EVIDENCE FOR STABLE ATTACHMENT OF DNA TO MEMBRANE AT THE REPLICATION ORIGIN OF *ESCHERICHIA COLI*

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The formation of pulse labelled DNA which remains firmly attached to membrane after further growth in unlabelled medium has been studied at different periods of the cell cycle using synchronized cultures of *Escherichia coli*. The maximal labelling of this species of membrane bound DNA occurs near the time of cell division. This provides evidence for association of DNA at the replication origin with the cell membrane.

In their replicon model for bacterial DNA replication, Jacob, Brenner and Cuzin proposed a membrane attachment site for DNA which permits orderly segregation of the daughter chromosomes (1). Subsequently, DNA at the replicating point was shown to be attached to a rapidly sedimenting structure, possibly membrane, in *Escherichia coli* by Smith and Hanawalt (2) and in *Bacillus subtilis* by Ganesan and Lederberg (3). This DNA is intimately attached only at the time of replication since the labelled DNA formed during a pulse incubation with ^{3}H-thymidine no longer sediments with the particulate fraction after additional growth in medium containing unlabelled thymidine. A different point of attachment of DNA to a particulate fraction was demonstrated in *B. subtilis* by Sueoka and Quinn who reported that DNA is attached to membrane at the replication origin and possibly at the terminus (4). In the present report, we present evidence for stable DNA attachment to membrane at the replication origin of *E. coli*.

METHODS. *E. coli* strain CR34-C416 thy^{-}, obtained from M. Goulian, was grown in a standard medium (S medium) consisting of medium A (5) supplemented with 4 mg/ml of succinic acid and 2 µg/ml of thymidine. Synchronized cultures were obtained by a modification of the Mitchison-Vincent procedure (6). Ex-

ponential phase cells at a density of 4 x 10^8 per ml were harvested at room temperature, suspended at a density of 4 x 10^{10} cells/ml, and agitated by vigorous vortex mixing in the presence of Superbrite glass beads type 150-5005 (Minnesota Mining and Manufacturing Co.) to dissociate aggregates of bacteria. A 0.5 ml portion of cells was layered on a 20 ml linear 15% to 5% Ficoll (Pharmacia Fine Chemical) gradient. Centrifugation was for 5.5 minutes at 2500 rpm in the Sorvall HB-4 rotor. In experiments where only the newly divided cells were used, the cells corresponding to 5% of the colony forming units applied to the gradient were pipetted from the top. Cell counts were obtained by plating appropriate dilutions on Difco nutrient agar.

Lysozyme and DNase were obtained from Worthington, thymidine from PL Biochemicals and ^{3}H-thymidine from Schwartz.

RESULTS. Our experiments are based on the rationale that initiation of chromosome replication at a membrane bound site in *E. coli* occurs shortly after cell division, and that the initiation origin remains attached to membrane throughout the cell cycle. When an exponential phase culture of *E. coli* is sedimented through a linear Ficoll gradient as described in Methods, the newly divided daughter cells remain near the top of the gradient. The more rapidly sedimenting fractions contain cells at intermediate stages of the cell cycle. Figure 1 shows the results of an experiment where cell fractions collected from a linear Ficoll gradient were first incubated for a pulse with ^{3}H-thymidine and then further incubated (chased) with a large excess of unlabelled thymidine prior to isolation of the particulate material containing membrane. The slowly sedimenting cell fraction which contains the newly formed daughter cells (fraction 1), has proportionately more labelled DNA in particulate material than do the next few more rapidly sedimenting fractions of the gradient.

In an extension of the experiment described above, a synchronized population of newly divided cells was grown in S medium, and aliquots were removed at intervals for "pulse-chase" incubations and for the determination of cell counts. One of six representative experiments is given in figure 2 and shows

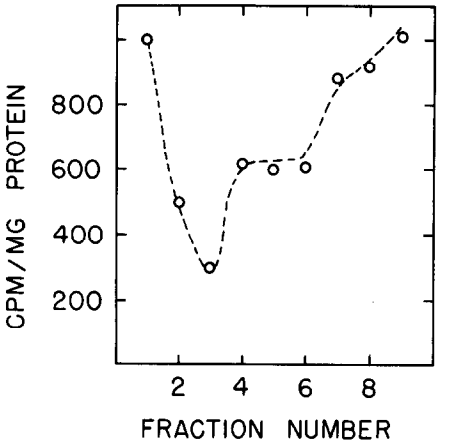

Figure 1. Relative amount of ^{3}H-thymidine incorporation into membrane associated DNA in cells fractionated on a Ficoll gradient. Cells were grown and sedimented through a linear Ficoll gradient as described in Methods. The contents of the tube were removed from top (fraction 1) to bottom (fraction 9). the cells of each fraction were collected on type HA Millipore filters, suspended in 1 ml of S medium supplemented with 50 µc/ml of ^{3}H-thymidine, and incubated at 37° for 2 minutes. At this point, unlabelled thymidine was added at a concentration of 500 µg/ml, and the incubation was continued for an additional 10 minutes. Unincorporated label was removed by collecting the cells on membrane filters, and the cells were washed on the filters with TE buffer (0.01 M Tris·HCl of pH 7.6 - 0.01 M sodium ethylenediaminetetraacetate of pH 7.6) and suspended in 5 ml of TE buffer. Following a sonication treatment calibrated empirically to reduce the membrane bound DNA to the lowest size limit, an aliquot was removed for protein determination (7), carrier membrane equivalent to 10 mg of protein from a 30 minute - 100,000 x g particulate fraction was added, and the samples were centrifuged for 30 minutes at 100,000 x g. The pellets were washed once by suspension in 10 ml of TE buffer followed by centrifugation, and the washed pellets were suspended in 1.5 ml of 5% aqueous Triton X-100 (Rohm and Haas) and added to 20 ml of Patterson-Green solution (8) containing 3:1 toluene:Triton X-100 for scintillation counting.

that the maximal labelling of DNA associated with particulate material occurs when the "pulse-chase" is performed near the time of cell division. This is the result expected if DNA at the origin (or terminus) of replication occupies a membrane bound site. In other experiments similar to the one described in figure 2, the duration of the chase incubation with unlabelled thymidine has been extended for as long as 3 generation times without a significant alteration of the results.

When particulate material from a fraction corresponding to the peak fraction (30 minutes) in figure 2 was subjected to isopycnic banding in a sucrose density gradient (9), the labelled DNA had the same bouyant density as did the membrane

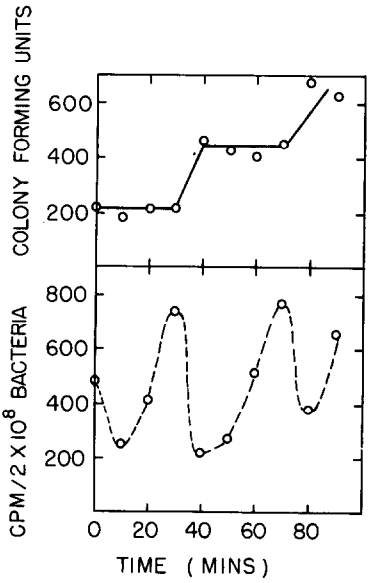

Figure 2. Periodic formation of membrane bound DNA during the cell cycle. A synchronized population of newly divided cells was prepared as described in Methods, and the cells were incubated with shaking at 37°. At 10 minute intervals, a sample was withdrawn, and pulse labelled for 10 minutes with ^{3}H-thymidine at 50 µc/ml. Unlabelled thymidine was then added at a concentration of 500 µg/ml, and the incubation continued for an additional 10 minutes. At the end of the ^{3}H-thymidine pulse incubation, a sample was removed for the determination of cell counts (colony forming units). ^{3}H-DNA bound to the particulate fraction was determined after the incubation with unlabelled thymidine as described for figure 1.

fraction which contains the cytochromes, transport proteins and succinate dehydrogenase, i.e., the cytoplasmic membrane. This labelled DNA is apparently released from purified membranes by detergent since after treatment with 0.1% Triton X-100 it remains near the top of a linear 20% to 5% sucrose gradient during a 5 hour sedimentation at 100,000 x g. While bound to membrane, it is insensitive to the action of pancreatic DNase alone, but is rapidly degraded by the concerted action of DNase and 0.1% Triton X-100.

DISCUSSION. The membrane associated DNA formed by *E. coli* near the time of cell division has properties similar to the DNA at the origin and terminus of replication[1] in *B. subtilis* (4), but unlike those of DNA at the replicating

[1]In the present report we do not distinguish between DNA at the origin and terminus of replication since it is probable that these segments of DNA bind to a single membrane structure.

point (2,3). A more recent study by Fuchs and Hanawalt (10) shows that newly replicated DNA is bound to a structure which no longer sediments with membranes after gentle sonication. In contrast, the putative DNA at the origin of replication described here remains firmly bound to membrane even after vigorous sonication. Taken together, these data suggest separate structures for replication on one hand, and for the determination of segregation of daughter chromosomes on the other.

Since the procedure used here reduces DNA to the limit of sonic degradation (approximately 200,000 daltons), it is possible to estimate the number of labelled DNA binding sites per cell. We make assumptions as follows: 1) 50% of the radioactive material in the peak fraction (30 minute) of figure 2 is DNA at the replication origin, 2) all cells of the 30 minute fraction are labelled at the origin, and 3) no loss of DNA at the replication origin occurs during isolation. Where one strand of each of the two duplexes of 200,000 daltons molecular weight at the origin is labelled, a figure of 0.8 labelled origins per cell is obtained. Other experiments yielded values ranging from 0.7 to 0.9. These figures, which can only be considered an approximation, are in reasonable agreement with the number of labelled replication origins expected for newly-formed daughter cells growing in succinate medium (11,12).

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