λ DNA–MEMBRANE COMPLEX ISOLATED IN THE CsCl DENSITY GRADIENT

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1. Introduction

It is currently accepted that the membrane of *Escherichia coli* cell plays a role in the replication of bacteriophage λ DNA. However, this opinion is based on experimental facts which cannot yet be interpreted with certainty. It has been shown that some of the phage DNA cosediments with the host membranes; this material is termed rapid sedimenting complex, RSC [1]. Intermediates of the first round of λ DNA replication are preferentially found in RSC [2] when bacteria are lysed 12 min after infection. However, the relation between the membrane-association and replication of λ DNA, could not be inferred from these experiments, because the RSC technique does not define any association between λ DNA and the cell membrane: cosedimentation cannot be considered as proof of a DNA–membrane association. The intracellular DNA engaged in the transcription and replication may sediment more rapidly than the DNA isolated from the mature phage [3]. In the search for a method unequivocally separating the postulated λ DNA–membrane complex from other forms of intracellular DNA we adopted the CsCl density gradient technique [4] which proved to be effective due to the considerable difference in buoyant density between DNA (1.7) and the membranes (1.3).

2. Methods

The bacteria (*E. coli* TC600) were grown in KMB-medium (modified K medium [5] containing maltose, instead of glucose, and additionally thiamine 2 μg/ml) at 37°C in a water-bath shaker and collected in the exponential phase of growth (A_{575} 0.3–0.5). After washing with TM buffer (0.05 M Tris–HCl/0.001 M EDTA, pH 7.4) the cells (5×10^9) were suspended in 0.5 ml same buffer and infected with [*methyl*-^{3}H]-thymidine-labeled phage λcI72 or λcI857 amS7 at m.o.i. 5, if not otherwise stated. After allowing adsorption at 37°C for 15 min, 5 ml warmed KMB medium was added and the suspension was incubated in the shaker at 37°C for 12 min, if not otherwise stated. Then the cells were lysed by the lysozyme-Brij-58 method according to [2]. After dialysis the volume of the cell lysate amounted to about 2 ml.

The cell lysate (0.2 ml) was layered on top of a preformed CsCl gradient in the 10 ml tube of the Beckman SW 27.1 rotor. The gradient consisted of 4 layers of CsCl solutions made on 0.05 M Tris–HCl/0.001 M EDTA (pH 7.4) buffer, the densities of which were 1.72 (2 ml), 1.50 (1.5 ml), 1.30 (1.5 ml) and 1.15 (1.5 ml). After centrifugation (6 h, 25 000 rev./min, 8°C) the gradients were approximately linear. The 15-drop fractions were collected in vials containing Whatman 3MM discs and dried. As a scintillation fluid, redistilled toluene, containing 4 g PPO/liter and 0.1 g POPOP/liter, was used. The radioactivity was counted in the Nuclear Chicago Isocap 300 scintillation spectrometer.

3. Results

The phospholipid components of the bacterial cell membrane are preferentially labeled with radioactive glycerol [2,4]. To detect an association of bacterial membranes with the infecting λ DNA, the bacteria, grown for two generations in KMB medium containing [^{14}C]glycerol were infected with [^{3}H]thymidine-labeled phage. The results of CsCl density gradient

sedimentation of the cell lysate are shown in fig.1. The membranes form a sharp ^{14}C peak at a density of 1.3; at the same position a turbid disc is visible by the naked eye. Part of the infecting phage DNA forms a sharp ^{3}H peak exactly at the density position of membranes, suggesting the existence of a specific λ DNA–membrane association. Another part of DNA, representing all other forms of intracellular λ DNA, forms a second peak close to the bottom of the centrifuge tube, at a density of about 1.6. In the experimental conditions applied (short sedimentation at 25 000 rev./min) the slowest sedimenting molecule involved, free λ DNA, cannot come to its position of equilibrium, nevertheless, it is well separated from the membrane-associated DNA.

Further proof concerning the identity of the partners forming the λ DNA–membrane complex ensues from the enzyme-sensitivity experiments. The combined action of Sarkosyl and proteinase K, destroying the membranes, results in the disappearance of λ DNA from the membrane peak (fig.2A); only the peak representing the free λ DNA is observed. After action of deoxyribonuclease (fig.2B), the amount of λ DNA in the membrane peak is strongly reduced and there is nothing left from the free λ DNA peak.

We wanted to ascertain whether the DNA of the cell-adsorbed phage is not trapped nonspecifically in membrane fragments during cell lysis. Phage λ becomes adsorbed to the bacterial cells in the cold, but the triggering of DNA injection and entry of the phage DNA into the cell does not occur until the temperature is raised [6]. In accordance with this

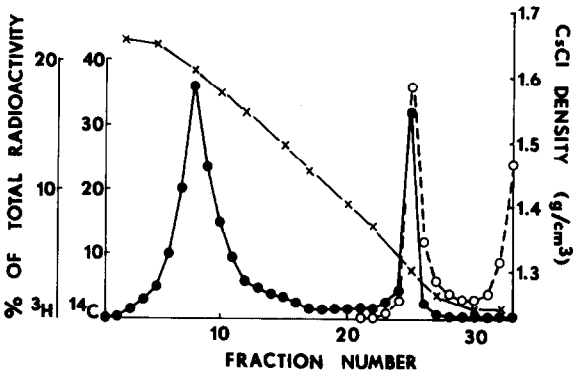
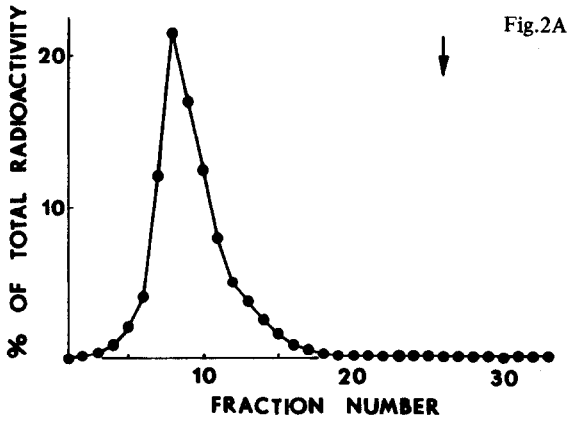


Fig.1. Double-label experiment. The bacteria, grown for two generations in KMB medium containing [2-^{14}C]glycerol (8.5 mCi/mmol, 0.5 μCi/ml), were infected with [^{3}H]-thymidine-labeled phage. Cell lysis and CsCl density gradient sedimentation were performed as in section 2. In each fraction the density (X), ^{14}C (o) total radioact. 2900 cpm, and ^{3}H (•) total radioact. 23 800 cpm, were estimated.



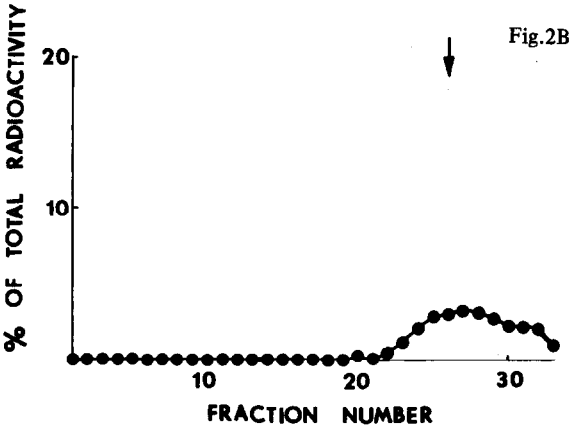


Fig.2. Enzyme-sensitivity of the λ DNA–membrane complex. The cell lysate (1 ml) was treated (A) with 10 μl 30% Sarkosyl for 15 min at both 4°C and 50°C, and with 10 μl 30 mg/ml proteinase K solution (self-digested for 4 h at 37°C, and at 80°C for 10 min in 0.005 M EDTA) for 3 h at 37°C, (B) with 10 μl 1 mg/ml deoxyribonuclease solution (in 0.05 M Tris–HCl/0.05 M NaCl/0.01 M $MgCl_2$, pH 7.4) for 30 min at 25°C. In B the cell lysate was pre-dialysed against the enzyme buffer. After CsCl density gradient sedimentation, the distribution of ^{3}H (•) total radioact. 17 000 cpm was determined. The position of the turbid membrane peak is shown by an arrow.

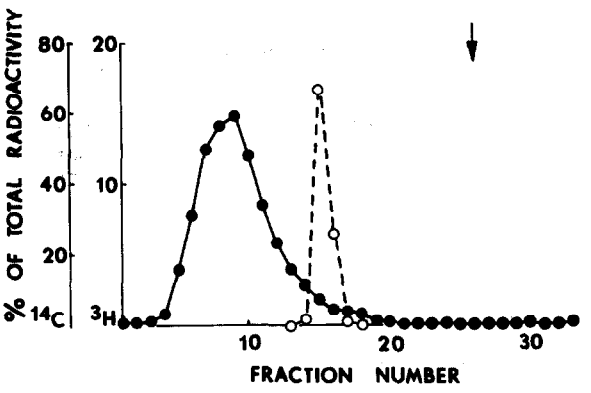
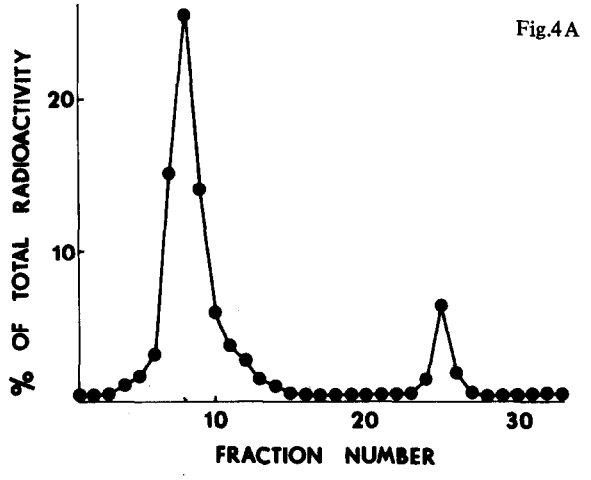


Fig.3. The fate of the adsorbed-phage DNA. The experiment was performed as in section 2, except that phage adsorption and incubation in KMB medium occurred at 2°C. The distribution of ^{3}H (•) total radioact. 25 500 cpm, in the CsCl density gradient was estimated. The position of the turbid membrane peak is shown by an arrow. A sample of [2-^{14}C]-thymidine labeled λ phage (○) total radioact. 5200 cpm, was added to the cell lysate immediately before sedimentation.

finding phage adsorption and incubation in KMB medium were performed at 2°C. The results (fig.3) clearly show that there is no radioactivity of adsorbed phage DNA at the membrane position of the gradient. The formation of the λ DNA–membrane complex is inhibited by chloramphenicol (fig.4) indicating that most of the intracellular phage DNA is not trapped nonspecifically in membrane fragments during cell lysis.

In our standard experiment the infected cells were incubated in KMB medium for 12 min, to allow for the first round of λ DNA replication [2]. It was interesting, however, to investigate the kinetics of formation of the λ DNA–membrane complex from the beginning of incubation. In the standard experiment (phage adsorption at 37°C) even at the very beginning (30 s) the complex appears, but it is practically absent when the phage adsorption has been performed at 2°C. After 2 min incubation in the KMB medium at 37°C it amounts to about half maximum value; however, it takes 4–6 min (depending on the temperature of phage adsorption) to attain the plateau of λ DNA attachment to the membrane.

Trying to reach a high efficiency of complex formation, we studied the effect of an increasing multiplicity of phage infection (m.o.i.), and have found that saturation occurs at much lower values (fig.5) than that claimed in [7]. In consideration of this discrepancy we performed parallel experiments, using the generally applied RSC technique (fig.5). However, both methods have shown that the membrane saturation occurs already about the m.o.i. 10. Besides, a comparison of both methods has revealed that there exists a class of λ DNA molecules which



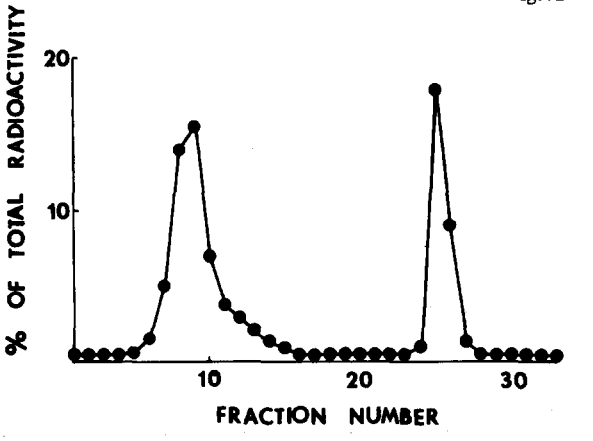


Fig.4. The effect of chloramphenicol. The experiment was performed as in section 2, except that in A chloramphenicol (200 μg/ml) was present during phage adsorption and incubation of infected cells. The distribution of ^{3}H (•) total radioact. 10 300 cpm, in the CsCl density gradient was estimated.

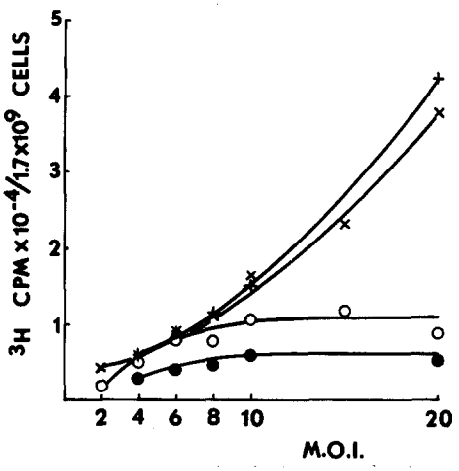


Fig.5. Influence of the multiplicity of infection. The cell lysate was analysed for the amount of the membrane-bound (•) and free (+) λ DNA, as in section 2. The same lysate was analysed by the RSC technique as follows: the lysate (0.2 ml) was applied to a 6 ml 20–30% sucrose gradient containing 1 M NaCl, 0.02 M Tris, 0.002 M EDTA, pH 8, over a cushion of 1.5 ml CsCl solution at a density of 1.7. The gradient was centrifuged at 23 000 rev./min for 1 h at 8°C in a SW 27.1 rotor, and in each fraction the radioactivity was estimated. The amount of λ DNA present in the rapid-sedimenting (○) and in the slow-sedimenting (×) peaks was calculated.

cosediment with membranes in the sucrose gradient (RSC technique), but did not form a λ DNA–membrane complex when analysed in our CsCl density gradient.

4. Discussion

We are describing a method for studying the attachment of infecting λ DNA to the host cell membrane, based on different buoyant densities of these structures. The amount of λ DNA found at the membrane position in the CsCl density gradient may represent a quantitative measure of the complex formation. The infecting phage cannot be mistaken for the complex, because their positions in gradient are wide apart; besides the λ DNA of the mature phage is liberated in the described procedure of cell lysis. This is important, because according to [8] 20–40% of the λ DNA of the purified phage preparation is present in the particles which adsorb on the cell, but do not inject their DNA. The comparison of methods (fig.5) reveals that the objections raised to the RSC technique were justifiable: there is a large amount of λ DNA cosedimenting with cell membranes in the sucrose gradient, which does not attach to the membranes, when examined in the CsCl density gradient. It is possible, that this unspecific binding is due to electrostatic and hydrogen-bond interactions which do not take place at the high ionic strength of the CsCl solution. It seems, therefore, that the material isolated by the CsCl technique is less contaminated and may represent a suitable object for the identification of the specific membrane components binding the parental λ DNA.

There have been several observations suggesting that most of the association is not due to injected phage DNA being trapped nonspecifically in membrane fragments during cell lysis. The formation of the complex:

(i) Is completed only after 4–6 min incubation in the culture medium (though λ DNA injection is a very rapid process at 37°C).
(ii) Is inhibited by chloramphenicol which does not interfere with λ DNA injection.
(iii) Attains plateau at low multiplicity of infection.

These results speak for the specificity of the λ DNA–membrane complex, but this problem requires further studies involving λ and *E. coli* mutants, the ones defective in the initiation of λ DNA replication [9] being the most interesting.

Our studies concerning the number of cell membrane attachment sites for parental λ DNA deserve a comment. In the experiment illustrated by fig.5 (CsCl density gradient technique), the saturation of cell membrane with λ DNA was attained at the ratio of 0.6×10^4 cpm/1.7×10^9 cells. The specific radioactivity of the phage preparation was 1.7×10^5 p.f.u./cpm. However, from 2.5–5-times higher value ought to be taken into account, because only 20–40% of the radioactivity represents phage particles injecting their DNA into the cells [8]. These calculations give a value of 1–3 ($5 \times 1.7 \times 10^5 \times 0.6 \times 10^4 / 1.7 \times 10^9$) cell membrane attachment sites for parental λ DNA. Our results are in contradiction with the data obtained in [7], where it was claimed that there is a minimum of 60–80 sites on the membrane for the attachment of parental λ DNA. The authors measured λ DNA retained on cellulose acetate filters, inferring that it is complexed with a host cell component, probably the cell membrane. Their method

seems to measure a less specific aggregation which does not occur with the sedimentation techniques [3,10].

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