

Deoxyribonucleic Acid-Membrane Interactions Near the Origin of Replication and Initiation of Deoxyribonucleic Acid Synthesis in *Escherichia coli*

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A previously reported salt-sensitive binding of deoxyribonucleic acid (DNA) to the cell envelope in *Escherichia coli*, involving approximately one site per chromosome near the origin of DNA replication, is rapidly disrupted *in vivo* by rifampin or chloramphenicol treatment and by amino acid starvation. DNA replication still initiates with this origin-specific binding disrupted, even when the disruption extends over the period of obligatory protein and ribonucleic acid synthesis that must precede initiation after release of cells from amino acid starvation. Thus the origin-associated membrane-DNA interaction is not necessary either for the initiation event itself or for the maturation of a putative initiation apparatus in *E. coli*.

It has been postulated that interaction of the origin of deoxyribonucleic acid (DNA) replication with the bacterial cell envelope may play a role in controlling initiation of DNA replication (4-6, 13, 20). This hypothesis is supported by findings that the origin is particularly enriched in DNA-membrane complexes isolated from *Bacillus subtilis* (16, 17) and *Escherichia coli* (3, 10), although no direct evidence connecting these DNA-membrane interactions with the actual initiation process has been offered. Such evidence may be hard to provide in the absence of methods for manipulating the membrane binding *in vivo* (there are, for example, no known temperature-sensitive binding mutants). The binding could, of course, have some different function, such as the orderly segregation of the replicated chromosome copies into daughter cells (5, 11).

We have recently described a unique type of membrane binding in *E. coli*. This binding is salt sensitive and protease labile *in vitro* and involves, on the average, one binding site per genome located near the origin of replication (1). Since such binding is clearly different from the salt-insensitive interactions occurring at many other regions of the chromosome (as well as from those induced under some circumstances by the lysozyme used to lyse the cells), it may have a special role. We report here an examination of several treatments that disrupt the binding *in vivo* and their effects on the subsequent initiation of DNA replication.

MATERIALS AND METHODS

Bacterial strains and growth medium. *E. coli* CT28 (requiring arginine, histidine, leucine, threonine, proline, thiamine, and thymine and carrying the temperature-sensitive *dnaC28* mutation) was obtained from C. I. Davern (15). This mutant is defective in initiation of DNA replication at a non-permissive temperature (e.g., 42°C). It was grown in Roberts minimal C-1 medium (12), supplemented with 0.5% Casamino Acids (Difco), thiamine (1 µg/ml), and thymine (20 µg/ml). During amino acid starvation, the Casamino Acids were replaced with methionine alone (40 µg/ml).

Radioactive labeling. To label the origin of replication, a 10-ml culture of *E. coli* CT28 was grown in a shaker water bath at 30°C to a titer of about 4×10^8 cells per ml (an absorbancy at 460 nm of 0.4 in a Zeiss PMQII spectrophotometer), and then shifted to 42°C for 65 min to allow all rounds of replication under way to be completed. The cells were collected on a membrane filter (Millipore) in a filter apparatus prewarmed to 42°C, suspended in 2.5 ml of medium containing 20 µg of thymine per ml plus 60 µCi of [*methyl*-³H]thymidine (56 Ci/mmol; Schwarz/Mann), and incubated for 3 min at 30°C with forced aeration to initiate replication and label the origin. The culture was refiltered, suspended in 10 ml of medium containing 500 µg of unlabeled thymidine per ml, and further incubated at 30°C with shaking for 15 to 20 min to chase the replication forks away from the labeled origin.

To label the non-origin regions of the chromosome, a 10-ml culture of CT28 was grown to an absorbancy at 460 nm of 0.4, shifted to 42°C for 65 min, and then shifted back to 30°C for 20 min before being collected by filtration and labeled for 3 min as described above.

For uniform labeling of the chromosome, an overnight culture of CT28 was diluted 1:300 into fresh medium containing 2.5 µCi of [³H]thymidine per ml and 200 µg of deoxyadenosine per ml. These cultures were grown for about 4 generations before further handling.

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DNA synthesis was monitored by either continuous labeling or pulse-labeling with [^3H]thymidine and measuring the acid-insoluble counts per minute. To determine the amount of label incorporated during continuous labeling, 0.2 ml of culture was added to 1.0 ml of 10% trichloroacetic acid containing 500 μg of unlabeled thymidine per ml. After about 4 h at 4°C, the precipitated sample was filtered onto a Millipore filter, washed with 0.1% cold trichloroacetic acid, dried, and counted in a toluene-based scintillation fluid (1).

To determine the DNA synthesis rate, 0.2 ml of culture was added to 0.1 ml of medium containing 2.5 μCi of [^3H]thymidine per ml at the indicated temperatures. After 3 min, 2 ml of 10% trichloroacetic acid containing 500 μg of unlabeled thymidine per ml was added, and the sample was treated as above for determining incorporated label.

Preparation of rapidly sedimenting complexes. This procedure has been described in detail elsewhere (1). Briefly, DNA-labeled cell preparations were added to KCN (final concentration 0.05 M) on ice. The cells were collected by centrifugation and suspended in 0.2 ml of TES buffer (0.05 M NaCl-0.05 M Tris, pH 8.0-0.005 M EDTA). The sample was alternately frozen and thawed eight times in a dry ice-alcohol bath and diluted with 0.5 ml of water to osmotically shock the cells. The lysate was sheared by forcefully passing it eight times through a 25-gauge, $\frac{5}{8}$ -inch (ca. 15.8-mm) needle, and centrifuged at low speed to remove unlysed cells.

The fraction of DNA present as rapidly sedimenting complexes, determined as described earlier (1), was taken to be the fraction bound to cell membrane, for reasons described in that reference. A sample (0.1 to 0.2 ml) of the lysate was layered onto a 3.2-ml, 5 to 20% sucrose gradient in TES buffer formed above a 0.8-ml 60% sucrose shelf. For samples that had been adjusted to a different ionic strength, the gradient was prepared from sucrose solutions having that same ionic strength. These gradients were centrifuged in an SW50.1 rotor at 31,000 rpm for 30 min at 20°C. Fractions were collected from the top, mixed with 125 μg of carrier ribonucleic acid (RNA), precipitated with trichloroacetic acid, and collected onto nitrocellulose membrane filters. The filters were dried and counted in the toluene-based scintillation fluid.

RESULTS

Effect of CAM on binding. DNA-membrane binding was detected here as a rapidly sedimenting complex in cell lysates containing cell DNA sheared to an average size of 8×10^6 daltons (1; Materials and Methods). The earlier work (1) showed two kinds of DNA-membrane binding in this complex. One type is stable to 1 M NaCl *in vitro*, and occurs at about 27 sites scattered around the *E. coli* chromosome, including one near the origin of DNA replication. A second type is disrupted by NaCl concentrations of 0.12 M or higher, and occurs at only a single site near the replication origin. Since Dworsky and Schaechter, using different procedures (2), found that chloramphenicol (CAM) treatment doubles the number of DNA-mem-

brane binding sites per chromosome, we investigated the effects of CAM on binding near the origin of replication, compared with the binding elsewhere in the chromosome.

E. coli CT28 cells, [^3H]thymidine-labeled in non-origin regions of the chromosome and exposed to 100 μg of CAM per ml, showed an increase in the fraction of labeled, membrane-bound DNA up to about 12% after a 5-min lag (Fig. 1). In contrast, when the DNA was labeled preferentially at the origin of replication, the label in this fraction decreased by about 50% during the first 4 min of treatment, a time somewhat shorter than that required for any effect on non-origin binding sites.

Figure 2A shows that about half the origin-associated binding before CAM treatment was salt sensitive, as found earlier (1), and that all such salt-sensitive binding disappeared within 4 min after the drug was added. In comparison (Fig. 2B), the complexes involving non-origin DNA (which are stable to 1 M NaCl) remained in the CAM-treated cultures for at least 30 min. This means that the origin-labeled complexes removed by exposure to CAM are predominantly of the salt-sensitive type.

The same results were obtained with cells labeled in the origin of replication and held at 42°C for 65 min before the CAM was applied (Fig. 1). This temperature regimen, used in the next experiment described below, had no effect on the CAM-induced release of origin binding.

Initiation of DNA replication in the pres-

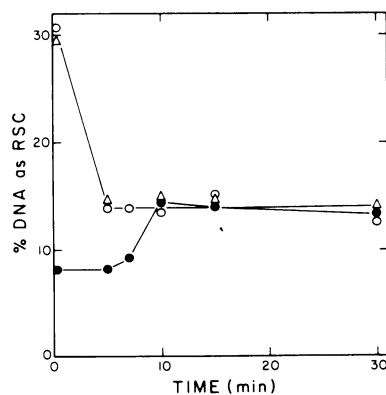


FIG. 1. Effect of CAM on membrane binding of origin and non-origin DNA. Cultures of CT28 at 30°C were exposed to 100 μg of CAM per ml from time 0, and samples removed at indicated times were assayed for the fraction of DNA in the form of rapidly sedimenting complexes (presumably membrane bound) as described in the text. (O) Cells labeled with [^3H]thymidine at the origin of replication; (●) cells labeled at non-origin regions of the chromosome; (Δ) cells labeled at the origin of replication, but incubated at 42°C for 65 min prior to the addition of CAM.

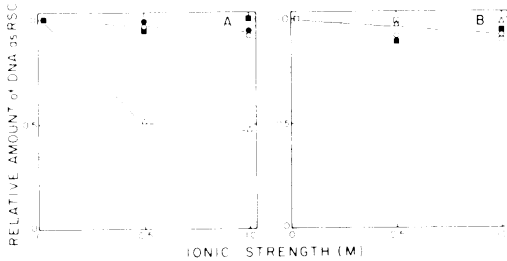


FIG. 2. Sensitivity of DNA-membrane complexes from CAM-treated cells to ionic strength. Samples of lysates from Fig. 1 were adjusted to the indicated ionic strength and assayed for the fraction of DNA bound to membrane, as described in the text. (A) Origin-labeled lysates for 0 (Δ), 4 (\blacksquare), 10 (\circ), and 30 (\bullet) min of exposure to CAM. (B) Non-origin-labeled lysates for 0 (\circ), 4 (\blacksquare), 10 (Δ), and 30 (\square) min of exposure.

ence of CAM. CAM treatment provides a way to remove the origin-associated, salt-sensitive binding sites at will. Figure 3 shows the effect of such treatment on the reinitiation of DNA replication. A culture of the temperature-sensitive initiation mutant CT28, growing in [^3H]thymidine medium to label the DNA, was brought to the nonpermissive initiation temperature (42°C), and all rounds of DNA synthesis were allowed to finish. CAM was added to samples, and, either 10 or 4 min later, the samples were returned to the permissive temperature (30°C) in parallel with a control not treated with CAM. The control promptly reinitiated DNA synthesis, as shown by the accumulation of acid-insoluble radioactive label. After about an 8-min delay, the treated cultures did likewise, proceeding at essentially the same rate as the untreated cells, at least for the next 20 min. Since a 4- or 10-min CAM treatment completely removed the salt-sensitive origin binding, regardless of the temperature shifts, this type of binding does not seem necessary for initiation of DNA replication.

Effect of RIF on initiation. A similar conclusion can be drawn from experiments with rifampin (RIF)-treated cultures. It has been reported that *E. coli* cells exposed to $100\ \mu\text{g}$ of RIF per ml for a short time show a reduction in DNA-membrane complexes (2). Figure 4 shows that exposure to $50\ \mu\text{g}/\text{ml}$ produced an initial reduction in numbers of all complexes (both those in origin and non-origin regions of the chromosome), but that the loss in membrane binding of non-origin DNA was transient. After 10 min only complexes involving the origin of replication were lost. As with CAM, holding the culture at 42°C for 65 min before applying RIF did not affect the kinetics of the origin-labeled complex disruption.

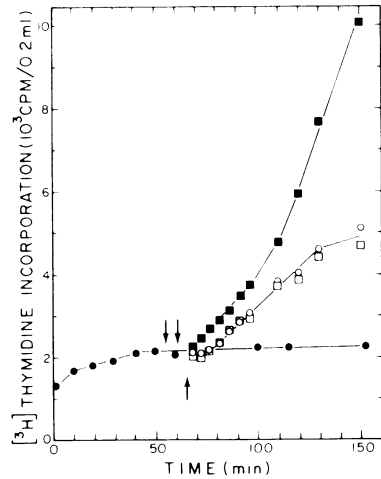


FIG. 3. Reinitiation of DNA replication in the presence of CAM. An exponentially growing culture in *C-1* medium, continuously labeled with $2.5\ \mu\text{Ci}$ of [^3H]thymidine per ml, was shifted from 30 to 42°C at time 0 (\bullet). CAM ($100\ \mu\text{g}/\text{ml}$) was added to samples as indicated by the upper arrows (\downarrow) at 55 (\circ) or 61 (\square) min. The treated cultures were then shifted back to 30°C at 65 min as indicated by the lower arrow (\uparrow), along with part of the untreated culture (\blacksquare), and 0.2-ml samples taken at the indicated times were assayed for incorporation of [^3H]thymidine.

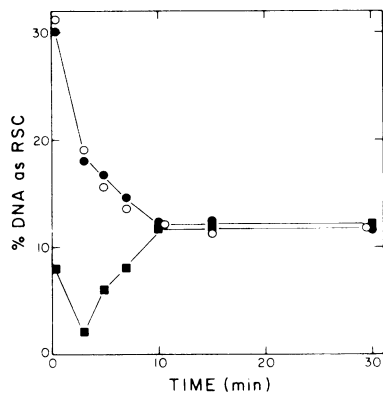


FIG. 4. Effect of RIF on DNA-membrane binding of origin and non-origin DNA. Cultures of CT28, preferentially labeled at the origin of replication (\bullet) or at non-origin regions of the chromosome (\blacksquare), were treated with $50\ \mu\text{g}$ of RIF per ml, and the fraction of DNA present as rapidly sedimenting complexes in 1 ml, removed at the indicated times, was determined as described in the text. A parallel culture, preferentially labeled at the origin, was incubated at 42°C for 65 min before its treatment with RIF was begun at time 0 like the others (\circ).

The salt sensitivity of complexes after RIF treatment was similar to that found in the CAM experiments. That is (Fig. 5), at time zero about

50% of the DNA-membrane complexes involving the origin of replication could be dissolved *in vitro* by high ionic strength, and this fraction was rapidly lost during RIF treatment. After 4 min of exposure about 35% of the complexes remained, after 7 min less than 5%, and after 30 min essentially none (Fig. 5A). In contrast, the non-origin DNA-membrane complexes were essentially salt insensitive (Fig. 5B). Figures 4 and 5 together show that the origin- and non-origin-labeled complexes remaining after 10 min of exposure were not distinguishable. Each amounted to 12% of the labeled DNA and was salt insensitive.

The effect of 50- μ g/ml RIF treatment on initiation of DNA replication was essentially the same as that found with CAM treatment; the data (not shown) closely resemble Fig. 3. Thus, again, initiation of DNA replication does not require that the origin-specific, salt-sensitive DNA-membrane complex be intact.

Initiation after amino acid starvation. Although the experiments with CAM and RIF show that the salt-sensitive binding of the origin region to membrane is not required for the actual initiation step in DNA replication, the accumulation of critical proteins and the maturation of some kind of "initiation apparatus" might require such an interaction. Since amino acid starvation disrupts the salt-sensitive binding in a fashion similar to the disruption following CAM treatment (unpublished data; Fig. 6), it is possible to test the requirement of the DNA-membrane binding during the putative maturation period. This is accomplished by allowing a CT28 culture that has been labeled at the origin to complete its rounds of replication at 42°C in the absence of the required amino acids. Under

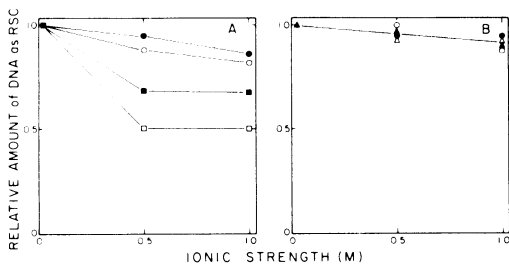


FIG. 5. Sensitivity of DNA-membrane complexes from RIF-treated cells to ionic strength. (A) Lysates from samples of the origin-labeled cultures in Fig. 4, taken after different times of exposure to RIF, were adjusted to the indicated ionic strength with NaCl and assayed for the fraction of DNA bound to membrane as described in the text: 0 (\square), 4 (\blacksquare), 7 (\circ), and 30 (\bullet) min. (B) Lysates from the non-origin-labeled cultures similarly tested after different times in RIF: 0 (\triangle), 4 (\circ), 7 (\bullet), and 30 (\blacktriangle) min.

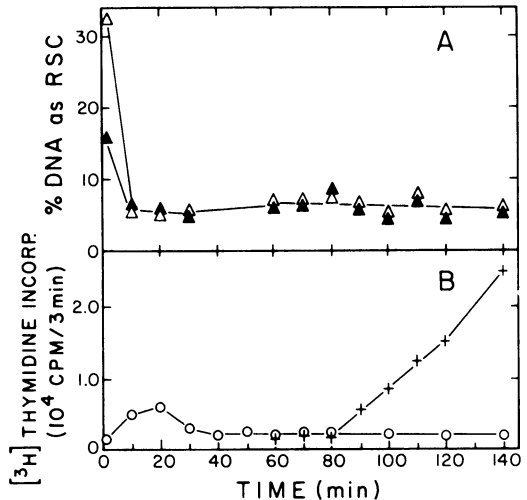


FIG. 6. Reinitiation of DNA synthesis after amino acid starvation. A culture of CT28, labeled at the origin of replication, was washed free of its required amino acids, suspended in minimal medium containing (per ml) 50 μ g of methionine, 1 μ g of thiamine, and 20 μ g of thymine, and shifted to 42°C at time 0. At 60 min, the required amino acids were restored, and the culture was returned to 30°C. (A) At the indicated times, samples were assayed for the fraction of DNA bound to membrane as described in the text, either after adjustment to 0.5 M NaCl (\blacktriangle) or without added salt (\triangle). (B) Samples of 0.2 ml of the culture were pulse-labeled with [³H]thymidine for 3-min intervals as described in the text: (\circ) a culture without amino acids (except methionine); (+) samples of the same culture after restoration of amino acids and shifting back to 30°C at 60 min. In all cases, the background due to prior labeling of the chromosomal origin of replication has been subtracted.

these conditions the maturation steps necessary for the next round of replication cannot occur, and the origin-specific binding is disrupted. Upon returning the culture to 30°C, and restoring the amino acids, one can then determine whether the origin-specific complex is restored prior to initiation of replication.

The results of such an experiment can be seen in Fig. 6, where the culture was deprived of amino acids and shifted to 42°C at time 0. The salt-sensitive, origin-specific membrane binding of DNA was rapidly disrupted (Fig. 6A), so that the lysate samples exposed to 0.5 M NaCl showed the same amount of binding of origin-labeled DNA as those not exposed to salt. This binding level remained around 5%, even after restoration of the amino acids and return of the culture to 30°C at 60 min, for the entire 140-min duration of the experiment.

The rate of DNA synthesis, measured by 3-min pulses with [³H]thymidine (Fig. 6B), be-

came constant at a low level after about 40 min, corresponding to the time at which the net accumulation of DNA ceased in Fig. 3. After the culture was shifted to 30°C and the amino acids were restored, a delay of 20 to 25 min ensued before a measurable increase in the rate of incorporation of [³H]thymidine occurred. This delay is the time presumably required for synthesis of initiation proteins and the putative maturation of any initiation apparatus. However, the initiation of replication clearly occurred before DNA-membrane binding at the origin was restored. Therefore, although such binding is present under normal growth conditions, it is required neither for the formation of an initiation apparatus nor for the actual initiation step itself.

DISCUSSION

The existence of a unique DNA-membrane binding site near the replication origin in *E. coli* (viz., salt sensitive and protease labile in vitro and persisting in the cell after termination of replication, as well as at stationary phase) (1) suggested its possible involvement in initiating DNA replication, in keeping with earlier postulates by others. However, when this site was disrupted by short exposure of cells to CAM or RIF, the cells could successfully reinitiate their DNA replication while the complex remained disrupted. The straightforward interpretation is that the membrane binding is not needed for initiation.

This type of DNA-membrane binding was also disrupted by starvation for a required amino acid. Since a period of protein and RNA synthesis is required before actual initiation can occur (8, 9, 19), some protein-nucleic acid assembly requiring support from the cell membrane might be necessary. However, when cells were subjected to amino acid starvation at the nonpermissive temperature, and were then returned to the permissive temperature with restoration of amino acids, initiation took place (after the expected delay) without reformation of the salt-sensitive binding. This not only supports the independence of initiation from the DNA-membrane binding, but further indicates that a complex is not required even for the maturation events that must precede initiation.

Cells are, of course, tightly integrated systems in which manipulation of one process has repercussions throughout the metabolic network. Thus it is always possible that unrecognized secondary effects of the drugs or amino acid starvation have influenced the experimental outcome. Such a possibility is, however, reduced by the fact that three different treatments, interrupting metabolic events at different points, led to the same conclusion.

We do not understand the modest delay in initiation of replication in RIF- or CAM-treated cells (shown in Fig. 3 for CAM). Our unpublished data show that shifting to the permissive temperature after only 4 min of RIF exposure (when only about 60 to 70% of the salt-sensitive origin binding had been removed) caused a delay of only 5 min. An 8-min delay occurred, however, after 10 min of exposure (when all the origin-specific binding had been disrupted). Figure 3 shows that reinitiation after CAM treatment occurred 8 min after the temperature shift for both 4- and 10-min treatments. Since at both these times 100% of the salt-sensitive complexes were gone, the 8-min delay may stem directly from the loss of all salt-sensitive binding sites, but it may also simply result from general physiological changes in the cell.

The fact that the distinctively different type of DNA-membrane binding (1) is not necessary for initiation of DNA replication reduces the likelihood that any membrane attachments are involved in this event. Although an average of one salt-insensitive binding site also exists near the origin of replication (1), its properties resemble those of the many other binding sites scattered around the chromosome. We earlier attributed it to the indirect consequences of the high transcription frequency in ribosomal RNA genes near the origin, particularly *rrnC* (1). If these ideas are wrong, this origin-associated membrane binding site could still participate in initiation, but otherwise there seems to be no site with the required properties. The absence of an obligate membrane binding for the initiation of DNA replication would, we hope, open the way for its biochemical investigation with entirely soluble components in vitro, as has already been done for bacteriophage (14) and plasmid DNAs (18) and for the elongation step of bacterial DNA replication (7).

The role of the unique, salt-sensitive DNA-membrane binding near the origin remains yet to be determined. An obvious possibility is to provide orderly segregation of replicated chromosomes into daughter cells, but systematic determination of the circumstances under which it is present should allow evaluation of this or other possible roles.

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