Association of Replicating Bacteriophage T7 DNA with Bacterial Membranes

RODULFO P. PACUMBABA AND MELVIN S. CENTER

Division of Biology, Kansas State University, Manhattan, Kansas 66506

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Infection of *Escherichia coli* with bacteriophage T7 leads to the formation of an association between host membranes and newly synthesized T7 DNA. Evidence for this conclusion is suggested by the findings that replicating T7 DNA cosediments with host membranes in sucrose and cesium chloride density gradients. Furthermore, the sedimentation rate of T7 DNA is dependent on the integrity of membrane structure. Finally, an association between DNA and membranes can be demonstrated by electron microscope studies.

Previous studies have shown that, after infection of *Escherichia coli* with bacteriophage T7, both the parental and newly synthesized DNAs assume a fast sedimentation rate in neutral sucrose gradients (2). Several lines of evidence indicated that the sedimentation rate of the DNA was due, at least in part, to its association with protein material. Further studies on this fast-sedimenting structure indicated that it was metabolically active and that it served as a precursor to material having sedimentation properties of mature T7 DNA (2).

In the present report these findings have been extended and evidence has been obtained that replicating T7 DNA is associated with host membranes. In the accompanying paper (3), some structural features of the DNA attached to membranes are examined and evidence is also presented that the T7 DNA polymerase is an integral component of the DNA-membrane complex and that this system can support a limited in vitro synthesis of T7 DNA.

MATERIALS AND METHODS

Bacterial strains. *Escherichia coli* B was used as the host for T7 phage infection.

Bacteriophage. Bacteriophage T7 was obtained from W. F. Studier.

Enzymes. Pronase type VI was obtained from Sigma Chemical Corp. Before use, the enzyme (5 mg/ml) was incubated for 3 h at 37°C. Phospholipase A and pancreatic DNase were from Worthington Biochemical Corp. Crystallized trypsin was purchased from Nutritional Biochemicals. Pancreatic RNase (grade 3) was from Gallard Schlesinger. Before use, the enzyme was heated for 5 min at 100°C.

Other materials. ³H-thymidine (15.6 Ci/mmol) and ¹⁴C-thymidine (35 mCi/mmol) were purchased from Schwarz BioResearch, Inc. 2-³H-glycerol (9 Ci/

mmol) was from New England Nuclear Corp.

Preparation of phage and phage DNA. Preparation of T7 phage was essentially as described by Studier (16).

Extraction of lipids. In the present experiments 2-³H-glycerol was used as a radioactive label specific for *E. coli* membranes (4). When cells are labeled with 2-³H-glycerol, we find that in uninfected or T7-infected cells, 90 to 95% of the ³H-label can be extracted into chloroform by using the procedure of Kanfer and Kennedy (7).

Electron microscopy. Isolated membranes prepared as described below were examined in the electron microscope by using the basic, protein film technique described by Kleinschmidt (9). The DNA-membrane complexes either undiluted or diluted 1:3 in 0.01 M Tris-hydrochloride (pH 7.6)-0.001 M EDTA were mixed with cytochrome *c* (100 µg/ml) and ammonium acetate (0.5 M). Spreading of this solution was onto a hypophase containing 0.25 M ammonium acetate. After picking up the specimen on the microscope grid, the material was treated with acid, uranyl acetate (0.5 mM in 90% ethanol), and isopentane. The grids were thereafter rotary shadowed with 80% Pt-20% Pd and examined in a Philips 201 electron microscope. The lengths of DNA molecules were determined with a K and E map measure (620300).

Labeling host membranes and intracellular phage DNA. *E. coli* B was grown at 30°C in M9 medium (1) supplemented with 0.2% Casamino Acids. Thirty minutes after the initial inoculation of the culture, 2-³H-glycerol was added to a final concentration of 1.6 µCi/ml. When the cell density reached 3.0×10^8 cells/ml, the culture was centrifuged and resuspended in fresh M9 medium containing 0.2% Casamino Acids. The cells were thereafter shaken at 25°C, and when the cell density reached 3.5×10^8 cells/ml, T7 was added at a multiplicity of 5.0. Under the conditions of these experiments the latent period for T7 at 25°C is about 35 min. Labeling of newly synthesized T7 DNA was initiated at 22 min after infection by the addition of ¹⁴C-thymidine to a final

concentration of 0.16 $\mu\text{Ci/ml}$. After a 6-min labeling period, the cells were lysed according to a modification of the procedure described by Strätling and Knippers (15). Infected cells from a 30-ml culture were centrifuged for 10 min at 4 C and 7,000 rpm in the Sorvall SS34 rotor. The pellet was suspended in 10 ml of a 20% sucrose solution containing 0.1 M Tris-hydrochloride (pH 8.0)-0.005 M ethylene glycol-bis(β -aminoethyl ether)- N,N' -tetraacetic acid (EGTA). The suspension was centrifuged, and the pellet was suspended in 0.5 ml of 0.1 M Tris-hydrochloride (pH 8.0)-0.005 M EGTA. Lysozyme in 0.25 M Tris-hydrochloride (pH 8.0) was added to a final concentration of 100 $\mu\text{g/ml}$. The cells were kept on ice for 30 min and Brij 58 was added to 0.5%. After about 10 min, lysis of the cells was complete. Loss of labeled phage DNA during the lysis procedure is minimal since it has been found that 95% of the labeled DNA present in the infected cell culture is recovered in the lysate prepared at 28 min after infection. Two additional techniques have also been used to lyse phage-infected cells. The infected cells from a 15-ml culture containing labeled host membranes and T7 DNA prepared as described above were centrifuged, and the pellet was suspended in 1.0 ml of 0.75 M sucrose in 0.01 M Tris-hydrochloride (pH 7.6). Spheroplasts were prepared with lysozyme-EDTA as described by Osborn et al. (12). The spheroplasts were dialyzed for 6 h against 0.01 M Tris-hydrochloride (pH 7.6)-0.001 M EDTA, during which time there was essentially complete lysis as determined by microscope observations. In an additional lysis procedure the cell pellet was suspended in 2.0 ml of 0.01 M Tris-hydrochloride (pH 7.6)-0.015 M EDTA, and the cells were lysed by freezing and thawing three times.

Isolation of DNA-membrane complex. Portions of the cell lysates (0.6–1.0 ml) were centrifuged through 24.5 ml of a 5 to 20% sucrose gradient which was formed over 6 ml of a 70% sucrose solution. The sucrose was prepared in 0.01 M Tris-hydrochloride (pH 7.6)-0.001 M EDTA. Centrifugation was for 30 min at 20,000 rpm and 5 C in the Spinco type SW27 rotor. Fractions of 1.0 ml were collected after puncture of the bottom of the tube with a 20-gauge needle. The distribution of radioactivity in the gradient was determined after acid precipitation of samples from each fraction (2). When the intracellular DNA was analyzed soon after infection, before the breakdown of host DNA, the lysates were sheared by four passages through a no. 25-gauge needle before centrifugation was carried out. Reducing the viscosity of the lysate by shearing the host DNA was essential to obtaining reproducible results in these experiments.

RESULTS

Evidence that replicating T7 DNA is associated with host cell membranes. (i) Sedimentation analysis of host membranes and T7 DNA. *E. coli* B in which the cell membranes were labeled with 2- ^3H -glycerol was infected with T7, and newly synthesized T7 DNA was labeled with ^{14}C -thymidine for 6 min beginning at 22 min after infection. Cell lysates were

prepared (by the lysozyme-Brij method) and fractionated by sedimentation in a 5 to 20% sucrose gradient. The distribution of radioactivity in the gradient is shown in Fig. 1. About 67% of the ^{14}C -labeled DNA and 60% of the ^3H -labeled cell membranes recovered from the gradient exhibited an extremely fast sedimentation rate and were recovered on the 70% sucrose shelf. An essentially identical sedimentation pattern was also observed for newly synthesized T7 DNA pulse-labeled from 20 to 26 min and 23 to 25 min after infection. The particular lysis technique used in these experiments did not seem to influence the sedimentation pattern of the pulse-labeled DNA, since identical results as those shown in Fig. 1 were obtained when cells were lysed by freezing and thawing or by disruption of spheroplasts. Additional studies have demonstrated that when *E. coli* B is infected with T7 phage containing ^{32}P -labeled DNA and the cells are lysed by the lysozyme-Brij method at 10 and 15 min after infection, about 35 to 40% of the intracellular parental DNA cosediments with host membrane material.

Several experiments have been carried out to eliminate the possibility that the fast sedimentation rate of parental or newly synthesized T7 DNA is simply due to trapping in the cell lysate.

FIG. 1. Sedimentation analysis of host cell membranes and T7 DNA. Labeling host cell membranes and intracellular phage DNA was carried out as described in Materials and Methods. Infected cells were taken at 28 min after infection and lysed by the lysozyme-Brij technique. The cell lysate was centrifuged in a 5 to 20% sucrose gradient containing a 70% sucrose shelf in the Spinco SW27 rotor for 30 min at 20,000 rpm and 5 C. Fractions (1 ml) were collected, and the acid-insoluble radioactivity in a 0.1-ml sample was determined. The arrow indicates the position to which marker T7 DNA sediments. Under these centrifugation conditions, mature T7 phage sediments to a position corresponding to fractions 15–20 of the sucrose gradient. Symbols: X, ^3H -labeled host membranes; ●, ^{14}C -labeled T7 DNA.

In these experiments, ^3H -labeled T7 DNA and infected cells containing unlabeled membranes were lysed in the presence of ^{32}P -labeled T7 DNA, and portions of the lysates were centrifuged in the usual manner. The results of these studies have shown that when cells are lysed by the lysozyme-Brij method or by dialysis of spheroplasts, only 1% of the exogenous DNA sediments to the 70% sucrose shelf. Identical results are also obtained when isolated DNA-membrane complexes are mixed with reference T7 DNA and sedimented in sucrose gradients.

(ii) Density gradient analysis of DNA-membrane complex. The results of the experiment described in Fig. 1 suggest that the fast sedimentation rate of the newly synthesized T7 DNA may be due to its association with host membrane material. Additional evidence in support of this was obtained by examining the sedimentation properties of isolated DNA-membrane complexes. The fast-sedimenting DNA (see Fig. 1) was isolated, dialyzed, and recentrifuged under conditions such that the membrane material reached equilibrium in sucrose density gradients. Membrane and DNA cosedimented after centrifugation in a 5 to 20% sucrose gradient containing a 70% sucrose shelf. Centrifugation of the isolated material in a 40 to 70% sucrose gradient showed that the major portion of the membrane and DNA sedimented to the position in the gradient corresponding to 60% sucrose (Fig. 2b). In this particular experiment, about 38% of the DNA recovered from the gradient exhibited a sedimentation rate similar to that of the T7 marker. The presence of this slower-sedimenting material under the conditions described in Fig. 2 is somewhat variable and is found to be about 20 to 40% in different experiments. Studies have also been carried out to determine whether the density of the membranes in sucrose gradients is appreciably influenced by an association with T7 DNA. This does not seem to be the case, since after treatment of the complex with pancreatic DNase the membranes have essentially the same sedimentation profile (Fig. 2b).

Additional evidence that replicating T7 DNA is associated with membranes is obtained by examining the sedimentation properties of labeled membranes and DNA in cell lysates prepared by disruption of spheroplasts (Fig. 3). Fig. 3a shows the sedimentation profile for the membrane complex in a stepwise cesium chloride gradient (density 1.3–1.7 g/ml). About 40% of the DNA was located near the top of the gradient at a density considerably less than that of the T7 marker. In these experiments, it was

FIG. 2. Sucrose density gradient analysis of isolated DNA-membrane complex. Centrifugation of cell lysates containing labeled host membranes and T7 DNA was as described in Fig. 1. Fractions 3–5 from the sucrose gradient (Fig. 1) were pooled and dialyzed for 5 h against 0.01 M Tris-hydrochloride (pH 7.6)–0.001 M EDTA. A portion of the dialyzed material (0.15 ml) was centrifuged in a 3.3-ml, 5 to 20% sucrose gradient overlaying a 1.0-ml, 70% sucrose shelf (panel a). A second portion of the dialyzed sample (0.15 ml) was centrifuged in a stepwise 40 to 70% sucrose gradient (panel b). The gradient was prepared by overlaying 1-ml 70, 60, 50 and 1.3-ml 40% sucrose solutions. The sucrose was prepared in 0.01 M Tris-hydrochloride (pH 7.6)–0.001 M EDTA. Centrifugation was for 3 h at 30,000 rpm and 5°C in the Spinco SW50.1 rotor. The membrane material appears to have assumed equilibrium under these conditions since centrifugation for longer time periods does not change the sedimentation profile. Three-drop fractions were collected from the bottom of the tube and the acid-insoluble radioactivity was determined. The position to which marker T7 DNA sediments in a parallel gradient is indicated by the arrow. Symbols: X, ^3H -labeled host membranes; ●, ^{14}C -labeled T7 DNA.

necessary to centrifuge for brief time periods in order to detect a cosedimentation of DNA and membranes in cesium chloride, since after prolonged incubation in high-ionic-strength solutions the DNA dissociates from the complex. These results are in contrast to those described by Miller (10), in which the parental T4 DNA-membrane complex was stable in cesium chloride gradients. It is interesting to note that a portion of the DNA sedimented at a rate faster than reference T7 (Fig. 3a) and may represent a concatemeric structure (8). This would agree with our electron microscope studies which

Fig. 3. Sedimentation of DNA-membrane complex from spheroplast lysates in density gradients. Labeling host cell membranes and intracellular T7 DNA and lysis of spheroplasts was carried out as described in Materials and Methods. A portion of the cell lysate (0.1 ml) was centrifuged in the Spinco SW50.1 rotor for 90 min at 40,000 rpm and 5°C in a stepwise cesium chloride gradient (panel a). Step gradients were prepared by layering 1.5 ml each of cesium chloride solutions having densities of 1.3, 1.5, and 1.7 g/ml. In a separate experiment a portion of the lysate (0.1 ml) was centrifuged in a stepwise 35 to 60% sucrose gradient in the Spinco SW50.1 rotor for 5 h at 30,000 rpm and 5°C (panel b). The gradient was prepared by overlaying 1 ml each of 60, 50, 40 and 1.3 ml 35% sucrose solutions. Acid-insoluble radioactivity was determined in three-drop fractions collected from the bottom of the tube. The position to which marker T7 DNA sediments in parallel gradients is indicated by the arrow. Symbols: X, ^3H -labeled host membranes; ●, ^{14}C -labeled T7 DNA.

indicate that a portion of the DNA found associated with the complex has a length two to three times that of mature T7 DNA. In addition, experiments described in the accompanying paper (3) indicate that a major fraction of the pulse-labeled T7 DNA which is attached to membranes has sedimentation properties characteristic of a concatemer. The DNA-membrane complex from a spheroplast lysate has also been analyzed after centrifugation to equilibrium in a 35 to 60% sucrose gradient (Fig. 3b). The membrane material separated into three discrete bands. The two major components sedimented to a position corresponding to 50 and 60% sucrose, whereas the third component, which represents about 10% of the total radioactivity recovered from the gradient, banded in the 35% sucrose. The major portion of the newly synthesized T7 DNA cosedimented

with the most dense membrane, although some of the DNA was also located in the 50% sucrose region. These results are closely similar to those obtained when isolated DNA-membrane complexes from cells lysed by lysozyme-Brij were centrifuged in sucrose density gradients.

(iii) **Disruption of the DNA-membrane complex.** In these experiments, DNA-membrane complexes isolated from T7-infected cells were incubated with various enzymes and detergents, and the effect of these treatments on the sedimentation rate of the complex was examined (Table 1). The results described in

TABLE 1. Disruption of DNA-membrane complex^a

Treatment	Percent of control exhibiting fast sedimentation rate	
	Membranes	DNA
Control	100	100
Pronase	49	12
Trypsin	62	34
DNase	100	20
RNase	100	100
Phospholipase A	56	57
Sarkosyl	1	1
Triton X-100	1	1

^a DNA-membrane complexes from T7-infected cells were prepared as described in Materials and Methods except that the complexes isolated from the initial centrifugation were dialyzed (5 h against 0.01 M Tris-hydrochloride [pH 7.6]-0.001 M EDTA) and recentrifuged under identical conditions. This material was isolated, dialyzed, and used in the incubations. In the absence of a second centrifugation, the DNA disassociated from the membrane after incubation in the presence of certain metal ions. After the second centrifugation the complex was considerably more stable. Incubation conditions and final concentration of reaction constituents: DNA-membrane complex, 0.15 ml; Pronase (125 μg), 0.02 M EDTA; trypsin (20 μg), 0.005 M CaCl_2 ; pancreatic DNase and RNase (20 μg), 0.005 M MgCl_2 , phospholipase A (10 μg), 0.005 M CaCl_2 , Sarkosyl and Triton X-100 (2%) in the presence of 0.005 M EDTA. Incubations were for 30 min at 32°C except with phospholipase A, which was for 2 h at 32°C. As a control for each experiment, the complex was incubated under identical conditions in the absence of enzyme or detergent. At the end of the incubations, EDTA was added to 0.005 M to those reactions containing CaCl_2 or MgCl_2 . The solutions were thereafter centrifuged in a 3.3-ml, 5 to 20% sucrose gradient overlaying a 1.0-ml, 70% sucrose solution. Centrifugation was in the Spinco SW50.1 rotor for 20 min at 20,000 rpm at 5°C. Three-drop fractions were collected directly into scintillation vials containing 2.4-cm glass fiber filter paper (Reeve Angel). The radioactivity located in the DNA-membrane fraction was set at 100%. Recovery of radioactivity for the control and treated sample was complete.

Table 1 suggest that the fast sedimentation rate of newly synthesized T7 DNA was due to its association with protein and lipid. Incubation of the complex with proteolytic and lipolytic enzymes or with certain detergents converted both membrane and DNA to slower sedimenting material. Treatment with pancreatic DNase but not RNase also released the DNA from the complex.

(iv) **Electron microscope examination of the DNA-membrane complex.** *E. coli* B was infected with T7, and at 28 min after infection the cells were lysed by the lysozyme-Brij method and the DNA-membrane complexes were isolated after centrifugation in a neutral sucrose gradient. The complexes were dialyzed for 5 h against 0.01 M Tris-hydrochloride (pH 7.6)-0.001 M EDTA and examined in the electron microscope by using the Kleinschmidt procedure (9). A systematic scan of the grid squares revealed that 30 to 60% of the membrane vesicles contained attached DNA. The general appearance of the DNA associated with the membrane is shown in Fig. 4A-C. Two types of DNA membrane association were found to be characteristic of essentially all complexes seen in the electron microscope. One type (Fig. 4A, B) consisted of a membrane vesicle attached near the end of the DNA molecule, whereas in the second type (Fig. 4C) the end of the DNA molecule was not visible at the attachment point to the membrane. This was due to either

the vesicle being located at an internal region in one continuous strand or to two different molecules being attached at their ends to the vesicle. All DNA molecules observed to be attached to membranes were linear structures and, in general, had a length equal to or two to three times as long as mature T7 DNA. The total lengths of those shown in Fig. 4 are about twice as long as mature T7 DNA. The DNA molecule in Fig. 4B contains a short segment of DNA ("stem") which extends out from the main strand. This structure was present, however, in only 10% of the DNA molecules examined. In the micrographs shown in Fig. 4A and B, "puddle"-like structures can be observed, and in some cases these are associated with the DNA molecules. These "puddles" are observed in most membrane preparations but are not seen when purified T7 DNA is examined in the electron microscope. The nature of the "stems" and "puddles" is not known at the present time.

(v) **Pulse-chase experiments.** In these experiments, *E. coli* B was infected with T7 and the newly synthesized DNA was pulse-labeled with ^3H -thymidine for 2 min beginning at 21 min after infection. Unlabeled thymidine was added to the culture, and at various times after infection samples were taken and the cells were lysed by the lysozyme-Brij technique. Portions of the cell lysate were centrifuged in a 5 to 20% neutral sucrose gradient containing a cesium chloride shelf. The sedimentation analysis was

FIG. 4. Electron micrographs of DNA associated with membranes. Electron microscopy of isolated DNA-membrane complexes was carried out as described in Materials and Methods. Complexes were isolated at 28 min after T7 infection. Final magnification is 34,250.

performed under conditions such that membrane-associated DNA, mature phage, and DNA sedimenting with reference T7 could be resolved in the sucrose gradients. A major portion of the newly synthesized T7 DNA analyzed in cell lysates prepared immediately after the 2-min labeling period exhibited a fast sedimentation rate and was recovered on the cesium chloride shelf (Fig. 5a). This DNA exhibited sedimentation properties characteristic of the DNA-membrane complex. The membrane-associated DNA appeared to be metabol-

ically active, since after a 6- and 12-min chase in the presence of unlabeled thymidine, about 55% of the DNA was released from the complex and then sedimented to a position corresponding to T7 phage and mature T7 DNA (Fig. 5b, c). In other experiments in which the T7 DNA was pulse-labeled for 2 min beginning at 18 min after infection and a chase was carried out for 15 min, about 70% of the fast-sedimenting DNA was released from the complex and sedimented with phage-specific components (Fig. 5c).

DISCUSSION

The results described in the present report suggest that the replication of T7 DNA occurs in association with the bacterial membrane. Several lines of evidence support this conclusion. First, a major portion of the newly synthesized T7 DNA cosediments with host cell membranes under a variety of centrifugation conditions. Second, treatment of the fast-sedimenting DNA with enzymes and detergents which disrupt membrane structure converts the DNA to a slower-sedimenting component. Finally a direct association between DNA and membranes has been observed by electron microscope studies. About 90% of the DNA molecules observed to be attached to membrane vesicles were either equal in length or two to three times as long as mature T7 DNA. Since the membrane complexes examined in the electron microscope were isolated from cells at a time when T7 DNA synthesis is maximal, and since T7 infection results in the degradation of host DNA to fragments considerably smaller than T7 (13), it would be indicated that most of the DNA observed to be attached to membrane vesicles is T7 and not host fragments. A recent electron microscope study by Griffith and Kornberg (5) using bacteriophage M13 has demonstrated an attachment between the replicative M13 DNA and the cell envelope. Pulse-chase experiments (Fig. 5) indicate that the replicating DNA associated with host membrane material is metabolically active and is utilized in the formation of mature phage particles. These results along with other observations obtained from reconstruction experiments strongly suggest that the fast sedimentation rate of newly synthesized T7 DNA is not due to trapping in the cell lysate.

Several workers have shown that in gram-negative bacteria the cell membranes can be separated into an outer and inner (cytoplasmic) membrane fraction by centrifugation of lysed spheroplasts in sucrose density gradients (11, 12, 14). The outer and inner membrane fractions which represent approximately equal

FIG. 5. Pulse-chase analysis of membrane associated T7 DNA. *E. coli* B was grown at 25°C in M9 medium (1) supplemented with 0.2% Casamino Acids. When the cell density reached 3×10^8 cells/ml, T7 was added at a multiplicity of 5.0. Newly synthesized T7 DNA was labeled from 21 to 23 min after infection by the addition of ^3H -thymidine to a final concentration of $0.30 \mu\text{Ci/ml}$. At the end of the labeling period, a 5-ml sample was taken and processed as described below. To the remaining culture unlabeled thymidine was added to a final concentration of 2.0 mg/ml. At 29 and 35 min after infection, 5.0-ml samples were taken and mixed with 5.0 ml of a 20% sucrose solution prepared in 0.1 M Tris-hydrochloride (pH 8.0)-0.005 M EGTA. The cells were centrifuged and lysed by the lysozyme-Brij method described in Materials and Methods. In this lysis procedure, washing of the cells was eliminated in order to prevent any possible breakage of cells and subsequent loss of phage DNA. Portions of the cell lysates were mixed with ^{32}P -T7 phage and centrifuged in a 3.3-ml, 5 to 20% sucrose gradient overlaying a 1.0-ml cesium chloride shelf (2). Centrifugation was in the Spinco SW50.1 rotor for 30 min at 20,000 rpm and 5°C. The position to which marker T7 phage sediments is indicated by the closed arrow. In separate experiments, cell lysates were mixed with ^{32}P -T7 DNA and centrifuged as described above. The position to which this DNA sediments is indicated by the open arrow.

amounts of 2-³H-glycerol-labeled material sediment to positions corresponding to 50% ($\rho = 1.22$) and 35% ($\rho = 1.16$) sucrose, respectively (12). In T7-infected cells the DNA membrane complexes from lysed spheroplasts and cells lysed in the presence of Brij sediment in sucrose density gradients to a position corresponding to 60% sucrose. At the present time it is not known whether this represents an attachment of T7 DNA to the outer membrane or whether the membrane components of the host exhibit altered densities after T7 infection. In a recent study, Miller (10) has shown that after infection with bacteriophage T4 the parental DNA associates with 2-³H-glycerol-labeled host membranes. This fraction has a density of 1.37 g/ml in cesium chloride gradients (10). In contrast, in studies carried out with bacteriophage M13 it has been shown by sucrose density gradient analysis that the infecting phage DNA is associated with the inner membrane of the host (6).

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