JOURNAL OF BACTERIOLOGY, June 1983, p. 1184–1194
0021-9193/83/061184-11$02.00/0

Vol. 154, No. 3

Site-Specific In Vitro Binding of Plasmid pUB110 to *Bacillus subtilis* Membrane Fraction

TERUO TANAKA† AND NOBORU SUEOKA*

Department of Molecular, Cellular, and Developmental Biology, University of Colorado, Boulder, Colorado 80309

Received 29 November 1982/Accepted 14 March 1983

The in vitro membrane binding of pSL103, a composite plasmid consisting of *Staphylococcus aureus* plasmid pUB110 and a *Bacillus pumilus* $trpC^+$ DNA fragment, to the *Bacillus subtilis* membrane fraction was studied with a total lysate of *B. subtilis* cells. The binding reaction required a heat treatment at 45°C and had an optimum KCl concentration of 60 mM. Nonradioactive pSL103, but not *Escherichia coli* plasmid pACYC184, competed with ^{3}H-labeled pSL103 for binding to the membrane. By the use of ^{32}P-labeled restriction fragments of pSL103 and pUB110, it has been found that only the pUB110 portion of pSL103 binds to the membrane and that there are four specific regions in pUB110 which bind to the membrane. Two of the four binding regions flank the replication origin. This in vitro binding was high-salt sensitive and apparently independent of the configurations of the plasmid. We have previously shown that the functional product of the initiation gene *dna-1* is required in vivo both for replication initiation and the binding of a DNA region near the replication origin to the membrane. Unlike in vivo binding, which is high-salt resistant and dependent on the product of *dna-1* gene (type-I binding), the in vitro binding reported in this paper was high-salt sensitive and independent of the *dna-1* gene product (type-II binding).

The possible implication of the membrane in the regulation of chromosomal and episomal replication has been proposed by Jacob et al. (12), and the association of bacterial nuclear body with the cytoplasmic membrane has been observed by electron microscopy (23). Since then, various reports have appeared on the membrane associations of the replication forks of *Bacillus subtilis* (7) and *Escherichia coli* (25); the replication origins of the *B. subtilis* chromosome (26, 27, 29, 35), the *E. coli* chromosome (4, 13, 20, 21) and the *Pneumococcus* chromosome (5); and the replication terminus of *B. subtilis* (27, 29, 36).

A direct relationship between the membrane association of the replication origin and the initiation of DNA replication has been demonstrated with *B. subtilis* (34), using the temperature-sensitive initiation mutants *dnaBI* and *dnaBII*, which are genetically distinct but closely located (10). Upon temperature shift from the permissive to the nonpermissive temperature, the replication origin DNA of *B. subtilis* was selectively released both in vivo and in vitro from the membrane of two temperature-sensitive initiation mutants, *dna-1* (*dnaBI1*; 32) and *dnaB19* (*dnaBII19*; 14). In contrast, the membrane attachment and replication of a chimeric plasmid, pSL103, between *Staphylococcus aureus* plasmid pUB110 (8) carrying the origin of replication and the $trpC^+$-containing DNA fragment from *Bacillus pumilus* (15), were affected in a *dnaBI* mutant but not in a *dnaBII* mutant (34). Moreover, pSL103 binds to the membrane through the pUB110 portion but not through the $trpC^+$ portion (17).

A direct answer to the question of whether or not the initiation of DNA replication at the origin is regulated by the membrane component might be provided when an in vitro initiation system, including the membrane fraction, is developed. In vitro initiation without the membrane fraction has been achieved in plasmid ColE1 (31), in R6K (11), in plasmid R1, (3), and in a plasmid carrying the replication origin of the *E. coli* chromosome (6). To study the nature of the membrane-replicon complex and the possible effect of the membrane on the in vitro initiation of DNA replication, we have examined the possibility of the in vitro binding of pSL103 to the membrane, and a specific in vitro binding between the pUB110 portion of pSL103 and the *B. subtilis*

† Present address: Mitsubishi-Kasei Institute of Life Sciences, 11 Minamiooya, Machida-shi, Tokyo, Japan.

membrane has been demonstrated (17). In the current studies, we simplified the binding reaction by the use of a total lysate of *B. subtilis* cells and established site-specific in vitro binding of the plasmid. We defined four regions in the pUB110 portion of pSL103 which include specific binding sites to the membrane and characterized the binding. This in vitro membrane binding of pSL103 and pUB110 is different from that which we found previously in vivo (34) in several important respects and is termed type-II binding in contrast to the in vivo binding, type-I. The possibility of the type-II binding of pUB110 and pSL103 as a necessary first step for type-1 binding and initiation is pointed out.

MATERIALS AND METHODS

Materials. *B. subtilis* 168 *trp thy dna-1* and *B. subtilis* 168 *trp thy* have been described previously (32). *Eco*RI, *Taq*I, *Hin*fI, *Hae*III, *Ava*I, *Ava*II, *Xba*I, and T4 DNA ligase were obtained from Bethesda Research Laboratories, Inc., and *Mbo*I and *Bst*NI were from New England Biolabs. [γ-^{32}P]ATP was obtained from New England Nuclear Corp. or was a gift from the National Jewish Hospital in Denver, Colo. The specific activity was between 2,000 and 3,000 Ci/mM. Polynucleotide kinase was obtained from P-L Biochemicals, Inc. TES buffer consists of 0.03 M Tris-hydrochloride (pH 8.0), 0.05 M EDTA, and 0.05 M NaCl. pUB110 was isolated as described previously (30).

Preparation of bacterial lysate. Cells of *B. subtilis* strains grown overnight in Penassay medium (1.75%; Difco Laboratories) supplemented with 10 μg of thymine per ml were transferred to a fresh medium to give a cell density of about 10^7 cells per ml (Klett units, 5) and allowed to grow at 32°C until the cell density reached 7×10^7 cells per ml (Klett units, 40). The cells were centrifuged at 8,000 rpm for 10 min and stored at −20°C until use. The lysates were made by the procedure of Korn et al. (17), slightly modified as follows. Frozen cells from 25 ml of culture were suspended in 0.5 ml of TKE (0.02 M Tris-hydrochloride [pH 8.0], 0.06 M KCl, and 0.001 M EDTA), and 0.1 ml of lysozyme (5 mg/ml in TKE) and 0.05 ml of 2-mercaptoethanol (0.1 M in TKE) were added. After the mixture was incubated at 32°C for 20 min, 0.1 ml of 5% Brij 58 in TKE was added. The viscous lysate thus obtained was put on ice, sheared by passing through an 18-gauge needle eight times, and stored on ice until use.

Radioactive DNA. pSL103 [^{3}H]DNA was prepared as described previously (17) and further purified by 5 to 20% sucrose gradient centrifugation to remove a rapidly sedimenting aggregate. The specific activity of pSL103 [^{3}H]DNA was 6.5×10^5 cpm/μg. [^{32}P]DNA fragments were prepared as follows. Two micrograms of pSL103 or pUB110 was digested by a restriction enzyme under the conditions described by the supplier in a total volume of 50 μl. After the reaction, 5 μl of 3 M sodium acetate (pH 7.0) was added, and the DNA fragments were precipitated by the addition of 2 volumes of ethanol in a dry ice-ethanol bath, followed by 10 min of centrifugation in a Beckman microfuge. The precipitates were dissolved in 23 μl of kination-exchange reaction mixture (18) containing 50 μCi of [γ-^{32}P]ATP and incubated at 37°C for 30 min after the addition of 2 μl (10 U) of polynucleotide kinase. TES (0.4 ml) and 5 M NaCl (0.05 ml) were added to the solution, which was mixed with 1 ml of ethanol in a polyallomer tube, and 3 ml of ethanol were layered on the mixture. After storage at −20°C overnight, [^{32}P]DNA fragments were precipitated in a Beckman SW50.1 rotor at 30,000 rpm for 30 min at 0°C. DNA was precipitated once more as described above and dissolved in 60 μl of buffer consisting of 0.1× SSC (1× SSC contains 0.15 M NaCl plus 0.015 M sodium citrate) and 1 mM EDTA. The specific activity of various restricted DNA fragments thus obtained ranged from 2.5×10^6 to 5.0×10^6 cpm/μg.

Binding of radioactive DNA to membrane fraction. The bacterial lysate (0.2 ml) was heated at 45°C for 10 min, and 5 to 10 μl of radioactive DNA (together with nonradioactive DNA for the competition experiments) was added and further incubated for 5 min at 45°C. The reaction mixture was transferred to a 32°C water bath, incubated for 20 min, chilled in an ice bath, and layered on a prechilled 5 to 20% sucrose gradient made in TKE with a 64% sucrose shelf (in TKE) at the bottom. The gradient was made in SW50.1 nitrocellulose tubes containing 0.9 ml of 64% sucrose at the bottom and approximately 4.0 ml of the 5 to 20% sucrose gradients. The gradients were centrifuged at 32,500 rpm for 30 min in an SW50.1 rotor and fractionated into 0.15-ml portions. Samples (20 to 40 μl) from each fraction were spotted on Whatman 3 MM papers (11.2 by 2.5 cm), and the papers were washed with cold 10% trichloroacetic acid and ethanol successively and counted in a scintillation counter.

Analysis of membrane-bound [^{32}P]DNA fragments. After sucrose gradient centrifugation, the fractions containing the membrane-[^{32}P]DNA complex were collected, and an equal volume of TKE solution was added. One volume of water-saturated phenol and a one-tenth volume of 10% sodium dodecyl sulfate were added. After centrifugation of the mixture at room temperature, the aqueous layer was taken, 1 μg of tRNA and a 1/10 volume of 3 M sodium acetate (pH 7.0) were added, and the DNA fragments were precipitated by the addition of ethanol and a subsequent centrifugation in an SW50.1 rotor as described above. The precipitates thus obtained were dissolved in 40 μl of 0.1× SSC plus 1 mM EDTA containing 1 μl of 10% sodium dodecyl sulfate. The DNA fragments were electrophoresed either in 0.7% agarose gels or in composite gels consisting of 0.5% agarose and 3% polyacrylamide (1). The amount of radioactivity loaded was adjusted so that each band contained 400 cpm. After electrophoresis, the gel was soaked in 10% trichloroacetic acid for 15 min, dried on Whatman 3 MM paper, and exposed to X-ray film (Kodak XAR-5) with an intensifying screen.

RESULTS

Binding of pSL103 to membrane fraction. In this study, we used total bacterial lysates for the tests of binding between the membrane fraction and pSL103 instead of purified membrane (17). The lysate prepared from *B. subtilis* 168 *thy trp dna-1* cells was incubated with pSL103

[^{3}H]DNA, followed by sucrose gradient centrifugation as described above. Some radioactive pSL103 appeared in the membrane fraction (Fig. 1A, fraction number 3 to 6). Under the conditions used, 17% of the input DNA was bound to the membrane, which corresponds to the binding of 29.6 molecules to a cell equivalent and is similar to the previous result (17). This number is probably underestimated since the calculation was based on the assumption that every cell was lysed during the preparation of the lysates. The plasmid is known to have a copy number of approximately 40 (15). The centrifugation of radioactive pSL103 alone did not show a peak at the membrane fraction (data not shown). *E. coli* plasmid pACYC184 did not compete significantly with the membrane association of pSL103 (Fig. 1B), whereas nonradioactive pSL103 competed for binding by 75% (Fig. 1C), indicating that the binding of pSL103 to the membrane is specific for pSL103. These results confirm our previous results that ColE1 or rat liver DNA does not compete with pSL103 in binding to a purified membrane fraction (17).

The membrane binding of pSL103 was not observed when $MgCl_2$ was added to the reaction mixture (Fig. 2B; only the membrane fraction is shown). However, once the membrane-pSL103 complex was isolated from the sucrose gradient, the radioactive peak of pSL103 [^{3}H]DNA in the membrane fraction did not change upon incubation with or without added $MgCl_2$ (data not shown). These results suggest that a nuclease in the total lysate was activated by Mg^{2+} and digested pSL103 [^{3}H]DNA added as a substrate. EDTA at a concentration of 10 mM did not affect the binding reaction (Fig. 2C). The binding of pSL103 [^{3}H]DNA to the membrane did not occur when the 45°C incubation was omitted (Fig. 2D).

We next examined whether the structural configuration of pSL103 affects the binding reaction. pSL103 [^{3}H]DNA was linearized by *Hin*dIII (pSL103 has a unique *Hin*dIII site in the *trpC*$^+$ fragment [16]) and used for binding. *Hin*dIII-treated linear DNA bound to the membrane at an efficiency of 77% of the membrane binding of supercoiled pSL103, indicating that the conformation of the plasmid DNA has little effect on the binding reaction.

The optimal salt concentration for the binding was determined by using ^{32}P-labeled *Mbo*I fragments prepared as described above. The optimal concentration of KCl was 60 mM; higher concentrations (above 1.0 M) were inhibitory to the binding (Fig. 3). This is in contrast to the stability of the in vivo membrane-pSL103 complex, which is resistant to 4 M CsCl (34).

Binding of specific DNA restriction fragments to membrane. To investigate whether a specific DNA region on pSL103 is responsible for the membrane binding, ^{32}P-labeled *Eco*RI fragments were prepared and bound to the membrane under standard conditions, and the membrane-bound DNA fragments were isolated as described above. Of the two *Eco*RI fragments of pSL103, the pUB110 fragment, but not the *trpC*$^+$ fragment, was bound to the membrane, indicating that the binding of pSL103 to the membrane is effected by the binding of the

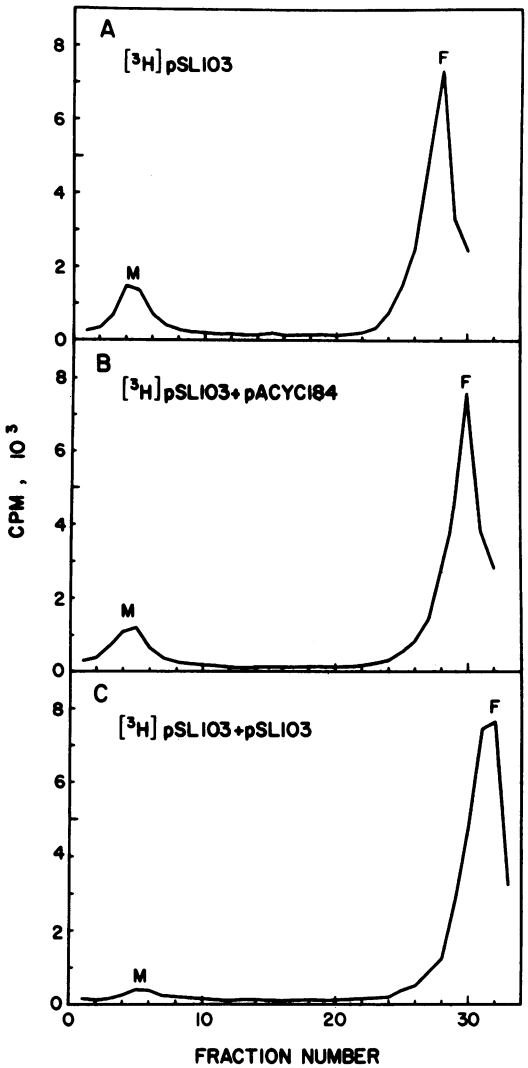

FIG. 1. Specific binding of pSL103 [^{3}H]DNA to the membrane fraction. (A) pSL103 [^{3}H]DNA (0.4 μg) was incubated with the cell lysate prepared from *B. subtilis* 168 *thy trp dna-1* and analyzed by 5 to 20% sucrose gradient centrifugation, as described in the text. (B and C) pACYC184 (11 μg) and pSL103 (10 μg), respectively, were added together with pSL103 [^{3}H]DNA. M, Membrane fraction; F, free fraction.

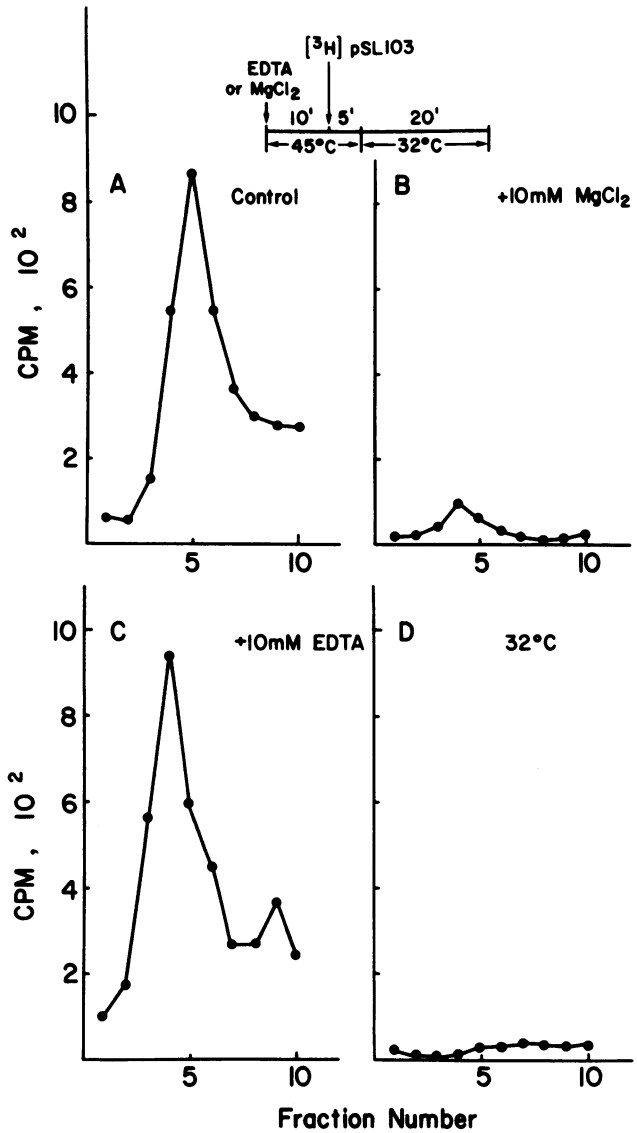

FIG. 2. Factors affecting pSL103 [^{3}H]DNA binding to the membrane fraction. The control (A) was handled by the standard procedure. $MgCl_2$ (B) and EDTA (C) were added to the reaction mixture at the onset of the reaction. A reaction mixture (D) was incubated at 32°C throughout the reaction. The reaction mixtures were centrifuged as described in the text. Only the radioactivity around the membrane fraction is shown.

pUB110 portion (Fig. 4). This result is in accord with the previous finding (17) that the linear *trpC*$^+$ fragment does not compete with pSL103 for binding to the membrane.

To localize the regions in pUB110 that bind to the membrane, we used various ^{32}P-labeled restriction fragments of pUB110. Four *Mbo*I fragments (A, B, C, and F) were bound to the membrane, and fragment E was bound very weakly although it is not visible in the figure (Fig. 5a). No detectable binding was observed for fragments D and G. The results indicate that there are at least four membrane binding sites in pUB110. Of the *Hin*fI fragments, only the largest DNA fragment (*Hin*fI-A) was bound to the membrane (Fig. 5b). At least two *Hin*fI fragments (~30 base pairs), in addition to those shown in Fig. 6, were generated, but they have not yet been mapped. Since *Mbo*I-A and *Mbo*I-F were not contained in *Hin*fI-A and no other *Hin*fI fragments were bound to the membrane, the results suggest that the binding ability of

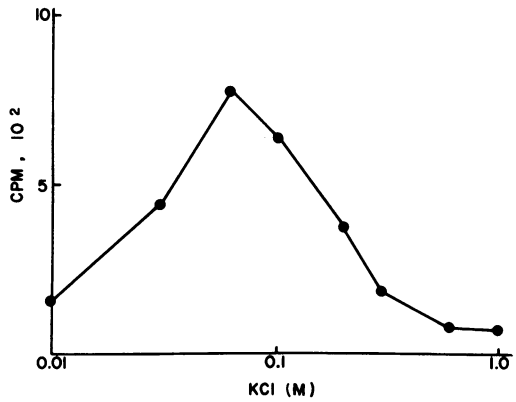

FIG. 3. Salt dependency of the membrane binding of *Mbo*I [^{32}P]DNA restriction fragments of pUB110. A lysate of *B. subtilis* 168 *thy trp dna-1* cells was made at the KCl concentration of 0.01 M, and the KCl concentration for the binding assay was adjusted between 0.01 M and 1.0 M. After incubation with [^{32}P]DNA in the standard reaction condition, the reaction mixtures were centrifuged, and the sum of the radioactivity in the membrane fraction was plotted against the KCl concentrations in the logarithmic scale.

these fragments was destroyed by *Hin*fI digestion. *Taq*I gave rise to three fragments, of which fragment A bound to the membrane, but fragments B and C did not (Fig. 5c). Since there is one *Taq*I site in *Mbo*I-C (Fig. 5a and c), these results suggest that the binding site in *Mbo*I-C was destroyed by *Taq*I; there must be one binding site around this *Taq*I site. To investigate which DNA region is involved in the binding of *Mbo*I-A (Fig. 5a), we further digested *Mbo*I [^{32}P]DNA fragments of pUB110 with *Hae*III and used them for the binding reaction. Of the radioactive *Hae*III-*Mbo*I fragments, only fragment E was bound; fragments D, F, and G were not (Fig. 5d). The result shown in Fig. 5i indicates that the three smaller *Hae*III fragments (E, F, and G) could not bind to the membrane and rules out the possibility that the junction region between *Mbo*I-A and *Mbo*I-D is involved in the binding reaction since *Hae*III fragment E (Fig. 5i) covers this junction area. It was still possible that there was another binding site in *Mbo*I-A which was destroyed by *Hae*III digestion. However, this possibility was ruled out by the findings that *Ava*II-C (Fig. 5g) and *Hin*fI-C (Fig. 5b), which cover the three *Hae*III cleavage sites (Fig. 6), did not bind to the membrane. These results indicated that the binding site(s) was within *Hae*III-*Mbo*I-E (Fig. 5d). This region was further narrowed down by *Taq*I digestion. *Mbo*I-A did not lose the binding ability by cleavage with *Taq*I (Fig. 5f). From the sequencing data (data not shown), the *Taq*I site was found to overlap the *Hin*fI site and is located 3 base pairs distal to one end of *Mbo*I-A (the end closer to the *Eco*RI site). These results show that the loss of the binding ability of *Mbo*I-A by *Hin*fI was not caused by cleavage in the overlapping region with *Taq*I. The compilation of these results suggests that the binding site in this region is around the *Hin*fI site at 3.15 kilo-bases (kb) from the *Eco*RI site (Fig. 6). By a similar strategy, the binding site in *Mbo*I-B (Fig. 5a) was narrowed down to the fragment around the *Ava*I site by the following observations: *Eco*RI-*Bst*NI-B (Fig. 5a) was bound to the membrane (Fig. 5e), and *Ava*I cleavage of *Mbo*I-B greatly reduced the binding ability of this fragment (Fig. 5h). Since *Hin*fI destroyed the binding ability of *Mbo*I-F (Fig. 5a and b), the membrane binding site may lie around the *Hin*fI site.

Figure 6 summarizes the results shown in Fig. 5. The solid and the dotted lines depict the restriction fragments which were bound or not bound, respectively, to the membrane. In this map, the location of the binding region can be narrowed down by overlapping solid lines. The common area in the solid lines is the shortest DNA fragment so far obtained (solid arcs around the concentric circles) that was bound to the membrane, and if this common area is destroyed by certain restriction enzymes, the area should correspond to the binding site.

Effect of KCl concentration on specificity of binding. The results shown so far were obtained at a KCl concentration where the maximum

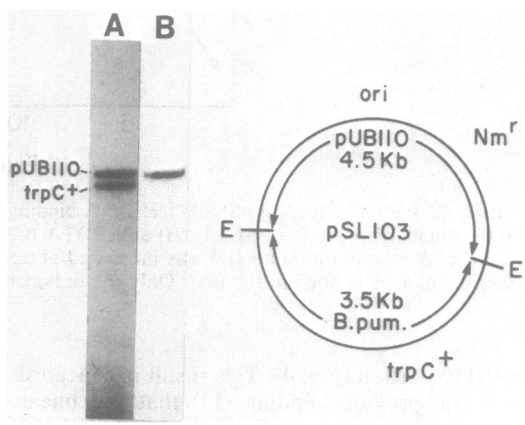

FIG. 4. Binding of the pUB110 fragment of pSL103 to the membrane fraction. pSL103 was digested with *Eco*RI, the 5′ ends were labeled with ^{32}P, and the fragments were bound to the membrane fraction as described in the text. The total fragments are shown in lane A, and the membrane-bound fragments are shown in lane B. E, *Eco*RI site; ori, origin; Nm^r, neomycin resistance gene; trpC$^+$, wild-type tryptophan C gene of *B. pumilus* (B. pum.).

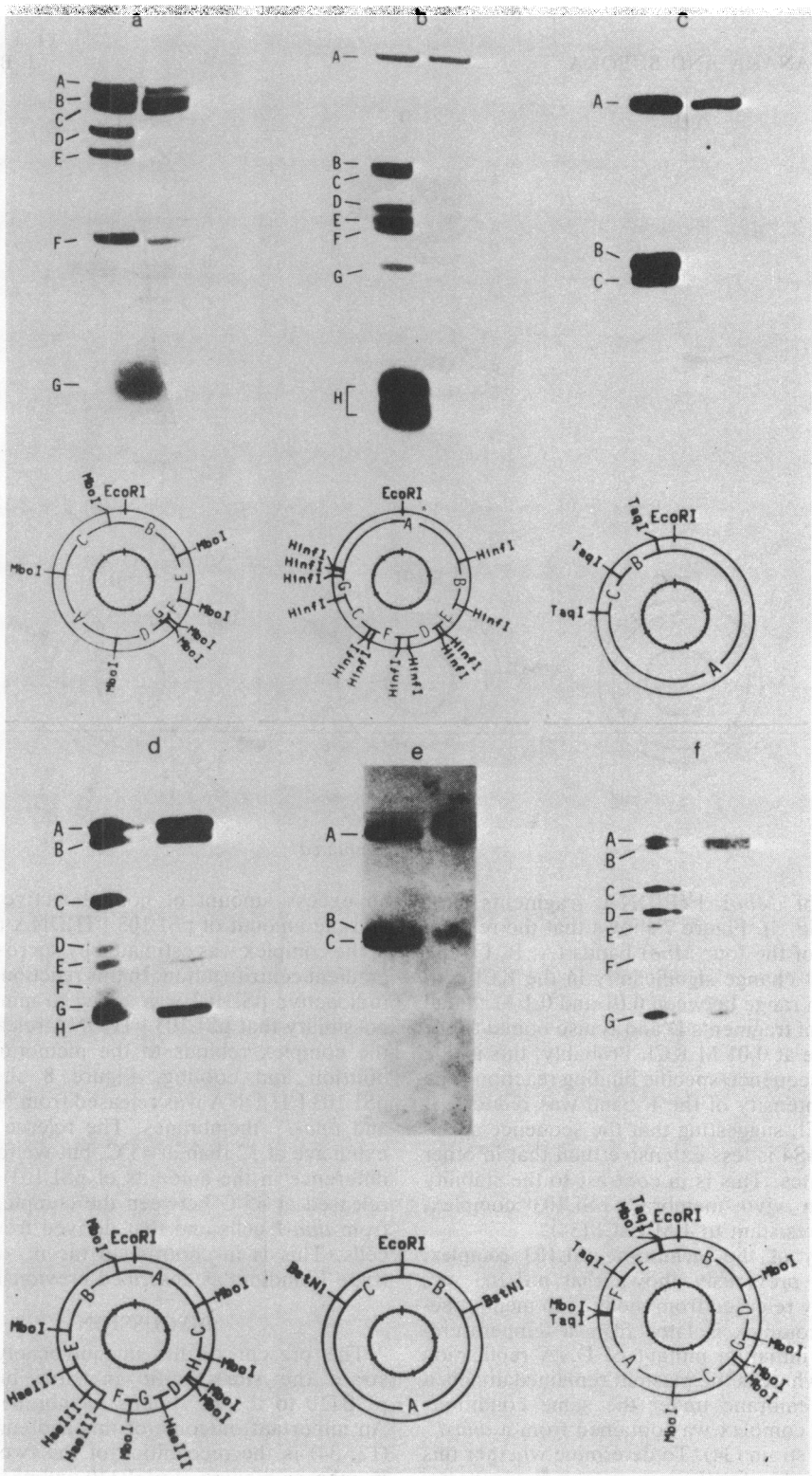

FIG. 5. Binding of [^{32}P]DNA restriction fragments to membrane fragment. Restriction fragments labeled at the 5′ ends with ^{32}P were prepared and incubated as described in the text. The [^{32}P]DNA in the membrane fraction was extracted and electrophoresed in composite gels. (a through i) The total DNA fragments used for the binding are shown in the left lane, and the membrane-bound fragments are shown in the right lane. The restriction enzymes used and their restriction sites on pUB110 are shown below each gel. The *Eco*RI site is shown in (a through i) for reference although this enzyme was actually used only for the experiments in (e), (g), and (i).

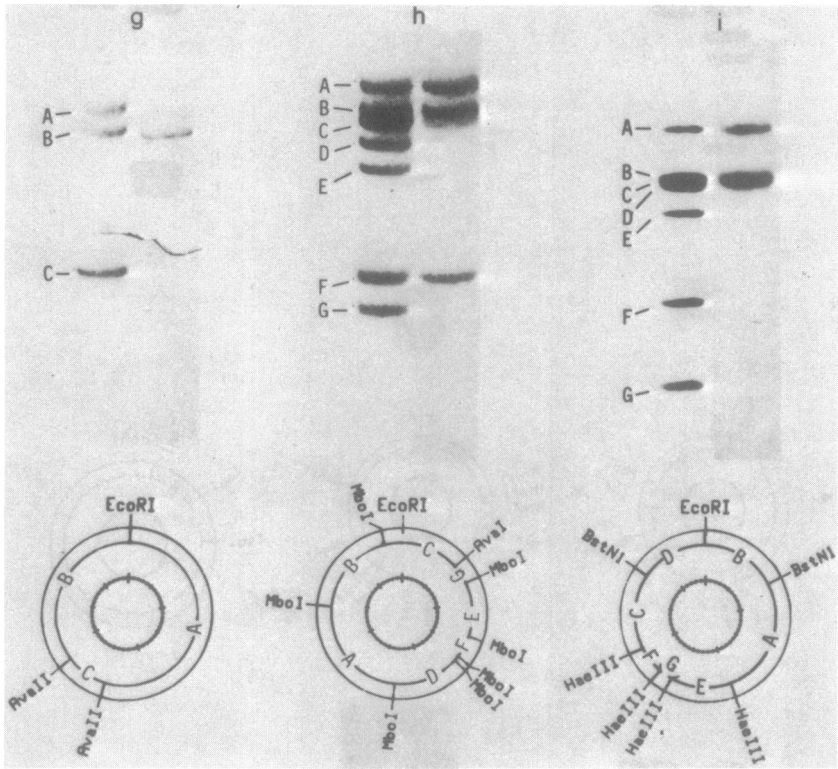

FIG. 5—*Continued*

amount of *Mbo*I [^{32}P]DNA fragments was bound (Fig. 3). Figure 7 shows that the relative intensity of the four *Mbo*I bands (A, B, C, and F) did not change significantly in the KCl concentration range between 0.01 and 0.1 M. Small amounts of fragments D and E also bound to the membrane at 0.01 M KCl. Probably, this is due to a non-sequence-specific binding reaction. The relative intensity of the F band was reduced at 0.2 M KCl, suggesting that the sequence specificity in BS4 is less extensive than that in other binding sites. This is in contrast to the stability of the in vivo membrane-pSL103 complex, which is resistant to 4 M CsCl (34).

Stability of the membrane-pSL103 complex. We have previously shown that pSL103 was selectively released from the in vivo membrane-pSL103 complex isolated from a temperature-sensitive initiation mutant of DNA replication (*dna-1*), whereas the plasmid remained attached to the membrane under the same conditions when the complex was obtained from a *dna-1*$^+$ wild-type strain (34). To determine whether this is also the case for the in vitro complex generated in this study, the membrane-pSL103 complexes were prepared from *dna-1* and *dna-1*$^+$ *B. subtilis* cells and incubated at both 32 and 45°C. After incubation, the reaction mixture was diluted 10-fold with ice-cold TKE buffer containing an excess amount of nonradioactive pSL103, and the amount of pSL103 [^{3}H]DNA remaining in the complex was estimated by sucrose density gradient centrifugation. In this reaction, the nonradioactive pSL103 was added to minimize the possibility that pSL103 [^{3}H]DNA released from the complex rebinds to the membrane during dilution and cooling. Figure 8 shows that pSL103 [^{3}H]DNA was released from both *dna-1* and *dna-1*$^+$ membranes. The release was less extensive at 32 than at 45°C, but we found little difference in the amounts of pSL103 [^{3}H]DNA released at 45°C between the complex derived from *dna-1* cells and that derived from *dna-1*$^+$ cells. This is in contrast to the in vivo result (type-I binding) as described previously.

DISCUSSION

The present results unequivocably demonstrate the site-specific in vitro binding of pUB110 to the *B. subtilis* membrane fraction. An important outcome of this and earlier work (17, 34) is the recognition of the two types of membrane binding of pUB110 and pSL103, i.e., type-I and type-II. Type-I binding is seen in the membrane-pUB110 or membrane-pSL103 complex which is formed in vivo; the complex is high-salt resistant and dependent on the functional product of *dna-1* (*dnaBI*) gene (34). Type-

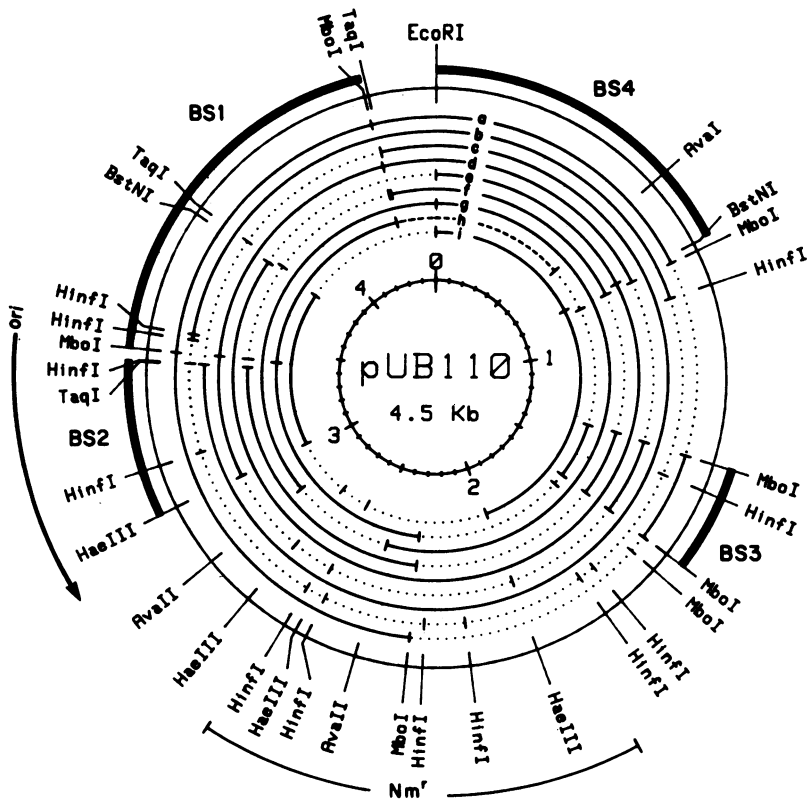

FIG. 6. Membrane-binding map of restriction fragments of pUB110. The solid and dotted lines in the concentric circles represent the DNA fragments bound and not bound, respectively, to the membrane, and the broken line designates a fragment which bound, but less efficiently. The thick arcs outside the concentric circles depict the length of the DNA fragments that can bind to the membrane. The current status of the localization of the binding sites (BS1 to BS4) within the corresponding regions is described in the text. The restriction enzymes used were: a, *Mbo*I; b, *Hin*fI; c, *Taq*I; d, *Mbo*I and *Hae*III; e, *Eco*RI and *Bst*NI; f, *Taq*I and *Mbo*I; g, *Eco*RI and *Ava*II; h, *Mbo*I and *Ava*I; i, *Eco*RI, *Bst*NI, and *Hae*III.

II binding, which is described in this paper, can be seen in vitro; the complex is high-salt sensitive (Fig. 3) and independent of the functional *dna-1* gene product (Fig. 8). By analysis with ^{32}P-labeled restriction fragments, this binding was found to consist of at least four membrane binding sites (BS1 to BS4; Fig. 6).

Type-II binding probably exists in vivo, since pSL103 was released almost completely from the membrane of *dna-1* cells when the membrane-pSL103 complex isolated from *dna-1* cells harboring the plasmid was heated at 45°C and centrifuged in a CsCl-sucrose gradient, whereas the release was not as extensive when the heated complex was centrifuged in the absence of CsCl (34). In addition, the membrane binding in vitro occurs at specific regions in pUB110 (Fig. 5). These results suggest that type-II binding may have a functional role in vivo, possibly to keep the plasmid bound to the membrane.

Scheer-Abramowitz et al. (24) have shown that the replication of pUB110 starts at 22% to the left of the *Eco*RI site (Fig. 6) and proceeds unidirectionally and counterclockwise. This initiation area is within *Taq*I-C and flanked by two membrane-binding regions (BR1 and BR2). After the reconstruction of pUB110 with *Taq*I and ligase, followed by selection for neomycin resistance, we examined 18 plasmids and found that all of the plasmids contained *Taq*I-A, -B, and -C (Fig. 5c). Fourteen plasmids were indistinguishable from pUB110, and the remaining four contained extra DNA fragments at the junction of *Taq*I-A and *Taq*I-B, but the junction of *Taq*I-B and *Taq*I-C and that of *Taq*I-C and *Taq*I-A were in the same orientation as those in pUB110 (data not shown). These results strongly suggest that the two *Taq*I cleavage sites flanking *Taq*I-C are in the region necessary for plasmid maintenance or replication. Since *Taq*I

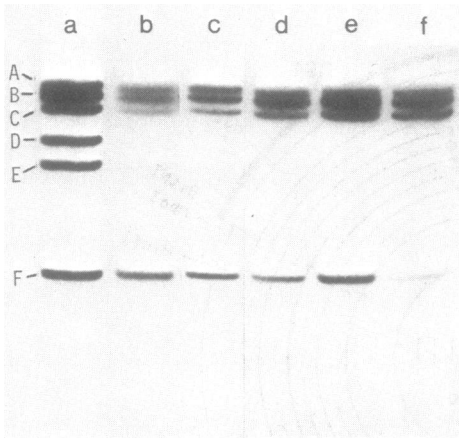

FIG. 7. ^{32}P-labeled *Mbo*I fragments bound to the membrane at various KCl concentrations. Lane a, *Mbo*I fragments used for the binding reactions. The KCl concentrations used were: 0.01 M (lane b); 0.03 M (lane c); 0.06 M (lane d); 0.1 M (lane e); 0.2 M (lane f).

abolishes the binding ability of BR1, the in vitro binding at this type-II binding site is, most likely, actually functioning and necessary in vivo. Scheer-Abramowitz et al. (24) have isolated two deletion plasmids, each of which is lacking either the BR3 or most of the BR4 region. We have also isolated a plasmid in which *Mbo*I-B (Fig. 5a) is lost (data not shown). These results show that the presence of both the third and the fourth binding region is not essential for the replication function, although the extent to which these binding sites contribute to the efficiency of replication remains unknown.

It is interesting to note that the capacity of the membrane for type-II binding turned out to be approximately 30 per cell, which is more or less equivalent to the capacity in vivo, which suggests that the membrane component(s) responsible for type-II binding is a normal constituent of the cell membrane which the plasmid recognizes and binds.

Although the function of type-II binding is not understood at the moment, several possibilities can be raised. The binding sites may be essential in keeping the plasmid on the membrane and, therefore, a necessary component for the initiation of replication. It is known that the mode of pSL103 replication is random (33); each molecule of this multicopy plasmid in the cell has an equal opportunity to replicate, irrespective of whether the molecule replicated recently or not (22). When pUB110 replicates, the molecule

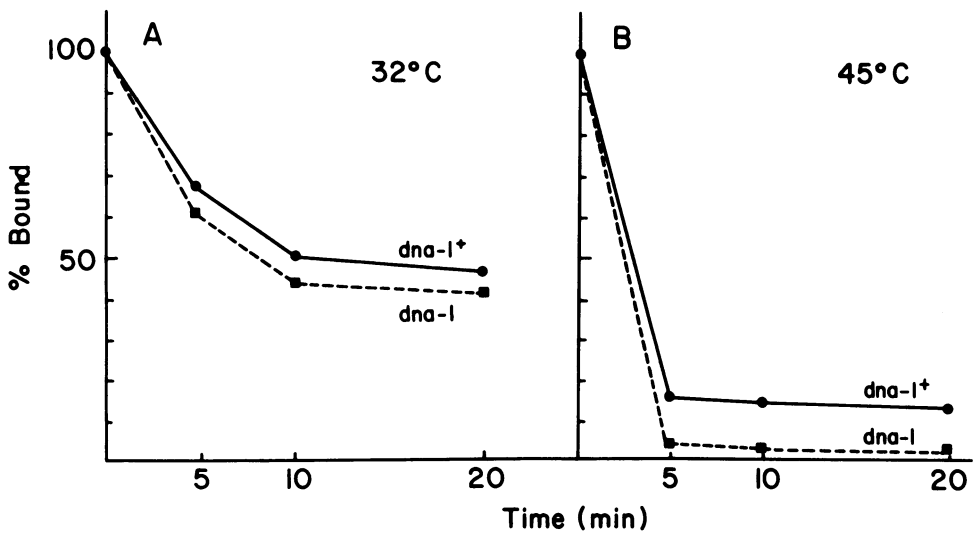

FIG. 8. Stability of ^{3}H-labeled pSL103-membrane complex derived from *dna-1* and *dna-1*$^{+}$ cells. The complexes were isolated from the membrane fraction after sucrose gradient centrifugation, and 0.4 ml of each complex was incubated at either 32 or 45°C for various lengths of time. At each time point, 0.02-ml samples were taken, diluted 10-fold with cold TKE containing 10 μg of nonradioactive pSL103, and centrifuged again in 10 to 20% sucrose gradients with a 64% sucrose solution at the bottom. The membrane fractions were collected, and after the addition of 2 μg of DNA as a carrier, samples were precipitated by an equal volume of cold 10% trichloroacetic acid. The precipitates were collected on HAWP filters (0.45 μm; Millipore Corp.) dried, and counted in a scintillation counter.

may be placed at the correct place on the membrane to initiate replication. Type-II binding that we observed in this study might help this site become accessible to pUB110 molecules. If this is the case and if the replication of the plasmid is not a rate-limiting factor, type-II binding should also be a primary factor in determining the copy number of the plasmid per cell. In any case, type-II binding should be a critical step in the determination of host-plasmid specificity, which should, in turn, be an important factor for incompatibility among different plasmids.

It is possible that the number of type-II binding sites per plasmid may have an influence on the efficiency of membrane binding. For example, pUB110 molecules carrying different numbers of type-II binding sites may bind to the membrane with different efficiencies. Another possibility is that type-II binding (at least at BR1 and BR2) is the necessary first step before type-I binding, which requires other reactions for pUB110 to start initiation. The type-II binding step could be essential to place the origin area on the membrane where the membrane complex can be built. The heat activation necessary for type-II binding (Fig. 2) might be one of the steps leading to type-I binding, and this heat activation may be substituted for in vivo by some biological reactions or conditions.

We cannot rule out the possibility that the type-II binding that we observed in this study is involved in the orderly partition of pUB110 molecules into daughter cells. Type-II binding sites may play a role similar to that of the *par* locus in the partition of pSC101 as reported by Meacock and Cohen (19). The *par* gene is located in the vicinity of the replication origin of the plasmid, but its function is independent of the replication itself. The indispensability of the two type-II binding sites flanking the origin of pUB110 for initiation, however, has not been unequivocably demonstrated. The possibility of a similar partition role has been suggested for the high-salt-sensitive complex between the membrane and the DNA region near the replication origin of *E. coli* (2).

ACKNOWLEDGMENTS

We thank Tim McKenzie and Lois Jones for their technical assistance.

This work was supported by Public Health Service grant GM 28133 from the National Institutes of Health and grant PCM 80-11549 from the National Science Foundation.

LITERATURE CITED

1. **Alwine, J. C., D. J. Kemp, B. A. Parker, J. Reiser, J. Renart, G. R. Stark, and G. M. Wahl.** 1979. Detection of specific RNAs or specific fragments of DNA by fractionation in gels and transfer to diazobenzyloxymethyl paper. Methods Enzymol. **68**:220–242.
2. **Craine, B. L., and C. S. Rupert.** 1979. Deoxyribonucleic acid-membrane interactions near the origin of replication and initiation of deoxyribonucleic acid synthesis in *Escherichia coli*. J. Bacteriol. **173**:740–745.
3. **Diaz, R., K. Nordstrom, and W. L. Staudenbauer.** 1981. Plasmid R1 DNA replication dependent on protein synthesis in cell free extracts of *E. coli*. Nature (London) **289**:326–328.
4. **Fielding, P., and C. F. Fox.** 1970. Evidence for the stable attachment of DNA to membrane at the replication origin of *E. coli*. Biochem. Biophys. Res. Commun. **41**:157–162.
5. **Firshein, W.** 1972. The DNA/membrane fraction of *Pneumococcus* contains a DNA replication complex. J. Mol. Biol. **70**:393–397.
6. **Fuller, R. S., J. M. Kaguni, and A. Kornberg.** 1981. Enzymatic replication of the origin of the *Escherichia coli* chromosome. Proc. Natl. Acad. Sci. U.S.A. **78**: 7370–7374.
7. **Ganesan, A., and J. Lederberg.** 1965. A cell membrane bound fraction of bacterial DNA. Biochem. Biophys. Res. Commun. **18**:824–835.
8. **Gryczan, T. J., S. Contente, and D. Dubnau.** 1978. Characterization of *Staphylococcus aureus* plasmids introduced by transformation into *Bacillus subtilis*. J. Bacteriol. **134**:318–329.
9. **Hye, R. J., M. A. O'Sullivan, K. Howard, and N. Sueoka.** 1976. Membrane association of origin, terminus, and replication fork in *Bacillus subtilis*, p. 83–90. *In* D. Schlessinger (ed.), Microbiology—1976. American Society for Microbiology, Washington, D.C.
10. **Imada, S., L. E. Carroll, and N. Sueoka.** 1980. Genetic mapping of a group of temperature-sensitive *dna* initiation mutants in *Bacillus subtilis*. Genetics **94**:809–823.
11. **Inuzuka, M., and D. R. Helinski.** 1978. Requirements of a plasmid-encoded protein for replication in vitro of plasmid R6K. Proc. Natl. Acad. Sci. U.S.A. **75**:5381–5385.
12. **Jacob, F., S. Brenner, and F. Cuzin.** (1963). On the regulation of DNA replication in bacteria. Cold Spring Harbor Symp. Quant. Biol. **28**:329–336.
13. **Jacq, A., H. Lother, W. Messer, and M. Kohiyama.** 1980. Isolation of a membrane protein having an affinity to the replication origin of *E. coli*, p. 207–219. *In* B. Alberts and F. Fox (ed.), Mechanistic studies of DNA replication and genetic recombination. ICN-UCLA Symposium on Molecular and Cellular Biology. Academic Press, Inc., New York.
14. **Karamata, D. and J. D. Gross.** 1970. Isolation and genetic analysis of temperature sensitive mutants of *B. subtilis* defective in DNA synthesis. Mol. Gen. Genet. **108**:277–287.
15. **Keggins, K., P. Lovett, and E. J. Duvall.** 1978. Molecular cloning of genetically active fragments in *Bacillus subtilis* and properties of the vector plasmid pUB110. Proc. Natl. Acad. Sci. U.S.A. **75**:1423–1427.
16. **Keggins, K. M., P. S. Lovett, R. Marrero, and S. D. Hoch.** 1979. Insertional inactivation of *trpC* in cloned *Bacillus trp* segments: evidence for a polar effect on *trpE*. J. Bacteriol. **139**:1001–1006.
17. **Korn, R., S. Winston, T. Tanaka, and N. Sueoka.** 1983. Specific *in vitro* binding of a plasmid to membrane fraction of *Bacillus subtilis*. Proc. Natl. Acad. Sci. U.S.A. **80**:574–578.
18. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65**:499–580.
19. **Meacock, P. A., and S. N. Cohen.** 1980. Partitioning of bacterial plasmids during cell division: a *cis*-acting locus that accomplishes stable plasmid inheritance. Cell **20**:529–542.
20. **Nagai, K., W. Hendrickson, R. Balakrishnan, H. Yamaki, D. Boyd, and M. Schaechter.** 1980. Isolation of a replication origin complex from *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. **77**:262–266.

21. **Parker, D. L., and D. A. Glaser.** 1974. Chromosomal sites of DNA-membrane attachment in *E. coli*. J. Mol. Biol. **87**:153–168.
22. **Rownd, R.** 1969. Replication of a bacterial episome under relaxed control. J. Mol. Biol. **44**:387–402.
23. **Ryter, A. and F. Jacob.** 1963. Etude au microscope electronique des relations entre mesosomes et noyaux chez *Bacillus subtilis*. C. R. Acad. Sci. Paris **257**:3060–3063.
24. **Scheer-Abramowitz, J., T. J. Gryczan, and D. Dubnau.** 1981. Origin and mode of replication of plasmids pE194 and pUB110. Plasmid **6**:67–77.
25. **Smith, D. W., and P. C. Hanawalt.** 1967. Properties of the growing point region in the bacterial chromosome. Biochem. Biophys. Res. Commun. **149**:519–531.
26. **Snyder, R. W., and F. E. Young.** 1969. Association between the chromosome and cytoplasmic membrane in *Bacillus subtilis*. Biochem. Biophys. Res. Commun. **35**:354–362.
27. **Streips, U. N., S. Horowitz, and R. J. Doyle.** 1980. Genetic analysis of DNA-surface interactions in *Bacillus subtilis*, p. 284–287. *In* D. Schlessinger (ed.), Microbiology—1980. American Society for Microbiology, Washington, D.C.
28. **Sueoka, N., and H. Hammers.** 1974. Isolation of DNA-membrane complex in *Bacillus subtilis*. Proc. Natl. Acad. Sci. U.S.A. **71**:4787–4791.
29. **Sueoka, N., and W. Quinn.** 1968. Membrane attachment of the chromosome replication origin in *Bacillus subtilis*. Cold Spring Harbor Symp. Quant. Biol. **33**:695–705.
30. **Tanaka, T., and N. Kawano.** 1980. Cloning vehicles for the homologous *Bacillus subtilis* host-vector system. Gene **10**:131–136.
31. **Tomizawa, J., T. Itoh, G. Selzer, and Y. Somi.** 1981. Inhibition of ColE1 RNA primer formation by a plasmid-specified small RNA. Proc. Natl. Acad. Sci. U.S.A. **78**:1421–1425.
32. **White, K., and N. Sueoka.** 1973. Temperature-sensitive DNA synthesis mutants of *Bacillus subtilis*—appendix: theory of density transfer for symmetric chromosome replication. Genetics **73**:185–214.
33. **Winston, S., R. Korn, and N. Sueoka.** 1980. DNA-membrane association and initiation of DNA replication of *Bacillus subtilis*, p. 207–219. *In* B. Alberts and F. Fox (ed.), Mechanistic studies of DNA replication and genetic recombination. ICN-UCLA Symposium on Molecular and Cellular Biology. Academic Press, Inc., New York.
34. **Winston, S., and N. Sueoka.** 1980. DNA-membrane association is necessary for initiation of chromosomal and plasmid replication in *Bacillus subtilis*. Proc. Natl. Acad. Sci. U.S.A. **77**:2834–1838.
35. **Yamaguchi, K., and H. Yoshikawa.** 1973. Topology of the chromosomal membrane junction in *Bacillus subtilis*. Nature (London) New Biol. **244**:204–206.
36. **Yamaguchi, K., and H. Yoshikawa.** 1975. Association of replication terminus of the *Bacillus subtilis* chromosome to the cell membrane. J. Bacteriol. **124**:1030–1033.