BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **221,** 186–192 (1996)
ARTICLE NO. 0567

# Characterization of Bacterial Cell Membrane Attachment Sites of Plasmid R6K

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Received February 15, 1996

*In vitro* binding studies revealed that plasmid R6K could attach to both inner and outer membrane fractions of its host cell, *Escherichia coli.* Derivatives of R6K carrying one or two of its three origins of replication could not bind stably to the same membrane fractions in the presence of salt. However, the derivative, pRK35, carrying the intact three origins of replication could bind stably to membrane fractions from its host in the presence or absence of salt. These observations suggest that the three origins of DNA replication must be contiguous for stable binding of the plasmid to the cell membrane. The results of binding experiments showed that plasmid R6K bound competitively with pRK35 as well as the heterologous plasmid, pl524. 

The mechanism by which DNA replicates in procaryotes has been well studied (18). However virtually little is known about the maintenance of these molecules in these organisms. Jacob *et al.* (14), proposed that bacterial DNA must attach to the cytoplasmic membrane for stable maintenance. However, it is not known whether the attachment serves to regulate initiation and replication of DNA. The attachment of bacterial chromosome to procaryotic membrane has been demonstrated by electron microscopy of ultra thin sections (13, 25) and by a variety of density gradient centrifugation techniques (3, 17, 27, 27).

Attempts have been made to determine if the DNA is attached to the cell membrane at one or many different sites. Reports by many investigators have shown that the outer membrane of *E. coli* is enriched with fragments containing the origin of replication of the chromosomal DNA (1, 18, 19, 22, 24). Studies with *Bacillus subtilis* have shown that fragments containing the origin of DNA replication could be found associating with the host cell membrane fractions (17, 20, 26). These studies of DNA-membrane complexes, have shown that specific proteins and RNA are involved in complexing the DNA to the membrane (1, 2, 6, 11, 20, 21, 22, 29). In the present study, we performed *in vitro* binding experiments to identify the sites of complexing, and to determine the possible role(s) of the membrane in the maintenance of this plasmid.

## MATERIALS AND METHODS

*Bacterial strains and plasmids.* The bacterial strains used in this study were plasmid negative *E. coli* strains, HB101, RS64, and an isogenic strain, RS50, harboring plasmid R6K. Other plasmids used were R6K derivatives, pRD17, pMF26, pMF34 and pRK35 (kindly supplied by Dr. Helinski, University of San Diego).

Nutrient-rich medium, L-broth, was used for growing the cells and it was supplemented with appropriate antibiotic, ampicillin or kanamycin to select against any plasmid-negative cells. The synthetic medium, minimum salt (5), was supplemented with 2 $\mu$g/ml thiamine hydrochloride, 0.5% casamino acid, and 0.5% glucose. In some experiments, [$^3$H]-thymidine (10 $\mu$Ci/ml) and [$^{14}$C]-glycerol (1 $\mu$Ci/ml) radioisotopes were used to label the plasmid DNA and the membrane components, respectively. To aid the uptake of [$^3$H]-thymidine, 20 $\mu$g/ml of 2′-deoxyadenosine was added to the medium.

*Purification of inner* (*IM*) *and outer* (*OM*) *membrane fractions.* Inner and outer membrane fractions were isolated according to the procedure of Osborn *et al.* 1972 (23). The inner and outer membrane fractions of the host cells, *E. coli*, were separated and purified using isopycnic sucrose density gradient centrifugation. In addition, crude membrane lysate was prepared by exposure of cell to lysozyme and sheared by passage through an 18-gauge needle. Unlysed cells were removed by low speed centrifugation.

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0006-291X/96 $18.00

*In vitro binding experiments.* Plasmid DNAs used in this study were isolated according to Triton X-100 lysis protocol (4). Using crude lysate, inner and outer membrane fractions, in vitro binding experiments (17, 28) were performed. The purified [$^3$H]-thymidine labeled covalently closed circular (CCC) plasmid R6K and its cloned derivatives were used to perform DNA binding experiments according to the procedure of Korn *et al.* 1983 (17). An aliquot of these lysates (200 $\mu$l) was incubated with [$^3$H]-dThd labeled plasmid R6K, and the sample was layered on 5–20% sucrose gradients. The gradients were centrifuged, fractionated, and radioactivity was determined.

## RESULTS

*Purification of inner and outer membrane fractions.* Purification of membrane fractions from *Salmonella typhimurium* has been demonstrated on isopycnic sucrose density gradient (23). Figure 1A represents the purified inner and outer membrane fractions from *E. coli* cells hosting plasmid R6K. Peak H represents the outer membrane while peak $L_1$ and $L_2$ represent the inner membrane.

*Analysis of plasmid R6K on 5–20% neutral sucrose density gradient.* We conducted experiments on 5–20% neutral sucrose gradients to determine the location of plasmid R6K free of membrane association (17). Figure 1B illustrates the profile of purified covalently closed circular molecules of plasmid R6K DNA on this gradient. As shown in the figure, essentially all of the [$^3$H]-dThd labeled plasmid DNA (98.9%) sedimented near the top of the gradient. The results demonstrate that

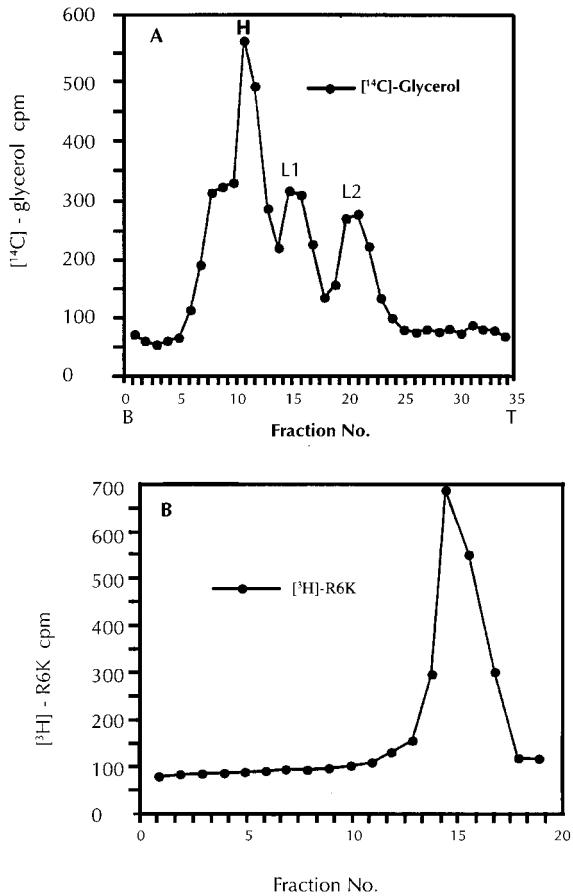


**FIG. 1.** Panel A. Profile of cell membrane fractions on an isopycnic sucrose density gradient. The profile was obtained when [$^{14}$C]-glycerol-labeled cells were sonicated and placed on an isopycnic density gradient. "H" refers to the position of the outer membrane fraction while $L_1$ and $L_2$ refer to the positions of inner membrane components. Panel B is the profile of [$^3$H]-labeled R6K on 5–20% sucrose density gradient. This is a profile of membrane-free CCC forms of [$^3$H]-labeled R6K plasmid on a 5–20% sucrose density gradient.

plasmid DNA molecules free of membrane association do not form a peak near the bottom of the gradient, as expected with DNA-membrane complexes (17, 21).

*In vitro binding profile of crude, IM and OM cell membranes.* Crude membrane lysate was prepared from cells hosting R6K DNA. As shown in Fig. 2A, incubation of R6K molecules with the crude lysate did not demonstrate a high degree of binding of the plasmid DNA (less than 1%). Because of the inability of the crude lysate to demonstrate significant binding, we performed the same *in vitro* binding experiments using purified OM and IM fractions. Figures 2B and C, illustrate the results of these experiments. As can be observed, binding was increased significantly when labelled R6K DNA was incubated in the presence of purified OM (39%) and IM (24%) fractions.

*Mapping of the attachment site*(*s*). Digestion of plasmid R6K with *Eco*R1 yields two fragments, a 22 Kb fragment which contains the two antibiotic resistant genes (Streptomycin and Ampicillin); and a 16 Kb fragment carrying the regulatory genes and the three origins ($\alpha$, $\beta$ & $\gamma$) of replication (15). The fragment containing the three origins of replication was cloned, and designated pRK35 (9). The three origins were also cloned separately as shown in Table 1 (9, 16). These cloned derivatives were analyzed for their binding potentials to both IM and OM fractions from their respective host cells. As shown in Table 1 our data illustrate that these plasmids could bind only

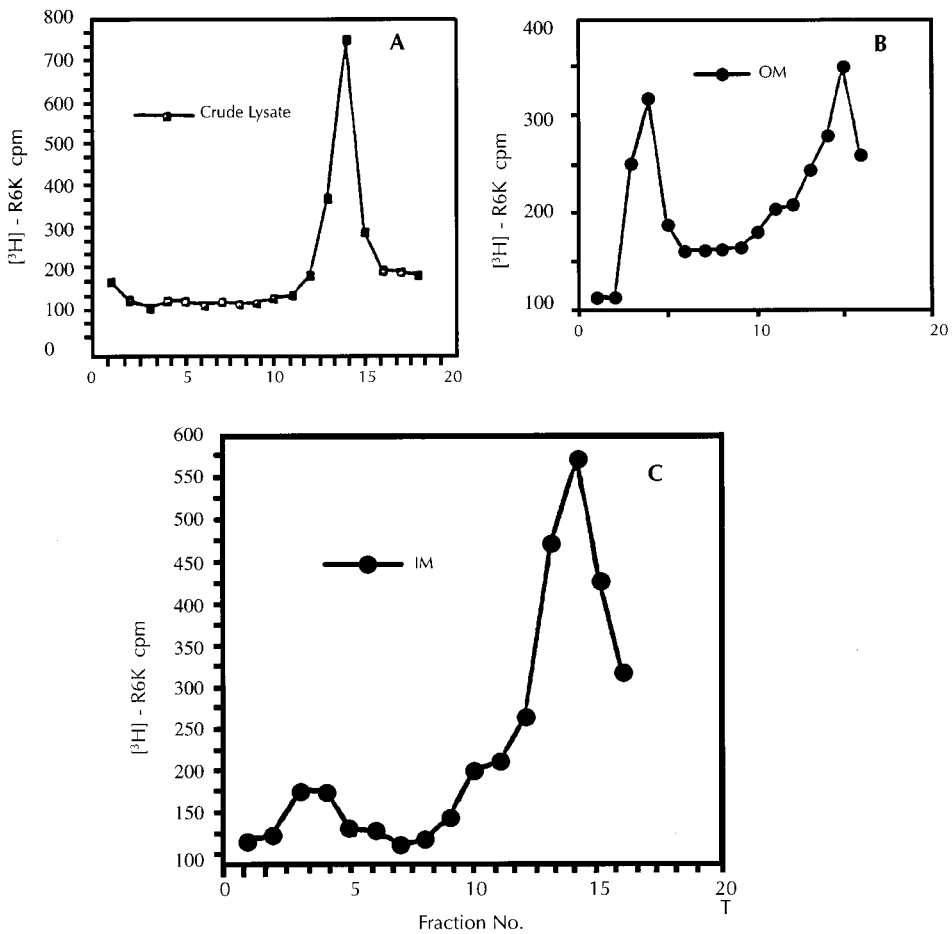


**FIG. 2.** Complexing of plasmid R6K DNA to crude lysate, OM, and IM. These represent the results obtained when the plasmid DNA–membrane complexes were analyzed on 5–20% sucrose density gradients. Panels A, B, and C represent crude lysate, OM and IM preparations, respectively.

TABLE 1
Binding of R6K and Its Derivative Plasmids to Their Host Membrane Fractions

| Plasmid | Genotype | Molecular Weight (Mdal) | Binding to membrane fractions | | | |
|---|---|---|---|---|---|---|
| | | | +KCl | | −KCl | |
| | | | OM | IM | OM | IM |
| pRD17 | α & γ | 4.2 | − | − | + | + |
| pMF26 | β & γ | 5.6 | − | − | + | + |
| pMF34 | γ | 6.3 | − | − | + | + |
| pRK35 | α, β & γ | 13.6 | + | + | + | + |
| R6K | α, β & γ | 25.0 | + | + | + | + |

This is a summary of the binding potential of plasmid R6K and its cloned derivatives in the presence and absence of 100 mM KCl. + = significant binding. − = no significant binding.

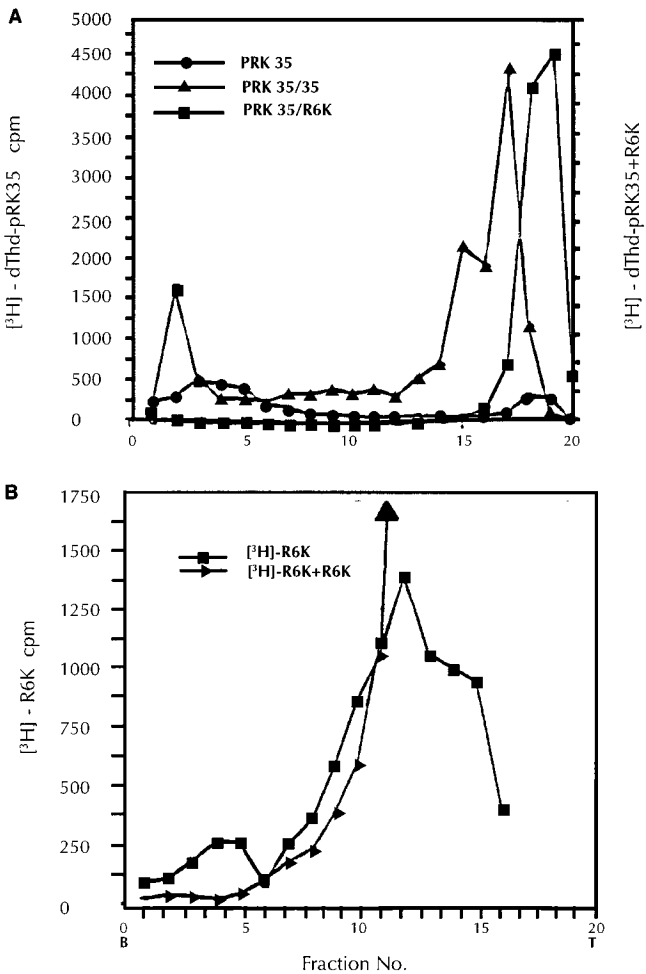


**FIG. 3.** Analysis of in vitro competitive binding of plasmids R6K and pRK35. Panel A illustrates the competitive binding of unlabeled plasmid R6K and labeled pRK35 DNA. Panel B demonstrates competitive binding for attachment sites of unlabeled R6K DNA with labeled R6K.

when KCl (100 mM) was omitted from the reaction mixture. However, when potassium ions were included in the reaction mixture, there was little or no binding potential of plasmid missing any one of the three origins of replication (Table 1).

*Competitive binding.* Binding of plasmid R6K at non-specific sites may be competed out by plasmid DNA from a different source. On the other hand, binding at specific sites may be competed out by plasmid R6K itself, or by a plasmid with similar binding sites.

Here, labeled R6K or pRK35 DNA was incubated in the presence of a 25-fold excess of unlabeled R6K and pRK35. The results obtained from these studies indicated that when [$^3$H]-labeled pRK35 was incubated in the presence of a 25-fold excess of unlabeled pRK35 or R6K, there was a significant reduction in the amount of DNA-membrane complexes formed (Fig. 3). This was illustrated by the reduction in the amount of labeled pRK35 recovered at the bottom of the gradient (Fig. 3A). Similar results were obtained when a 25-fold excess of unlabeled R6K was

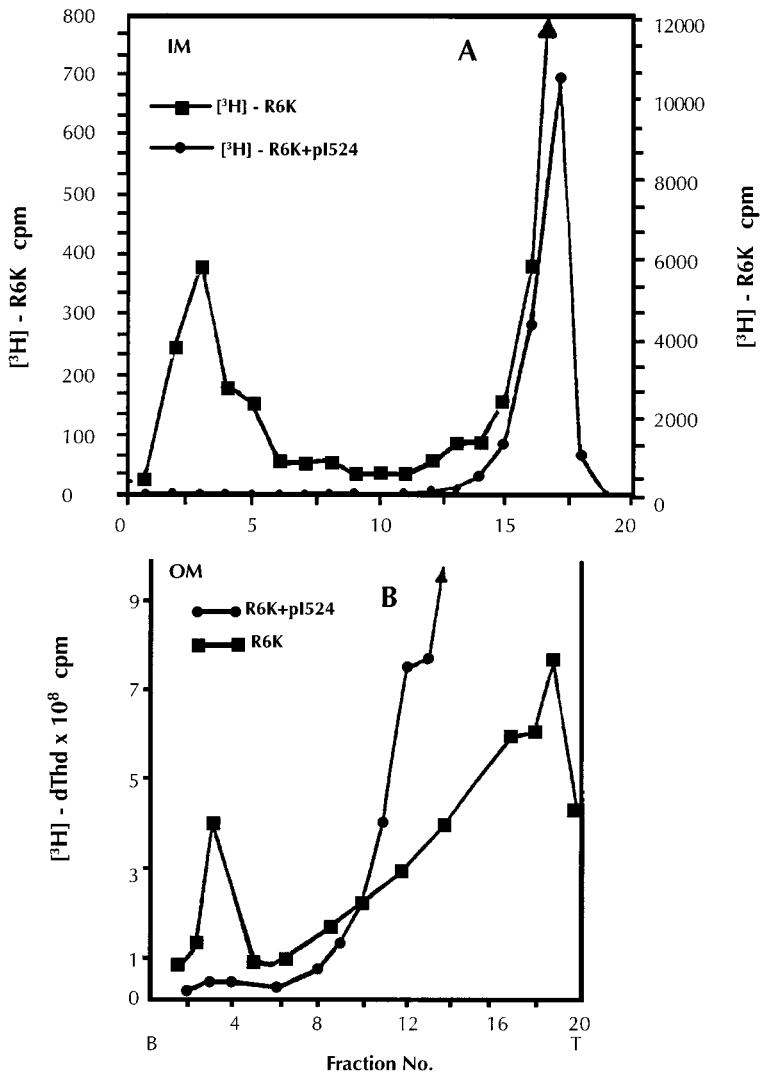


**FIG. 4.** Profiles of competitive binding between plasmids R6K and pl524. Panel A illustrates the competitive binding of unlabeled plasmid pl524 DNA with [$^3$H]-labeled R6K for inner membrane attachment sites. Panel B shows the competition between plasmid pl524 DNA and plasmid R6K DNA for outer membrane attachment sites.

added to the incubation mixture (Fig. 3B). These results are consistent with previous data, (3) and indicated that pRK35 and R6K were competing for the same binding sites on the membrane fraction. Unexpectedly, however, a 25 fold excess of unlabeled plasmid pl524 also showed competition with the labeled plasmid R6K for attachment sites (Fig. 4). Panel A and B illustrate competitive binding between plasmids R6K and pl524 for attachment sites on IM and OM respectively. These results indicate that plasmids R6K and pl524 share some similarities in the binding sites or binding properties.

## DISCUSSION

Our studies of plasmid R6K in relationship with its host (*E. coli*) cell membrane revealed that under the *in vitro* binding conditions, the plasmid DNA could bind to both purified outer and inner membrane preparations. On the other hand, only a very small degree of binding was observed with the crude membrane preparation. This could have resulted from nucleases in the crude extract. Alternatively, the isolation and purification of the two components separately (inner and outer membranes) exposed DNA-binding proteins which resulted in increase binding of R6K DNA.

The plasmid R6K derivatives, lacking one or more origins of replication exhibited Type-II binding (26), i.e., nonspecific in terms of their function. This type of binding would represent an unstable complexing, and could be easily destroyed by salt. On the other hand, the derivative containing all three origins of R6K replication as well as the parental plasmid R6K exhibited stable binding in the presence of 100 mM KCl. This type of binding is referred to as Type-I binding (26), and it is speculated to be genetically determined by the plasmid for a specific function. In addition, these results indicate that under certain experimental conditions, the presence of all the three origins presents a conformation that is favorable for plasmid R6K DNA binding. Our experimental data on competitive binding gave interesting results. Both plasmids R6K and pRK35 competed with each other, as well as individually, for the binding sites on cell membrane fractions.

The R6K DNA-membrane complexing may be mediated via integral membrane proteins. An analysis of R6K replication has shown that the pi protein, the product of the *pir* gene, is necessary for the replication of this plasmid (7, 8, 9, 10, 12). Thus, it is feasible that this protein could also play an essential role in complexing of R6K DNA to its host membrane fractions. These results are in support of the concept that complexing of DNA to membrane may be to promote equal partitioning and segregation of DNA to progeny cells in addition to its role in DNA replication.

Finally, we have established that plasmid R6K DNA could bind to both IM and OM based on our *in vitro* studies. However, the role of membrane in the maintenance of plasmid R6K needs further investigation to ascertain if attachment is necessary for DNA replication or segregation or for both. In addition, the involvement of the pi protein, if any, in plasmid R6K DNA-membrane complexing should be investigated.

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