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Direct Evidence for Specific Binding of the Replicative Origin of the *Escherichia coli* Chromosome to the Membrane

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The origin of replication of the *Escherichia coli* chromosomal DNA binds with high affinity to outer membrane preparations. This specific binding requires a 463-base-pair region of origin DNA between positions −45 and +417 of the *oriC* map. We show that binding does not require the presence of adjacent regions. From further analysis, we conclude that more than one binding site resides within the 325-base-pair fragment between positions +38 (*Bam*HI) and +417 (*Xho*I). When this fragment is cut, two pieces bind with high affinity and one binds with lesser affinity. The binding ability of one of the high affinity sites is abolished by cutting it at position +92 with *Bam*HI.

It was recently reported that origin DNA from the *Escherichia coli* chromosome (5) or from a *Bacillus subtilis* plasmid (8) binds specifically to purified membranes. This has helped add credibility to the conjecture that DNA in bacteria is bound to the membrane. Work from this laboratory has shown that when *E. coli* DNA is cut with restriction enzymes and added to membranes in the presence of an excess of heterologous competitor DNA, a single restriction fragment is preferentially bound (5). This fragment contains the origin of *E. coli* chromosome replication, *oriC*. The DNA used in these experiments was prepared by CsCl density gradient centrifugation of initiation complexes previously isolated by fractionation on sucrose gradients. We showed that these preparations contain two unidentified proteins bound to the DNA. Their apparent molecular weight is 55 and 75 kilodaltons (5). Deproteinization decreased the binding ability of the DNA considerably.

In our previous work, we reported that specificity of binding resides within 463 base pairs (bp) of the *oriC* region. Here we present evidence that this fragment alone, without flanking DNA, is capable of membrane binding. In addition, we show that this fragment contains two, or possibly three, distinct binding sites.

MATERIALS AND METHODS

Cell strains and media. All bacterial strains are derivatives of *E. coli* K-12. A temperature-sensitive initiation mutant, PC-2 (*dnaC*(Ts) *leu thy* Str^r) was provided by Y. Hirota. Strain JF411 (*galE mtl lacY* Tsx^r *glmS argE proA* B1 Str^r [λY199, λ d*glm411*]) was a source of phage λ411 and was provided by J. Felton and A. Wright of our department. Strain CM993 (carrying pCM959) was generously provided by K. von Meyenburg.

Mutant PC-2 was grown at 30°C in M9 minimal medium containing 0.2% glucose supplemented with 20 μg of leucine, 0.4 μg of thiamine, and 2 μg of thymine per ml. Strain JF411 was grown at 30°C in L broth plus 0.2% glucose. Strain CM993 was grown in M9 minimal medium supplemented with 15 μg of thiamine per ml.

Isolation of origin complexes. The procedure has been described in detail previously (9). A brief description follows. To isolate the origin complexes, mutant PC-2 cultures were grown at 30°C until the optical density reached 0.3, and then chromosome replication was allowed to terminate by treatment for 100 min at the nonpermissive temperature (40°C). Cultures were shifted back to 30°C to allow the initiation of DNA replication for 1.5 min. Cells were harvested, washed, and resuspended in TKE buffer (20 mM Tris-hydrochloride [pH 7.2], 0.1 M KCl, 1 mM Na_2-EDTA). Cells were disrupted by passage through a French pressure cell, intact cells were removed by low-speed centrifugation, and membrane material was pelleted by centrifugation at $2 \times 10^5 \times g$ for 45 min. The resulting material was resuspended in TKE and layered on 30 to 50% (wt/wt) sucrose linear gradient cushioned by 60% (wt/wt) sucrose. After centrifugation at 150,000 × *g* for 2.5 h, DNA was found in three fractions: at or near the shelf, in the middle of the gradient, and near the top. The material above the cushion is termed origin complex H and is the starting material in most of the following experiments. The fraction in the middle of the gradient, sedimenting at 250 to 350S, is termed origin complex L.

CsCl purification of DNA. Origin complex H or L was dialyzed overnight against TKE buffer, and the volume was reduced to 1 ml by dialysis against polyethylene glycol ($M_w \simeq 20{,}000$ daltons). Complexes were layered on 4.2 ml of 1.63 g of CsCl per ml of TE buffer (20 mM Tris [pH 7.2], 1 mM Na_2-EDTA). After centrifugation for 24 h at 35,000 rpm in a Beckman SW 50.1 rotor, outer membrane material was removed from the top of the gradient, and the DNA band was removed by dripping from the bottom. These fractions were dialyzed against two changes of TNE (20 mM Tris [pH 7.4], 50 mM NaCl, 1 mM Na_2-EDTA) or TE buffer.

Isolation of plasmid and phage DNA. Cells of strain CM993 were harvested at an optical density of 0.9 at 600 nm. pCM959 DNA was isolated by the alkaline-sodium dodecyl sulfate procedure of Birnboim and Doly (1). As a size marker we used, among others, DNA from λ411, an *oriC*-containing phage. The isolation procedure of this phage was described previously (5). Its DNA was purified by CsCl centrifugation after treatment of phages with 1% sodium dodecyl sulfate

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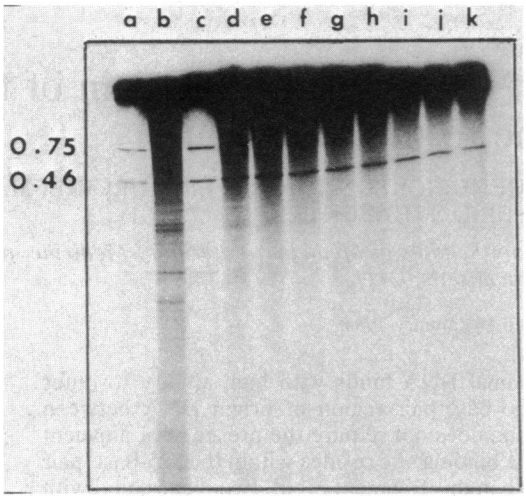

FIG. 1. Filter binding of *AvaI* restriction fragments from the whole *E. coli* chromosome. Origin complex H was used to prepare CsCl DNA. This DNA was restricted with *AvaI* and its 5′ termini were labeled. It was then incubated for 60 min at 4°C with 6 μg of protein equivalents of outer membrane in the presence of different amounts of calf thymus DNA. After filter binding, eluates from the filters were applied to a 5% acrylamide-0.5% agarose gel and electrophoresed. Lanes a and c, λ411 DNA cut with *AvaI* and end labeled. Lanes b and d through k, Origin complex H CsCl DNA cut with *AvaI* and end labeled. Lane b, Control, without filter binding. Lanes d through k: Filter binding in the presence of calf thymus DNA: (d) 0, (e) 2, (f) 5, (g) 10, (h) 20, (i) 50, (j) 100, and (k) 200 μg/ml, respectively.

and 50 μg of proteinase K per ml (37°C for 60 min) followed by phenol extraction.

Restriction endonuclease digestion. Samples were digested at 37°C for 60 min, using the buffer suggested by the suppliers. For the complete digestion, a 10-fold excess of restriction enzyme was added. In the case of combined digestions, all enzymes were simultaneously added in a buffer of 70 mM Tris (pH 7.6), 50 mM NaCl, and 6 mM $MgCl_2$, unless described otherwise. In the case of the *SmaI* and *XhoI* combination, CsCl DNA was first digested with *SmaI* in the appropriate low-salt buffer, and then Tris and NaCl were brought up to 50 and 100 mM, respectively, and the digestion continued with *XhoI*.

The resulting DNA fragments were analyzed by electrophoresis on 1.2% agarose gel in E buffer (11) or on 5% acrylamide-0.5% agarose gel in Tiollais buffer (see details below).

Acrylamide-agarose gel electrophoresis. Acrylamide-agarose gel electrophoresis was carried out as follows: 10% acrylamide (acrylamide-bisacrylamide, 20:1) in doubled Tiollais buffer (40 mM Tris, 20 mM sodium acetate, 2 mM Na_2-EDTA [pH 8.0] was heated to 60°C under vacuum. An equal volume of 1% agarose solution was added, and the mixture was degassed once again. A 40-μl protein of a freshly prepared ammonium persulfate solution (15 mg/ ml) and 7 μl of *N,N,N′,N′*-tetramethylethylenediamine were added, and the gel was poured immediately.

^{32}P-end labeling of the restriction pieces with Klenow fragment. Fragments were labeled at their termini by the method of Brown and Smith (2). DNA was dissolved in the followng solution: 35 mM Tris-hydrochloride (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol, 70 mM NaCl, and 50 μM each dATP, dGTP, and dTTP. One unit of Klenow fragment (New England Biolabs, Inc.) and 25 μCi of [α^{32}P]dCTP (600 to 800 Ci/mmol) were added to initiate the labeling reaction. Incubation was performed at room temperature for 60 min. Unincorporated nucleotides were removed from labeled DNA by passage through a Sephadex G-50 minicolumn equilibrated with TNE buffer.

Filter binding assay. Filters (6-mm) were punched out of BA85 nitrocellulose paper (Schleicher & Schuell, Inc.). Nonspecific binding of DNA to the filters was reduced to less than 1% of the input by boiling the filters in distilled water for 15 min and then soaking them in TNE plus 5 mM $MgCl_2$ for at least 30 min before use.

Standard incubation volumes were 50 μl. Mixtures contained ^{32}P-labeled DNA (350 to 450 ng/ml), calf thymus DNA, 5 mM $MgCl_2$, and 4 to 6 μg of membrane protein (BioRad assay) in TNE buffer. After adding the outer membrane (from the top of the CsCl gradients), incubation was carried out on ice for 60 min. Mixtures were then passed slowly through filters, at a rate of 50 μl/30 s. Filters were washed with a total volume of 100 μl of TNE plus 5 mM $MgCl_2$. Filters were then placed in a 0.5-ml microcentrifuge tube with a hole in the bottom and incubated for 60 min at room temperature in 20 to 25 μl of 20 mM Tris (pH 7.2)–0.2% Sarkosyl. Tubes containing the filters were then placed inside 1.5-ml microcentrifuge tubes, and the DNA-containing solution was removed by centrifugation for 1 min. For electrophoresis, the sample was adjusted to 2% Ficoll–50 mM NaCl–0.0025% bromophenol blue.

Densitometry of autoradiographs. Autoradiographs obtained were copied on Kodak duplicating film, and the sample tracks were cut into strips. Density of the bands was determined with an LKB Instruments, Inc., scanning densitometer.

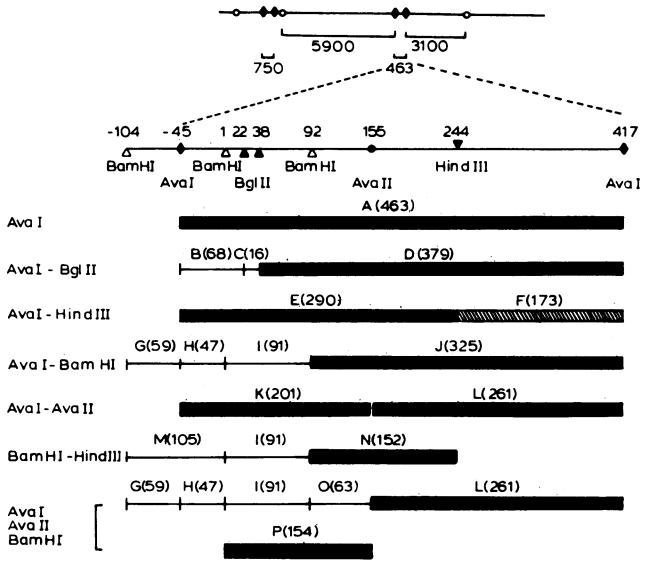

FIG. 2. Restriction map of the *E. coli oriC* region. This region and some of its restriction sites are redrawn from several papers (10, 12, 13). Restriction endonuclease cutting sites between −110 and +420 are shown. Some of the pieces expected from combination of digestion with more than one enzyme are also depicted. Heavy black boxes, strongly binding fragments. Hatched box, weakly binding fragment. Lines, nonbinding fragments. Symbols: ○, *Eco*RI site; ◆, *Ava*I site; △, *Bam*HI site; ▲, *Bgl*II site; ▼, *Hin*dIII site; and ●, *Ava*II site.

RESULTS

The region of DNA from −45 (*Sma*I site) to +417 (*Xho*I site) has been previously shown to be necessary for the specific binding of chromosome origin DNA to the outer membrane (5). In these earlier studies, DNA fragments containing this region were flanked by long stretches of DNA. We now attempted to determine whether this region can bind alone without flanking DNA. The amount of [^{3}H]thymidine incorporated in an in vivo pulse in such small fragments makes it difficult to detect this region on a gel. We therefore end labeled DNA with the Klenow fragment of DNA polymerase I. This method requires little handling and avoids phenol extraction which would remove the protein components of CsCl DNA (5).

DNA used for labeling was prepared from origin complex H by CsCl centrifugation (see above). Although enriched several-fold for the origin region (9), the bulk of this material is DNA from the whole chromosome. Therefore, use of this material allowed the comparison of origin DNA not just with neighboring DNA from the origin region, but also with the rest of the chromosome. The results of such an experiment, with DNA digested with *Ava*I, are shown in Fig.1. Treatment with this enzyme produced a large number of fragments, most of which were not resolved by acrylamide gel electrophoresis. Among several fragments distinct enough to be visible was the 463-bp origin-containing piece. Binding was carried out with 0.35 to 0.4 μg of restricted CsCl DNA per ml. The addition of increasing amounts of calf thymus DNA to the binding mixture resulted in decreased retention of all other fragments, whereas the origin fragment was bound to the same extent in all samples up to a concentration of 120 μg of calf thymus DNA per ml. Thus, calf thymus DNA competes with DNA for nonspecific binding to membranes, as previously reported (5). Enrichment for the origin fragment was not detected when the membrane preparation was omitted from the binding mixture or when it was heated to 80°C for 5 min. Similar results were obtained with DNA digested by a combination of *Sma*I and *Xho*I enzymes, whose cuts result in the same 463-bp fragment (data not shown).

The behavior of the *oriC* fragment could also be compared with that of another specific fragment. Digestion with *Ava*I generated a 750-bp fragment located close to the left side of the origin region. This fragment provides a suitable control because it is similar in size to the *oriC* fragment and can be readily distinguished from other fragments. It did not bind in the presence of calf thymus DNA. From density estimation of autoradiographs exposed for different times, we conclude that the 463-bp *oriC* fragment binds to the membrane at least 100 times more strongly than does the 750-bp fragment or any other fragment from the *E. coli* chromosome.

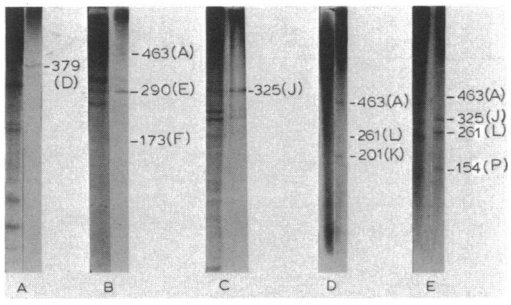

FIG. 3. Localization of binding activity within *oriC* DNA. Membrane binding was carried out as in the legend to Fig. 1 but with DNA restricted with different enzymes. For each pair of lanes, the one on the left shows the control not subjected to membrane binding DNA, and the one on the right shows the DNA bound to membranes in the presence of 100 μg of calf thymus DNA per ml. A, *Ava*I-*Bgl*II; B, *Ava*I-*Hin*dIII; C, *Ava*I-*Bam*HI; D, *Ava*I-*Ava*II; and E, *Ava*I-*Bam*HI-*Ava*II.

TABLE 1. Enzyme analysis results

Fragment	Map coordinates	Membrane binding[a]
A	−45 to +417	+++
D	+38 to +417	+++
E	−45 to +244	+++
J	+92 to +417	+++
K	−45 to +155	+++
N	+92 to +244	+++
P	+1 to +155	+++
L	+155 to +417	+++
F	+244 to +417	+
B	−45 to +22	−
C	+22 to +38	−
G	−104 to −45	−
H	−45 to +1	−
I	+1 to +92	−
M	−104 to +1	−
O	+92 to +155	−

[a] +++, Strong binding; +, weak binding; −, no detectable binding.

Further analysis of DNA binding to the membrane was carried out with various restriction enzymes which cut inside the 463-bp origin fragment. Figure 2 shows the restriction sites of several enzymes in the origin region between bases −104 and +417. The enzyme *Bgl*II cuts at +22 and +38. *Hin*dIII cuts at +244, *Bam*HI at −104, +1, and +92, and *Ava*II at +155. A preparation of CsCl DNA was cut with *Ava*I and labeled with ^{32}P. It was then divided into five samples. One sample was not cut further, the other four were further digested with *Bgl*II, *Bam*HI, *Hin*dIII, and *Ava*II, respectively.

The results of cutting with *Ava*I plus other enzymes are shown in Fig. 3. In each case, a large number of bands was generated from cutting the whole chromosome. Membrane binding in the presence of increasing amounts of calf thymus DNA resulted in decreased binding of all but a few fragments. In each case, fragments that remained bound were from the 463-bp *oriC* fragment. When this fragment was cut with *Hin*dIII at +244, the leftward fragment (E) bound about three times more strongly than did the rightward one (F), as determined by scanning densitometry of autoradiographs. The results of analysis with these enzymes are shown in Table 1. They show that binding of *oriC* takes place within a 379-bp stretch from +38 to +417.

Fragment E, which binds strongly, was studied in greater detail by using fragments generated with *Ava*I and *Ava*II (Fig. 3) and incompletely digested with *Bam*HI. Strong binding was seen with fragment P (+1 to +155). Thus, a strong binding region resides between +38 (*Bfl*II) and +155 (*Ava*II). Cutting at +92 with *Bam*HI impaired the binding of the resulting fragments. Fragments to the left of +92 did not bind. We conclude that the +92 site is essential for binding of this region. On the other hand, fragment N (+92 to +244; *Bam*HI-*Hin*dIII) or L (+155 to +417; *Ava*II-*Ava*I) binds strongly, indicating that there is a second strong binding site to the right of +92. Since fragment O (+92 to +155) did not bind, this region must reside between +155 and +244.

DISCUSSION

We have localized the site of membrane binding of *E. coli* origin DNA to a stretch of several hundred bases. A fragment of DNA containing this region binds at least 100 times more strongly than does any of several thousand fragments generated from the *E. coli* chromosome. We have now shown that the specificity resides within a 379-bp region between positions +38 and +417 on the *oriC* map.

We have also found that, by further cleaving the *oriC* region, we can define three regions capable of binding. Two of these (+38 to +155 and +155 to +244) bind with high affinity, whereas the third, from +244 to +417, binds about three times less strongly. We have further shown that binding of the first fragment is abolished by cutting at +92 with *Bam*HI. The +92 *Bam*HI site is not highly conserved and appears in three out of six *oriC* sequences of the gram-negative bacteria examined by Zyskind et al. (13). Site-specific mutagenesis showed that base deletions or insertions at this site suffice to render *oriC* nonfunctional, whereas several single-base substitutions have no effect (6, 10). These authors conclude that this site is in a region that serves as a spacer rather than as a protein recognition site.

How do our findings correlate with what is known about the interaction of proteins with *oriC* DNA? A protein derived from membrane preparations was shown to bind to two regions of single-stranded *oriC* DNA, between +35 and +270 and between +417 and +488 (7). The function of this B′ protein is not known. The protein product of *dnaA* protein, which is involved in chromosome initiation in vivo as well as in vitro (4), binds specifically to an *oriC* fragment 641 bp in size (3). Cutting at +244 with *Hin*dIII abolishes this binding (4). Thus, at a first glance, binding of B′ protein at its left site may be related to one of our membrane-binding sites. It is not known whether strong binding of *dnaA* protein has any relation to the membrane binding described here.

In previous work, we showed that membrane binding of origin DNA is enhanced by the presence of two peptides (55 and 75 kilodaltons) which remain attached to DNA after treatment with 5.5 M CsCl (5). We are trying to determine the sites of attachment of these proteins on *oriC* DNA.

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