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13. ABSTRACT *(Maximum 200 words)*

This proposal has focused on a broad host range plasmid, RK2, as a model system to study how a pair of initiation proteins encoded by the plasmid for DNA replication function when replication occurs associated with the membrane of its bacterial host Escherichia coli. It was found that the initiation proteins were very resistant to agents that dissociate weakly bound (peripheral) membrane associated proteins. In this respect, they resemble strongly bound (integral) membrane proteins. Yet, there is only one short region of hydrophobic amino acids that might be involved in such membrane association. To determine the topology of these proteins in the membrane, additional studies involving site directed mutagenesis of the small domain, and the construction of protein fragments demonstrated not only that the small region was essential for membrane binding but also for viability of the plasmid. In addition, two other membrane binding domains were detected, one near the beginning (amino terminal) of the protein, the other, adjacent to the short hydrophobic domain. Finally, anionic phospholipids (part of the membrane) may be involved in maintaining plasmid viability by anchoring the initiation proteins to the membrane.

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Topology of a Membrane Associated Regulator of Prokaryotic DNA Replication

August 1, 1994 - July 31, 1998

I) Summary Statement

Previous research supported by DOD grants have concerned the significance of membrane associated DNA complexes in DNA replication of prokaryotes. Several different experimental approaches were developed to study this complex in two model systems, the broad host range plasmid RK2 cultured in its Escherichia coli host, and Bacillus subtilis. This report deals with the plasmid model system because it has been possible to provide an in depth analysis of how and why the plasmid is able to replicate as a membrane associated replicon (genetic unit of replication).

One of the most important criteria for membrane association may be that some of the proteins encoded by plasmid, specifically a pair of initiation proteins which control the beginning of plasmid replication (called the TrfA proteins) bind both to the plasmid origin of replication (a small region of plasmid DNA where this process occurs) and to the membrane of their E. coli host. These plasmid encoded initiation proteins, therefore, seem to have two incompatible domains: one interacting with a hydrophobic (membrane) environment, and one interacting with a hydrophilic (cytoplasmic) environment.

Such conclusions were borne out by the following observations. At first, plasmid specific DNA replication was found to be associated with the inner membrane fraction of its Escherichia coli host (Michaels et al, 1994) although the initiation proteins were detected in both the inner and outer membrane fractions of E. coli (Michaels et al, 1994). Further dissection of the membrane fractions into six subdomains by the methods of Ishidate et al, (1986) revealed that a subdomain derived from the inner membrane fraction representing less than 10% of the total membrane was responsible for binding the plasmid origin of replication (oriV) in vitro and contained significant amounts of the plasmid encoded initiation proteins (Mei et al, 1995). There was a strong correlation between the association of the TrfA initiation proteins with this submembrane domain and the binding of oriV in vitro or plasmid DNA in vivo.

Of importance was the fact that this correlation was obtained only when the membrane subfractions were extracted from E. coli cells containing the transformed plasmid. In contrast, when the subfractions were extracted from E. coli cells that had not been transformed, only non-specific binding of the origin region was noted. It was observations such as these which suggested strongly that the initiation proteins could be responsible, in part, for the association of plasmid DNA to the membrane.

In analyzing the structure and sequence of amino acids that comprised the TrfA initiation proteins, it was observed they contained only one relatively short domain consisting of hydrophobic amino acids that could be responsible for membrane binding (Kostyal et al, 1989). This domain (consisting of 13 amino acids, termed the HR region) is common to both initiation proteins and is located towards the carboxyl end of the proteins. However, they do not appear to be integral membrane proteins since they do not meet an essential property of such proteins, namely, a region sufficiently long to traverse the membrane (Singer et al, 1987). Nevertheless, some membrane proteins also do not satisfy the membrane spanning rule and yet are able to span the membrane (More and Miura, 1987). Alternatively, it was possible that the TrfA proteins, like several others (Ulbrandt et al, 1992) could bind to anionic phospholipids in the membrane.

A two fold strategy of site directed mutagenesis and construction of protein fragments was used to analyze this HR region and other regions. First, a series of mutations was created within the region. These mutants were assessed for their ability to complement an oriV plasmid and for membrane association (complementation is defined as the ability of the initiation protein [mutated as well as wild type] to act in trans and support the ability of an RK2 miniplasmid containing just oriV to survive in the same cell). It was found that there was a strong correlation between the ability of the HR region mutants to bind stably to the membrane of E. coli and the ability of the mutant to complement an oriV plasmid. Instability of membrane binding resulted in an inhibition of complementation.

In a second approach, a series of TrfA fragments were constructed by PCR (polymerase chain reaction) in a special plasmid vector which permits inducible expression of the fragments and detection by a specific antibody. A TrfA peptide fragment representing 80 amino acids including the 13 residue HR region was present primarily in the membrane fractions, but not the soluble fraction, whereas two other 80 residue TrfA peptides were found predominately in soluble fractions. However, when different fragment associations were constructed, two other membrane binding domains were revealed, one near the amino terminal portion of the TrfA protein, and one adjacent to the HR region. These latter two binding domains had been separated in the initial

constructions, but when rejoined to their respective domains, they exhibited membrane binding capabilities.

In a final study, it has been observed that anionic phospholipids are important in maintaining viability of the RK2 plasmid. Using E. coli mutants conditionally defective in synthesizing such anionic phospholipids (Xia and Daihan, 1995); it was found that the mutant could not be transformed under restrictive conditions, (i.e., without the inducer being added), whereas a control plasmid could be transformed regardless of the presence of sufficient phospholipids. In additional experiments, it was observed that when the RK2 plasmid was transformed (under permissive conditions) and then incubated under restrictive conditions, it was lost, suggesting that maintenance required anionic phospholipids. Correlating these results, decreasing concentrations of the inducer resulted in less anionic phospholipids being synthesized and a decreased ability of the TrfA initiation proteins to bind to the membrane.

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Xia, V., and Dowhan, W. (1995). Proc. Natl. Acad. Sci. USA. 92: 783-787.

II) List of Publications

1. Michaels, K., Mei, J., and Firshein, W. (1994). TrfA-dependent inner membrane-associated plasmid RK2 DNA synthesis in Escherichia coli maxicells. Plasmid 32: 19-31.

2. Mei, J., Benashki, S., and Firshein, W. (1995). Interactions of the origin of replication (oriV) and initiation proteins (TrfA) of plasmid RK2 with submembrane domains of Escherichia coli. J. Bacteriol. 177: 6766-6772.

3. Firshein, W., and Kim P. (1997). Plasmid replication and partition in Escherichia coli: Is the cell membrane the key? Mol. Microbiol. 23: 1-10.

*4. Moriya, S., Firshein, W., Yoshikawa, H., and Ogasawara, N. (1994). Replication of a Bacillus subtilis oriC plasmid in vitro. Mol. Microbiol. 12: 469-478.

III. List of Participating Scientific Personnel

1. Peter Kim, Graduate Student
2. Katerina Michaels, Graduate Student (MS, 1994)
3. Sharon Benashki (MS, 1995)
4. Julia Mei, Graduate Student (Ph. D., 1995).

*5. Shigeki Moriya was a visiting assistant professor from the University of Osaka in 1993. A paper (no. 4, above) was published in 1994 on a topic supported by a previous DOD grant.