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Purification and Characterization of a Low-Molecular-Weight Membrane Protein with Affinity for the *Escherichia coli* Origin of Replication

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A purification procedure was devised for a low-molecular-mass (about 10-kilodalton) membrane protein from *Escherichia coli* that was shown to bind specifically to the chromosomal replication origin region (*oriC*). Nitrocellulose membrane retention assays showed the binding site to be adjacent to the right boundary of the *oriC* minimal sequence. We determined the amino acid sequence of the N-terminal and C-terminal regions as well as the global amino acid composition of this membrane protein. Specific antibodies against the protein were produced and used to confirm the cell membrane location of the protein. These results demonstrate that this is a new membrane protein, different from the previously described B′ protein, with specific binding activity for the *oriC* region. We propose that this protein be called membrane *oriC*-binding protein 2 (MOB2 protein).

It has been conjectured that the bacterial origin of replication is associated with the membrane. This attachment, as postulated in the replicon hypothesis (11), could play a role in ensuring proper partitioning of daughter chromosomes at the time of cell division. Membrane-DNA complexes enriched in DNA from the region of the origin of replication have been isolated in *Escherichia coli* (7). More recently, Kusano et al. identified several binding sites in the *E. coli* chromosomal origin of replication (*oriC*) region which were important for specific association of the DNA to the membrane fraction (15). To study the interaction between *oriC* and the membrane, we used a different approach and isolated membrane proteins having a specific affinity for DNA containing the *oriC* region. We obtained a membrane fraction containing a 60-kilodalton (kDa) protein called B′ which showed preferential affinity for single-stranded *oriC* DNA (14). Two specific binding sites were identified for protein B′, one located in the minimal *oriC* sequence, around the *Bam*HI position 92 [*Bam*HI(92)] site on the strand reading 3′-OH–5′-P in the direction of the *E. coli* genetic map (site I), and the other located between positions 417 and 488 on the strand reading 5′-P–3′-OH (site II) (13, 14).

Some of the B′ preparation contained an additional protein of 12 kDa (13). This finding led us to question the role of this protein in the interaction of the B′ preparation with the *oriC* region. In a preliminary report, we described the purification of a heat-stable, low-molecular-weight membrane DNA-binding protein that bound specifically to an *oriC*-containing plasmid DNA in either the double-stranded or the single-stranded state (4). Its apparent molecular size of 12 kDa suggested that it may be identical to the protein seen in the B′ preparation. It was therefore important to examine more precisely the binding properties of the 12-kDa protein to determine whether they could account for properties attributed to the B′ preparation. Consequently, we decided to characterize in more detail the biochemical properties of this low-molecular-weight protein (referred to herein as MOB2 protein) and determine its specific binding site(s) in the *oriC* region.

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MATERIALS AND METHODS

Purification of the MOB2 protein. All procedures were carried out at 4°C unless otherwise specified. A 210-g portion of cell paste, obtained from a 40-liter culture of *E. coli* K-12 PA3092 and harvested during exponential growth, was suspended in 420 ml of 20 mM Tris hydrochloride (pH 8.3) containing 10 mM magnesium acetate, 10 mM 2-mercaptoethanol, 0.5 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), and 10% glycerol. After centrifugation at 10,000 × *g* for 10 min, cells were suspended in 210 ml of 50 mM Tris hydrochloride (pH 8.3) containing 5 mM EDTA and 0.1 mM dithiothreitol. Cells were lysed by the addition of lysozyme to a final concentration of 1 mg/ml and incubation for 30 min at 0°C in the presence of 0.5 mM phenylmethylsulfonyl fluoride. Pancreatic DNase I and $MgCl_2$ were then added to final concentrations of 10 μg/ml and 5 mM, respectively, followed by incubation at 37°C for 15 min. The lysate was centrifuged at 12,000 × *g* for 20 min to remove cell debris, and the supernatant was centrifuged at 120,000 × *g* for 60 min after the final concentration of $MgCl_2$ was adjusted to 20 mM. The pellet (total membrane fraction) was suspended in 100 ml of 10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (pH 6.5) containing 1 mM dithiothreitol, 5 mM EGTA, and 10% glycerol. This material was then successively solubilized by 1% Triton X-100 (solubilized membrane fraction 1), 1% Triton X-100 and 2 M urea (solubilized membrane fraction 2), and 1% Triton X-100 and 4 M urea (solubilized membrane fraction 3) as previously described (4). Fraction 3 was used for further purification.

Solubilized membrane fraction 3 containing 175 mg of protein in 35 ml was dialyzed at room temperature against 10 mM MES buffer (pH 6.5) containing 1 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 10% glycerol, and 0.1% Triton X-100 (buffer A) to eliminate urea. The fraction was heated at 100°C until a precipitate became visible and then further

incubated for 3 min. After centrifugation at 10,000 × g for 15 min, the pellet was washed with 10 ml of the dialyzing buffer by an additional centrifugation step, and both supernatants were pooled.

Boiled solubilized membrane fraction 3 containing 18 mg of protein in 45 ml was passed over a 6-ml (9.4- by 0.9-cm) phosphocellulose column (P11; Whatman, Inc., Clifton, N.J.) previously equilibrated in buffer A containing 0.1 M NaCl. The column was washed with 10 bed volumes of 0.15 M NaCl in buffer A and then eluted with a linear salt gradient of 0.15 to 1.5 M NaCl (120 ml) in the same buffer. The main products obtained were two close peaks of binding activity to supercoiled pOC42 [^{3}H]DNA (18), which were eluted between 0.5 and 0.75 M NaCl. Each peak was assayed for specific binding to *oriC* DNA by competition experiments between supercoiled pOC42 [^{3}H]DNA (*oriC*$^+$) and supercoiled pBR322 cold DNA. Both peaks showed specific recognition (i.e., preferential binding to pOC42 DNA in the presence of an excess of pBR322 DNA) for supercoiled pOC42 DNA and contained the MOB2 protein as judged from immunoblotting experiments with anti-MOB2 antibodies. Peak 2 appeared purer, as judged from its specific activity and from sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (see Fig. 1B). This material was used for further purification by chromatography on a Blue Trisacryl (I.B.F., France) column; 13 ml of peak 2 containing 845 μg of protein (65 μg/ml) was dialyzed for 2 h against buffer A containing 0.4 M NaCl and then loaded onto the column (0.2 ml) equilibrated in dialyzing buffer. The column was washed with a 20-fold volume of 0.5 M NaCl in buffer A. DNA-binding activity specific for supercoiled pOC42 DNA was eluted stepwise with 10 times the column volume of 0.75 M NaCl in the same buffer. Specific activity of the purified MOB2 protein was 1,300 U/mg.

Application of the fraction to the phosphocellulose column rapidly after heat treatment is necessary because the protein is rapidly inactivated, presumably because of aggregation. Lower specific activity than what we report here obtained for previous preparations (4) is also thought to have been due to such inactivation.

The MOB2 protein could be purified by the same procedure starting from membrane fractions solubilized by 1% Triton X-100 and 2 M urea (solubilized membrane fraction 2). The DNA-binding activity profile after chromatography on phosphocellulose was very similar to the profile obtained with solubilized membrane fraction 3. The two peaks were pooled and loaded onto a Blue Trisacryl column. DNA-binding activity was eluted with a salt gradient of 0.2 to 1.2 M NaCl. *oriC*-specific DNA-binding activity eluted as a single peak between 0.7 and 1 M NaCl (data not shown). Active fractions contained two smaller bands in addition to the MOB2 protein, contaminants which could be eliminated by chromatography on single-stranded DNA Ultrogel (I.B.F.), where they appeared in the 0.1 M NaCl wash; the MOB2 protein eluted at 0.3 M NaCl. The protein so purified was recognized by the anti-MOB2 antibodies.

Assay of DNA-binding activity. The protein fraction to be assayed was added to 0.2 ml of reaction mixture containing 10 mM MES buffer (pH 6.5), 1 mM $MgCl_2$, 50 mM NaCl, 100 μg of bovine serum albumin per ml (fraction VI; Sigma Chemical Co., St. Louis, Mo.), and supercoiled pOC42 [^{3}H]DNA (18). After incubation at 37°C for 5 min or at 0°C for 15 min, radioactive DNA-protein complexes were filtered through a nitrocellulose membrane (Millipore Corp., Bedford, Mass.) as described elsewhere (12). Background DNA retained in the absence of protein (100 to 200 cpm) was subtracted from the results. In competition experiments, the protein fraction was added to a mixture of labeled DNA and an increasing amount of cold competitor DNA. Results were expressed as the percentage of labeled DNA retained when the binding assay was performed in the absence of competitor DNA.

One unit of DNA-binding activity was defined as the retention of 1 nmol (as nucleotide) of supercoiled pOC42 [^{3}H]DNA on the filter in the assay carried out at 37°C in the absence of competitor DNA.

Determination of amino acid composition. For determination of amino acid composition and for assays of each amino acid, 1 μg of protein was hydrolyzed with 50 μl of a 2:1 mixture of hydrochloric acid and trifluoroacetic acid containing 0.05% phenol at 166°C for 25 and 50 min (26). The hydrolysates were dried and dissolved in 0.01 N HCl (made from constant-boiling HCl; Pierce Chemical Co., Rockford, Ill.) and applied to a Durram D500 amino acid analyzer set to a sensitivity of 500 pmol of amino acid. Cystine was analyzed after performic acid oxidation of the protein (8).

For N-terminal sequence determination, 200 pmol of protein was boiled in 1% SDS at 100°C for 5 min and added to the spinning cup of a Sequanator (model 890C; Beckman Instruments, Inc., Fullerton, Calif.) precoated with 3 mg of Polybrene (Pierce). Sequencing and identification of phenylthiohydantoin amino acid were performed as described previously (24).

To determine the C-terminal sequence, 200 pmol of protein was digested with 5 μg of carboxypeptidase A and carboxypeptidase B (both were diisopropylphosphate [Sigma] treated) in 0.1 N collodine-pyridine acetate buffer (pH 8.3) for 3 and 8 h (25). The digests were analyzed with a Durram analyzer set at 500 pmol of amino acids full scale.

Other protein assays. Protein concentration was determined by the method of Bradford (3). SDS-PAGE was carried out according to Laemmli (16); gels were stained either with Coomassie blue or with a silver stain kit (Bio-Rad Laboratories, Richmond, Calif.) according to the instructions of the manufacturer.

Plasmids and DNA labeling. pBR322 and the *oriC*-containing plasmid pOC42 DNA (18) were prepared after amplification by a scale-up of the alkaline extraction method (2) followed by chromatography on hydroxyapatite.

Preparations of tritiated pOC42 plasmids were obtained from pOC42-containing cells grown in supplemented medium 63 (20). [^{3}H]thymidine (4 μCi/μg per ml) (Amersham Corp., Arlington Heights, Ill.) was added 1 h after addition of chloramphenicol (80 μg/ml), and amplification was carried out overnight. Specific activity was between 0.2 and 0.3 μCi per μg of plasmid DNA.

For preparation of ^{32}P-labeled DNA fragments, 5 μg of pOC42 DNA was digested with the appropriate restriction endonuclease(s) (Boehringer GmbH, Mannheim, Federal Republic of Germany, or Bethesda Research Laboratories, Inc., Rockville, Md.). The 3′ ends were labeled with the Klenow fragment of *E. coli* DNA polymerase I (4,000 U/mg; Boehringer) and an appropriate ^{32}P-deoxynucleotide triphosphate (3,000 Ci/mmol; Amersham) according to the method of Maniatis et al. (17). Labeled fragments were then separated on a polyacrylamide gel run in 45 mM Tris borate buffer (pH 8.3) containing 0.5 mM EDTA. After autoradiography, DNA bands were cut out of the gel and electrophoretically extracted from the polyacrylamide in an ISCO sample concentrator (model 1750). The extracted DNA was purified by chloroform treatment in the presence of 1 M NaCl and 1% SDS before ethanol precipitation.

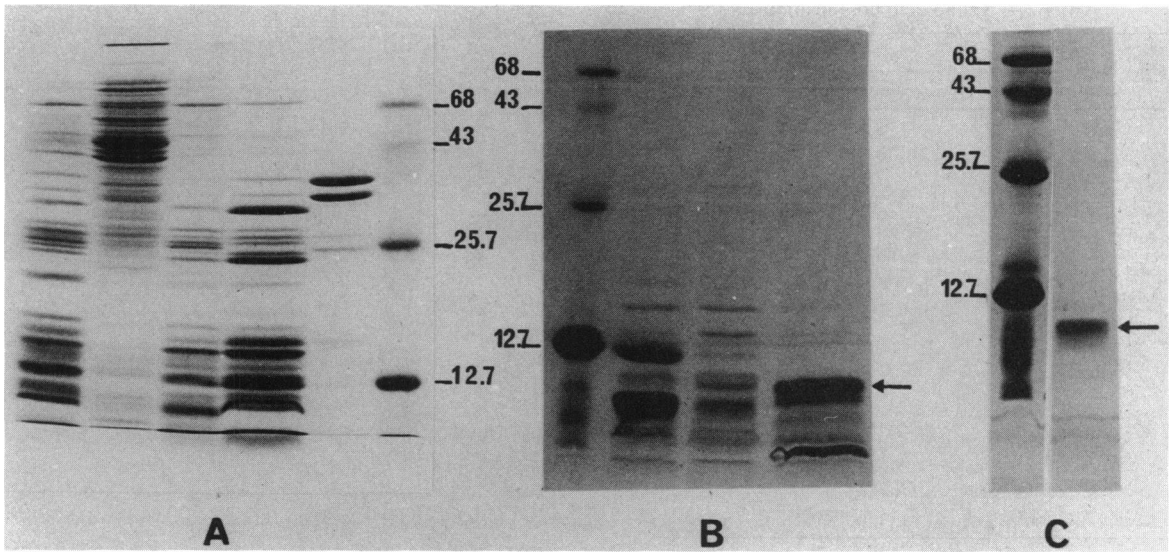

FIG. 1. SDS-PAGE of the MOB2 protein. Molecular mass markers: bovine serum albumin (68 kDa), ovalbumin (43 kDa), chymotrypsinogen (25.7 kDa), cytochrome *c* (12.7 kDa). Gel concentration was 16% acrylamide–0.47% bisacrylamide. (A) From left to right: Total membrane fraction (100 μg), solubilized membrane fraction 1 (220 μg), solubilized membrane fraction 2 (110 μg), solubilized membrane fraction 3 (220 μg), Triton X-100–urea-insoluble membrane fraction (100 μg). (B) Phosphocellulose chromatography of solubilized membrane fraction 3. From left to right: Molecular weight markers, phosphocellulose fraction 18 (20 μg), fraction 26 (15 μg), fraction 40 (18 μg). (C) Blue Trisacryl chromatography of the MOB2 protein. From left to right: Molecular weight markers, Blue Trisacryl–0.75 M NaCl fraction (1.7 μg). Gel in panel A was stained with Coomassie blue; gels in panels B and C were stained by the silver stain technique. Arrows indicate positions of the MOB2 protein.

Immunological methods. To prepare anti-MOB2 protein antibodies, 100 μg of purified MOB2 protein (200 μg/ml) was emulsified with complete Freund adjuvant (Difco Laboratories, Detroit, Mich.) and injected intradermally into a rabbit for the primary injection. One booster injection was administered after 1 month, using incomplete Freund adjuvant and 100 μg of protein. Blood was collected from ear blood vessels.

Fractions to be assayed for immunoblotting were subjected to SDS-PAGE (16% acrylamide) and then electrophoretically transferred on a positively charged nylon membrane (Biodyne TM transfer membrane; Pall Ultrafine Filtration Corp., N.Y.). Procedures recommended by the manufacturer were followed. The MOB2 protein was immunologically detected by incubation with anti-MOB2 polyclonal antibodies and subsequent incubation with goat anti-rabbit immunoglobulin G peroxidase conjugate (Miles Laboratories, Inc., Elkhart, Ind.); 4-chloro-1-naphthol was used as a substrate (23). Molecular weight markers, run on the same gel as the samples, were also transferred, and the corresponding part of the membrane was stained by the ferridye technique (21).

For quantification of the relative amount of protein present in the various cellular fractions, a photograph of the immunoblot was scanned with a densitometer (Shimadzu dual-wavelength thin-layer chromatography density scanner).

RESULTS

Modification of MOB2 protein purification procedure. As in the procedure previously described (4), the MOB2 protein was purified on the basis of its DNA-binding activity. DNA-binding activity was determined by retention of labeled DNA-protein complexes on nitrocellulose filters. ^{3}H-labeled pOC42 (an *oriC*-containing plasmid [18]) DNA was used as a substrate. Since membrane fractions contained unspecific DNA-binding proteins (12; this report) and nonspecific binding of MOB2 protein contributed to the measured activity, each active peak or fraction was further assayed for ability to bind preferentially to pOC42 [^{3}H]DNA in the presence of an excess of cold competitor, non-*oriC* DNA, as determined by competition binding experiments. In addition, each step was monitored by use of anti-MOB2 antibodies (see Results).

The new procedure for purification was designed to solve aggregation problems often encountered with the procedure previously described (4). Addition of Mg^{2+} ions (1 mM) and of NaCl (50 mM) to all buffers after MOB2 solubilization was found to be critical for reducing aggregation of the protein. The DNA-binding assay was also modified, taking into account determination of optimal conditions and optimal substrate for binding activity of the MOB2 protein. Another modification was the introduction of a boiling step, which relied on the stability of the protein when heated at 100°C (4). The complete procedure is detailed in Materials and Methods.

Figure 1 shows SDS-PAGE of various fractions obtained during the purification procedure. The purified MOB2 protein fraction contained only one band, with an apparent molecular mass of 9 kDa (Fig. 1C), and had preferential affinity for supercoiled pOC42 [^{3}H]DNA (see Fig. 5).

Analysis of N-terminal and C-terminal sequences and of amino acid composition. The N-terminal sequence was identified as Lys-Val-Ile-Ala-Glu-Asp-Asn-Gly by Edman degradation. Glycine and a small amount of isoleucine were also found at the first step of Edman degradation. C-terminal analysis revealed that glycine was a contaminant in the protein preparation. The N-terminal sequence was determined by two independent analyses of MOB2 preparations, the second being carried out with the peptide extracted from

TABLE 1. Amino acid composition[a]

Amino acid	Residue		
	25 min	50 min	Integrated value
Asx	7.6	7.6	8
Thr	3.5	3.1	4
Ser	8.4	7.2	10
Glx	9.0	9.0	9
Pro	3.0	3.0	3
Gly	15.1	14.8	15
Ala	12.0	12.1	12
Val	3.5	5.2	5
Met[b]	1.1	1.2	1–2
Ile	2.7	3.0	3
Leu	5.6	5.5	6
Tyr	3.5	3.2	4
Phe	2.6	2.7	3
His[c]	5.4	5.5	<5
Lys	9.5	9.4	10
Arg	7.3	6.6	7
Cys[b]	1.0		1

[a] Tryptophan was not analyzed.
[b] Between Asp and Thr there was a peak corresponding to methionine sulfon (about 1 mol/mol of protein). This was not confirmed.
[c] There was an unidentifiable peak near the histidine peak. The histidine value may therefore be less than 5.

the band in a preparative acrylamide gel. No differences were found between the two determinations, which confirmed their purity.

The C-terminal sequence, deduced from carboxypeptidase digestion, was found to be -Glu-Leu-Ala. The protein preparation used (200 pmol) contained the following free amino acids: 40 pmol of glycine, 25 pmol of lysine, and less than 5 pmol each of threonine, serine, alanine, and valine.

The amino acid composition of the protein is given in Table 1. The average values of two independent analyses are listed as the integrated value based on glutamic acid = 9. The histidine value was not accurate because of a neighboring peak. Tryptophan was not analyzed. The polar index was 50%. The amino acid composition and at least one amide group found in the N-terminal sequence indicated, and isoelectrofocusing electrophoresis (IEF) confirmed, that this was a basic protein; in equilibrium IEF, the MOB2 protein migrated to the cathode, and in nonequilibrium IEF it migrated slower than cytochrome *c*, which indicated that its pI was between 8.5 and 9.2 (data not shown).

Cellular localization of the MOB2 protein. Several hydrophobic properties of the MOB2 protein, such as solubility in Triton X-100, urea, and acetone (unpublished observation), suggested that it may be associated with the membrane. However, its global amino acid composition did not indicate that this was a particularly hydrophobic protein. We decided to use antibodies to determine its cellular localization.

The purified MOB2 protein was injected into a rabbit to produce polyclonal antibodies. We first verified that the antiserum obtained contained antibodies against the protein. A total membrane fraction was subjected to SDS-PAGE and immunoblotted. The immunoblot showed that the antibodies reacted with a protein with an apparent molecular mass of 9 kDa (Fig. 2A). Three other bands, corresponding to proteins of 70, 60, and 31 kDa, were also repeatedly found. One additional minor band was also seen (37 kDa; Fig. 2A), and there was a faint band at a position of 14 kDa (Fig. 2B).

When the purified protein (Blue Trisacryl–0.75 M NaCl fraction) was immunoblotted, antibodies revealed one single band with the same apparent molecular weight as that

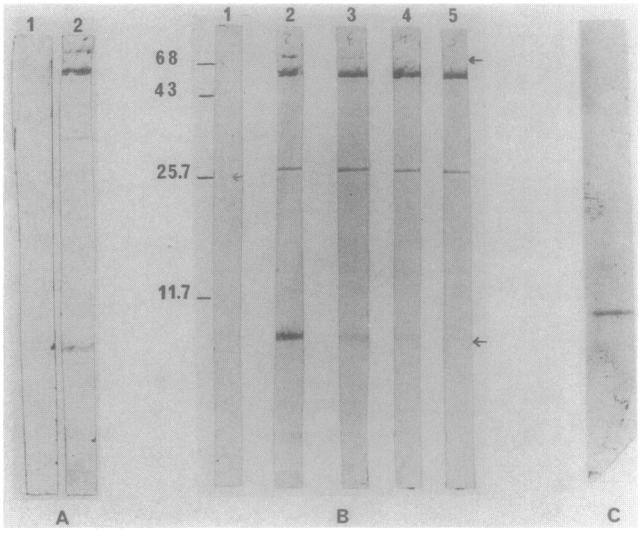

FIG. 2. Immunoblot analysis of specificity of anti-MOB2 protein antiserum. (A) Total membrane fraction was subjected to electrophoresis on a 16% polyacrylamide gel in denaturing conditions and electrophoretically transferred (0.5 A, 45 min) to a nylon membrane (Biodyne). The blot was then incubated with antiserum as described in Materials and Methods. Lanes: 1, preimmune serum, dilution of 1:50; 2, anti-MOB2 serum, dilution of 1:50. (B) Experimental procedures were as described for panel A. Lanes: 1, preimmune serum, dilution of 1:50; 2, anti-MOB2 antiserum, dilution of 1:50; 3, anti-MOB2 antiserum, dilution of 1:50, preincubated overnight at 4°C with 2.5 μg of purified MOB2 protein (Blue Trisacryl–0.75 M NaCl fraction); 4, same as lane 3, using 5 μg of MOB2 protein; 5, same as lane 3, using 10 μg of MOB2 protein. Positions of molecular mass markers (in kilodaltons) are indicated on the left. Arrows indicate bands that disappeared after preincubation with the purified MOB2 protein. (C) Immunoblot analysis of purified MOB2 protein. An immunoblot obtained after SDS-PAGE of purified MOB2 protein (Blue Trisacryl–0.75 M NaCl fraction) was incubated with a 1:50 dilution of anti-MOB2 antiserum.

detected in the total membrane fraction (Fig. 2C). When the antiserum was preincubated with the purified MOB2 protein before incubation with the immunoblot, the two bands at 9 and 70 kDa disappeared, whereas the 60- and 31-kDa proteins still reacted with the antibodies (Fig. 2B). We conclude from these results that the 70-kDa protein was immunologically related to the MOB2 protein, whereas the 60- and 31-kDa proteins were immunogenic contaminants present in at least one of the preparations used for immunization.

Antibodies were then incubated with immunoblots of the cytoplasmic fraction (120,000 × *g* supernatant) and the total membrane fraction (120,000 × *g* pellet). Densitometric analysis of a photograph of the immunoblot showed that 80 to 90% of the MOB2 protein was in the membrane fraction and that 10 to 20% was in the soluble protein fraction (Fig. 3). Treatment of the membrane pellet with 1% Triton X-100 was not sufficient to completely release the MOB2 protein from the membrane; up to 4 M urea was needed in addition to Triton X-100 to fully solubilize the protein. No MOB2 protein was detected in the membrane fraction resistant to solubilization by 1% Triton X-100 and 4 M urea (outer membrane fraction). These results suggest that this protein is strongly associated with the *E. coli* inner membrane.

Interaction of the MOB2 protein with the *oriC* region. We first studied the general binding properties of the MOB2 protein. The protein bound more efficiently to supercoiled

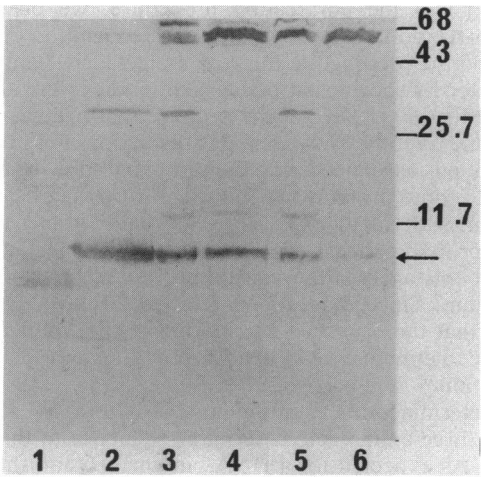

FIG. 3. Cellular location of the MOB2 protein. Various cellular fractions were subjected to electrophoresis and immunoblotted as described in the legend to Fig. 2. Lanes: 1, membrane proteins not solubilized by 1% Triton X-100–4 M urea (25 μg); 2, solubilized membrane fraction 3 (60 μg); 3, solubilized membrane fraction 2 (70 μg); 4, solubilized membrane fraction 1 (385 μg); 5, total membrane protein fraction (70 μg); 6, cytoplasmic fraction (70 μg).

DNA than to linear double-stranded (Fig. 4A) or single-stranded (Fig. 4B) DNA; in the case of supercoiled DNA, 1 nmol of protein was needed for retention on the filter of 7 nmol of DNA as nucleotide, whereas in the case of single-stranded or double-stranded linear DNA, the stoichiometry was four to five times lower (2 nmol of DNA as nucleotide retained per nmol of protein added). The same difference was found for binding to pBR322 DNA.

Competition experiments between labeled pOC42 DNA and cold pBR322 for binding of the MOB2 protein indicated that the MOB2 protein bound preferentially to supercoiled pOC42 [^{3}H]DNA in the presence of an increasing amount of cold pBR322 DNA as competitor, whereas cold pOC42 DNA could compete efficiently for binding of the MOB2 protein (Fig. 5A). pBR322 DNA was also found to compete efficiently for binding when a nonspecific DNA-binding fraction was used as a control (Fig. 5A). This preferential binding of the MOB2 protein to *oriC*-containing DNA was also found in the case of single- or double-stranded linear pOC42 DNA (Fig. 5B). The kinetics of competition indicated that the specificity might be stronger with single-stranded DNA than with double-stranded or supercoiled DNA. In the case of single-stranded DNA, both strands were labeled; the kinetics of competition indicated that both strands were recognized by the protein (Fig. 5B).

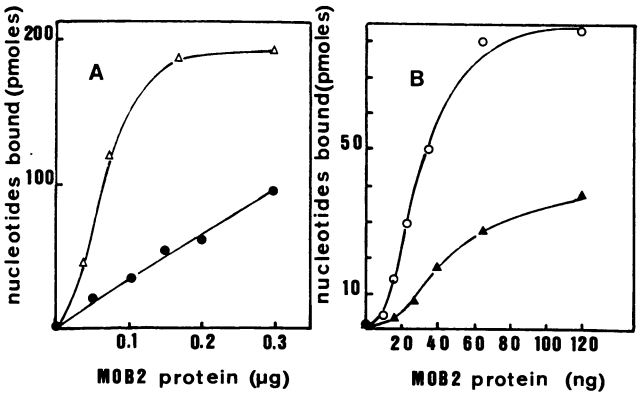

FIG. 4. DNA-binding properties of the MOB2 protein. (A) Binding of the MOB2 protein to supercoiled (△) and linear double-stranded (●) pOC42 DNA. Binding to [^{3}H]DNA was assayed in both cases in 10 mM MES buffer (pH 6.5) in the presence of 1 mM $MgCl_2$. pOC42 DNA was linearized by cutting with *Eco*RI. (B) Binding of the MOB2 protein to supercoiled (○) and single-stranded (heat-denatured) (▲) pOC42 [^{3}H]DNA. Conditions were identical to those described for panel A.

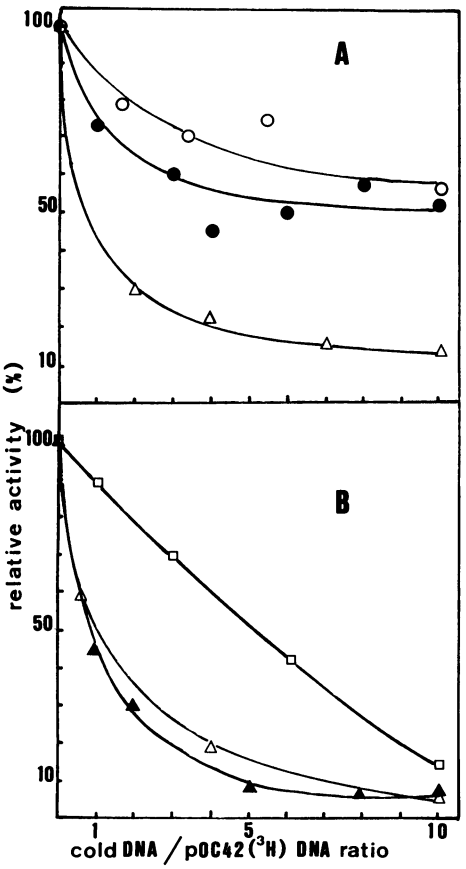

FIG. 5. Specific binding of the MOB2 protein to pOC42 DNA. The fraction to be assayed was added at a nonsaturating level to the reaction mixture containing pOC42 [^{3}H]DNA and an increasing amount of cold competitor DNA. Results are expressed as percentage of binding of the fraction to pOC42 DNA in the absence of competitor DNA. (A) Specific binding of the MOB2 protein to single-stranded (heat-denatured) (○) and double-stranded (●) pOC42 DNA. POC42 DNA was linearized by *Eco*RI. Linearized pBR322 single-stranded or double-stranded DNA was used as competitor DNA. In the case of single-stranded DNA, a control experiment (△) was done with a nonspecific membrane DNA-binding fraciton. (B) Specific binding of the MOB2 protein to supercoiled (□) pOC42 DNA. Supercoiled pBR322 DNA was used as a competitor. The same experiment was performed with a nonspecific membrane DNA-binding fraction (△) or with MOB2 protein, using unlabeled supercoiled pOC42 DNA as competitor (▲).

Since plasmid pOC42 consists of a 2,000-base-pair insert into pBR322 (18) and the minimal *oriC* sequence consists of 245 base pairs (22), it was necessary to pinpoint the specific binding site of the MOB2 protein. To do so, we performed competition experiments between various fragments from the *oriC* region, ^{32}P labeled at the 3′ ends as described in

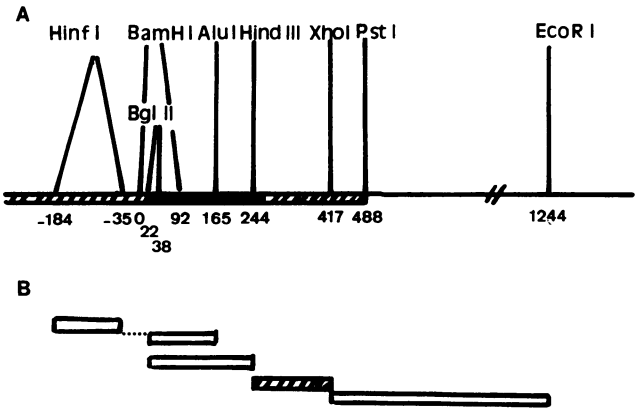

FIG. 6. Specific binding of the MOB2 protein in the *oriC* region. (A) Restriction map of the *oriC* region. Only restriction sites relevant to this study have been indicated. Symbols: ▨, chromosomal DNA; ▬, pBR322 DNA; ■, minimal *oriC* sequence. Nucleotide coordinates are numbered from conventional *oriC* coordinates: +1 is the first guanine of the *Bam*HI site (GGATC). (B) Binding specificity of the MOB2 protein for various fragments from the *oriC* region. Symbols: □, nonspecific binding; ▨, specific binding; · · ·, no binding detected by membrane filtration, presumably due to the small size of the protein. In all cases, specific binding was assayed with single- and double-stranded DNA. Unlabeled pBR322 DNA linearized by cutting with *Eco*RI, denatured or not, was used as competitor DNA. *E. coli* SSB protein was used as a control (nonspecific DNA-binding protein) in the case of the single-stranded DNAs.

Materials and Methods, and linear pBR322 cold DNA (Fig. 6).

Only the *Hin*dIII(244)-*Xho*I(417) fragment was recognized specifically by the MOB2 protein. Again, the relative affinity seemed higher for single-stranded DNA than for double-stranded DNA (data not shown). The protein appeared to bind nonspecifically to the *Bgl*II(38)-*Alu*I(165) fragment, which contains site I of the B′ protein, or the *Xho*I(417)-*Eco*RI(1244) fragment, which contains site II of the B′ protein (13, 14), as either single-stranded or double-stranded DNA. From these results, we concluded that the recognition site of the MOB2 protein was located between the *Hin*dIII(244) and *Xho*I(417) restriction sites.

DISCUSSION

We have described a membrane DNA-binding protein of low molecular weight that binds to a specific *E. coli* DNA site adjacent to the minimal chromosomal origin of replication (*oriC*). We propose that this protein be called MOB2 protein, for membrane origin-binding protein 2. This protein was purified to near homogeneity; purified fractions showed a single band in SDS-PAGE with an apparent molecular mass of approximately 9 kDa. Early preparations of the MOB2 protein were found to migrate in SDS-PAGE as a 12-kDa protein, as previously observed (4), in contrast to later preparations such as the one described here, which migrated as a 9-kDa protein. Antibodies prepared against those early preparations reacted specifically with the 9-kDa protein. The reasons for these variations in apparent molecular weight are not understood; they were observed with various preparations purified by the procedure described here and thus cannot be accounted for by the modification of the purification procedure. These variations make it difficult to assign a precise molecular mass to the protein; it is likely to be around 10 kDa.

Throughout the purification procedure, we observed a strict correlation between the specific recognition of pOC42 DNA and the presence of this low-molecular-weight protein as detected by specific antibodies. Antibodies were also used to show that 80 to 90% of the protein fractionated with the membrane fraction. However, this result does not prove that the protein is associated with the cell membrane in vivo. For instance, some proteins are known to form aggregates that sediment with membranes (9). We have found that the MOB2 protein forms aggregates in the presence of EDTA and that this aggregation can be prevented or reversed by magnesium. Since our procedure used 20 mM $MgCl_2$, we believe that the observed association of the MOB2 protein with the membrane is probably not due to this type of aggregation.

We determined the eight-amino-acid N-terminal sequence and the three-amino-acid C-terminal sequence of the MOB2 protein. As expected for a DNA-binding protein, the amino acid composition shows that this is a basic protein, which was confirmed by IEF. One surprising finding was that the N-terminal sequence of the protein (Lys-Val-Ile-Ala-Glu-Asp-Asn-Gly) shows a good homology with amino acids 92 to 100 (Lys-Ile-Ile-Ala-Ala-Asp-Asn-Gly) of the *dnaK* gene product (1). This is a 70-kDa heat shock protein (6) implicated in bacteriophage lambda replication (5) as well as in host DNA replication and transcription (10). It has been reported to bind to the membrane of *E. coli* (27), and it is more than 50% homologous to the eucaryotic heat shock protein HSP70 (1). However, the MOB2 protein is not a proteolytic product of the DnaK protein, since the C-terminal sequence was not found downstream from the analogous amino acids of the DnaK protein amino acid sequence. Furthermore, assuming that the MOB2 protein is about 100 amino acids long, we calculated the amino acid composition of a putative peptide of that length derived from the DnaK protein. Its composition is significantly different from that of the MOB2 protein. We do not know if this homology is significant or merely coincidental.

We can expect that a membrane DNA-binding protein would have both hydrophobic regions that allow interactions with the membrane and hydrophilic regions in contact with the cytoplasm and DNA. Although the percentage of hydrophobic amino acid of the MOB2 protein is rather low (30%) compared with that of other intrinsic membrane proteins, a cluster of hydrophobic amino acids might be sufficient to anchor it to the membrane. Determination of the complete primary sequence of the protein should give more information in this respect. Association with the membrane could also be mediated by amino acid residue modification such as acetylation or lipid addition or by interaction with another membrane protein(s). The fact that up to 4 M urea is required in addition to Triton X-100 to completely release the MOB2 protein from the membrane supports this view.

Several membrane DNA-binding proteins that are specific for DNA containing the *oriC* region have been described (4, 13). A protein fraction, called B′, purified from the *E. coli* membrane was found to bind specifically to two sites in (site I) and near (site II) the minimal origin of replication (13). From the results presented here, we conclude that the binding properties of the MOB2 protein cannot account for properties attributed to the B′ protein for the following reasons. The MOB2 binding site is located between nucleotides 244 and 473, a region that was not recognized by the B′ protein (13). Neither site I nor site II of the B′ protein is recognized by the MOB2 protein. (ii) The B′ protein could recognize only single-stranded DNA (14), whereas the

MOB2 protein can identify its specific binding site in single-stranded, double-stranded, or supercoiled DNA. (iii) The B′ protein was unstable when kept on ice or after repeated thawing, and the MOB2 protein is extremely stable, retaining activity even after 3 min of boiling.

Some of the B′ preparation contained, in addition to a 60-kDa protein, a protein with an apparent molecular mass of 12 kDa (13). However, the B′ preparation that we used for localization of the specific binding sites showed only a 60-kDa band and no trace of a low-molecular-weight protein after SDS-PAGE in conditions where, if present, the MOB2 protein should have been seen (unpublished observation). Therefore, we tentatively conclude that the 60-kDa (B′) protein was responsible for binding to sites I and II, whereas the MOB2 protein binds to a third site (site III) adjacent to the right boundary of the minimal origin of replication.

We have also shown that the MOB2 protein is specific for supercoiled *oriC*$^+$ DNA, although to a somewhat lesser extent than for single-stranded DNA. More definite conclusions concerning this finding await experiments more precise than the filter binding assay. Our results also showed that in the case of single-stranded DNA, apparently both strands are recognized. The 173-base-pair region containing the MOB2-specific binding site contains several inverted repeats, especially between positions 290 and 370 (19). These palindromes could be important for specific binding of the protein.

Hendrickson et al. isolated *oriC*-membrane protein complexes (7) that were tightly associated with an outer membrane fraction. Kusano et al. dissected the *oriC* DNA fragments that were important for specific association with the complex (15). They concluded that one binding site lay between position *Bgl*II(38) and *Ava*II(155) and was destroyed by cutting at position 92 with *Bam*HI. This site could correspond to the B′ binding site I, since we also found that specific recognition was abolished when site I was cleaved at position 92 (13). The *Hin*dIII(244)-*Xho*I(417) fragment was described as carrying a weaker affinity site in their study. We did not find specific binding to this fragment with the B′ preparation. However, it is recognized by the MOB2 protein. Thus, both fragments containing the B′ binding site I and those containing the MOB2 binding site were found important in their study for specific association of the *oriC* region with their membrane complex. This result suggests that both the MOB2 protein and the B′ protein, in addition to other proteins, may have been in this complex.

The fact that a protein that could be identical to the MOB2 protein was found in some preparations of the B′ protein and that a 60-kDa protein, which could be the B′ protein, copurified with the MOB2 protein, especially when the boiling step was omitted (4), suggests that the two proteins interact. We would like to speculate that the MOB2 protein, in association with the 60-kDa B′ protein and possibly other *E. coli* proteins, participates in a chromosomal segregation complex. This hypothesis is currently being investigated, primarily by studying *E. coli* partition mutants. In any event, identification of the gene encoding the MOB2 protein should allow mutants useful for determining the exact in vivo function of this protein to be obtained.

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