Membrane-Bound Fractions of R6K Plasmid DNA in *Escherichia coli*

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The intracellular location of plasmid DNA has been of interest in an effort to understand the maintenance of these molecules. We have employed a simple procedure which enables us to isolate from exponentially grown cells on sucrose gradients membrane-complexed forms of R6K plasmid DNA. Electron micrographs identified the complexing of membrane fractions to circular forms of R6K DNA. Biochemical studies of the complexed R6K molecules showed the presence of membrane-specific proteins and suggested that complexing of R6K DNA was primarily with inner membrane fractions of *Escherichia coli*.

The maintenance of bacterial plasmid DNA requires a precise relationship of these molecules with other cellular constituents. Jacob et al. (8) postulated that DNA molecules must attach to membrane to be stably maintained, i.e., to replicate and partition themselves into daughter cells. There is substantial evidence that bacterial chromosomes and some viral genomes bind to membrane for replication (3, 5, 16). However, the evidence that bacterial plasmids attach to membrane for replication or segregation or both during cell growth has been indirect (7, 11, 15, 16).

The observations that a variety of bacterial plasmids can be found to cosediment with their host folded chromosomal complex (chromosome plus membrane [1, 10]) supports the notion of a physical relationship necessary for intracellular maintenance of plasmid DNA. Nevertheless, the cellular components—chromosomes or membrane or both—of the observed cosedimentation have not been determined. Examinations of ColE1 (15) and RK2 (7) plasmid DNA-membrane complexes in minicells suggest that these plasmids complex to membrane during normal cell growth and division. In addition, an analysis of plasmid maintenance in *Staphylococcus aureus* suggests that the bacterial membrane is involved (11). The findings that F plasmid DNA can be found associated with membrane-free folded chromosomes without linear insertion have led to the possibility of a new model for F DNA replication and segregation involving bacterial chromosomes (9).

In this study, we have employed a procedure previously developed for purifying R6K DNA

from *Escherichia coli* (1, 14). Cells were lysed as they sedimented through a layer of detergent, and thereby the solubilization or mechanical disruption, or both, of putative plasmid membrane complexes was reduced. The release of membrane-complexed molecules of R6K plasmid DNA on cleared lysate on sucrose gradients was followed by differential labeling with [¹⁴C]glycerol and [³H]deoxythymidine during exponential growth to identify the membrane and DNA components, respectively (Fig. 1). Spheroplasts were made in a top layer of a neutral sucrose gradient and then lysed while sedimenting through a layer of detergent. The ¹⁴C-labeled glycerol material formed a distinct peak coincident with the 58S peak of ³H-labeled R6K plasmid DNA, indicating the cosedimentation of a putative plasmid-membrane complex. Analyses of these plasmid forms on isopycnic sucrose gradients (12) indicated that approximately 25% of the molecules isolated on sucrose were complexed to membrane material (Fig. 2). A comparison of the sedimentation values of cleared lysate on sucrose-purified forms (Fig. 1) to purified covalently closed circular (CCC) monomer of R6K isolated on dye-buoyant density gradients, showed that the gradient-isolated complex had a higher S value at 58S as compared with the CsCl-isolated form (51S) (14). The density of the 58S form as CCC molecules of R6K was previously confirmed by cesium chloride-ethidium bromide density equilibrium gradients and agarose gel electrophoresis (13, 14).

R6K plasmid DNA was prepared from gradients (Fig. 1) and examined by dark-field electron microscopy for the presence of membrane-plasmid complexes. The procedure used was essentially that described by Dubochet et al. (4). This

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FIG. 1. Cleared lysate on sucrose gradient sedimentation profiles of [³H]deoxythymidine-labeled DNA (●) and [¹⁴C]glycerol-labeled membrane (○) fractions from cells harboring R6K plasmid DNA. Cells were grown exponentially in a minimal salts medium, concentrated by centrifugation (5°C), and suspended in one-tenth the original volume in cold buffer consisting of 0.2 M KCN–0.25 M NaCl–0.01 M Tris-hydrochloride made up in 5% sucrose at pH 8.0. A 0.4-ml portion of the concentrated cell suspension was mixed with 0.1 ml of a 5 mg/ml stock solution of freshly prepared lysozyme in 0.01 M EDTA, 0.01 M Tris-hydrochloride, and 0.25 M NaCl, pH 8.0. A 0.2-ml sample of this mixture was immediately layered onto a 0.2-ml layer of detergent (4% Brij 58) made up in 5% sucrose on top of 11 ml of 10 to 30% neutral sucrose gradient maintained at 5°C. All solutions were made up of the same buffer described for the lysozyme suspension. The gradients were then left undisturbed for 10 min at 5°C to allow for spheroplast formation in the top layer. Immediately thereafter, the gradients were centrifuged at 5°C in a Spinco SW41.1 swinging bucket rotor. The gradients were then collected from the top (15 drops per fraction) with the aid of an Auto Densi-flow II onto filter strips or in trays and were spotted. The strips were then washed in trichloroacetic acid and ethanol, air dried, and counted. R6K circular duplex DNA extracted under these conditions sedimented at 58S.

FIG. 2. Isopycnic sucrose gradient centrifugation of R6K DNA complexes from cleared lysates on sucrose gradients. Plasmid forms pooled from the 58S region of gradients described in Fig. 1 were layered on a step sucrose density gradient. Step gradients were prepared by layering 2 ml of 50, 45, 40, 35, and 30% sucrose solutions (5 mM EDTA, pH 7.5) over a 0.5-ml 60% sucrose shelf as described by Osborn et al. (12). The gradients were then centrifuged in a SW41.1 rotor at 38,000 rpm at 4°C for 16 h. Fractions of 15 drops were collected by puncturing from the bottom onto filter strips; these were washed and counted as described in the legend to Fig. 1. The arrow refers to membrane-complexed form of R6K DNA. Peak at fraction 23 represents membrane-free circular form of this plasmid. Symbols: ●, [³H]deoxythymidine-labeled DNA; ○, [¹⁴C]glycerol-labeled membrane.

FIG. 3. DNA-membrane samples were dialyzed extensively against 0.01 M Tris-hydrochloride-0.001 M EDTA (pH 8.0). The samples were diluted 1:10 into glass-distilled H_2O and mounted on carbon films supported on 400-mesh copper grids made hydrophilic by glow discharge in amylamine (4). After being rinsed with H_2O , the samples were stained with 1% aqueous uranyl acetate and rotary shadowed with platinum at an angle of 7° . Micrographs were made on a Siemens 101 electron microscope. The bar represents $0.5 \mu m$.

FIG. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of proteins complexing to R6K DNA from gradients. ^3H -labeled R6K-containing cells were grown exponentially and lysed on top of 11-ml gradients overlaid with Brij 58, Sarkosyl, and Triton X-100 as described in the text. The gradients were collected (15 drops) by puncturing from the bottom into trays and then were spotted and counted as described in the legend to Fig. 1. Plasmid regions were pooled and analyzed for protein content. The gels were stained with Coomassie blue. Lanes: (a) Brij sample; (b) Triton sample; (c) Sarkosyl sample; (d) cytoplasmic membrane; (e) outer membrane *E. coli*.

method generated a very high contrast and resolution of samples which facilitates successful visualization of protein-nucleic acid interactions. Shown in Fig. 3 are characteristic membrane vesicles observed in these fractions. A number of supercoiled duplexed DNA molecules appeared to be attached to these vesicles (Fig. 3a). Figure 3b shows several open circular molecules with membrane fragments attached. Observations of over 50 DNA molecules indicated that approximately 1 in 6 was complexed with membrane material. Since many forms were observed with only one membrane fragment per molecule, it is possible to infer that there is one attachment site per complexed molecule; however, our data are incomplete in this regard.

To further characterize R6K plasmid-membrane fractions, samples of these forms (58S) were subjected to polyacrylamide gel electrophoresis in sodium dodecyl sulfate (Fig. 4). In these analyses, three different detergents (Brij 58, Triton X-100, and Sarkosyl), which had different effects on membrane solubilization (6), were used as described in the legend to Fig. 1 to isolate plasmid-membrane fractions. The protein

banding profiles obtained under this lysis condition were essentially the same for all detergents used. A protein of approximately 82,000 molecular weight was predominant in plasmid samples obtained with Triton and Sarkosyl (lanes b and c). This protein was also present in relatively low concentration in purified inner membrane fractions (Fig. 4d). In addition, there was a protein of approximately 61,000 molecular weight which appeared to be plasmid specific (Fig. 4a to c), i.e., it was not found in purified membrane fractions. A comparison of proteins derived from plasmid-containing fractions with those found in both the inner and outer membrane fractions of *E. coli* showed many proteins in common. However, there were more proteins in common with inner membrane than with outer membrane proteins.

In earlier studies (1, 14) in which the relationship of R6K DNA to its folded chromosomal complex (chromosome plus membrane) was investigated, approximately 80% of nonreplicating and 100% of the replicating molecules of this plasmid were found associated to this complex. However, the cellular component to which these forms associate themselves was not determined. From the findings presented here, it would appear that at least a portion of 10 to 15 copies (14) of the nonreplicating molecules of R6K DNA per cell is associated to the membrane of *E. coli*. The role of this association in regulating initiation of R6K synthesis and segregation of replicas into progeny cells has not been determined. Nonetheless, in view of the stringent complexing of the replicating forms to the folded chromosomal complex (1, 2), it would be appealing to suggest that this association is for the purpose of replication. This interpretation would be consistent with the replicon model of Jacob et al. (8) and would support similar observations with plasmid ColE1 (15) and RK2 derivatives (7). Further, the identification of a membrane-specific binding site on the bacterial nucleoid at the point of origin for chromosomal replication (5, 16) lends support to a specific membrane binding site for plasmid DNA attachment. Whether the association of R6K DNA to membrane serves to fulfill the various functions involved in plasmid maintenance is not clear.

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LITERATURE CITED

1. Archibold, E. R., C. W. Clark, and R. J. Sheehy. 1978. Relationship of R6K replicating forms to the folded chromosome of *Escherichia coli*. *J. Bacteriol.* 135:476-482.
2. Archibold, E. R., R. Johnson, and R. J. Sheehy. 1981. Effects of Rifampicin on the relationship of R6K replicating forms to the folded chromosomal complex of *E. coli*.

- Biochim. Biophys. Acta 653:294-298.
3. Drlica, K., E. Burgi, and A. Worcel. 1978. Association of the folded chromosome with the cell envelope of *Escherichia coli*: nature of the membrane-associated DNA. J. Bacteriol. 134:1108-1116.
  4. Dubochet, J., J. Ducommun, M. Zollinger, and E. Kellenberger. 1971. A new preparation method for dark-field electron microscopy. J. Ultrastruct. Res. 35:147-167.
  5. Dworsky, P., and M. Schaechter. 1973. Effect of rifampin on the structure and membrane attachment of the nucleoid of *Escherichia coli*. J. Bacteriol. 116:1364-1374.
  6. Filip, C., G. Fletcher, J. L. Wulff, and C. F. Earhart. 1973. Solubilization of the cytoplasmic membrane of *Escherichia coli* by ionic detergent sodium-lauryl sarcosinate. J. Bacteriol. 115:717-722.
  7. Firshein, W., P. Strumph, P. Benjamin, K. Burnstein, and J. Kornacki. 1982. Replication of a low-copy-number plasmid by a plasmid DNA-membrane complex extracted from minicells of *Escherichia coli*. J. Bacteriol. 150:1234-1243.
  8. Jacob, F., S. Brenner, and F. Cuzin. 1963. On the regulation of DNA replication in bacteria. Cold Spring Harbor Symp. Quant. Biol. 28:329-340.
  9. Kline, B. C., and J. R. Miller. 1975. Detection of nonintegrated plasmid deoxyribonucleic acid in the folded chromosome of *Escherichia coli*: physiochemical approach to studying the unit of segregation. J. Bacteriol. 121:165-172.
  10. Kline, B. C., J. R. Miller, D. E. Cress, M. Wlodarczyk, J. J. Manis, and M. R. Otten. 1976. Nonintegrated plasmid-chromosome complexes in *Escherichia coli*. J. Bacteriol. 127:881-889.
  11. Novick, R., C. Sanchez-Rivas, A. Gruss, and I. Edelman. 1980. Involvement of the cell envelope in plasmid maintenance: plasmid curing during the regeneration of protoplasts. Plasmid 3:348-358.
  12. Osborn, M., J. Gander, E. Parish, and J. Carson. 1972. Mechanism of assembly of the outer membrane *Salmonella typhimurium*. J. Biol. Chem. 247:3964-3972.
  13. Sheehy, R. J., C. W. Clark, and E. R. Archibald. 1977. A technique for the analysis of nonreplicating and replicating forms of R6K plasmid DNA in *Escherichia coli*: relationship to the folded chromosome, p. 265-275. In S. Mitsuhashi, L. Rosival, and V. Kremery (ed.), Plasmids: medical and theoretical aspects. Springer-Verlag, New York.
  14. Sheehy, R. J., C. W. Clark, and E. R. Archibald. 1978. Isolation of nonintegrated plasmid DNA in *Escherichia coli* free from host folded chromosomes, p. 112-114. In D. Schlessinger (ed.), Microbiology—1978. American Society for Microbiology, Washington, D.C.
  15. Sparks, R., and D. Helinski. 1979. Association of cellular membrane of *E. coli* minicells with the origin/terminus of replication of plasmid ColE1 DNA. Nature (London) 277:572-575.
  16. Winston, S., and N. Sueoka. 1980. DNA membrane association is necessary for initiation of chromosomal and plasmid replication in *Bacillus subtilis*. Proc. Natl. Acad. Sci. U.S.A. 77:2834-2838.