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Association of Replicative T4 Deoxyribonucleic Acid and Bacterial Membranes

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Experiments utilizing CsCl density gradient analysis and radioactive labels specific for bacteriophage T4 deoxyribonucleic acid (DNA) and membranes have shown that replicative T4 DNA is associated with host membranes. The association is inhibited by chloramphenicol and takes place just prior to semi-conservative replication of the phage DNA.

Soon after infection of *Escherichia coli* by bacteriophage T4, the injected deoxyribonucleic acid (DNA) of the phage becomes associated in a rapidly sedimenting protein-DNA complex (13). The formation of this complex is dependent on protein synthesis after infection and is specific for replicative DNA (13). Newly synthesized phage proteins are associated with the complex and form a special class of proteins synthesized after infection (14). Rapidly sedimenting complexes with some of these properties have been described for other phage systems (3, 4, 9, 11, 15). Many authors have speculated that these fast-sedimenting complexes represent association of phage DNA with bacterial membranes (1, 3, 4, 9, 15).

The purpose of the experiments reported in this communication was to investigate the hypothesis that intracellular T4 DNA is attached to membranes during replication. Radioactive labels specific for DNA and membranes have been employed in conjunction with density gradient centrifugation. The results confirm the hypothesis that one component of the fast-sedimenting T4 protein-DNA is host membrane.

MATERIALS AND METHODS

The procedures used for radioactive labeling of phage DNA and gradient centrifugation have been described elsewhere (12). Cells were lysed by the lysozyme-Triton X-100 procedure (13) or by the lysozyme-Brij 58 procedure (8).

The linear CsCl density gradients were produced in a standard, two-chamber mixing vessel. The heights of the liquid in the chambers were adjusted to compensate for the difference in density of the initial CsCl concentrations. The refractive indexes of the fractions were calibrated in a Bellingham and Stanley refractometer after collection of the gradients. The gradients were spun in an SW50.1 rotor in a Spinco ultracentrifuge for 4.5 hr at 35,000 rev/min at 5 C. The gradients were buffered with 50 mM tris(hydroxymethyl)aminomethane (Tris; *p*H 7.4) and contained 1 mM ethylenediaminetetraacetic acid (EDTA).

Enzymatic digestions were conducted with pancreatic ribonuclease A and pancreatic deoxyribonuclease I purchased from Worthington Biochemical Corp. Lysates were incubated with 100 μg of ribonuclease per ml (heated for 2 min at 100 C) for 30 min at 25 C in 0.05 M Tris-0.05 M NaCl-0.01 M EDTA, *p*H 7.4. Lysates were incubated with 100 μg of deoxyribonuclease for 30 min at 25 C in 0.05 M Tris-0.05 M NaCl-0.01 M $MgCl_2$, *p*H 7.4.

Samples of radioactive samples were transferred to glass-fiber filters and counted in a Nuclear Chicago Isocap 300 scintillation spectrometer.

RESULTS

Development of density gradients. Previously, DNA-protein or "membrane" complexes had been isolated on the basis of sedimentation velocity characteristics. Lysates were layered on sucrose gradients, and complexes were sedimented to a CsCl or saturated sucrose "shelf" or "pad," which collected any material with a high sedimentation coefficient. This technique was inadequate to separate "DNA-membrane" complexes and membrane fragments. Since the objective of the following experiments was to detect a specific association of DNA and membranes, a method was needed which would separate membranes and DNA-membrane complexes and free DNA. The method of choice was CsCl density gradient centrifugation. CsCl gradients were produced which were approximately linear between densities 1.72 and 1.15. The gradients were centrifuged for a time long enough for the slowest sedimenting molecule involved, free DNA, to come to its position of equilibrium at the bottom of the tube, but a time short enough for the gradient to be stable. Figure 1 shows that DNA of 63*S* and T4 phage ~1,000*S* come to their

appropriate positions in the gradient under our standard conditions (Fig. 1). Since the T4 complex sediments faster than phage (13), and membranes have a very light density, all the macromolecular components in which we are interested should come to a position corresponding to their appropriate density in the gradient.

Density of the T4 DNA-protein complex. The average density of replicative DNA-protein complexes was determined by density gradient analysis of lysozyme-Triton lysates of *E. coli* B23 infected with ^{32}P-labeled parental phage (Fig. 1). Intracellular phage DNA was found to band in two major peaks in the gradient, one at the bottom of the tube in the position of free DNA and another broad peak at a density of approximately 1.37 g/cm (Fig. 1, 2A, 3C). This peak represents DNA in the fast-sedimenting complex. This average density varies somewhat as a function of time after infection, but indicates that there are about equivalent amounts of DNA and protein in the complex during the early stage of replication.

Detection of a membrane moiety in the complex. To detect a specific association of replicative DNA and membrane after T4 infection, cells were grown for two generations in glycerol-2-^{3}H-labeled medium and infected with ^{32}P-labeled T4 phage. Glycerol-2-^{3}H is known to label membranes specifically (4). At 8 to 10 min after infection, the cells were lysed and analyzed in a 1.7 to 1.1 CsCl density gradient (Fig. 2A). As expected, two major peaks of ^{32}P-labeled DNA were found, as well as several peaks of glycerol-2-^{3}H-labeled material. This pattern was characteristic for lysates of cells prelabeled for one-half to three generations before infection. The densest peak of glycerol-2-^{3}H-material banded exactly with the lightest of the ^{32}P-labeled DNA peaks. This indicated a specific association of DNA and membrane. When the material from this peak was isolated, dialyzed, and rebanded, the recovery of the material was quantitative, and the material banded at the original location in the gradient. This indicates that the complex consists of a stable association of phage DNA and membranes.

Extraction of the glycerol-2-^{3}H-labeled moiety with chloroform-methanol. The material in the CsCl density gradient peak characteristic of the DNA-membrane complex was extracted by a chloroform-methanol extraction for lipids (2). This was done to show that the glycerol-2-^{3}H had been incorporated into a lipid moiety and not into nucleic acid. Consequently DNA-membrane complexes were isolated with a CsCl density gradient as in Fig. 2A; ^{32}P-labeled DNA then was added to the complex to act as a control in the extraction. The experiment shows (Table 1) that the overwhelming proportion of the ^{3}H-label in the DNA-membrane complex has been incorporated in a lipid moiety and not into a nucleic acid. While almost 70% of the input ^{3}H is recovered in the chloroform phase of the first extraction, practically none of the ^{32}P contaminates this phase. This conclusion is consistent

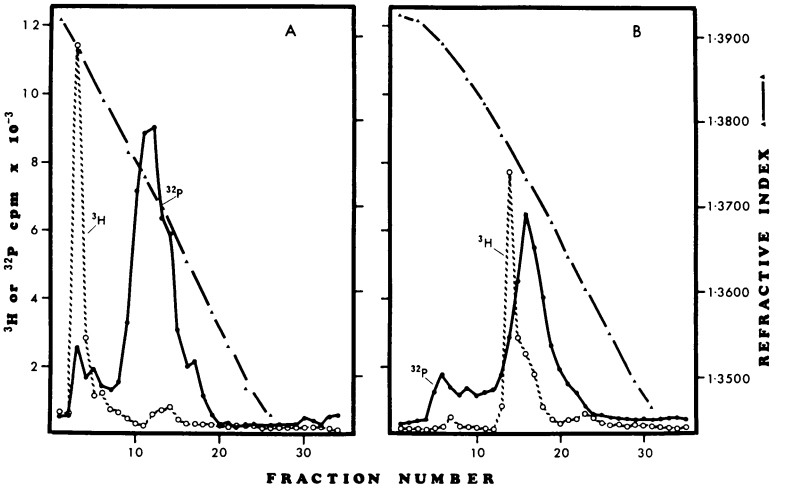

FIG. 1. *CsCl density gradient analysis of T4 protein-DNA complexes. E. coli B23 was grown to 3 × 10^{8} cells/ml in 10 × PO$_4$-TCG and was infected with an MOI = 5.0 with ^{32}P-labeled T4B0$_1^r$. At 8 min after infection, the cells were lysed by the lysozyme-Triton X-100 procedure. Phenol-extracted ^{3}H-labeled reference T4 DNA (A) or ^{3}H-labeled T4 phage (B) were mixed with the lysates, and the mixtures were analyzed by CsCl density gradient centrifugation as described in Materials and Methods.*

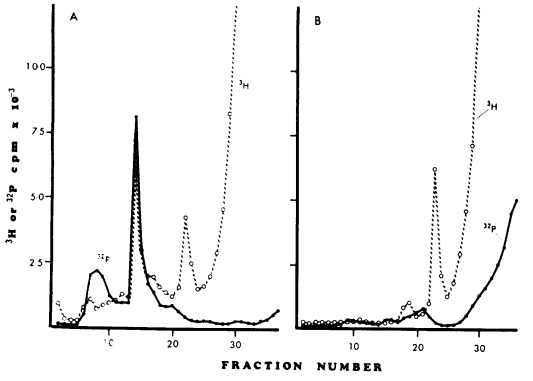

FIG. 2. *CsCl density gradient analysis of T4 DNA-membrane complexes. E. coli B23 was grown to 3 × 10^8 cells/ml in 10 × PO_4-TCG and 100 μg of glycerol-2-^3H per ml. They were infected with an MOI of 5.0 with ^{32}P-labeled T4BO$_1^r$. At 8 min after infection, the cells were lysed by the lysozyme-Triton X-100 procedure. Lysates were layered directly on 1.72 to 1.15 CsCl density gradients (A) or were incubated with ribonuclease and deoxyribonuclease before layering on the gradients (B).*

with known characteristics of glycerol-2-^3H incorporation (5).

To confirm that the ^3H label extracted by chloroform was indeed present in lipid compounds, the extract was analyzed by thin-layer chromatography (2). The extract was co-chromatographed with authentic standards, and the overwhelming majority of the label was found in phosphatidylethanolamine and phosphatidylglycerol.

Nuclease sensitivity of the DNA-membrane complex. Previously, we had shown that the fast-sedimenting characteristics of newly synthesized proteins in the DNA-protein complex were dependent on the integrity of the DNA in the complex (14). In the present experiments, we have shown that the density of the glycerol-2-^3H-labeled moiety is dependent on the integrity of the nucleic acid of the complex. After incubation of appropriately labeled lysates with ribonuclease and deoxyribonuclease, the peak which is characteristic of the DNA-membrane complex is no longer present after CsCl density gradient analysis (Fig. 2B). Incubation in the absence of ribonuclease and deoxyribonuclease did not alter the pattern described by Fig. 2A. Though incubation of a lysate of infected cells with deoxyribonuclease leads to a large reduction in the size of the peak, only incubation with deoxyribonuclease and ribonuclease leads to its complete obliteration. This is consistent with previous results indicating the presence of RNA components in fast-sedimenting complexes (7, 8). Our results have shown that the density of the 2-^3H-labeled moiety is dependent on the integrity of phage DNA. This supports the contention that the peak of ^{32}P-labeled DNA and glycerol-2-^3H-labeled membranes represents the association of replicative DNA and membranes.

Chloroamphenicol (CM) inhibition of the DNA-membrane association. If CM is added to infected cells earlier than 3 min after infection, formation of the DNA-membrane complex is inhibited (Fig. 3A). ^{32}P-labeled, injected, parental T4 DNA is found in the peak characteristic of free DNA after CsCl density gradient analysis, if CM is added within 3 min of the time of infection (Fig. 3A). This inhibition is coincident with CM inhibition of the previously described fast-sedimenting complex (13). The timing of the formation of the DNA-membrane complex was examined by analyzing samples of infected cells lysed at various times after infection. The timing of the formation of the DNA-membrane peak is coincident with formation of the fast-sedimenting complex (13) and occurs just prior to the detection of semi-conservative replication in vivo (13). At later times after infection, the density of the complex shifts towards a higher density in the gradients; this may be because the average size of the membrane moiety remains constant, whereas the DNA moiety gradually increases in size due to the formation of concatenates (Fig. 3E, 3F). Because of the inhibition of DNA-membrane associated by CM, it can be suggested that association is dependent on synthesis of phage protein. This inhibition also supports the contention that the association is not an artifact of cell lysis, because under the appropriate conditions injected DNA is not attached to cell membrane.

DISCUSSION

The replicon model for the control of DNA replication in bacteria required that replicating DNA be attached to cell membranes. This hypothesis was made for two reasons: (i) it provided a mechanism for the segregation of progeny DNA molecules to daughter bacterial cells during division and (ii) it provided a point of attachment thought to be necessary for the autonomous replication of F (10).

When Smith and Hanawalt (16) and Ganesan and Lederberg (7) found fast-sedimenting replicative DNA complexes in bacterial cells, it was attractive to assume that this represented membrane attachment of the bacterial DNA. Replication of bacteriophage T4, however, does not need a mechanism for segregation of progeny DNA molecules to separate daughter cells. Therefore, it was not

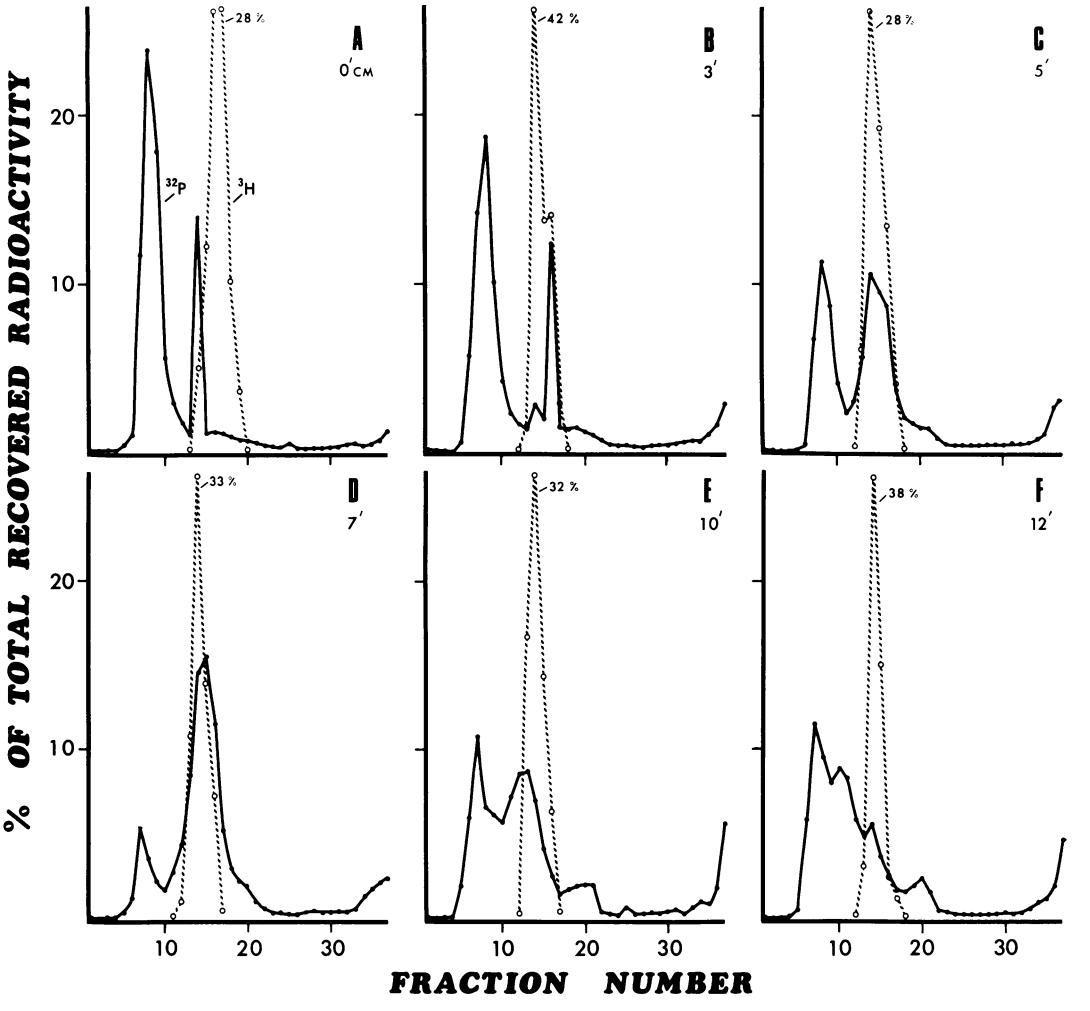

FIG. 3. *Chloramphenicol (CM) inhibition of the formation of T4 DNA-membrane complexes and timing of the formation cells per milliliter. E. coli B23 was grown to 3 × 10⁸ in 10 × PO₄-TCG and infected with ³²P-labeled T4B0ᵢʳ. At 0 min after infection, a sample was transferred to CM. At 3, 5, 7, 9, and 12 min after infections, samples with and without CM were lysed by the lysozyme-Triton X-100 procedure. ³H-labeled reference T4 phage were added to the lysates, and the mixtures were analyzed by the CsCl density gradient technique. A, CM added at 0 min after infection and incubated for 10 min before lysis. B–F, No CM added. Samples lysed at 3, 5, 7, 9, and 12 min after infection. Percent at the top of each peak of ³H-labeled phage refers to percent of recovered activity in the highest fraction.*

clear that T4 replicative DNA should be attached to host membranes, nor was it obvious that fast sedimentation alone indicated that the DNA was attached to something large, e.g., host membrane. Very compact structures containing 63*S* DNA (i.e., bacteriophage) can sediment almost as fast as the DNA-protein complex. Therefore, it was important to obtain direct evidence that replicative T4 DNA is attached to host membranes during bacteriophage development. Double-label experiments specifically labeling DNA and membranes show that there is an association of replicative DNA and membranes simultaneous with the formation of the previously described fast-sedimenting DNA-protein complex. This membrane association is dependent on phage protein synthesis and takes place just prior to the time when semi-conservative replication can be detected. These two facts demonstrate that the association is not due to injected phage DNA being "trapped" nonspecifically in membrane fragments during the process of cell lysis.

TABLE 1. *Chloroform-methanol extraction of the glycerol-2-^{3}H-labeled moiety of the DNA-membrane complex*[a]

Time	Total radioactivity (counts/min)	
	^{3}H	^{32}P
Before extraction	8.5×10^4	11.9×10^4
After extraction, in chloroform phase	5.68×10^4	0.01×10^4
After extraction, in water phase	0.69×10^4	6.86×10^4

[a] *E. coli* B23 was grown to 3×10^8 in $10 \times$ PO$_4$-TCG with glycerol-2-^3H at 100 μCi/ml. The bacteria were infected with ^{32}P-labeled T4 phage at multiplicity of infection of 5.0 and incubated at 37 C. At 8 min after infection, the infected cells were lysed, and the lysate was subjected to CsCl density gradient sedimentation. The material in the peak characteristic of the T4 DNA-membrane complex was pooled and dialyzed against 50 mM Tris-50 mM NaCl-1 mM EDTA at 4 C. Phenol-extracted ^{32}P-labeled T4 phage DNA was added to the dialysate at 10 times the internal ^{32}P counts, and the mixture was extracted by standard procedures (2). Only one extraction of the aqueous phase was carried out instead of the four normally used for maximum recovery of the lipid material.

Since the original reasons for postulating membrane-DNA association do not readily apply to T4, some other rationale must be supplied for this phenomenon. It is probably pertinent that semiconservative replication in vitro has not been achieved in preparations free of membranes. It seems likely that the membrane structure itself may play some fundamental part in the replication process.

Further studies to define the nature of the DNA-membrane association and the characteristics of the lipid fractions involved are currently underway.

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