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Identification of a Biochemically Unique DNA-Membrane Interaction Involving the *Escherichia coli* Origin of Replication

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DNA-membrane complexes have been obtained from *Escherichia coli* by using a freeze-thaw lysis procedure that avoids lysozyme and detergents. Complexes made in this manner and containing DNA near the origin of replication are uniquely sensitive to ionic strength, Pronase, and trypsin. There is approximately one such complex per chromosomal origin. The sensitivities suggest that origin-specific binding is mediated by a protein. By using these unique characteristics to distinguish origin-specific complexes from the majority of DNA-membrane binding sites, it was found that the origin-specific binding persists after termination of chromosomal replication.

The initiation of DNA replication in *Escherichia coli* occurs at a genetically heritable locus that is near the *ilv* operon (2, 19) and most probably is between *dnaA* and the *bgl* loci (11, 21). Despite the central role that the initiation event plays, very little is known about the molecular events involved. In addition to the apparent requirement for RNA polymerase activity (17), initiation requires protein synthesis, including at least the *dnaC* (40), *dnaA* (40), *dnaI* (1), *dnaP* (39), and *dnaE* (40) gene products. It has also been postulated that a DNA-cell envelope interaction plays an enzymatic and/or regulatory role in the initiation of replication (10, 13, 14, 29, 31, 40). This hypothesis has gained credence through the finding that the origin of replication is specifically associated with the cell envelope in *Bacillus subtilis* (38) and *E. coli* (6, 27).

The present paper describes isolation of a DNA-membrane complex containing DNA near the origin of replication whose properties are distinguishable from the majority of the DNA-membrane complexes. This not only suggests that its function may be different from that of complexes containing other regions of the chromosome, but opens the way for a separate investigation of its behavior.

MATERIALS AND METHODS

Bacterial strains and growth medium. *E. coli* CT28 (requires arginine, histidine, leucine, threonine, proline, thiamine, and thymine and carries the *dnaC28* mutation) was obtained from C. I. Davern (33). The medium used was Robert minimal C-1 medium (28) supplemented with 0.5% Casamino Acids (Difco Laboratories), thiamine (1 μg/ml), and thymine (20 μg/ml).

Radioactive labeling. To label the origin of replication, a 10-ml culture of *E. coli* CT28 was grown in a shaking water bath at 30°C to a titer of about 3×10^8 cells per ml (an absorbance at 460 nm of about 0.4 in the Zeiss PMQII). The culture was then shifted to 42°C for 65 min and collected on a filter (45-μm pore size; Millipore Corp.) in a filter apparatus prewarmed to 42°C. The cells were suspended in 2.5 ml of medium containing 0.06 mCi of [*methyl*-^{3}H]thymidine per ml (56 Ci/mmol; Schwarz/Mann) at 30°C with forced aeration. After 3 min the culture was refiltered and resuspended in 10 ml of medium containing 500 μg of unlabeled thymidine per ml. The culture was then incubated at 30°C in a water bath shaker for 15 to 20 min before further treatment.

To label the nonorigin regions of the chromosome, a 10-ml culture of CT28 was grown at 30°C to an absorbance at 460 nm of 0.4. The culture was shifted to 42°C for 65 min, then shifted back to 30°C, and 20 min later was 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 [^{3}H]thymidine per ml and 200 μg of deoxyadenosine per ml. This culture was grown for about four generation times before further handling.

Preparation of sheared lysates. Labeled cell preparations (about 5×10^8 cells per ml) were added to KCN (final concentration, 0.05 M) on ice. The cells were collected by centrifugation (4,000 × *g* for 10 min at 4°C). The cells were suspended in 0.2 ml of TES buffer [0.05 M NaCl-0.05 M tris(hydroxymethyl)-aminomethane base-0.005 M ethylenediaminetetraacetic acid, pH 8.0] and frozen and thawed eight times in a dry ice-alcohol bath. Lysis was completed by

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diluting with 0.5 to 1.0 ml of water for osmotic shock that resulted in maximum lysis as monitored by the decrease in turbidity at 600 nm. Separate measurements indicate that 70 ± 2% of the cells were lysed, as measured by release of DNA. The viscous lysate was sheared by passing eight times through a 25-gauge, 0.625-inch (ca. 1.59-cm) needle, and after a low-speed centrifugation (4,000 × *g*, 5 min, 4°C) to remove the unlysed cells, the supernatant was analyzed immediately or stored at −70°C. Storage for up to several weeks had no observable effect.

Neutral sucrose gradient sedimentation. To determine the fraction of DNA present as rapidly sedimenting complexes (RSC), a portion (0.1 to 0.2 ml) of the lysate was layered onto a 3.2-ml 5 to 20% sucrose gradient in TES buffer layered over a 0.8-ml 60% sucrose–CsCl (1.2 g/ml) in TES buffer shelf. The gradients were prepared in a cellulose nitrate centrifuge tube (0.5 by 2 inches [ca. 1.27 by 5.08 cm]; Beckman no. 305050). For experiments involving different ionic strengths, the gradients were adjusted to the same ionic strength as the sample with NaCl. Centrifugation was carried out at 31,000 rpm at 20°C for 30 min in an SW50.1 rotor. Alternatively, a larger volume of lysate was layered on a 12-ml 5 to 20% gradient with a 3-ml shelf of the same components as above. These gradients were centrifuged for 90 min at 25,000 rpm in an SW27 rotor at 20°C.

Fractions were collected from the bottom of the tube, precipitated with 10% trichloroacetic acid, filtered onto nitrocellulose membrane filters, dried, and counted in a toluene-based scintillation fluid [2,5-diphenyloxazole, 8 g/liter, and 1,4-bis-(5-phenyloxazolyl)benzene, 0.8 g/liter]. At times, peaks were collected in bulk from the top, mixed with 125 μg of carrier RNA, trichloroacetic acid precipitated, and treated as above. This gives a very sensitive measure of the amount of DNA as RSC, even when low numbers of counts per minute are loaded onto the gradient. Recovery was essentially 100%.

To determine the molecular weight of the fragments, 0.1 ml of lysate was mixed with 0.01 ml of 10% sodium lauryl sulfate, 0.01 ml of TES-saturated phenol, and 0.01 ml of [^{14}C]thymidine-labeled T7 phage. After a 10-min incubation at room temperature, the lysate was layered on a 4-ml 5 to 20% sucrose gradient (TES buffer) and centrifuged for 55 min at 45,000 rpm at 20°C in an SW50.1 rotor. Seven-drop fractions were collected from the bottom onto filter paper strips, treated with 10% trichloroacetic acid and ethanol in succession, dried, and counted in a toluene-based scintillation fluid. The number average molecular weight was calculated after the method of Ivarie and Pène (12), by using 25 × 10^6 as the molecular weight of the T7 DNA (16) as a standard.

Chemicals and enzymes. The enzymes used were bovine pancreatic trypsin (10,000 U/mg) from Sigma, egg white lysozyme (29,600 U/mg) from Calbiochem, and deoxyribonuclease I (3,840 Kunitz units/mg) from Sigma. Spermidine was obtained from Calbiochem.

RESULTS

Preparation of rapidly sedimenting complexes. A critical step in isolating DNA-membrane complexes is the lysing of the cells. We have employed a freeze-thaw method to eliminate artifacts stemming from detergents (22, 23, 30) or lysozyme (15, 35; see also Fig. 4) that have caused misinterpretations of the data of others. Although the efficiency of freeze-thaw lysis is lower than that of the lysozyme and detergent methods, any unlysed cells can be removed by low-speed centrifugation. This procedure has resulted in lysates that (after shearing, as described above) contain DNA with a reproducible number average molecular weight of about 8 × 10^6, as judged by sucrose density gradient centrifugation.

The typical pattern produced by sedimentation of such a sheared lysate in a 5 to 20% neutral sucrose gradient over a high-density shelf is shown in Fig. 1A, where most of the DNA re-

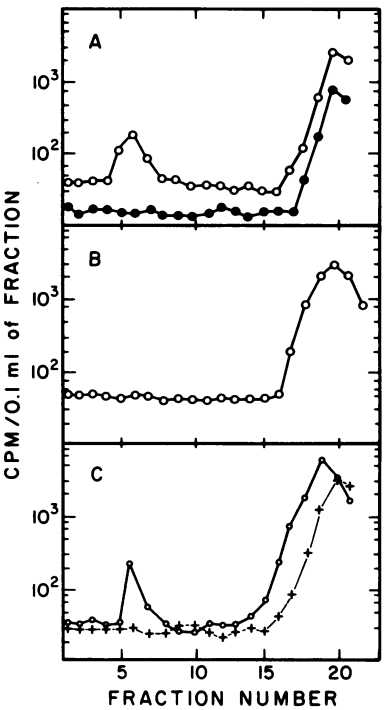

FIG. 1. *Sedimentation characteristics of radioactively labeled DNA in differently prepared cell lysates. Counts per minute in fractions from a sucrose gradient, centrifuged as described in the text and plotted versus fraction number. (A) Cells uniformly labeled with [^{3}H]thymidine (○) mixed with purified [^{14}C]thymine DNA (●) before lysis. (B) Sheared lysate from uniformly labeled culture, adjusted to 1% sodium lauryl sulfate before centrifugation. (C) Composite of two gradients run in the same rotor at the same time: (i) Lysate of a uniformly labeled culture treated with deoxyribonuclease I (91 Kunitz units per ml for 15 min at room temperature) (+) and (ii) untreated control* (○).

mains at the top of the gradient and only a small percentage moves to the shelf as an RSC. The distribution and number average molecular weight for DNA isolated from the RSC are essentially the same as those of the total lysate. Purified ^{14}C-labeled DNA, added to the cell suspension before lysis, does not contribute to the RSC fraction, showing that the DNA in the RSC is not first released by lysis and then trapped in cell debris. Addition of sodium lauryl sulfate (1% final concentration) to a sheared lysate results in a complete loss of rapidly sedimenting DNA (Fig. 1B), consistent with the interpretation that the RSC involves cell membrane. Figure 1C shows that all of the detectable DNA in the RSC fraction is sensitive to deoxyribonuclease I, indicating that all unlysed cells have been effectively removed. The sensitivity to DNase also argues against entrapment of DNA in vesicles formed during lysis (24). All of these results indicate that DNA-membrane complexes obtained by freeze-thaw lysis are qualitatively similar to those found by others using lysozyme and detergents (8, 12, 18, 27, 37).

To determine the number of DNA-membrane binding sites present, cultures of CT28 were labeled continuously with [^3H]thymidine for about four generations at 30°C and used to prepare sheared lysates. The fraction of DNA as RSC under these growth conditions was 0.08 ± 0.002 (see Table 1). By using the number average molecular weight of the fragments (8×10^6) and the molecular weight of the *E. coli* chromosome (2.7×10^9), we calculate at least 27 attachments per chromosome. This agrees well with estimates obtained for other strains of *E. coli* (5) and for *B. subtilis* (12).

Labeling of the origin. The origin of replication can be preferentially labeled by aligning DNA replication in a culture so that all chromosomes initiate within a very short period of time. This can be accomplished with the *dnaC* mutant CT28 by holding it at the nonpermissive temperature (42°C) until it completes all the rounds of replication in progress and accumulates the products necessary for initiating new rounds (32) when subsequently returned to the permissive temperature (30°C). Completion of all rounds of replication after shifting to 42°C was shown by a plateau of [^3H]thymidine incorporation. Rapid reinitiation of [^3H]thymidine incorporation took place upon the shift back to 30°C, as demonstrated earlier by Schubach et al. (34). The origin region was preferentially labeled, as described above, by a 3-min pulse-labeling of cells that had reached their plateau period and had then been shifted back to 30°C. Replication forks were chased away from the origin by washing the radioactivity, replacing it with nonradioactive thymidine, and growing the cells an additional 15 to 20 min. It has been shown that the above protocol does, in fact, label the origin of replication in CT28 (20).

TABLE 1. *Fraction of DNA as RSC after various labeling schemes*

Lysate	Fraction of DNA as RSC	Avg ± SD[a]	Enrichment ratio[b]
Uniformly labeled in expt:			
1	0.082		
2	0.080		
3	0.078	0.080 ± 0.002	1.0
4	0.079		
5	0.081		
6	0.077		
Random pulse[c] in expt:			
1	0.081		
2	0.078	0.079 ± 0.002	0.99
3	0.079		
Nonorigin pulse in expt:			
1	0.076		
2	0.081		
3	0.080	0.079 ± 0.002	0.99
4	0.079		
Origin pulse in expt:			
1	0.32		
2	0.30		
3	0.31	0.30 ± 0.01	3.75
4	0.29		
5	0.30		

[a] SD, Standard deviation.

[b] Enrichment ratio = percent DNA as RSC (pulse)/percent DNA as RSC (uniform).

[c] An exponential culture of CT28 was pulse-labeled at 30°C in an identical fashion as the nonorigin pulse.

Membrane binding of the origin. Preferential labeling of the origin region in CT28 results in about 30% of the label being represented in the RSC fraction (Table 1). In contrast, the fraction of DNA as RSC from uniformly labeled chromosomes or randomly labeled chromosomes was about 8%. Pulse-labeled nonorigin regions of the chromosome were also represented in the RSC fraction at about 8%. The enrichment for origin-labeled fragments was thus about 3.75-fold, compared to no enrichment for non-origin-labeled regions.

Sensitivity of DNA-membrane complexes to ionic strength. Increased ionic strength has a different effect on complexes involving the origin of replication than on complexes involving other chromosomal regions. When portions of a

single lysate adjusted to the desired ionic strength were compared for the amount of DNA appearing as RSC, the majority of the DNA-membrane complexes (represented effectively by uniformly labeled chromosomes) were virtually unaffected by ionic strengths up to 1.0 M NaCl (Fig. 2). Complexes specifically removed from the origin of replication (by labeling 20 min after chromosomal alignment) also were not affected by increases in ionic strength. However, complexes involving regions near the origin exhibit marked sensitivity, about 50% of the DNA-membrane complexes being disrupted by the highest ionic strength employed. More detailed study (see below) shows that the complexes sensitive to ionic strength are disrupted by as little as 0.12 M NaCl. Preferential labeling of the origin region, using amino acid starvation with a *dnaC*$^+$ strain to produce the chromosomal alignment before labeling, gave similar results, showing that the effect is not peculiar to *dnaC* mutants or to the temperature regimen used for chromosomal alignment in CT28.

Protease sensitivity of DNA-membrane complexes. The sensitivity of the origin-specific complex to an increase in ionic strength suggests that the DNA-membrane interaction may be mediated by a protein-DNA interaction. To test this possibility, lysates were incubated with Pronase, and the effect on the fraction of DNA as RSC was determined. Figure 3A shows that the majority of the binding sites from a lysate with uniformly labeled DNA are relatively refractory to the Pronase digestion (solid squares). However, the lysate containing label only in the origin region was reduced by about 60% after treatment with Pronase (open squares). Because increasing the concentration to 500 μg/ml caused no further decrease than treatment with 250 μg/ml, it is likely that all Pronase-sensitive sites had been removed.

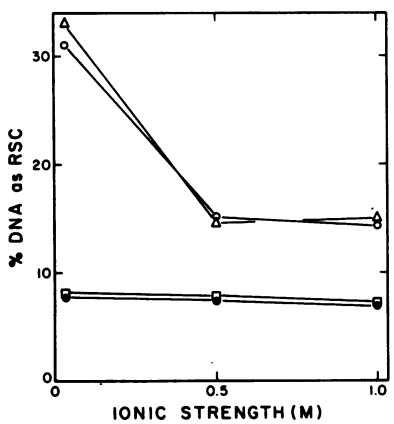

FIG. 2. *Effect of ionic strength on DNA-membrane binding. Sheared lysates of appropriately labeled cells were adjusted to the indicated ionic strength by addition of concentrated NaCl solution, kept on ice for 10 min, and layered onto sucrose gradients of the same ionic strength as the lysate before centrifuging in an SW50.1 rotor. The fraction of total labeled DNA as RSC is plotted versus ionic strength. Symbols: (□) uniformly labeled lysate; (●) lysate labeled in nonorigin regions; (○) lysate labeled in the origin region; and (△) lysate from a culture of CT28 preferentially labeled at the origin of replication and reincubated at 42°C for 65 min before lysis.*

Portions of lysates after Pronase treatment were adjusted to higher ionic strengths, and the fraction of the DNA remaining as RSC was determined. Figure 3B shows that the salt-sensitive sites present before Pronase treatment (open circles) have been completely removed by Pronase treatment (closed circles). Because only 50% of the DNA was salt sensitive before Pronase digestion and the removal of a little more than 50% of the DNA from the RSC fraction with Pronase treatment left no salt-sensitive complexes, we conclude that Pronase specifically attacks the salt-sensitive sites.

Trypsin has been reported to attack preferentially the lipoprotein component that helps maintain the structure of the wall-membrane adhesion zones present in *E. coli* (9). Trypsin treatment of DNA-membrane complexes gives qualitatively the same effect as Pronase treatment: complexes from uniformly labeled lysates are little affected, whereas complexes from lysates preferentially labeled at the origin of replication show sensitivity. Again, the salt sensitivity of the origin-labeled complexes after trypsin treatment was greatly reduced, indicating that the origin-specific binding had been preferentially disrupted by the proteolytic enzyme.

Binding after termination of replication. Placing a culture of CT28, labeled at the origin of replication, back to 42°C for 65 min results in the termination of all rounds of replication and a plateau of [^{3}H]thymidine incorporation, because no new initiations can occur without a functional *dnaC* gene product. DNA-membrane complexes isolated from such a culture show no change in the fraction of DNA present as RSC or in the sensitivity of the complexes to ionic strength (Fig. 2), showing that the complexes persist after termination of replication.

Effects of lysozyme and spermidine. When lysozyme is mixed with a freeze-thaw lysate at low ionic strength (0.015 M), a considerable amount of DNA is found in the RSC fraction compared to that of the lysate with no additions (see Fig. 4). Essentially all of this binding can be subsequently removed by raising the ionic strength to greater than 0.05 M. Thus, although this lysozyme-mediated binding is su-

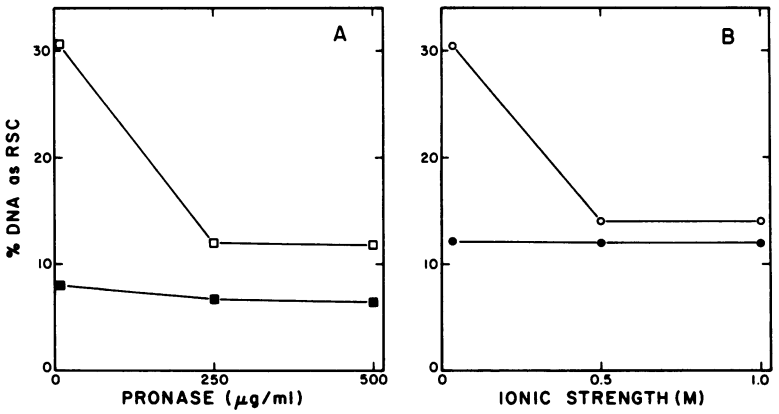

FIG. 3. *Effect of Pronase on DNA-membrane complexes. (A) Lysates from either uniformly labeled chromosomes (■) or from origin-labeled chromosomes (□), treated with the indicated concentrations of Pronase for 15 min at 30°C before determining the fraction of labeled DNA as RSC in the SW50.1 rotor. (B) Lysate labeled in the origin of replication tested for sensitivity to ionic strength without Pronase treatment (○) and after a 15-min incubation with 500 μg of Pronase per ml (●).*

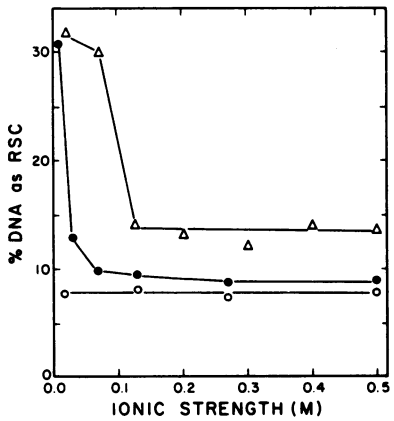

FIG. 4. *Effect of lysozyme on DNA-membrane interaction. Sheared lysate from a uniformly labeled culture was adjusted to the indicated ionic strength with NaCl either in the presence of 500 μg of lysozyme per ml (●) or in the absence of lysozyme (○). After incubation on ice for 10 min, the fraction of DNA present as RSC was determined by sedimentation in the SW50.1 rotor. A lysate from a culture labeled at the origin of replication, similarly adjusted to the indicated ionic strength with NaCl and centrifuged after 10 min, is shown for comparison (△).*

TABLE 2. *Effects of spermidine on the salt-sensitive binding near the origin of replication*[a]

Addition to lysate	% DNA remaining as RSC
None	30.0
Spermidine (15 mM)	29.0
Spermidine (30 mM)	30.5
NaCl (0.5 M)	14.5
NaCl (1.0 M)	14.5

[a] Portions of a sheared lysate obtained from a culture of CT28 preferentially labeled at the origin of replication were adjusted to the indicated concentration of NaCl or spermidine. After 10 min on ice, the fraction of DNA as RSC was determined as described in the text.

perficially similar to that of the origin-specific binding, the origin-specific binding is slightly more stable to increases in ionic strength.

Recent studies on the role of membrane binding in relation to the chromosome replication cycle have utilized membrane-bound folded chromosomes isolated in 1.0 M NaCl (14, 40). This ionic strength would, however, disrupt completely the origin-specific binding. A potential substitute for the 1 M NaCl is spermidine (7). Table 2 shows that exposure of origin-labeled lysates to 15 or 30 mM spermidine does not affect the amount of the pulse-labeled DNA present in the RSC fraction. Thus, it may be possible to isolate membrane-bound folded chromosomes with the origin-specific binding intact by stabilizing the chromosome structure with spermidine instead of 1.0 M NaCl.

DISCUSSION

Specific labeling of the origin of replication in *E. coli* has allowed detection of a unique DNA-membrane complex that possesses physiochemical properties distinct from the majority of detectable complexes. The origin-specific binding (i.e., the binding disrupted by 0.12 M NaCl) is disrupted completely by treatment with Pronase and trypsin, unlike the majority of DNA-membrane complexes that are unaffected by protease or by 1 M NaCl (36, 41; also Fig. 2 and 3). These

features suggest that a protein mediates the DNA binding. Because binding occurs only in a unique region of the chromosome, it is also possible that the protein recognizes a specific DNA sequence near the origin of replication and promotes interaction of this DNA segment with the cell envelope. The salt-sensitive binding persists after termination of DNA replication, suggesting that detachment and reattachment of the origin do not play an important role with respect to initiation of DNA replication during the cell cycle, as surmised by others (14, 41). The same conclusion was reached by Ryder and Smith (31), although they were not able to relate their data to the binding of a particular region of the chromosome.

The number of uniquely bound DNA fragments per origin can be calculated from the amount of DNA replicated during the pulse-labeling time, the average size of fragments, and the fraction of DNA bound in salt-sensitive complexes. The DNA replicated during a particular time interval can be determined from the replication period, or C period (3), and the value of C under the growth conditions employed can be obtained from the accumulation of DNA after initiation of replication is stopped at a nonpermissive temperature. The fractional increase F in DNA after cessation of initiation is related to the C period and the growth rate by $F = e^{k(C/2)}$, where k is the inverse of the generation time (26). Because the average accumulation of DNA under these conditions is 1.54 ± 0.04-fold, whereas the generation time is 75 min, the C period is 60 min. Therefore, in 3 min about 3/60 of the *E. coli* chromosome, or 1.2×10^8 daltons, would be replicated. Because the number average fragment molecular weight is 8×10^6, about 15 fragments are labeled, of which 30% or about 4.5 are present in rapidly sedimenting complexes. Of these, about half, or slightly more than two, are salt sensitive (Fig. 2). Because two origins are labeled by each initiation event (i.e., the origin is duplicated), it would appear that there is one unique, salt-sensitive site per origin region. This value is an estimate due to some asynchrony in initiation of replication.

On the average, there is also one site with properties similar to the majority of the sites (salt insensitive) that is close to the origin of replication.

Existence of one salt-insensitive complex near the origin of replication can be explained by a mechanism not related to initiation of DNA replication. Regions of the chromosomes that are being transcribed may have a much higher probability of associating with the cell membrane than nonactive regions (18, 33). Because the RNA genes are among the most highly active regions of the chromosome (4) and are clustered near the origin in *E. coli* (25), one might expect the origin region to be enriched in the membrane-bound DNA and to have properties similar to the majority of DNA-membrane complexes at sites scattered around the chromosome.

The properties of the origin-specific binding site that we observe suggest that some changes in traditional techniques might be advisable in studying the role of DNA-membrane binding during the replication cycle (14, 41). For example, 1 M NaCl as a source of stabilizing counterions in isolation of membrane-bound folded chromosomes would disrupt the sites described here. Because we find that spermidine does not affect the binding at concentrations that stabilize the folded chromosome (7), its use would seem preferable. Most lysis methods utilize lysozyme, which we find leads to additional, nonspecific binding at low salt concentrations (34; see Fig. 4), whereas high salt leads to disruption of the origin-specific binding. From Fig. 4 it is apparent that the ionic strength must be carefully controlled between 0.05 and 0.07 M, when lysozyme is used to avoid both disruption of complexes and artifactual binding. It is possible that a small amount of lysozyme-induced binding may persist even at the higher ionic strengths.

The biochemical properties of the origin-specific complex characterized here (i.e., salt sensitivity, trypsin sensitivity, and Pronase sensitivity) are identical to those properties of the DNA-membrane complexes isolated by Olsen et al. (24). However, we have seen the salt-sensitive sites only when the origin of replication was preferentially labeled, whereas Olsen et al. (24) isolated their complexes from uniformly labeled chromosomes. These differences could be explained by assuming that Olsen et al. enriched for the salt-sensitive complex. We have found that the origin-labeled DNA retains its high probability of interacting with the membrane when the cells enter stationary phase, in contrast to the majority of complexes which decrease in number (unpublished data). It is possible that harvesting large batch cultures at a relatively high cell density as reported by Olsen et al. produces a condition similar to stationary phase resulting in an enrichment for the salt-sensitive binding. If our complexes are indeed the same as those of Olsen et al., then their data indicating that wall-membrane adhesion zones are the points of DNA attachment apply to our salt-sensitive complexes as well.

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