Purification and Characterization of SopA and SopB Proteins Essential for F Plasmid Partitioning*

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Mini-F plasmid has the trans-acting genes sopA and sopB and the cis-acting site sopC which are essential for accurate partitioning of plasmid DNA molecules into both daughter cells. In this study, we purified independently SopA and SopB proteins, analyzed the in vitro DNA-binding activity of these proteins by the gel retardation assay, and determined the precise binding sites of DNA by the footprinting method. SopA binds to four repeated sequences (CTTTGC) located in the promoter-operator region of the sopAB operon. The SopA binding activity is enhanced by the addition of SopB protein. SopB protein itself does not bind to this DNA region. These results suggest that the complex of SopA and SopB proteins autoregulate the expression of the sopA-sopB operon. On the other hand, SopB protein binds to the sopC region, in which 12 direct repeats of 43-base pairs nucleotides exist. SopB protein recognizes the inverted repeats of 7 base pairs in each direct repeats. SopA protein does not affect the SopB binding activity to the sopC DNA segment.

There is little information about chromosome partitioning in prokaryotes. It is, however, revealed that low copy number plasmids in bacteria have their own partition mechanism: F plasmid (Ogura and Hiraga, 1983), p1 (Austen and Abeles, 1985), R1 (Gerdes and Molin, 1986), and pSC101 (Meacock and Cohen, 1980) in Escherichia coli, and pTAR in Agrobacterium tumefaciens (Gallie and Kado, 1987). The F plasmid has two trans-acting genes, sopA and sopB, and the cis-acting DNA site sopC, which are essential for accurate partitioning of plasmid DNA molecules into both daughter cells in cell division (Ogura and Hiraga, 1983). The complete DNA sequence of the segment containing sopA, sopB, and sopC was determined (Mori et al., 1986). The sopA and sopB genes code for 43.7- and 35.4-kDa proteins, respectively (Wehmann and Eichenlaub, 1980; Komai et al., 1982; Phua and Sauer, 1982; Bex et al., 1983; Austin and Wierzchicki, 1983; Hayakawa et al., 1985; Mori et al., 1986). The result of DNA sequencing suggests that the sopA and sopB genes construct an operon, and this operon is transcribed in the direction sopA to sopB (Mori et al., 1986). The sopC region consists of 12 direct repeats of the 43-base pairs motif without any spacer sequences (Mori et al., 1986; Helsberg and Eichenlaub, 1986; Lane et al., 1987). The SopB protein binds to the sopC region (Hayakawa et al., 1985). Although the SopA protein is essential for plasmid partitioning, there are no informations for the function of SopA protein so far.

In this study, we found that the purified SopA protein effectively binds in vitro to the regulatory region of the sopAB operon, and the binding is enhanced by the addition of the SopB protein. Autoregulation of the operon by these proteins is discussed. We also found that SopB protein binds the inverted repeats of 7 bp (base pair) in each of the 43-bp direct repeats of the sopC region.

**EXPERIMENTAL PROCEDURES**

Chemicals and Enzymes

DE52 and cellulose phosphate P-11 were obtained from Whatman, Blue-Sepharose CL-6B, heparin-Sepharose CL-6B, DEAE-Sephacel, and Sephacryl S-200 were obtained from Pharmacia LKB Biotechnology Inc. The low molecular weight electrophoresis calibration kit obtained from Pharmacia LKB Biotechnology Inc. was used as the molecular weight standards of protein in SDS-polyacrylamide gel electrophoresis. The silver staining kit was obtained from Daichi-Kagaku Co., ampicillin from Meiji Seika Co., Tokyo, and tetracycline from Sigma. CBB color solution for determination of protein concentration was from Nakarai Chemical Co., and [γ-32P]ATP (8000 Ci/mmole) was obtained from Amersham International. Restriction enzymes were obtained from Takara Shuzo Co., Boehringer-Mannheim, or New England Biolabs. Ribonuclease-free deoxyribonucleic acid (DRF) from bovine pancreas was obtained from Cooper Biochemical. Lysozyme and bovine serum albumin were obtained from Sigma. Calf intestine alkaline phosphatase was obtained from Boehringer-Mannheim and T4 DNA polymerase, T4 DNA polynucleotide kinase, and T4 DNA ligase from Takara Shuzo Co.

**Bacterial Strains and Plasmids**

The used strains of *E. coli* K12 were P678-54 (Adler et al., 1967), DH1 (Low, 1968), HB101 (Boveri and Backman, 1979), and KY7231 (Ogura and Hiraga, 1983). pBR322 (Boliver et al., 1977) was used as a vector. pXX167 is a pBR322 derivative carrying the *PstI-EcoRI* segment (5788–9564; according to the complete nucleotide sequence, 1986, EMBL Data Base) of F plasmid, which contains sopA, sopB, and sopC (Ogura and Hiraga, 1983). Bacterial strain P678-54 carrying pXX529 (this paper) was used for purification of SopA protein. Strain KY7231 carrying pXX523 (Kusukawa et al., 1987) was used for purification of SopB protein.

**Construction of Plasmids**

pBR322 DNA was digested with *PstI* and the *PstI* site was changed to an EcoRI site using T4 DNA polymerase and an EcoRI linker. The resulting plasmid, designated pXX524, lost the *bla* gene conferring ampicillin resistance. The *Bsp1298* (6531)·*HindIII* (7783) fragment contains the sequence used for the preparation of sopA and sopB genes.

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1 The abbreviations used are: bp, base pair(s); SDS, sodium dodecyl sulfate.
DNA Binding of F Plasmid Partitioning Proteins

taining the sopA gene and the operator-promoter region was isolated from pX167 and inserted into pXX529 using an EcoRI linker. The resulting plasmids was named pXX529. The construction of pXX529 was described previously (Kusukawa et al., 1987). These plasmids were introduced into the bacterial strain P678-54, HB101, DH1, and KY7231 by transformation. Transformants were confirmed for production of the SopA or SopB proteins and compared the amounts of these proteins by SDS-polyacrylamide gel electrophoresis.

Buffer Solutions Used for Protein Purification

Buffer solutions (SPB; Sop protein purification buffer) utilized throughout the purification procedures contained 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10 mM β-mercaptoethanol, 10% glycerol and various amounts of NaCl. These buffers are referred to as 50 mM NaCl-SPB, 100 mM NaCl-SPB, etc., to indicate the concentration of NaCl present in the buffer.

Cell Cultures Used for Protein Purification

One liter of Luria broth (1% Bacto-tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.4) with 15 µg/ml tetracycline in 2-liter flasks were inoculated with 50 ml of fresh overnight cultures and shaken at 37°C for 15 h. The cultures were centrifuged at 8000 g for 15 min. The cells were suspended in 20 ml of 50 mM NaCl-SPB, transferred into a 50-ml tube, and recentrifuged at 8000 x g for 10 min. The supernatant fluid was removed, and the pellets were stored at -70°C.

Gel Electrophoresis of Proteins

SDS-polyacrylamide gel electrophoresis was carried out according to Laemmli (1970) with 10.5% acrylamide used. Samples were boiled for 5 min in SDS sample buffer (0.125 mM Tris-HCl, pH 6.8; 4% SDS; 40% glycerol; 0.14 M β-mercaptoethanol; 0.01% bromphenol blue). Gels were run in a Tris glycine buffer (25 mM Tris-HCl; 0.19 mM glycine; 1% SDS) at 40 mA until bromphenol blue reached to the end of the gels. The gels were stained with a silver staining kit.

Determination of Protein Concentration

Protein concentration of SopA and SopB was measured by the CBB color solution.

Assay of Proteins of Bacterial Cells by Electrophoresis

5 ml of Luria broth containing 15 µg/ml tetracycline was inoculated with a small portion of a desired strain. The culture was grown, shaken, collected by centrifugation, and resuspended in a test tube. The culture was transferred into a test tube and centrifuged to collect cells. Cells were suspended with 500 µl of sample buffer and boiled for 5 min, followed by SDS-polyacrylamide gel electrophoresis to test the efficiency of the SopA and SopB productions.

Gel Retardation Assay

Assays were carried out essentially as described by Abeles (1986) with several modifications. Each binding reaction contained 10 mM Tris-HCl at pH 7.6, 3 mM MgCl₂, 5 mM CaCl₂, and 50 mM NaCl in a volume of 20 µl containing 3²P-labeled DNA fragments and various concentrations of SopA or SopB protein. The components were allowed to react at 0°C for 50 min. 5 µl of the stop dye solution (40% glycerol, 0.05% bromphenol blue, and xylene cyanol) was added. The samples were immediately loaded on a prerun 6% polyacrylamide gel (20 x 20 cm) in ¼ x TBE buffer (45 mM Tris base, 45 mM boric acid, and 1 mM EDTA, pH 8.0) and submitted to electrophoresis at 40 mA for about 45 min. The gels were dried and autoradiographed with Fuji RX film without an intensifying screen at room temperature for about 12 h.

DNa 1 Footprinting

The protection experiments against digestion by DNase I were performed principally according to Alba (1983). DNA binding reactions were carried out in 100 µl of reaction mixture containing 3²P-labeled DNA fragment, 20 mM Tris-HCl (pH 7.9), 3 mM MgCl₂, 5 mM CaCl₂, 100 mM NaCl, 0.1 mM dithiothreitol, 0.1 mM EDTA, 50 µg/ml of bovine serum albumin, and various concentration of SopA, SopB, or both the proteins. After a 30-min preincubation at 0°C, five µl of RNase-free DNase I (0.1 mg/ml) was added and further incubated for 20 min. After adding 50 µl of stop solution (10 mM sodium acetate, 20 mM EDTA, and 100 µg/ml tRNA), the mixture was treated with phenol. The extracted DNA was precipitated with ethanol. The precipitate was rinsed with ethanol and dissolved in 3 µl of TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). 3 µl of loading buffer (95% of deionized formamide, 0.1% xylene cyanol, 0.1% bromphenol blue) was added. After 2-min boiling, samples were loaded on a gel of 8% polyacrylamide and 8 M urea in TBE buffer (90 mM Tris base, 89 mM boric acid, 2 mM EDTA, pH 8.0) and submitted to electrophoresis at 50 volumes/cm for about 2.5 or 5 h. The gels were dried and autodiradiographed with Fuji RX film with an intensifying screen at -70°C for about 15 h.

Overproduction of SopA and SopB Proteins

To get large amounts of the SopA protein, the plasmid pXX529 carrying the sopA gene was constructed as shown in Fig. 1. This plasmid was introduced into bacterial strains, DH1, HB101, P678-54, and KY7231 by transformation, and cell extracts were analyzed by SDS-polyacrylamide gel electrophoresis to compare the amounts of the SopA protein. An SDS-polyacrylamide gel of total cellular proteins revealed novel proteins from these strains with plasmids but not from those without plasmids (data not shown). Since the amount of SopA protein was highest in the P678-54 strain carrying pXX529, this strain was chosen for purification of the SopA protein. On the other hand, the strain KY7231 carrying the plasmid pXX523, containing the sopB gene (see Fig. 1), was chosen for purification of SopB protein from the highest production of SopB protein. SopA or SopB protein was distinguished as an additional band in SDS-polyacrylamide gel electrophoresis comparing with the parallel sample obtained from plasmid-free bacteria in all following purification steps.

Purification of the SopA Protein

First step: Preparation of the Cell Extract—The cell pellet (about 50 g of wet weight) from a 20-liter culture of P678-54 carrying pXX529 was thawed on ice for several hours and then suspended in 90 ml of 50 mM NaCl-SPB, 10 ml of 5 mg/ml lysozyme was added. After 1-h incubation on ice, the cell suspension was sonicated with a Branson sonifier. 30-s sonication was performed five times, interspersing with 1-min cooling periods in ice water. The sonicated sample was centrifuged at 10,000 x g for 1 h at 4°C. A small portion of the supernatant was analyzed by SDS-polyacrylamide gel electrophoresis.

Step 2: Bio-Rex-70 Column—The supernatant was loaded onto a Bio-Rex-70 column (100 ml, 2.5 x 20 cm) equilibrated with 50 mM NaCl-SPB. The SopA protein was not adsorbed to the Bio-Rex-70 column. The flow-through fractions were pooled. A small portion of the pooled fractions was analyzed by electrophoresis.

Step 3: DE52 Column—The flow-through fraction was loaded on a DE52 column (75 ml, 2.5 x 15 cm) equilibrated with 50 mM NaCl-SPB. The column was washed with 300 ml of 50 mM NaCl-SPB, and then adsorbed proteins were eluted with a 300 ml gradient of 50 mM to 0.5 M NaCl-SPB. Fractions were collected and analyzed by SDS-PAGE.

FIG. 1. pBR322 derivatives carrying the sopA or sopB gene and the 3²P-labeled DNA fragments used for the DNA-protein binding experiments and the footprinting experiments. The top bar represents the Psii-EcoRI fragment containing the sop genes. The numbers in parentheses denote base pair coordinates on the mini-F plasmid (Eichenlaub, 1986, EMBL Data Bank). Two hatched boxes under the top thick bar represent the fragments carrying sopA or sopB. These fragments were cloned into the pBR322 vector using an EcoRI linker. The resulting plasmids pXX529 and pXX523 carry sopA and sopB, respectively. Both plasmids carry the tet gene. Open bars represent the DNA fragments labeled with 3²P at one or both ends (asterisk).
polyacrylamide gel electrophoresis. The fractions containing the SopA protein were pooled and dialyzed overnight at 4 °C against 50 mM NaCl-SPB 3 changes.

**Step 4: Phosphocellulose P-11 Column.** The dialyzed sample was loaded on a phosphocellulose column (50 ml, 2.5 × 10 cm) equilibrated with 50 mM NaCl-SPB and washed with 200 ml of the same buffer. The SopA protein was eluted with a 200-ml gradient of 50 mM to 0.5 M NaCl-SPB. Fractions (100 drops) were collected and analyzed by the electrophoresis. The SopA containing fractions were pooled and dialyzed overnight against three changes of 50 mM NaCl-SPB at 4 °C.

**Step 5: Blue-Sepharose CL-6B Column.** The dialyzed sample was loaded on a blue-Sepharose CL-6B column (30 ml, 2.5 × 7 cm) equilibrated with 50 mM NaCl-SPB and was washed with 100 ml of the same solution. The proteins adsorbed to the column were eluted with a 100-ml gradient of 50 mM to 1.25 M NaCl-SPB, and fractions (50 drops) were collected and analyzed by the electrophoresis. The SopA containing fractions were pooled and dialyzed against 50 mM NaCl-SPB. This solution was concentrated by a ultrafiltration apparatus with a hollow fiber membrane tube. The final protein concentration of the SopA protein solution was about 1 mg/ml. About 5 mg of SopA protein was purified from 50-g cells. The proteins present in each steps are shown in Fig. 2A.

**Purification of SopB Protein**

**Step 1: Preparation of the Cell Extract.** The cell extract of KY7231 carrying pXX523 was prepared as described in the Step 1 of SopA protein.

**Step 2: DEAE-Sepahcell Column.** The supernatant was loaded on a DEAE-Sepahcell column (100 ml, 2.5 × 20 cm) equilibrated with 50 mM NaCl-SPB. The SopB protein does not charge to this DEAE-Sepahcell column. The flow-through fraction was pooled and submitted to the next step.

**Step 3: Phosphocellulose P-11 Column.** The flow-through fraction was loaded on a P-11 column (75 ml, 2.5 × 15 cm) equilibrated with 50 mM NaCl-SPB, washed with 300 ml of 50 mM NaCl-SPB, and eluted with a 300-ml gradient of 50 mM to 0.5 M NaCl-SPB. Fractions (150 drops) were collected and analyzed by the electrophoresis. The fractions containing SopB protein were pooled and dialyzed at 4 °C overnight against three changes of 50 mM NaCl-SPB.

**Step 4: Blue-Sepharose CL-6B Column.** The SopB solution was loaded on a blue-Sepharose CL-6B column (30 ml, 2.5 × 7 cm) equilibrated with 50 mM NaCl-SPB and washed with 100 ml of the same buffer. The column was eluted with a 100-ml gradient of 50 mM to 1.25 M NaCl-SPB. Fractions (50 drops) were collected and analyzed by the electrophoresis. The SopB fractions were pooled and dialyzed against 50 mM NaCl-SPB.

**Step 5: Heparin-Sepharose CL-6B Column.** The pooled solution was loaded on a heparin-Sepharose CL-6B column (20 ml, 1.8 × 7.5 cm) equilibrated with 50 mM NaCl-SPB and was washed with the same buffer. It was eluted with a 100-ml gradient of 50 mM to 1.25 M NaCl-SPB, and fractions (50 drops) were collected and analyzed by the electrophoresis. The SopB solution was further concentrated by Centricon-30 from Amicon until about 1 ml.

**Step 6: Sephacryl S-200 Column.** The concentrated solution was loaded on a Sephacryl S-200 (2.5 × 100 cm) column and eluted with 0.5 M NaCl-SPB. Fractions (80 drops) were collected and analyzed by the same way as described above. The SopB fractions were pooled and dialyzed against 50 mM NaCl-SPB. After dialysis, the solution was concentrated by the ultrafiltration with hollow fiber membranes until about 4 ml. The SopB solution was divided into small aliquots and stored at −20 °C. The final concentration of the SopB protein solution was about 1 mg/ml. The proteins present in each steps are shown in Fig. 2B.

A portion of each purified protein was used to analyze for amino terminus sequence analysis. The results showed that the sequence of the first 20 amino acids of SopA protein was Met-Lys-Leu-Met-Glu-Thr-Leu-Asn-Glu-(Cys)-Ile-Asn-Ala-Gly-His-Glu-Met-Thr-Lys-Asp-Ala and that the first 20 amino acids of SopB protein was Met-Lys-Arg-Ala-Pro-Val-Ile-Pro-Lys-His-Thr-Leu-Asn-Thr-Glu-Pro-Val-Glu-Asp-Thr. These results are completely consistent with those from nucleotide sequences (Mori et al., 1986). The amino acid compositions of the two proteins were also determined. The results are consistent with those predicted from nucleotide sequences (Table I).

### Table I

<table>
<thead>
<tr>
<th>Amino acid composition of SopA and SopB proteins</th>
<th>SopA</th>
<th>SopB</th>
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<tbody>
<tr>
<td>Found</td>
<td>Predicted</td>
<td>Found</td>
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<tr>
<td>Ala</td>
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<td>30</td>
</tr>
<tr>
<td>Arg</td>
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<td>27</td>
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<tr>
<td>Asx</td>
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<td>42</td>
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<tr>
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<tr>
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<tr>
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<tr>
<td>Lys</td>
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<td>17</td>
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<tr>
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<tr>
<td>Val</td>
<td>24.8</td>
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</table>

<sup>a</sup>The amino acid analysis was based on one 22-h hydrolysis.
<sup>b</sup>Prediction based on the nucleotide sequence.

<sup>A</sup>Asn and Asp.
<sup>G</sup>Gln and Glu.
<sup>ND</sup>Not determined.

**Fig. 2. Electrophoresis of samples in various steps of purification.** A, SopA protein in the cell extract of the strain P678-54 carrying pXX529 was purified as described in the text. Samples of various purification steps were analyzed by electrophoresis. Lanes 1–5, Steps 1–5. B, SopB protein in the cell extract of the strain KY7231 carrying pXX523 was purified as described in the text. Lanes 1–6, Steps 1–6.
RESULTS

Binding of the SopA Protein to the Promoter-Operator Region of the sopAB Operon—In the amino acid sequence of SopA protein deduced from DNA sequence (Mori et al., 1986), we found an amino acid sequence which was similar to the consensus sequence of the DNA binding domain in various DNA binding proteins (Pabo and Sauer, 1984). To test the binding ability of SopA protein to DNA segments, we used the polyacrylamide gel electrophoresis technique described under “Experimental Procedures.” The DNA-protein complex migrates more slowly through the gel than the free DNA fragment. In the binding assay, we used the HaeII(6331)-HpaII(6597) DNA fragment carrying the promoter-operator region and the promoter proximal part of the sopA gene and the BclI(7565)-HpaII(7718) DNA fragment carrying the putative promoter of the sopB gene (see Fig. 1). As shown in Fig. 3A, when 1 mM of SopA protein was added, SopA protein bound to the HaeII-HpaII DNA fragment (lane 5 of Fig. 3A), but not to the BclI-HpaII DNA fragment (data not shown).

When low concentrations of SopA were added, the DNA-protein complex was not observed (lanes 2–4 in Fig. 3A).

Effect of SopB Protein on Binding of SopA Protein to the Promoter-Operator Region—We observed that SopA protein was overproduced in vivo when the sopA gene and its promoter region, but not the sopB gene, was cloned into pHBR322 vector and that SopA was not overproduced when both the sopA and sopB genes were cloned together (data not shown). These phenomena suggest that the SopB protein has a role for the regulation of expression of the sopA gene. To test the possibility that SopB protein acts as the modulator of SopA protein in the SopA-DNA binding, we assayed in vitro the effect of the SopB protein on the formation of the DNA-protein complex. The SopB protein itself bound neither the HaeII-HpaII DNA fragment (Fig. 3A, lanes 6–9) nor the BclI-HpaII DNA fragment (data not shown). When SopA and SopB proteins (0.5 μM each) were added to the HaeII-HpaII DNA fragment, DNA-protein complexes were detected (Fig. 3A, lane 12). This result suggests that SopB protein enhances the formation of the SopA protein-DNA complex.

To confirm this result, the HaeII-HpaII DNA fragment and various amounts of the SopA protein were incubated in the presence of 0.5 μM of SopB protein. After electrophoresis, the ratio of slow migrated DNA to total DNA were densitometrically analyzed. As shown in Fig. 3B, the addition of SopB protein clearly stimulated the DNA-protein binding. In the presence of 0.5 μM SopB protein, 20% of labeled DNA formed the DNA-protein complex even in 0.01 μM SopA protein. We performed similar type of experiments in the presence of bovine serum albumin (0.5 μM) instead of SopB protein. Bovine serum albumin had no effects, suggesting that the stimulation was specific for SopB protein.

The addition of high amounts (over 2 μM) of SopB protein in the DNA-protein binding assay caused nonspecific aggregation of DNA and SopB protein in any DNA fragments. The aggregates remained at the top of gel after electrophoresis.

Footprinting: Protection of the Promoter-Operator Region by SopA Protein—As described above, SopA protein at high concentrations binds specifically to the HaeII-HpaII DNA fragment of the promoter-operator region. To determine the precise binding sites, we performed footprinting experiments with DNase I. As shown in Fig. 4 (lanes 2–5) and summarized in Fig. 5, three sites, 6406–6412 (ACTTTGC), 6424–6431 (ACTTTGC), and 6443–6450 (GCTTTGC) were clearly protected by SopA protein against the DNase I digestion. These protected sequences contain the 6-bp sequence CTTTGC. In addition, the 6479–6486 region was also protected by SopA protein. This region also contains the 6-bp consensus sequence in the complementary strand. Nucleotide bands at the positions 6432–6433 and 6471–6472 became hypersensitive to the DNase I digestion in the presence of SopA.

Footprinting: Protection of the Promoter-Operator Region in the Presence of Both SopA and SopB Proteins—We analyzed protected sites of the operator-promoter region in the presence of both SopA and SopB proteins. As shown in lanes 6–9 of Fig. 4, protection patterns were similar to those in the presence of SopA alone (see lanes 2–5 of Fig. 4); however, the nucleotide band at position 6484–6485 became hypersensitive to DNase I digestion in the presence of SopA and SopB proteins.

Binding of SopB Protein to the sopC DNA—We have described previously the complete DNA sequence of the sop region and the striking features were revealed in the sopC region (Mori et al., 1986). There are 12 43-bp direct repeats and every repeat has a pair of 7-bp inverted repeats. Hayakawa et al. (1985) showed that the SopB protein bound specif-
to the PstI-EcoRI DNA fragment (nucleotide No. 7329–9564 in Fig. 1). To demonstrate that the SopB protein binds to specific sites of the sopC region, we performed the binding assay using the purified SopB protein and the AccI(8660)-HinflI(9216) DNA fragment, which carries the first to eleventh direct repeats, the 52-bp upstream region of the direct repeats, and a half of the twelfth direct repeat (see Fig. 1). The result is shown in Fig. 6. When SopB proteins were used 100 times

\[ \begin{array}{c|cccccccccc} \text{Lane} & 1 & 2 & 3 & 4 & 5 & 6 & 7 & 8 & 9 & 10 \\ \hline \text{SopA} & 0 & 0 & 5 & 20 & 0 & 1 & 5 & 20 & 0 & \mu M \\ \text{SopB} & 0 & 0 & 0 & 0 & 5 & 5 & 5 & 5 & 0 & \mu M \\ \end{array} \]

\[ \text{SD} \]

\[ \begin{array}{c|cccc} \text{molar ratio} & 20 & 40 & 100 & 200 \\ \hline \text{molar} & 3 & 2 & 1 & 0.5 \\ \end{array} \]

Fig. 4. Footprinting pattern of the HaeII(6331)-HpaII(6597) DNA fragment in the presence of SopA protein or both SopA and SopB proteins. ~10 nM HaeII(6331)-end-labeled DNA fragment was used. Lane 1, standard markers by the Maxam-Gilbert sequencing G + A reaction. Lanes 2 and 10, control without proteins. Lane 3, 1 µM SopA. Lane 4, 5 µM SopA. Lane 5, 20 µM SopA. Lane 6, 5 µM SopB. Lane 7, 5 µM SopB plus 1 µM SopA. Lane 8, 5 µM SopB plus 5 µM SopA. Lane 9, 5 µM SopB plus 10 µM SopA. The -10 and -35 regions are shown as open bars. The sopA structural gene is shown by a thin arrow. The thick arrows with numbers shown in the left side represent the repeated sequences (CCTTTGC). Horizontal arrows shown in the right side indicate hypersensitive sites against DNase I digestion in the presence of protein. An open arrow indicates the sensitive sites in the presence of both SopA and SopB proteins. The numbers denote base pair co-ordinates on the mini-F plasmid (Eichenlaub, 1986, EMBL Data Bank).

Fig. 5. Summary of footprinting experiments. Four arrows shown under the nucleotide sequences represent the repeated sequences (CCTTTGC). Black bars are the regions protected against the DNase I digestion by SopA protein (or the complex of SopA and SopB). Hypersensitive sites are indicated by the vertical arrows on the sequences. An open arrow shows the sensitive site against DNase I digestion in the presence of both SopA and SopB proteins. The -35 and -10 regions and Shine-Dalgarno sequence (SD) are surrounded by boxes. The numbers denote base pair co-ordinates on the mini-F plasmid (Eichenlaub, 1986, EMBL Data Bank).

Fig. 6. Specific binding of SopB protein to the sopC DNA fragment. ~5 nM 32P-labeled AccI(8660)-HinflI(9216) DNA fragment was mixed with SopB protein and allowed to bind for 30 min at 0 °C as described under “Experimental Procedures.” The concentration of SopB added was 0.1 (lane 1), 0.2 (lane 2), 0.5 (lane 3), 1 (lane 4), and 1.7 µM (lane 5).
molar more than sopC DNA, DNA-protein complexes were formed (see Fig. 6, lane 3). Many distinct retarded bands could be observed. It might be due to the difference in the number of SopB protein molecules binding to a DNA molecule.

To determine the precise DNA-binding sites, we performed the DNase I footprinting experiments using the AccI(8735)-HinflI(9216) DNA fragment labeled with 32P at the Hinfl site. Results in Fig. 7 (lane 2) clearly shows that the 24-bp sequence containing a pair of inverted repeats in each direct repeat was protected by SopB protein. At least 10 regions were observed to be protected in this gel. The first protected region corresponds to the eleventh direct repeat. Similar results were obtained using the AccI(8660)-Sau3AI(9130) DNA fragment labeled with 32P at the AccI site (Fig. 7, lane 7). DNA sequences of the first pair of inverted repeats and the twelfth one are slightly different from the others (Mori et al., 1986; Helsberg and Eichenlaub, 1986). Fig. 7 (lane 7) shows that the first one was weakly protected from DNase I digestion, whereas the other repeats were strongly protected. The results are summarized in Fig. 8.

The individual repeats at least from second to eleventh one had similar affinities for the SopB protein when the experiment was carried out using the lower amounts of the protein (data not shown).

Effects of SopA Protein on Binding between SopB Protein and the sopC DNA—As shown in lanes 3 and 8 in Fig. 7, the addition of purified SopA protein had no effect on the binding between SopB protein and the sopC DNA. When SopA protein was mixed with the sopC DNA, but not SopB protein, protec-
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**Fig. 7.** Footprint analysis of the sopC DNA fragment in the presence of SopB protein, SopA protein, or both proteins. Lanes 1-5, the 10 nM 32P-labeled Apal(8735)-HindIII(9216) DNA fragment. Lanes 6-10, the 10 nM 32P-labeled AccI(8660)-Sau3AI(9130) DNA fragment. No proteins were added in lanes 1, 5, 6, and 10. 2.5 μM of each protein was added as follows; SopB protein (lanes 2 and 7), SopA and SopB proteins (lanes 3 and 8), and SopA protein (lanes 4 and 9).

**DISCUSSION**

Results presented here indicate that the purified SopA protein binds specifically to the sites in the operator-promoter region of the sopAB operon. Moreover, in the presence of both SopA and SopB proteins, the DNA-protein complex formation is enhanced. This result suggests that the complex of SopA and SopB proteins regulates the expression of the sopAB operon. Wehlmann and Eichenlaub (1980) showed that sopB mutants overproduced SopA protein (A protein) at least 5-fold that of the parental strain. Kusukawa et al. (1987) described that SopA protein is overproduced when the DNA segment containing the intact sopA gene and its operator-promoter region is cloned on a vector plasmid. In addition, a mutation occurred in the sopB gene of a plasmid carrying both the sopA and sopB genes stimulates the production of SopA protein. The simplest explanation for these results is that SopB protein acts as the repressor of the sop operon. However, as shown in Fig. 3A, the purified SopB protein does not bind to the operator-promoter fragment of the sopA operon and the possible promoter fragment of the sopB gene. Based on these results, we propose a model that the complex of SopA and SopB proteins acts as the autorepressor of the sopB operon (Fig. 8). Our recent results, in vitro, indicate that both Sop proteins are essential for the repression of the sopAB operon. The autoregulation of the sopAB operon is important to insure plasmid partitioning, because overproduction of SopB protein causes plasmid instability and deletions of the sopC region (Ogura and Hiraga, 1983; Kusukawa et al., 1987).

Recently, we performed a preliminary experiment to determine whether SopB protein was included in the SopA-promoter complexes. Proteins that migrated with protein-DNA complexes were transferred to a nitrocellulose filter and visualized by anti-SopA IgG or anti-SopB IgG. The result seems to indicate that the SopB protein is contained in the complex.

Although the SopA protein may collaborate with the SopB protein in the autorepression of the sopAB operon, this function may not be the sole role of SopA protein. This protein may also have another function essential for plasmid partitioning. Ogura and Hiraga (1983) described previously that an oriC plasmid carrying the sopB and sopC genes, but neither the sopA gene nor the promoter-operator region of the sopAB operon, is unstable in nonselective media and that a pBR322 derivative carrying the sopA gene stabilizes this plasmid. This indicates that the SopA protein has another function which is different from the autorepressor function. There are two possibilities: (i) SopA protein is one of the important components of the partition apparatus, partisome (Hiraga et al., 1985), and (ii) SopA protein molecules bind to free SopB protein molecules to avoid the IncG incompatibility caused by excess amounts of SopB protein (Kusukawa et al., 1987).

The structural similarity in arrangements of genes and functional sites of replication and partition between P1 and F plasmids was described (Abeles et al., 1984; Abeles et al.,

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1985; Mori et al., 1986). Davis and Austin (1988) described recently that the purified ParB protein bound specifically to the cis-acting parS site of P1 and that no binding to parS was evident when purified ParA protein was used. ParA and ParB proteins are required in vivo for the autorepression of the parAB operon (Friedman and Austin, 1988). We found three direct repeats of the nucleotide sequence AAGCAT and a similar sequence AAAGCAT which was located in the opposite direction against those repeats in the promoter region of the P1 parAB operon. The arrangement of these sequences is similar to that of F (see Fig. 5). We suppose that ParA protein recognizes these repeated sequences and that the complex of ParA and ParB acts as the autorepressor of the parAB operon.

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