Evidence for the Specific Association of the Chromosomal Origin with Outer Membrane Fractions Isolated from *Escherichia coli*

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DNA-envelope complexes isolated from osmotically lysed spheroplasts of *Escherichia coli* contained 0.2 to 1% of the total cellular DNA after labeling with [3H]thymidine. Molecular weight determinations indicated that the amount of bound DNA was equivalent in most cases to a maximum of three binding sites per chromosome. Bound DNA from *E. coli B/r* was distributed approximately equally between inner and outer membrane components when envelopes were fractionated on sucrose equilibrium gradients. Outer membrane-DNA complexes, in particular, fraction H1, with a density of 1.24 g/cm³, were quite stable against shearing and against Sarkosyl NL87. In the case of *E. coli B/r*, H1-DNA was also relatively resistant to deoxyribonuclease. Inner membrane-DNA complexes, in contrast, were quite labile and readily dissociated to release free DNA. The outer membrane fractions did not appear to contain replication fork DNA, but small amounts may have been present in the inner membrane complexes. A two- to eightfold enrichment for chromosomal origin DNA in the envelope was obtained when cultures of *E. coli K-12*, synchronized for DNA replication, were pulse labeled at different times in the replication cycle. This enrichment was found invariably in the outer membrane fractions. However, the data do not exclude the possibility that this DNA is bound to regions of adhesion between inner and outer membranes which sediment with a density indistinguishable from that of the outer membrane.

Van Iterson (43) and Ryter and Jacob (37, 38) provided the first electron micrographic evidence that the chromosome of *Bacillus subtilis* was bound to the bacterial surface. Subsequently, numerous workers have reported the association of bacterial DNA in cell lysates with "fast-sedimenting" (26) or with particle-bound DNA isolated on filters (41). Such bound DNA has variously been claimed to be enriched, after being pulse-labeled for replication forks (15, 40), chromosome origins (12, 16, 31, 42), or other structural regions of the chromosome (1, 10, 32, 36, 44). In addition, Sueoka and Quinn (42) and Yamaguchi and Yoshikawa (44) have obtained evidence that transformable genetic markers, associated with both the origin and terminal regions of the *B. subtilis* chromosome, are both enriched in fast-sedimenting, particle-bound fractions from lysed bacteria. In a different approach, Earhart et al. (11) and Ballesta et al. (3) have isolated from cell lysates DNA which attaches to magnesium-Sarkosyl crystals together with envelope material. In addition, Olsen et al. (29) have isolated *Escherichia coli* DNA attached to a particular envelope component of high density, which they have suggested to be junction regions between inner and outer membrane. A similar conclusion was reached by Portalier and Worcel (32), working with whole chromosomes isolated together with the cell envelope. Finally, while this work was in progress, Gomez-Eichelmann and Bastarrachea (16) reported the association of an origin-like DNA with an isolated inner membrane fraction.

In the present investigation, we attempted to isolate envelope-bound DNA free from bulk DNA, to assess the stability and yield of the complexes, to test for the presence of replication fork or origin DNA and, finally, to localize the latter in either the inner or the outer membrane. The results obtained show that less than 1% of the DNA of *E. coli B/r*, corresponding to one to three binding sites per chromosome, could be isolated in association with both inner and outer membrane fractions after separation by equilibrium centrifugation in sucrose gradients. A low-molecular-weight DNA in the outer membrane fractions was more stably attached and enriched for deoxyribonuclease (DNase)-resistant DNA.
than was the inner membrane DNA. In addition, in cultures of E. coli K-12 synchronized for DNA replication, the outer membrane fraction was significantly enriched for "origin"-labeled DNA.

**MATERIALS AND METHODS**

**Chemicals.** All chemicals employed were analytical grade and were obtained from Sigma Chemical Co., Fisons, Scientific Apparatus and Co. Ltd., or British Drug House. Egg white lysozyme (EC 3.2.1.17) was obtained from Sigma, and bovine pancreas DNase I (EC 3.1.4.5), Pronase, and bovine pancreatic ribonuclease (RNase) A were obtained from Worthington Biochemicals Corp. Restriction endonucleases were purchased from Miles Laboratories, Inc. [methyl-3H]thymidine (18.5 Ci/mmol), [14C]thymidine (59 mCi/mmol), and [U-14C]leucine (324 mCi/mmol) were purchased from the Radiochemical Centre, Amersham, England.

**Bacterial strains.** The strains used were: E. coli B/r strain LEB18 F' lacZ str, obtained from P. A. Meacock; E. coli K-12 strain P162-8, F' leu thr thi thyA dnmA rma and E. coli 15'T strain PA718 arg met thyB (low requiring) obtained from R. H. Pritchard (34); and E. coli K-12 strain GI 1000 F' leu thr thi thyA deoB (λφ857), obtained from B. M. Wilkins and used as a source of labeled λ.

**Growth conditions.** Cells were grown in M9-glucose minimal medium. Auctinfic strains were supplemented with the required amino acids and with thymine (40 μg/ml) and thiamine (10 μg/ml). In certain cases, to facilitate sporulation conditions, PPB medium (0.1% beef extract [Difco], 1% peptone, and 0.5% NaCl) was used. We added 5 ml of this PPB stock to 100 ml of M9-glucose minimal medium.

**Labeling conditions.** Normally, 10-ml cultures were grown for several generations in steady-state exponential growth to an absorbance at 450 nm of 0.7. For long-term labeling, 50 μCi of [3H]thymidine per ml (18.5 Ci/mmol) was included in the medium, and uridine (final concentration, 1.5 mM) was added as a competitive inhibitor of thymidine phosphorylase. In pulse-labeling experiments, uridine was omitted and cultures (absorbance at 450 nm, 0.7) were mixed with 50 to 90 μCi of [3H]thymidine per ml for the appropriate period. Pulse labeling was terminated by adding a double volume of cold cyanide buffer [10 mM tris(hydroxymethyl)aminomethane (Tris), 10 mM NaCl; pH 7.0].

In long-term double-labeling experiments in which membrane proteins were also labeled, cold leucine (5 μg/ml) and 4 μCi of [14C]leucine per ml (324 mCi/mmol), together with 50 μCi of [3H]thymidine and 1.5 mM uridine, were added to the medium. In all labeling experiments, radioactive cultures were mixed with cold carrier cells (grown in identical conditions) in a ratio of 1:5 before envelope preparation.

**Preparation of DNA-membrane complexes.** Cells labeled as described and harvested in an absorbance at 450 nm of 0.7 were collected by centrifugation at 2,000 × g at 4°C for 15 min, suspended in 0.75 M sucrose–10 mM Tris–hydrochloride (pH 7.8), and treated with lysozyme-ethylenediaminetetraacetic acid (EDTA), under optimal conditions exactly as described by Osborn et al. (30). With this method, almost 100% of the cells yielded spheroplasts. Spheroplasts were gently broken by the addition of 4 volumes of cold distilled water. The lysate obtained was centrifuged twice at 3,000 × g for 10 min at 4°C to sediment any remaining whole cells. The clarified lysate was then spun at 40,000 × g at 4°C for 30 min to sediment the envelopes. The envelopes were usually washed in TM buffer (10 mM Tris, 1 mM MgSO4, 7H2O; pH 7.0). Alternative buffers TE (20 mM Tris, 1 mM EDTA; pH 8.0), or TEK (20 mM Tris, 1 mM EDTA, 0.1 M KCl; pH 8.0) were also used, but the yield of DNA recovered was similar in all cases. The washing was carried out two or three times with centrifugation at 40,000 × g to pellet the envelopes. Between washings, the DNA-membrane complexes were resuspended by using a glass rod and approximately 30 s of blending in a Vortex mixer. The final DNA membrane pellet was usually resuspended in a small volume (0.2 ml) of the wash buffer.

**DNase treatment of lysates.** The clarified lysate, containing 5 mM MgSO4, 7H2O, was treated with DNase I (final concentration, 5 μg/ml). The mixture was incubated at 37°C until solubilization of DNA ceased (20 min); 5 mM EDTA was then added to stop the reaction. The DNase-treated lysate was then processed as the untreated preparation described above.

**Separation of DNA-membrane complexes in isopycnic sucrose gradients.** DNA-membrane complexes were sedimented on isopycnic sucrose gradients as described by Osborn et al. (30). Gradients (13 ml) were spun at 35,000 rpm in an SW40.1 rotor in a Beckman ultracentrifuge for 18 h at 4°C; 15-drop fractions were collected from the bottom of the tubes and counted.

In experiments in which individual peaks were required from the gradient for further identification or recentrifugation, a quantitative method was employed in which the ratio of carrier cells to labeled cells was increased to 30:1. The visible individual bands were then collected from the top of the gradient with a syringe, diluted in TM buffer, and centrifuged to pellet form at 40,000 rpm in a 50 Ti rotor. The polyacrylamide content of different membrane fractions was analyzed by slab gel electrophoresis in the sodium dodecyl sulfate–acylamide system described previously (8, 24).

**Molecular weight determination.** Membrane complexes were adjusted to a final concentration of 0.5% sodium dodecyl sulfate and incubated for about 20 min at room temperature. For low-molecular-weight DNA, E. coli RNA was used as a marker. DNA preparations were layered onto linear 2.5 to 15% (wt/vol) sucrose gradients (in TM buffer); a 62% (wt/vol) sucrose (0.2 ml) shelf was included at the bottom of the tube. Gradients were spun at 45,000 rpm in an SW 50.1 rotor for 2.5 h at 4°C.

For high-molecular-weight DNA, λ [14C]DNA or colicin E1 DNA was used as standard, and the DNA preparations were layered onto linear 5 to 20% (wt/vol) sucrose (in 0.1 M NaCl, 0.01 M Tris, 0.01 M EDTA buffer; pH 8.0) with a 62% (wt/vol) sucrose (0.2 ml) shelf. Gradients were centrifuged at 37,000 rpm in an SW50.1 rotor for 2 h at 20°C and fractionated, and molecular weights were calculated (14).

**Base analysis of labeled membrane DNA.** The labeled DNA-membrane complexes (1 ml in TM buffer) were mixed with 10 mg of calf thymus DNA
and freeze-dried; 0.2 ml of HClO₄ was added to the lyophilized extracts, and the solutions were heated at 100°C for 60 min. The mixtures were diluted to 1.0 ml with water and homogenized, and the clear solution was separated from the particulate residue by centrifugation; 20-µl samples were then applied to thin-layer chromatography plates (Schleicher & Schuell Co., PE1-cellulose plates with luminescer), and the bases were separated by an isopropanol-hydrochloride solvent system (5).

**Alignment for chromosome replication.** Steady-state, exponentially growing cells were filtered through a membrane filter (0.45 µm; Sartorius), washed thoroughly with M9 buffer, and suspended in prewarmed medium lacking the required amino acids; incubation was continued for 90 min to complete ongoing rounds. The culture was again filtered, washed, and suspended in prewarmed medium containing the required amino acids but lacking thymine, and incubation continued until a mass doubling was attained. Synchronous reinitiation occurred immediately upon restoration of thymine, and the cultures were pulse-labeled at this time with [³H]thymidine (for amounts see Results) and also after a further 30 min of incubation.

**Restriction enzyme analysis.** [³H]thymidine-labeled DNA-envelope complexes were digested with EcoRI or HinIII endonuclease for 30 min at 37°C as described by Greene et al. (17). Digested samples were subjected to electrophoresis in 0.9% agarose horizontal slab gels by the method of McDonell et al. (27) and, finally, the [³H]-labeled bands were detected by fluorography as described by Laskey and Mills (25).

**RESULTS**

Isolation of membrane associated with DNA on sucrose equilibrium gradients. By using the procedure described by Osborn et al. (26), it is possible to separate various outer and inner membrane fractions of the envelopes on a density basis (Fig. 1). This procedure yields a variable (strain-dependent) number of distinct envelope fractions, usually assigned as heavy (H) or light (L), which Osborn et al. demonstrated to correspond to outer and inner membranes, respectively. The polypeptide content of inner and outer membranes is quite distinct as shown in Fig. 2 (2, 24) and, by using this criterion for *E. coli* B/r, we identified two outer membrane (H1 and H2) and two inner membrane (L1 and L2) fractions (24). The basis for the separation of the outer membrane into two fractions is not known but may reflect differences in lipopolysaccharide content or greater contamination of H2 by inner membrane fragments. No significant levels of unseparated envelope material (fraction M), as judged by polypeptide content, are visible in gradient profiles from *E. coli* B/r, although such a fraction is present in *E. coli* K-12 envelope profiles (24; see Fig. 8).

When exponential cultures of *E. coli* B/r were labeled for several generations with radioactive thymidine and the washed envelopes were fractionated on discontinuous sucrose gradients, all heavy and light bands were found to contain labeled DNA (Fig. 1). The amount of DNA recovered in the envelope fraction varied between 0.3 and 0.95% of total DNA, and this was consistently distributed approximately equally between the heavy and light fractions. When cell lysates were treated with DNase, a small amount of DNA was still recovered in the envelope fraction, and the major portion of this material was associated with the heavy membrane fractions (Fig. 1b).

Control experiments showed that unbroken *E. coli* cells sedimented to the bottom of the gradient (Fig. 1), whereas free, unbound DNA normally sedimented to a position close to L1.
Distribution of pulse-labeled DNA in envelope fractions. Exponential cultures of E. coli B/r were briefly pulse-labeled with thymidine, and envelopes were isolated and fractionated on sucrose equilibrium gradients. As shown in Fig. 3a, the distribution of labeled DNA was very similar to that obtained after long-term labeling, as was the amount of labeled DNA (as a percentage of total labeled thymidine incorporated during the pulse) recovered in the envelope under these conditions. This result indicates that very little DNA actually present in replicating forks was recovered in the envelope isolated by our procedure. This is largely confirmed by the pulse-chase experiment described in Fig. 3b. These results show that the bulk of pulse-labeled DNA recovered remained associated with the envelope during the subsequent 15-min chase. Inspection of Fig. 3b, however, does show that some DNA was preferentially chased from the inner membrane fraction L1, whereas DNA in the outer membrane fraction was particularly stable during the chase period.

Stability of membrane-DNA complexes. Isolated DNA-envelope complexes from E. coli B/r were disrupted by homogenization with a glass homogenizer, resulting in the release of free DNA particularly from the lighter, inner membrane complexes. In contrast, DNase-resistant

This low density of free DNA is to be expected in gradients lacking salt (7), and we have confirmed that both E. coli DNA and λ DNA float in a broad band near the top of the gradient even after centrifugation for 36 h. Other control experiments (data not shown) showed that the great majority of the DNA sedimented with the envelope fractions on Urografin gradients, a procedure which readily separates bound DNA from free forms of DNA (23). We conclude, therefore, that the equilibrium sucrose gradient technique provides a reliable method for the analysis of those DNA fractions which form some kind of association with envelope components.

![Figure 2](http://jb.asm.org/)

**Fig. 2.** Analysis of the polypeptide content of membrane fractions. Outer and inner membrane fractions were first isolated from a discontinuous sucrose gradient similar to that shown in Fig. 1. Samples were then subjected to electrophoresis in the presence of sodium dodecyl sulfate in a 10% acrylamide slab gel and finally stained with Coomassie brilliant blue.

![Figure 3](http://jb.asm.org/)

**Fig. 3.** Stability of pulse-labeled DNA in complexes. An exponential culture of E. coli B/r was pulse-labeled with [3H]thymidine for 30 s at 30°C. (a) From half the culture, envelopes were immediately isolated and analyzed as described in the legend to Fig. 1. (b) Remaining culture was immediately filtered to remove isotope and was chased in fresh, unlabeled medium for 15 min at 30°C before isolation of envelopes.
DNA, which associates primarily with the outer membrane fraction, was resistant to this treatment (data not shown). All of the DNA was apparently released from the complex by treatment with Triton X-100, a detergent which disrupts both inner and outer membranes in this organism (Fig. 4c). When complexes were isolated from lysates treated with Sarkosyl (a detergent which solubilizes inner but not outer membranes [13, 8]), an H-DNA complex was isolated apparently intact, whereas other DNA complexes were disrupted (Fig. 4b). Similar results were obtained when the DNA-envelope complexes were first isolated and then treated with Sarkosyl. It was not possible to positively identify the outer membrane fraction in Fig. 4b as H1 or H2 because density changes due to some loss of phospholipid could occur during Sarkosyl treatment.

Properties of DNA associated with outer membrane fractions of E. coli B/r. The relative instability of DNA-inner membrane complexes indicated above was further confirmed when L1 and L2 complexes isolated from the Osborn gradient were recentrifuged to equilibrium after being layered on a continuous sucrose gradient. Very little DNA remained associated with the sedimenting membrane fraction in these experiments (Fig. 5b and c); most of it remained behind as free DNA at the top of the gradient. The DNA in L1 and L2 complexes had a molecular weight of about $5 \times 10^6$, corresponding to a maximum of three bound DNA sites per chromosome (as calculated in Table 1). After release from the membrane by detergent, L1- and L2-DNA banded with a density of 1.72 g/cm$^3$ on CsCl gradients.

Properties of DNA associated with outer membrane fractions of E. coli B/r. The majority of DNA associated with H1 (as shown in Fig. 5a) still sedimented coincidentally with the membrane fraction when this material was recentrifuged to equilibrium on a 40 to 55% linear sucrose gradient. Results with the H2 fraction were variable, but in some experiments the majority of the DNA appeared free at the top of the gradient after recentrifugation (data not shown). The labeled material extracted from both H1 and H2 banded with a density of 1.72 g/cm$^3$ on CsCl gradients. H2-DNA had an average molecular weight of $1.5 \times 10^6$ to $3.5 \times 10^6$, whereas H1-DNA appeared smaller and more heterogeneous, with some major species having molecular weights of $6 \times 10^5$ or $2.5 \times 10^5$ (Table 1).

DNase resistance of H1-DNA. Specific attachment of the bacterial chromosome to the surface envelope might be expected to provide protection to at least some regions of the DNA against the action of added DNase. Thus, clarified lysates prepared as for the experiment of Fig. 1 were treated with DNase I until essentially no further solubilization of DNA could be detected. Under these conditions, the total amount of DNA recovered in the envelope fraction varied widely (between 5 and 50% of that recovered without DNase treatment). Nevertheless, in all experiments H1-DNA was enriched two- to threefold after the addition of DNase, compared with other envelope DNA fractions. Thus, H1-DNA constituted at least 50% of the envelope DNA after DNase treatment (Fig. 1b).

The amount of label recovered in the H1 fraction after DNase digestion of lysates was in some cases as little as 0.03% of the lysate DNA. Nevertheless, this material was confirmed as DNA, since more than 80% of the label was rendered acid soluble by further treatment with DNase after removal of membrane material by detergent. Furthermore, more than 95% of the recovered tritium label in acid hydrolysates was shown to co-chromatograph with thymine in silica thin-layer plates (5). The molecular weight of the DNase-resistant fraction from H1 was shown to be approximately $2.2 \times 10^6$, or about one-tenth the size of fragments from untreated...
FIG. 5. Resedimentation of isolated complexes. Labeled H1-DNA and L1· and L2-DNA complexes first collected from sucrose gradients similar to that shown in Fig. 1a. The H1 complex was pelleted and recentrifuged to equilibrium after being layered on a 40 to 55% linear sucrose gradient; (b) L2 fraction recentrifuged on a 25 to 40% linear sucrose gradient; (c) L1 fraction rerun on 25 to 40% sucrose. The density (grams per cubic centimeter) of the membrane complex is indicated beside each curve.
complexes. This corresponds to an average fragment size of approximately 36 base pairs for a double-stranded DNA molecule. This result indicates that, although the DNA associated with the H1 envelope fraction in the bacterial lysate was in fact accessible to endonuclease cleavage, only limited digestion took place, and the resulting small fragments remained firmly bound to some envelope component.

Analysis of envelope-DNA complexes in 

**Table 1. Calculated number of DNA-binding sites associated with various membrane fractions**

<table>
<thead>
<tr>
<th>Membrane fraction*</th>
<th>[3H]-thymidine-labeled DNA (% total in lysate)</th>
<th>Binding sites per some complexes.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L1</td>
<td>0.07</td>
<td>6.5 x 10^6</td>
</tr>
<tr>
<td>L2</td>
<td>0.15</td>
<td>4.7 x 10^6</td>
</tr>
<tr>
<td>H2</td>
<td>0.15</td>
<td>3.0 x 10^6</td>
</tr>
<tr>
<td>H1</td>
<td>0.18</td>
<td>6.0 x 10^5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.5 x 10^5</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L1 plus L2</td>
<td>0.07</td>
<td>10^6</td>
</tr>
<tr>
<td>H2</td>
<td>0.02</td>
<td>1.5 x 10^5</td>
</tr>
<tr>
<td>H1</td>
<td>0.02</td>
<td>3.0 x 10^5</td>
</tr>
<tr>
<td>Total envelope DNA</td>
<td>1.0</td>
<td>1.7 x 10^5</td>
</tr>
<tr>
<td>Total envelope DNA (aligned culture)</td>
<td>4.0</td>
<td>1.7 x 10^5</td>
</tr>
</tbody>
</table>

* The molecular weight and the yield (percentage of total DNA) of each DNA species was determined after release from membrane fractions. Assuming the average molecular weight of the partially replicated E. coli chromosome to be 3.9 x 10^6, we determined the number of fragments of DNA and hence the apparent number of binding sites per chromosome in each complex.

* Experiments were with E. coli B/r and were conducted in a manner similar to that described in Fig. 1.

* In this experiment, H1-DNA consisted of two major fractions: molecular weight 6 x 10^6 constituted about 20% and that of 2.5 x 10^5 constituted about 50% of the total.

* Isolated from E. coli K-12 P162-8 grown exponentially for several generations in the presence of [3H]thymidine.

* Envelope DNA recovered from E. coli K-12 P162-8 and pulse-labeled during initiation of an aligned culture. In this case, more than 80% of the label sedimeted with the H-fractions on discontinuous sucrose gradients. The recovery data (4%) are the average of 14 experiments.

**E. coli K-12 synchronized with respect to DNA replication.** Previous workers (12, 31) have reported some evidence for the binding of origin DNA to unfractionated surface membranes of E. coli. In addition, Gomez-Eichemann and Bastarrachea (16) recently reported the association of the chromosomal origin with an inner membrane fraction. Experiments were therefore carried out to determine whether specifically labeled origin DNA was preferentially bound to either the inner or the outer membrane fractions. Preliminary experiments to align the chromosomes of E. coli B/r strains were unsuccessful; the results have indicated that termination of DNA replication, induced by amino acid starvation, is abnormal in these strains (unpublished data; 35). E. coli K-12 P162-8 was chosen as an alternative, and control experiments demonstrated that exponential cultures grown in M9 glucose medium and starved of required amino acids gave an approximately 50% increment of DNA before replication ceased. After a subsequent period of thymine starvation, an apparently synchronous reinitiation of DNA synthesis could be observed upon restoration of thymine (Fig. 6). The bacteria are not synchronized with respect to size by this procedure and, therefore, after the initial burst of initiation, the rate of DNA synthesis continued to increase exponentially as progressively more and more cells in the population acquired the mass necessary to trigger a second round of replication (Fig. 6, 22, 33). Restriction enzyme analysis of the DNA made during initiation confirmed the presence of unique sequences, and the major EcoRI fragments labeled had molecular weights of approximately 9 x 10^6 and 6 x 10^6, similar to the origin fragments of 8.2 x 10^6 and 5.7 x 10^6, described by Marsh and Worcel (28), in cultures aligned by an identical procedure. Our results (Fig. 7) were obtained by analysis of labeled DNA still bound to envelope material, in which case some EcoRI sites may have been masked. This may explain the absence of another major band of about 7.2 x 10^6 daltons described by Marsh and Worcel (28). Nevertheless, our results further demonstrate the accessibility of large regions of the bound DNA to added DNase.

Using such aligned cultures of E. coli K-12, we pulse-labeled cells with [3H]thymidine for the first 30 s after restoration of thymine (origin pulse) and again for 30 s after a further 30 min of DNA synthesis (late chromosome pulse). Envelopes were isolated and subfractionated on sucrose equilibrium gradients and gave a normal complement of inner and outer membrane protein fractions (data not shown). In contrast, the overall distribution of [3H]thymidine label in
samples labeled at both early and late times showed the great majority of label in the outer membrane fractions (Fig. 8d). In addition however, it is clear that there was a fourfold enrichment for that DNA (expressed as the percentage of the labeled thymidine incorporated into the culture) recovered in association with the outer membrane which was labeled during initiation (as compared with DNA labeled at a later time). In some experiments (Fig. 8b), origin DNA appeared especially enriched in the H1 fraction, compared with H2, but this effect was not always obtained. Similar overall results were obtained with an aligned culture of two other E. coli strains, and, in a total of 10 experiments with strain E. coli K-12 P162-8, the enrichment factor varied between two- and eightfold. These experiments included one in which, after pulse labeling of the origin, a chase period was inserted so that envelopes could be isolated from bacteria of the same physiological age as those labeled late in the replication cycle. Finally, exhaustive washing of the envelopes from the aligned cultures was required to remove nonorigin-labeled DNA, thus leaving the outer membrane fractions enriched for origin DNA (Fig. 8c and d).

**DISCUSSION**

DNA-membrane complexes isolated from exponential cultures by the procedure described above contained 0.3 to 1% of the total DNA, corresponding to one to three envelope-bound fragments per bacterium. In E. coli B/r LEB18, this DNA was distributed about equally between inner (L2, L1) and outer (H2, H1) membrane fractions; in E. coli 15T- and E. coli K-12 P162-8, 80 to 90% of the envelope DNA fractionated with the outer membrane. In our study (see also 24), outer membranes separated on discontinuous sucrose gradients into at least two fractions, H1 and H2, with approximate densities of 1.24
and 1.22 g/cm³, respectively, and another fraction (density, 1.25 g/cm³) appeared occasionally. Both H1 and H2 appeared to contain an identical set of polypeptides, comparable to outer membrane profiles published by others (2, 24).

The strategy adopted in this study was to minimize nonspecific binding of DNA to envelope material by avoiding detergents, excessive amounts of lysozyme (39), or sonic oscillation to break open cells. Exhaustive washing was also used to remove loosely associated DNA. This step was found to be especially important for obtaining envelopes enriched for origin DNA. Great care was taken to separate any unbroken cells from the complexes, and the presence of DNA in all complexes was confirmed by several methods in addition to its identification by labeled thymidine. Finally, by avoiding detergents we hoped to maximize the recovery of membrane-associated DNA. The strategy adopted appears to have been justified by the consistent finding that origin-labeled DNA was enriched two- to eightfold in the isolated envelope. This result, together with the accessibility (albeit restricted to small oligonucleotide segments in the case of the H1 fraction) to added DNase I, appears to rule out gross trapping of nonspecific DNA fragments in membrane vesicles which may have been present in either the inner or the outer membrane fractions.

The isolation of a membrane-bound origin DNA confirmed the previous findings of Parker and Glaser (31), who used quite different procedures to demonstrate origin DNA in whole envelope fractions. However, our results, which demonstrate the close association of origin DNA with outer, rather than inner membranes, was unexpected and in complete contradiction to the results recently reported by Gomez-Eichelmann and Bastarrachea (16). Nevertheless, we confirmed this result in many experiments and with three different bacterial strains. These results are also consistent with the observation that, in a dnaA mutant that is defective in initiation of DNA replication, DNA can be recovered in the outer membrane after DNA synthesis at permissive temperatures but not at the nonpermissive temperature (E. Orr and V. Darby, personal communication). In addition, Doyle et al. (9) have reported that the origin of the chromosome of B. subtilis may be bound to the peptidoglycan skeleton, external to the cytoplasmic membrane. Although we cannot explain the reason for the different results obtained by Gomez-Eichelmann and Bastarrachea, we suggest that a crucial factor may be the ability of the alignment technique to label specifically the origin DNA. We found that efficient synchronization of DNA replication by the alignment technique was strain dependent and that the conditions used had to be carefully established for a given strain. In the case of strain P162-8, the kinetics of DNA synthesis, both during and immediately following alignment, clearly indicated that synchronous initiation was obtained. In addition, analysis by restriction enzymes of the DNA made after synchronization showed that a unique section of the chromosome was replicated coincidentally with initiation. Strain P162-8 has been thoroughly characterized in this laboratory over several years, and there is no evidence for the presence of plasmids or inducible prophages in this strain. Therefore, we conclude that the unique sequences detected did occur in the bacterial chromosome.

Quantitative analysis of the DNA labeled during the first 30 s of initiation and recovered in envelope fractions showed that the average yield (4% of total DNA labeled in a 30-s pulse over 14 experiments; average molecular weight, 1.5 × 10⁶) represents only 10% of that estimated to be present in vivo (Table 1). This apparently low recovery from aligned cultures of strain P162-8 suggests that the bulk of outer membrane DNA isolated from exponential cultures may not be origin DNA. Replication fork DNA did not appear to be present, but nonspecific fragments cannot be ruled out. Some of this DNA might also represent the remnants of the class of 20 or so chromosome-membrane-binding sites described previously by several groups (10, 32, 36).

Two points concerning the properties of DNA-membrane complexes from exponentially growing cultures require further elaboration. First, the extreme lability of inner membrane complexes suggests that, if they exist in vivo, DNA binding to the complexes is weak and readily dissociated, as suggested previously for mem-

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**FIG. 8.** Analysis of DNA-envelope complexes after chromosomal alignment. A culture of E. coli K12 P162-8, aligned as shown in Fig. 6, was pulse-labeled with [³H]thymidine at 37°C during the first 30 s of reinitiation and pulsed again after an additional 30 min. Pulse-labeling was terminated as described in the text, in two separate experiments (a and b). The envelopes were isolated and analyzed on sucrose equilibrium gradients as described in the legend to Fig. 1. (c) Envelope material in TK buffer was centrifuged for only 6 h and after only one high-speed spin; (d) as in (c), but envelopes were washed twice more in TK buffer before centrifuging. The data are presented as superimposed plots of origin-labeled material (——) and of envelope DNA, labeled 30 min after reinitiation (-----). The inset shown in (d) is the envelope profile for strain P162-8 after pulse-labeling of an exponential culture with [³H]thymidine. For this strain, the densities of membrane fractions are (grams per cubic centimeter): H1, 1.25; H2, 1.23; M (unseparated envelope), 1.2; L2, 1.18; and L1, 1.15.
brane-attached replication forks (19). Second, the relative resistance of outer membrane DNA to endonuclease I with the protection, at least in H1-DNA complexes, of bound oligonucleotide fragments of about 36 base pairs is consistent with the binding of the DNA to membrane polypeptides.

Finally, we would like to emphasize that the coincident banding of origin DNA in density gradients with outer membrane fractions does not rule out the possibility that the origin is bound to the outer membrane via an area of adhesion between inner and outer membranes (4). This possibility has also been proposed by Heidrich and co-workers (21, 29) and by Portalier and Worcel (32). More specifically, these workers have provided evidence for the presence of an 8 \times 10^4-dalton polypeptide at the DNA-membrane binding site, which they have suggested is identical to protein D, an envelope protein reported to be synthesized at one specific time in the cell cycle of E. coli (8, 18). However, this polypeptide (molecular weight, 8.1 \times 10^4) has recently been identified as the feuB gene product, an inducible, iron-binding protein in the outer membrane (6). The previously observed periodic mode of synthesis of this protein during the cell cycle is probably an artifact (6) and, moreover, mutants lacking this protein appear to grow normally (20). Isolation of the adhesion zones themselves may now be required to identify any polypeptides specifically involved in binding the origin or any other region of the chromosome to the membrane.

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