MEMBRANE ATTACHMENT OF REPLICATING PARENTAL DNA MOLECULES OF BACTERIOPHAGE M13

Walter L. Staudenbauer and Peter Hans Hofschneider

Max-Planck-Institut für Biochemie,
Munich 15, Goethestr. 31, Germany

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Summary: Early in the infection with bacteriophage M13 the infecting parental DNA strand becomes attached to the host cell membrane. Using a gentle lysis procedure followed by sucrose gradient centrifugation, up to 80% of the parental DNA cosediments with the bacterial membranes. The membrane fraction was deproteinized by phenol extraction and the solubilized DNA was further analysed by band sedimentation in neutral and alkaline CsCl gradients. Between 5' and 15' after infection at least half of the membrane bound parental DNA was found to be incorporated into replicative intermediates with viral strands of more than unit length.

INTRODUCTION

During infection with ØX 174 the semiconservative replication of the parental replicative form molecule (RF) occurs at a special site on the bacterial membrane (1). Thereby the parental DNA strand is irreversibly bound to the membrane and is not transferred into progeny phages. In the case of the filamentous phage M13, however, almost 50% of the parental DNA is transferred to progeny phages (2). This high transfer could result either from a reversible binding of the parental DNA to the host membrane or from a different mechanism of DNA replication not requiring a membrane site. It will be shown that early in infection with M13 the parental DNA can be isolated as a membrane-bound replicative intermediate.

MATERIALS AND METHODS

E. coli K12 KMBL 42 F' was provided by Dr. A. Roersch, M13 phage was our own stock. Preparation of ^{32}P-labeled phages and ^{3}H-labeled single stranded phage DNA (ss DNA), determination of the effective multiplicity of infection (m.o.i.), and assay of radioactivity have been described previously (2).

Cells were grown in M9 medium (3) to 2 x 10^{8} cells/ml and infected with ^{32}P-labeled phages (5 x 10^{-5} cpm/pfu) at a m.o.i. of 5. After 5 min., samples were removed to determine the effective m.o.i.. The cells were separated from unadsorbed phages by low-speed centrifugation and resuspended in an equal volume of prewarmed medium. 10 ml aliquots were removed at the times indicated and cooled in an ice bath after addition of 0.1 ml 2 M KCN. The cells were sedimented by low-speed centrifugation, suspended in TEN-buffer (0.05 M Tris-HCl, pH 7.5, 0.005 M EDTA, 0.1 M NaCl) and treated twice for one minute in a Waring Blendor to shear off F-pili and adsorbed phage particles (4). After two additional washings the cells were resuspended in TEN-buffer, containing 0.01 M EDTA and 15% (w/w) sucrose, at a density of 2 x 10^{9} cells/ml. Lysozyme was added to 0.5 mg/ml and the samples were incubated for 2-3 hours at 0^{o}C. Then cells were lysed by one cycle of freeze-thawing using an acetone-dry ice bath. Lysis was checked under the light microscope and was more than 95%.

Fractionation of the lysate was carried out by sucrose gradient centrifugation. 3 ml of lysate were layered on a Spinco SW 27 gradient consisting of an 8 ml cushion of 60% sucrose under a 20-40% sucrose gradient (26 ml) in TEN-buffer.

For further analysis fractions from the sucrose gradient were pooled, dialysed for 3 hours against TEN-buffer and con-

centrated by dialysis against aquacide (Calbiochem). The concentrated fractions were deproteinized by phenol extraction (2). 0.2 ml of the deproteinized DNA were layered on linear neutral CsCl gradients (4.3 ml) in 0.01 M Tris-HCl, pH 7.2, 0.001 M EDTA formed from CsCl solutions of the density 1.2 g/ml (2.8 g CsCl/10 ml buffer) and density 1.4 g/ml (6.1 g CsCl/10 ml buffer). For alkaline CsCl gradients 0.2 M NaOH was added to the buffer and the samples were denatured for 15 min. at room temperature in 0.2 M NaOH.

RESULTS AND DISCUSSION

Isolation of membrane bound parental DNA. In a typical sedimentation profile of a cell lysate early after infection (Fig. 1), the cell membranes form a turbid, viscous band on top of the 60% sucrose cushion (fraction 4), whereas the soluble components

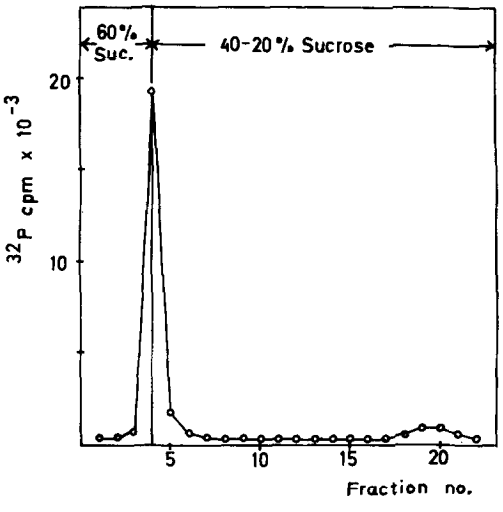

Fig. 1. Distribution of parental DNA in a sucrose gradient of gently lysed cells, isolated 5' after infection. The gradient was spun in a SW 27 rotor at 25,000 rpm for 1 hour at 4°C. 2 ml fractions were collected from the bottom of the tube and assayed for TCA precipitable radioactivity. The recovery of label was 88%. o——o, ^{32}P-labeled parental DNA (cpm/fraction).

of the cytoplasma stay near the top of the gradient. Non-lysed cells sediment through the cushion (5). 70-80% of the parental DNA was found to co-sediment with the bacterial membrane. Similar results were obtained after lysing the lysozyme-treated cells with Brij 58, or after gentle osmotic lysis by removing the osmotic stabilizer through dialysis (1). Lysis by freeze-thawing was preferred, since it avoids the addition of detergent. A further advantage of this method is that the lysate can be stored frozen before further manipulation.

To exclude that the membrane-bound DNA was not due to incomplete removal of adsorbed phages, infection was carried out in the presence of 20 mM KCN. Under these conditions phages can adsorb to the cells, but penetration is inhibited (6). Only less than 3% of the radioactivity of the unpoisoned control was found in the membrane fraction.

To test whether the distribution shown in Fig. 1 is really due to in vivo-events and not to unspecific aggregations during lysis, ^{3}H-labeled ss DNA was added to the cell suspension before lysis. Only 2% of the ^{3}H-labeled ss DNA co-sedimented with the cells membranes during sucrose gradient centrifugation. Similar results using radioactive labeled ØX 174 RF were reported previously (1).

From the relative amount of infected cells, and from the amount of intracellular parental label, an effective m.o.i. of 0.3 was calculated independently. Under these conditions only a small portion of the cells is infected by more than one phage. The membrane-bound parental label must therefore be due to the only parental molecule present, which carries the infection. Whether the small amount of the parental DNA found in the cytoplasma fraction reflects a possible bypass of the membrane

when the amount of 70S ribosomes increased to 48 μg.

Kinetics of polyphenylalanine synthesis in the absence and in the presence of 30S ribosomal subunits were presented in Fig. 4. It is clear from this figure that both the rate and the yield of polyphenylalanine synthesis were stimulated by the addition of 30S ribosomal subunits.

Effect of 50S ribosomal subunits on polyphenylalanine synthesis

Experimental data presented in Fig. 5 show that the addition of 50S ribosomal subunits to 70S ribosomes did not stimulate polyphenylalanine synthesis. The amount of polyphenylalanine synthesized in the presence of 80 μg of 50S ribosomal subunits was almost the same as that synthesized without adding 50S ribosomal subunits.

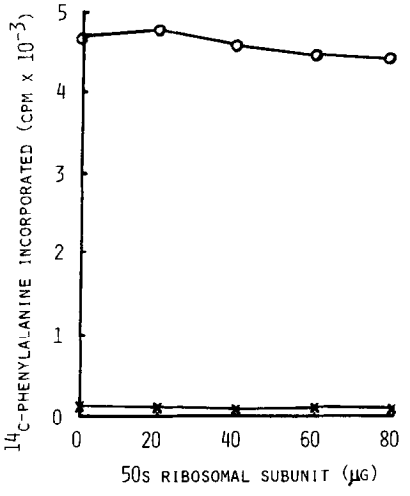

Fig. 5. Effect of 50S ribosomal subunits on polyphenylalanini synthesis. The experimental conditions are the same as described in the legend of Fig. 3 except the amounts of ribosomes and ribosomal subunits. 24 μg of pure 70S ribosomes and several amounts of 50S ribosomal subunits as indicated are added in 0.1 ml of the reaction mixture. x — x, without 50S; o — o, with 50S.

DISCUSSION

Conventional ribosome preparations consist of mainly 70S ribosomes. These preparations have been used for cell free polypeptide synthesis directed by both synthetic and natural mRNAs. Recent studies on the role of 30S ribosomal subunits in polypeptide synthesis demonstrated that the ini-

Gradient analysis of membrane-bound DNA. To obtain definite evidence that the membrane-bound DNA is actually involved in replication, the membrane fraction was isolated 5' and 15' after infection, deproteinized by phenol extraction, and the DNA subjected to neutral and alkaline CsCl gradient centrifugation (Fig. 3A-D). Most of the parental label from the 5' sample sediments in the neutral CsCl gradient in the region of RF I and RF II with a small shoulder in the position of ss DNA (Fig. 3A). At 15', however, most of the radioactive label sediments like ss DNA, and even faster (Fig. 3B). This material might represent replicative intermediates having the parental label incorporated into viral strands of several times unit length (2) as can be seen from the alkaline CsCl gradients: In the alkaline gradient from the 5' sample about half of the parentally-labeled DNA strands sediment faster than ss DNA (Fig. 3C). At 15' this shoulder has developed into a broad peak (Fig. 3D). For the maximum of this peak a sedimentation constant of 27 S can be calculated, corresponding to viral strands of 4 times unit length (7). Degradation and reincorporation into bacterial DNA can be excluded, since it has been shown previously that the parental DNA strand is transferred intact with high efficiency into progeny phages (2). It should be noted that in both alkaline gradients only a small amount of label sediments in the position of denatured RF I. This indicates that the material resembling RF I in the neutral gradients is actually not RF I but a replicating molecule possessing a parentally-labeled viral strand of twice unit length (8).

From these data it can be concluded that early in M13 infection the parental DNA becomes temporarily attached to the cell membrane and is part of a replicating intermediate. Evi-

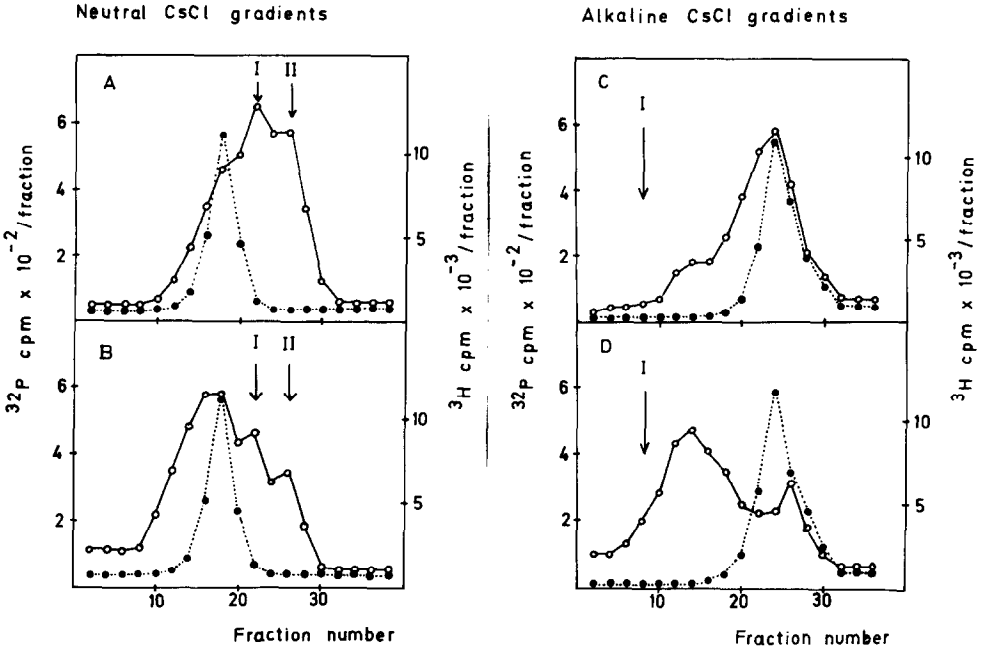

Fig. 3. CsCl gradient analysis of ^{32}P-labeled membrane-bound parental DNA. Samples were isolated 5' (A,C) and 15' (B,D) after infection, deproteinized and mixed with ^{3}H-labeled M13 ss DNA. Centrifugation was performed in the Spinco Ti 56 rotor at 45,000 rpm for 1.5 hours at 4°C. The tubes were punctured, 38 fractions collected on filter paper disks and assayed for radioactivity. Sedimentation is from right to left. Arrows indicate the positions of RF I and RF II, as calculated from the position of the ss DNA marker. o——o, ^{32}P-labeled parental DNA (cpm/fraction); ●····●, ^{3}H-labeled ss DNA (cpm/fraction).

dently, as shown by the elongation of the parentally-labeled DNA, viral strand material is synthesized in a membrane-bound, "rolling circle"-like structure. Whether complementary strands are also synthesized in this structure, remains to be studied.

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