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6. AUTHOR(S)
William Firshein

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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Wesleyan University
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Membrane associated DNA replication in prokaryotes has been studied intensively using two model systems, *Bacillus subtilis* and plasmid RK2 cultured in its *Escherichia coli* host. In the former a new membrane protein that had previously been found to act as an inhibitor of DNA replication was identified (by gene cloning procedures) to be an enzyme of the pyruvate dehydrogenase complex, the E2 subunit dihydrolipoamide acetyl transferase. The identification of this inhibitor is representative of an increasingly recognized phenomenon, namely the existence of multifunctional proteins. The temporal expression of the protein during the growth cycle has also been elucidated. Its action appears to be controlled by proteases which degrade the protein prior to a round of DNA replication.

In the latter, plasmid DNA replication has been found to be associated with the inner but not outer membrane of its *E. coli* host, while plasmid encoded initiation proteins have been found to be associated with both membrane fractions. Although sequence analysis of the genes encoding the proteins predict them to be peripheral (loosely associated) membrane proteins, they can not be dissociated by treatments designed to remove such peripheral proteins, suggesting that part of this association is integral in nature (strongly associated).

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Structure-Function Aspects of Membrane Associated Prokaryotic DNA Replication

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I. Summary Statement

This problem, in its broad view, has concerned the role of membrane associated DNA complexes in DNA replication of Prokaryotes. Its importance lies in the increasingly recognized fact that membrane associated regulation of specific metabolic process is a moderator of normal cell function. Both in prokaryotes and eukaryotes, a plethora of evidence has emphasized this view. These include the activation of protein kinases as metabolic signaling devices in the latter, and a variety of gene products in the former which must associate with the membrane in order to activate a specific response such as the recA protein (in the SOS repair phenomenon) and the CPX protein (which affects the syntheses or stability of a number of envelope proteins). However, the apparent requirement of specific initiation proteins for DNA replication to function in a membrane environment such as the dnaA protein in E coli and the dnaB protein of B. subtilis has focused attention on DNA replication and its link to this fundamental cell structure (the cell membrane).

Our own investigations over the past three and a half years supported by the USA Army Research Office has been built on a foundation of results achieved over a ten year period prior to the grant. Such previous studies have yielded considerable information concerning the macromolecular composition of the complexes, their synthetic capabilities in vitro and the kinetics involved, the physical state of the endogenous DNA template, and the discovery that certain putative or known regulatory proteins for DNA replication other than those described above are either derived from the membrane or function in a membrane environment.

Investigating these latter proteins has been the main but not sole focus of the grant. In the first case we have tried to clone and characterize a gene from Bacillus subtilis that codes for a 64 kda membrane protein that acts as a repressor or negative regulator for DNA replication (Laffan and Firshein, Proc. Natl. Acad. Sci. USA 85: 7452-7456, 1988). In addition, we attempted to determine its temporal expression during the growth cycle of the bacterium. Finally, as a byproduct research project carried out by a visiting assistant professor from Osaka University, Japan, Dr. Shigeki Moriya, we tried to develop an in vitro membrane associated DNA replication system using an OriC plasmid developed by Dr. Moriya before he came to my laboratory.

The most important discovery we have made, a startling one in our opinion, has been the identification (through gene cloning procedures) of the protein that acts as the negative regulator for B. subtilis DNA replication. It is the E2 subunit of the pyruvate dehydrogenase complex (PDH) (dihydrolipamide acetyl transferase, which catalyzes a transfer of acetyl groups via CoenzymeA to dihydrolipoate and dihydrolipamide in the complex).

Earlier, preliminary results seemed to negate this possibility, but more accurate sequence analysis of the cloned gene gave a clear answer as to its identity. Why this protein should apparently also be involved in possibly regulating DNA replication is unknown as yet. However, it is interesting that within the operon of the subunits of the complex, the E2 subunit has its own promoter, associates with membrane bound ribosomes and is absolutely required by the cells, whereas the other subunits (pyruvate decarboxylase and dihydrolipoyl dehydrogenase) can be substituted by other related enzymes.

It is becoming increasingly evident that multifunctional proteins exist for reactions in addition to those for which they have been originally identified. Recently, Seror et al (EMBO J. 13: 2472-2480, 1994) found that a cysteinyl-tRNA synthetase affects the timing of replication initiation in B. subtilis by possibly affecting the synthesis of specific cysteine rich proteins normally involved in such initiation. Additionally Dr. Moriya has informed me that a "new" replication protein (dnaR) in B. subtilis discovered by a colleague of his binds to a known initiation protein dnaA to aid in its activation. This new protein has been identified as PRPP (5' phosphoribosylpyrophosphate) synthetase. Whether these multifunctionalities are unique to spore formers like B. subtilis is not known but it is an intriguing observation in which we are highly interested.

With respect to the E2 subunit, attempts are underway to confirm its mode of action in vivo by genetic experiments (supported by an augmentation grant from the U. S. Army Research Office) (31174-LS-AAS). These include site directed mutagenesis procedures in which various single site mutations and short deletions of the gene will be used to ascertain whether specific domains are involved in its action as a repressor. However, it will be important to distinguish between the activity of the protein in respiration and in replication, not an easy task. Nevertheless, one possible way this may be accomplished is to take advantage of a second discovery made during the period of the grant, namely the elucidation of the temporal expression of the repressor protein during the growth cycle of B. subtilis. The most important observation was that the protein was degraded by proteases just after germination of spores followed by a round of DNA replication. If protease inhibitors were present, however, the inhibitor protein was not degraded and DNA replication did not occur. However, if protease inhibitors were removed long after untreated cells had begun to enter the log phase, the repressor protein was degraded followed by a round of DNA replication. Thus, there was a strong correlation between the necessity to degrade the repressor protein (E2 subunit) and the onset of DNA replication. Such observations may, therefore, be concerned primarily with the activity of the E2 subunit in DNA replication rather than respiration, since it is difficult to conceive of the E2-subunit being degraded by proteases during respiration. Thus, it would be ideal to test mutant proteins for their ability to act in the appropriate manner during growth of the organism to determine whether they are degraded after germination, etc. Finally, it is important to recognize that the E2-subunit may interact with another as yet unidentified protein which is the true "repressor" since the E2 subunit has a multidomain structure which can bind to the other proteins of the PDH complex (or the unknown repressor protein).

The last discovery made with B. subtilis during the grant period concerns the development of an in vitro replicating system using an OriC

plasmid constructed by Dr. Shigeki Moriya. At first an attempt was made to develop one that was membrane associated. However, considerable repair activity was detected in membrane fractions extracted from cells that had been transferred with the OriC plasmid. As a result, a soluble replicating system was developed instead which was absolutely dependent upon the presence of the OriC plasmid and had little repair activity. This new replication system (albeit in a soluble form) represents the first such system outside E. coli. We are still hopeful of developing a useful membrane replicating system using the OriC plasmid since there could be an enrichment of two origins in such a system, one from the chromosome and one from the OriC plasmid. This could help us study membrane associated DNA replication more efficiently, if repair activity can be reduced and if both origins are firing (initiating DNA replication) at the same time.

With respect to the second case we have investigated the membrane association of a known pair of plasmid initiation proteins which are encoded by overlapping genes from the broad host range plasmid RK2. The basic problem has been to determine how it is possible for the initiation proteins to bind to the origin of plasmid replication (OriV) and at the same time associate with the membrane.

That such an association occurs and that plasmid replication is associated with a specific membrane domain of its E. coli host have been the major discoveries during the grant period. Thus, plasmid replication occurs in the inner membrane fraction of its E. coli host but not the outer membrane as shown by a variety of experiments. These include the inhibition of such replication by antibody directed against the initiation (trfA) proteins in the inner but not outer membrane fraction, the lack of any antibody effects in plasmid-free cells, the inhibition of plasmid replication by an antibiotic (rifampicin) that affects several reactions involving initiation in the inner but not outer membrane fraction, and the hybridization of newly synthesized DNA with known plasmid restriction fragments, when such newly synthesized DNA is extracted from the inner but not outer membrane fractions.

The second major discovery has been the fact that although the known sequence of the proteins predict that they are peripheral membrane proteins (loosely associated), treatments designed to remove such proteins failed to do so completely indicating that part of their association is integral in nature (strongly associated). Moreover, when the genes encoding the proteins were cloned into another plasmid (PBR322, a well known high copy number plasmid), that is, removed from their 'normal' genome site, they still acted in part as integral proteins suggesting that other plasmid encoded proteins, or the origin (OriV) of the plasmid played no role in their membrane association. Thus, some property of the proteins themselves render them with an affinity for the membrane. Two such properties include a short region of 13 amino acids (common to both proteins) which are highly apolar about a third of the distance from the C-terminus of the protein, and three cysteine moieties which are very close to the C-terminus. The former could provide capabilities for membrane phospholipid association, while the latter, by disulfide linkages, could provide association with other (host) membrane proteins.

Finally, the third discovery relating to the localization of the plasmid encoded initiation proteins in the membrane is one which, at first,

contradicted earlier results. Such earlier results showed that the trfA initiation proteins were present in the inner but not outer membrane fractions. These results were highly supportive of the fact that plasmid replication was also detected in the inner but not outer membrane fraction. However, current results by more sensitive detection procedures and another method for membrane isolation that preserves the supercoiled endogenous plasmid DNA template revealed that the trfA initiation proteins were present in both inner and outer membrane fractions (with little in the cytosol). We are not disturbed by such observations because they could indicate 1) that other plasmid encoded or host proteins required for DNA replication are only present in the inner but not outer membrane fraction, and/or 2) that the actual domain for replication is one that contains either elements of both the inner and outer membrane fraction, or a smaller fraction trapped in the larger membrane fractions. A number of observations of other investigators notably Rothfield and colleagues) (Ishidate et al, J. Biol. Chem. 261: 428-443, 1986) have shown that the concept of two membrane fractions in Gram negative bacteria is simplistic and that additional subdomains exist. One of these subdomains could represent the actual site of plasmid DNA replication and trfA protein concentration.

In conclusion with this plasmid system, if a molecular model can be developed that shows the topology of the initiation proteins in the membrane of their E. coli host, it could represent the first physical example of the validity of the replicon model proposed by Jacob et al (Cold Spring Harbor Symposium Quant. Biol., 28: 329-348, 1963) more than thirty years ago.

II. List of Publications

- 1) Wilkinson, B.E., Mele, L., Laffan, J. and Firshein, W. 1992. Temporal expression of a membrane-associated protein putatively involved in repression of initiation of DNA replication in Bacillus subtilis. J. Bacteriol., 174: 477-485.
- 2) Moriya, S., Firshein, W., Yoshikawa, H., and Ogasawara, N. 1994. Replication of a Bacillus subtilis oriC plasmid in vitro Mol. Microbiol., 12: 469-478.
- 3) 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.

III. List of Abstracts

- 1) Moriya, S., and Firshein, W. 1992. Studies of in vitro synthesis of a Bacillus subtilis oriC plasmid in soluble and membrane associated systems. In: Molecular mechanisms in DNA Replication and Recombination. Keystone symposia on Molecular and Cellular Biology J. Cell Biochem. Supplement 16B, F318, p 44.
2. Mei, J., Michaels, K., and Firshein, W. 1992. Membrane associated plasmid RK2 DNA replication: Binding of OriV to subdomains of the Escherichia coli membrane and development of a more efficient membrane replicating system using maxicell mutants. In: EMBO

workshop on promiscuous plasmids in gram positive and negative bacteria. Spain.

IV. List of all Participating Scientific Personnel

- | | |
|-----------------------------|---|
| 1. Bethany Eident Wilkinson | Ph.D, 1992 |
| 2. Katerina Michaels | M. S. 1993 |
| 3. Julia Mei | Graduate Student |
| 4. Andrew Stein | Graduate Student |
| 5. Loretta Mele | Graduate Student |
| 6. Sharon Benashki | Graduate Student |
| 7. Shigeki Moriya | Visiting Assistant Professor
Osaka University, Japan |