Volume 4 Number 5 1977 Nucleic Acids Research

Isolation of folded chromosomes from Mycoplasma hyorhinis*

Marlyn Teplitz

Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, CA 91125, USA

Received 1 February 1977

ABSTRACT

Folded chromosomes were isolated from Mycoplasma hyorhinis. When examined by electron microscopy, these molecules show variability of loop size, number of loops, total contour length and degree of twisting of the DNA. Sedimentation velocity was unaltered after treatment with RNase, proteinase K, SDS, temperatures up to 65°C and NaCl concentrations from 0.1 M to 4 M.

INTRODUCTION

The folded chromosome of E. coli has been isolated and several reports indicate that RNA and protein may be involved in maintaining the structural integrity of the DNA complex (1-7). Electron microscopic studies of Mycoplasma hominis H39 (8) show that the DNA of this organism is a circular molecule which also exists in a similar folded state, and which replicates according to the model of Cairns (10). Mycoplasma genomes which have been studied have molecular weights of about 4×10^8 to 8×10^8 (9, 11, 12), or about one fourth the size of the E. coli genome. We have isolated folded chromosomes from Mycoplasma hyorhinis, and electron microscopy has shown the structure to be similar to that reported for M. hominis H39. This paper describes experiments to characterize the nature of the folded chromosome and the relationship of RNA and protein to it.

METHODS

Growth and Lysis of Organisms and Isolation by Sucrose Sedimentation

Mycoplasma hyorhinis was grown on a culture of BHK cells shown to be free of previous Mycoplasma infection by examination of cell surface replicas (13). This infected cell line was carried and used as a source of organisms for experiments.

Cells were removed from the dish by brief incubation at 37° with 0.025% trypsin. All supernatant medium and cells were combined and

centrifuged at 2,000 rpm for 10 min in a PR6 International Centrifuge. The resultant cell pellet was discarded, and the supernatant centrifuged at 20,000 rpm for 30 min in an SW 27 rotor. (Trypsinization removes the organisms from the cell surfaces and permits this separation.) Folded chromosomes were isolated by a modification of the method of Stonington and Pettijohn (1). Pelleted Mycoplasma were collected in 0.3 ml of lysing buffer (0.1 M NaCl, 10 mM EDTA, 15% sucrose, 30 mM Tris, pH 8.3). 0.1% diethyl oxydiformate (Eastman Kodak Co.) was added, and the organisms lysed by the addition of 0.3 ml of lysing solution (1% Brij 58, 0.4% sodium deoxycholate, 2 M NaCl, 10 mM EDTA) for 10 min at room temperature. Approximately 10^8 - 10^9 cells were lysed at once. Since the organism lacks a mucopolysaccharide cell wall, lysozyme treatment prior to lysis was omitted. The lysate was layered onto a 10 - 30% sucrose gradient (1) and centrifuged at 18,000 rpm for two hours in an SW 41 rotor. In some experiments ^{14}C-T7 phage particles were included on the gradient as a marker. Fractions were collected through a large needle (1 mm diameter) and aliquots were counted on filter papers.

Radioactive Labelling of DNA

Infected BHK cells were labelled with 5 μC/ml of ^{3}H-thymidine (New England Nuclear, sp. act. 40-60 Ci/mmol) 24 hours before harvesting. To be certain that nuclear DNA from cells was not being isolated along with the Mycoplasma folded chromosomes, an uninfected culture of BHK cells was labelled with 0.01 μC/ml of ^{14}C-thymidine (Schwarz-Mann, sp. act. 40 mC/mmol). After 24 hours these cells were trypsinized, mixed with an infected culture labelled with ^{3}H-thymidine, and treated as described above (Figure 1a).

Sucrose Gradient Sedimentation of Isolated Folded Chromosomes

Aliquots of peak fractions from sucrose gradients described above (corresponding to fractions 28 and 29 of Figure 1a) were diluted twice with water and rerun on similar gradients after incubation at 37° for one hour with 400 μg/ml pancreatic RNase (Sigma), 200 μg/ml proteinase K (EM Laboratories) or 2% SDS, or after incubation at 65° for one hour with no previous treatment. Similar aliquots were centrifuged without previous treatment in sucrose gradients with NaCl concentrations of 0.1 M up to 4 M.

Electron Microscopy

DNA from peak fractions of preparative sucrose gradients was prepared for electron microscopy by the aqueous method of Davis et al. (16). Grids were stained with alcoholic uranyl acetate and rotary shadowed with

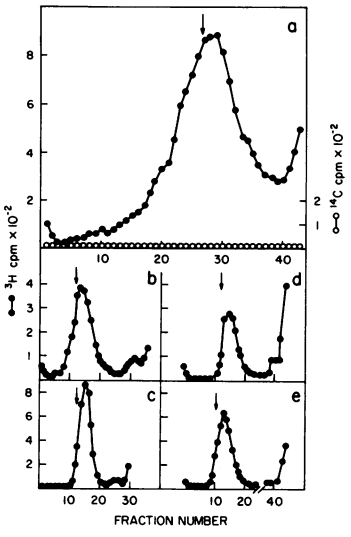

FIGURE 1. Sedimentation Profile of Folded Chromosomes in Sucrose Gradients. (a) Lysate of a Mycoplasma pellet from a mixture of BHK cells infected with M. hyorhinis (●——●) and uninfected BHK cells (○——○). (b-e) Results of several experiments in which aliquots of peak fractions (corresponding to 28 and 29 of (a)) were diluted, treated as described and rerun on similar gradients: (b) 37° one hour; (c) 400 μg/ml pancreatic RNase, 37° one hour; (d) 200 μg/ml proteinase K, 37° one hour; (e) 2% SDS, 37° one hour. The arrows indicate the position of ^{14}C-T7 phage particle marker.

platinum-palladium (80 - 20%). In some experiments 100 μg/ml of ethidium bromide was added to the hypophase solution.

Cesium Chloride - Ethidium Bromide Density Centrifugation

Aliquots of sucrose gradient fractions corresponding to fractions 20, 29, and 35 of Figure 1a were centrifuged to equilibrium in cesium chloride (density 1.55 gm/ml) and ethidium bromide (300 μg/ml) at 36,000 rpm in an SW 50.1 rotor. ^{14}C-SV40 I DNA and II DNA were included as markers. Fractions were counted on filter papers after washing with cold TCA and ethanol.

RESULTS AND DISCUSSION

Figure 1a shows that when lysates were centrifuged through sucrose gradients most of the ^{3}H counts sedimented in a single broad peak. No ^{14}C counts appeared in ^{3}H peak region. Some ^{3}H counts were always found at the top of the gradient; this region was shown to contain small DNA fragments when these fractions were examined by electron microscopy. As

compared with the internal marker, T7 phage particles (435S) (14), the sedimentation coefficient of the peak fraction was about 430, while the entire range was about 340 to 575. Therefore, under these conditions of lysis and sucrose sedimentation, it was possible to isolate heterogeneous folded chromosomes from M. hyorhinis.

Isolated folded chromosomes were subjected to a number of enzymatic and chemical treatments in order to determine a possible association of RNA and protein with the folded structure, as in the case of E. coli (1-7). Samples from peak fractions in the gradient were combined and aliquots were incubated at 37° for periods up to one hour with 400 μg/ml of pancreatic RNase, 200 μg/ml of proteinase K, or 2% SDS. There was no significant change in sedimentation velocity, as indicated by the position of the T7 marker (Figure 1b-e). The same results were observed when aliquots were incubated at 65° for one hour. The sedimentation velocity also remained the same when the DNA was isolated or rerun on high salt (up to 4 M NaCl) or low salt (0.1 M NaCl) sucrose gradients. These results indicate that, unlike the case of E. coli, conditions expected to dissociate protein and RNA from DNA do not change the sedimentation properties of these folded chromosomes.

Representative electron micrographs of folded DNA from peak fractions are shown in Figure 2. They are similar to those seen by Bode and Morowitz in M. hominis H39 by direct observation after osmotic shock or lysis (8), and also to those described by Delius and Worcel in E. coli (5). In general they appear to consist of varying numbers of loops of DNA which converge at a small locus or core. All of the DNA loops were extended in about 70% of the molecules (Figure 2a), while 17% had varying numbers of twisted loops, ranging from a few to all (Figure 2b). In addition to these two types of molecules, which had a single core region, 11% showed no intact core (Figure 2c) and 2% had two distinct cores and completely open loops (Figure 2d). Of six measurable molecules with contour lengths ranging from 178 to 300 microns, the average length was 220. These measurements indicate molecular weights of 3.6×10^8 to 6×10^8, averaging 4.4×10^8, within the range reported for molecular weights of Mycoplasma DNA (11, 12). The numbers of loops in individual molecules were extremely variable. They could be counted in 34 cases, and ranged from 25 to 96, with a mean of 40 ± 26. The large range of contour lengths and numbers of loops may be a reflection of the fact that the organisms used in these experiments were in various stages of their life cycle, and it is

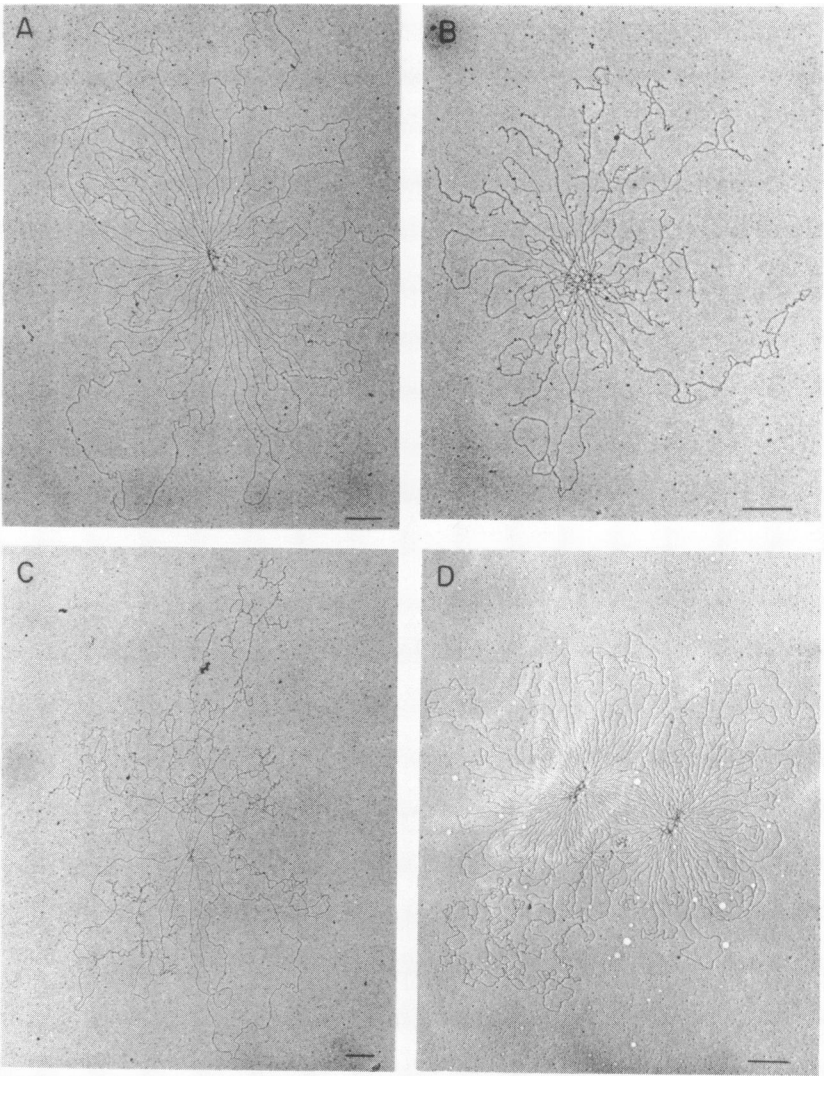

FIGURE 2. Electron Micrographs of Folded Chromosomes of M. Hyorhinis. (a) Folded DNA with completely relaxed loops; (b) Folded DNA showing both twisted and relaxed loops; (c) DNA without distinct core region; (d) Folded DNA showing two core regions. Bars represent 1 micron.

expected that the DNA recovered would range in size from single genome to fully replicated. No obvious replication forks were observed, but they may occur in the core region. In addition to variation in size, the number of loops and degree of relaxation of the DNA must also contribute to the

broad sedimentation profile in neutral sucrose.

The heterogeneous sedimentation profile was reproduced when individual fractions were rerun on a second sucrose gradient; fractions from the leading, center and trailing portions of the peak maintained the same relative positions (data not shown). In a further effort to characterize molecules from different regions of the broadly sedimenting peak, fractions corresponding to numbers 20, 29, and 35 in Figure 1a were centrifuged to equilibrium in cesium chloride-ethidium bromide. As can be seen in Figure 3, in each of these cases all ^{3}H counts were recovered in the upper

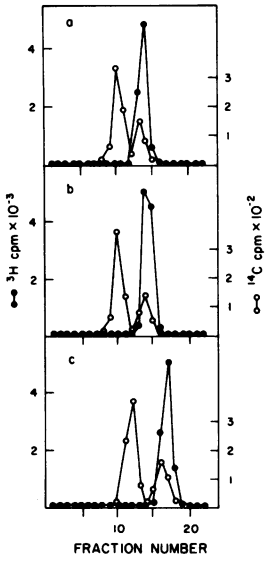

FIGURE 3. Cesium Chloride-Ethidium Bromide Density Centrifugation of Isolated Folded Chromosomes. Aliquots of preparative gradient fractions corresponding to fractions 20, 29, 35 of Figure 1a were centrifuged to equilibrium in cesium chloride and ethidium bromide. (a) Fraction 35; (b) Fraction 29; (c) Fraction 20. ^{14}C-SV40 DNA I and DNA II were included as markers. ^{3}H (•——•); ^{14}C (○——○).

band with SV40 II DNA. The same was true when the lysate was centrifuged directly in such gradients, though the peak was slightly broader (data not shown). These results suggest that loops must contain at least one nick. However, as described above, a significant number of molecules had loops which exhibited the properties of closed circular DNA (Figure 2b) when prepared for electron microscopy by the aqueous spreading technic (15). When folded chromosomes from peak fractions of sucrose gradients were prepared for electron microscopy in the presence of 100 μg/ml of ethidium bromide, most molecules showed tightly twisted structures, as was seen in E. coli (5), suggesting that most of the loops have the properties of

covalently closed circular DNA (Figure 4). The reason for the discrepancy between these two results is still unclear.

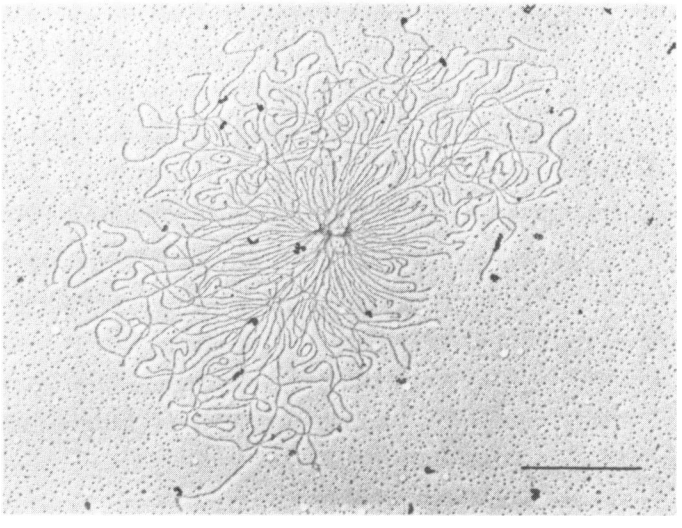

FIGURE 4. Electron Micrograph of Folded Chromosome of M. Hyorhinis in the Presence of Ethidium Bromide. DNA was prepared for electron microscopy in the presence of 100 μg/ml of ethidium bromide in the hypophase.

These folded chromosomes resemble those of E. coli structurally, but there appear to be differences in the factors involved in maintaining their physical integrity. It is still possible that RNA and protein are involved, but for some reason not yet elucidated, they are protected from the enzymatic and physical treatments described.

ACKNOWLEDGEMENTS

We are grateful to Dr. Harumi Kasamatsu for valuable discussions and suggestions. This work was supported by National Institutes of Health grant CA08014. This is contribution No. 5502 from the Division of Chemistry and Chemical Engineering.

REFERENCES

* Most of these experiments were done under the supervision of Dr. Jerome Vinograd. The manuscript was written after his death and is dedicated to his memory.

1. Stonington, O. and Pettijohn, D. E. (1971) Proc. Nat. Acad. Sci. USA 68, 6-9.
2. Pettijohn, D. E., Hecht, R. M., Stonington, O. G. and Stamato, T. D. (1973) in DNA Synthesis In Vitro, eds. Wells, R. D. and Inman, R. B. (University Park Press, Baltimore, Md.) pp. 145-162.

3. Worcel, A. and Burgi, E. (1972) J. Mol. Biol. 71, 127-147.
4. Worcel, A. and Burgi, E. (1974) J. Mol. Biol. 82, 91-105.
5. Delius, H. and Worcel, A. (1974) J. Mol. Biol. 82, 107-109.
6. Dworsky, P. (1975) Z. fur Allg. Microbiologie 15, 231-241.
7. Drlica, K. and Worcel, A. (1975) J. Mol. Biol. 98, 393-411.
8. Bode, H. R. and Morowitz, H. J. (1967) J. Mol. Biol. 23, 191-199.
9. Morowitz, H. J. and Wallace, D. C. (1973) Ann. N. Y. Acad. Sci. 225, 62-73.
10. Cairns, J. (1963) Cold Spring Harbor Symp. Quant. Biol. 28, 43-46.
11. Bak, A. L., Black, F. T., Christiansen, C. and Freundt, E. A. (1969) Nature 224, 1209-1210.
12. Morowitz, H. J. (1969) in Mycoplasmatales and the L-phase of Bacteria, Ed. Hayflick, L. (Appleton-Century-Crofts, New York) pp. 405-412.
13. Brown, S., Teplitz, M. and Revel, J-P. (1974) Proc. Nat. Acad. Sci. USA 71, 464-468.
14. Dubin, S. B., Benedek, G. B., Bancroft, F. C. and Freifelder, D. (1970) J. Mol. Biol. 54, 547-556.
15. Upholt, W. B., Gray, H. B., Jr. and Vinograd, J. (1971) J. Mol. Biol. 62, 21-38.
16. Davis, R. W., Simon, M. and Davidson, N. (1971) in Methods in Enzymology, eds. Grossman, L. and Moldave, K. (Academic Press, New York) Vol. 21, pp. 413-428.