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Mini-F Protein that Binds to a Unique Region for Partition of Mini-F Plasmid DNA

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A mini-F-coded protein, named F2 protein, binds specifically to mini-F DNA. This protein has a molecular weight of 37,000 and is coded by the A2 segment of the mini-F genome (47.3 to 49.4 kilobases on the F coordinate map). The binding site is located also in the A2 segment of mini-F. This binding site is lost by spontaneous deletion when the A2 segment alone, but not A2 together with its neighboring segment, is cloned in a multicopy plasmid pBR322. These data are discussed in connection with incompatibility and plasmid stability.

The regulation of cell division is a central problem in biology. Bacterial plasmids are the best models for this problem because they are small but stably inherited, and their gene products can be assigned across the genome.

The F plasmid of *Escherichia coli*, which is 94.5 kilobases (kb) in size, belongs to the FI incompatibility group of plasmids and is stably perpetuated in one or two copies per host chromosome (for reviews, see references 9 and 16). Dissection of F plasmid by *Eco*RI restriction enzyme showed that a 9-kb fragment of F plasmid (f5; 40.3 to 49.4 kb on the F coordinate map) is able to replicate autonomously (10, 17). This small plasmid, named mini-F, retains the FI-specific incompatibility and the copy number of the parental F plasmid and is perpetuated stably in *E. coli*. Therefore, in place of the parental F plasmid, mini-F has been the subject of extensive studies of its replication, incompatibility, and partition.

A much smaller derivative of mini-F plasmid carrying only the region from 44.87 to 46.35 kb on the F plasmid, hereafter referred to as 44.87-46.35F, can also replicate autonomously (13). This plasmid carries a replication origin (*oriS* or *ori-2*) and incompatibility regions (*incB* and *incC*), along with a gene that codes for a 29-kilodalton (kd) protein (9; Fig. 1). However, this small derivative was found to be unstable, indicating that machinery essential for the stable perpetuation of F plasmid has been lost. Recently, Ogura and Hiraga (14) and Austin and Wierzbicki (1) reported that the A2 fragment (47.3 to 49.4 kb on the F coordinate map), with its associated region in mini-F, affects plasmid stability. According to Komai et al. (7), this region codes for a polypeptide of 41 kd, called F1 protein, and another polypeptide of 37 kd, called F2 protein. It also carries the *incD* locus (5), which is responsible for the FI group-specific incompatibility. Ogura and Hiraga (14) compared their results of functional analyses with the protein assignment data of Komai et al. (7) and inferred that *trans*-acting *sopA* and *sopB* products that stabilize an *oriC* plasmid may be the F1 and F2 proteins. They further showed that in the A2 fragment is an additional, *cis*-acting locus called *sopC*.

In this paper, we will demonstrate that the F2 protein binds specifically to a unique region in the A2 fragment. The binding site matches the site assigned to *sopC*.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains used, Km55 and Km1196, are derivatives of *Escherichia coli* K-12 and have been described previously (7). Strain Km55 is a *gal*$^-$ derivative, and strain Km1196 was used to prepare minicells. λ *imm*^{21} dv was described previously (12). MacConkey-galactose agar, Davis-Casamino medium, and Davis-19AA medium for isotope labeling have been described elsewhere (7).

Preparation of plasmid DNA. Preparation of plasmid DNA was described previously (7). Essentially, DNA was extracted from a plasmid-harboring *E. coli* strain by use of lysozyme and Triton X-100 followed by centrifugation in CsCl containing ethidium bromide.

Preparation of minicells, labeling of proteins with [^{35}S]methionine, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and fluorography. The methods used in this work have been described previously (7). Minicells were prepared from an overnight culture of strain Km1196 harboring a plasmid. Proteins labeled with [^{35}S]methionine were analyzed by slab gel electrophoresis with 10% polyacrylamide containing sodium dodecyl sulfate. Fluorography was carried out with En^{3}Hance (New England Nuclear Corp., Boston, Mass.).

Preparation of DNA-binding protein. DNA-binding protein was prepared by the method of Kwoh and Zinder (8) with slight modifications. Purified minicells, labeled with [^{35}S]methionine, were suspended in 130 μl of freezing buffer (10 mM Tris-hydrochloride [pH 8.0], 5 mM EDTA, 50 mM NaCl, 10% sucrose) to give a suspension with an optical density at 550 nm of between 5 and 20. To this solution was added 15 μl each of 3 M NaCl and lysozyme (5 mg/ml). The mixture was kept on ice for 1 h and then frozen and thawed six times in a dry ice-ethanol bath and ice water. After the addition of 2 μl of 35% Triton X-100 by a 30-min incubation in an ice bath, the lysate was centrifuged at 10,000 × *g* for 30 min by an Eppendorf Microfuge. The supernatant was recovered, and its NaCl concentration was adjusted to 0.6 M by the addition of the 3 M solution. The sample was loaded on the top of a 5 to 20% sucrose gradient in buffer A (10 mM Tris-hydrochloride [pH 8.0], 1 mM EDTA, 0.1 mM dithiothreitol, 50 μg of bovine serum albumin per ml) and

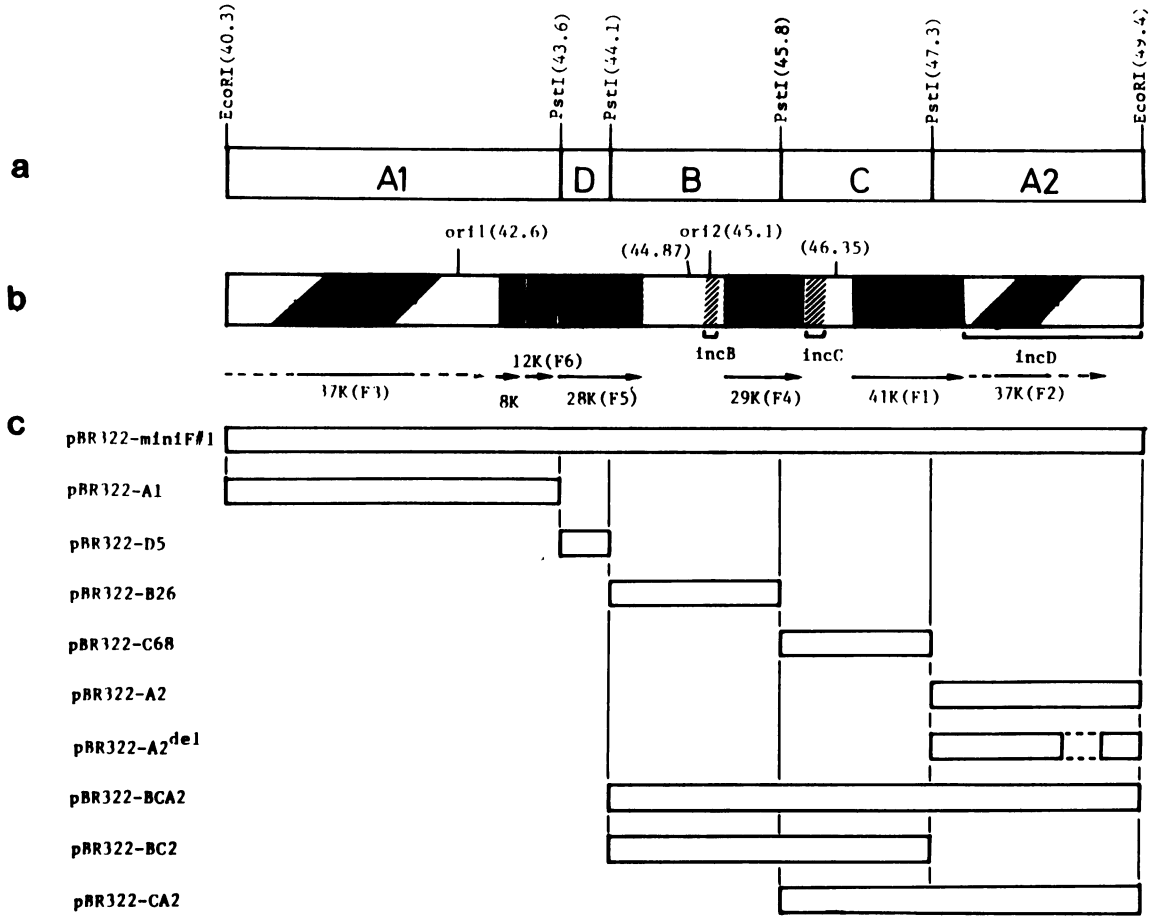

FIG. 1. Genetic structure of mini-F genome and the set of recombinant plasmids carrying various segments of mini-F DNA used in this study. (a) Restriction map of mini-F (12). The numbers in parentheses denote kb coordinates on the mini-F genome. (b) Coding regions of mini-F encoded proteins. Since the endpoints of coding regions of F2 and F3 have not been determined, in these cases the solid lines represent the expected size of the gene, and the dashed lines cover possible ranges of coding regions. Arrows represent the direction of transcription. Filled boxes indicate the coding regions (2, 7). *ori-1* and *ori-2* are the replication origins of mini-F (3, 4, 13). Shaded areas represent regions where a tandem repetitive sequence appears and where *incB* and *incC* have been mapped (18). (c) Various segments of mini-F DNA cloned in pBR322 vector. The dashed region in pBR322-A2^{del} indicates a region of deletion, 450 bp in size, in the A2 segment.

centrifuged at 80,000 × *g* for 2.5 h at 4°C in a Beckman SW50.1 rotor. After centrifugation, fractions of 0.5 ml were collected from the bottom. We observed that the binding proteins were in the top fraction. This fraction was stored in ice and used for a binding assay.

DNA-protein-binding assay. ^{35}S-labeled protein samples (10 to 20 μl) were mixed with 5 × 10^{11} molecules of DNA in 100 μl of buffer B (buffer A containing 0.1 M NaCl and 7.5 mM $MgSO_4$). The mixture was kept on ice for 30 min and layered over a 5 to 20% sucrose gradient in buffer B. It was centrifuged at 100,000 × *g* for 2 h at 4°C and fractionated. In some experiments, the centrifugation step was replaced by Sepharose CL-4B (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) column chromatography. A column (0.7 by 13.0 cm) was preequilibrated with buffer B, the DNA-protein mixture was loaded onto the column, and elution was performed with the same buffer. Fractions of 150 μl were collected, and samples were removed and put on a paper filter. The filters were dried, washed with cold 5% trichloroacetic acid, and counted with a liquid scintillation counter.

Incompatibility test. To test FI-specific incompatibility, $CaCl_2$-treated strain Km55 harboring F'8*gal* plasmid was transformed with pBR322-cloned mini-F fragment DNA. Transformants were selected on a MacConkey-galactose agar plate containing tetracycline (7 μg/ml). When incompatible, the resident F'8*gal* plasmid is lost, and a white colony appears on the MacConkey-galactose agar plate.

RESULTS

A protein which binds to mini-F DNA is coded by mini-F. Mini-F and pBR322 plasmids replicate under a stringent and a relaxed control in which 1 to 2 and 20 to 30 copies per chromosome, respectively, are perpetuated. The chimeric plasmid pBR322–mini-F replicates under a relaxed control, allowing a higher-than-normal level of accumulation of mini-F coded proteins by a gene dosage effect (7, 18). This feature was utilized in testing the mini-F-coded proteins for DNA-binding activity. Minicell-producing *E. coli* Km1196 carrying a chimeric pBR322–mini-F plasmid (pBR322–mini-F#1 in Fig. 1) was grown, and minicells were purified and labeled with [^{35}S]methionine. The minicells were lysed as described above and the ^{35}S-labeled extract was mixed with circular pBR322–mini-F DNA and sedimented in a sucrose gradient. If this extract contains DNA-binding protein(s), the protein should sediment along with the fast-sedimenting circular

DNA molecules, which produce two peaks ascribable to closed circular and open circular forms.

The extract did indeed bind to both closed circular and open circular forms of pBR322–mini-F DNA, but not to pBR322 DNA at all (Fig. 2). Nicking or strand breaking of the DNA molecule under the conditions used was unlikely, since the proportion of closed circular and open circular molecules remained unchanged after the addition of the extract or after storage at 0°C for several hours before centrifugation (data not shown). The protein also bound to the DNA in a linear form (data not shown).

In an attempt to identify the region that codes for the DNA-binding protein(s) on the mini-F genome, we constructed minicells harboring pBR322-based recombinant plasmids with various segments of mini-F (Fig. 1). Unexpectedly, we noticed that pBR322-A2 plasmid was rather unstable in *E. coli*; after growth of transformants with the freshly constructed recombinant DNA in vitro for 30 generations, about half of the pBR322-A2 DNA recovered was a deletion derivative. Upon further incubation, the proportion of the deletion derivative increased to more than 99% after 90 generations. The deletion was not caused by use of the minicell producer strain, because other strains showed the same effect. The plasmid with the deletion was designated pBR322-A2^{del}. Restriction enzyme analyses demonstrated that the deletion extends about 450 base pairs (bp) in a unique region of the A2 fragment, covering the *Ava*II sites at around 48.9 kb on the F coordinate map but not the *Hpa*I site at 49.2 kb. Ogura and Hiraga (14) independently observed

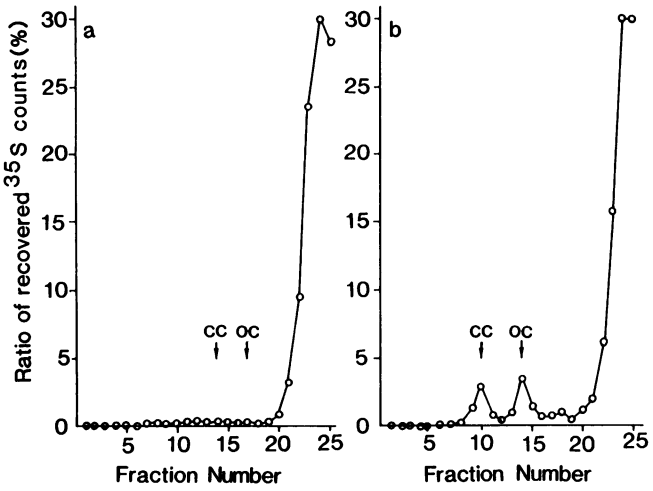

FIG. 2. Sucrose gradient sedimentation of mini-F-coded protein-DNA complex. Minicells carrying pBR322–mini-F#1 plasmid were purified and labeled with [^{35}S]methionine. The minicells were lysed, mixed with [^{3}H]thymidine-labeled plasmid DNA in closed circular (cc) and open circular (oc) forms, and centrifuged through a sucrose gradient as described in the text. (a) pBR322 DNA. Arrows at fractions 14 and 17 represent the positions of closed circular and open circular forms, respectively, of DNA molecules of pBR322, as determined by counting ^{3}H. For clarity, sedimentation profiles of the DNA have been omitted from the figure. The bulk, unbound ^{35}S-labeled protein sedimented slowly and stayed in the top fraction. (b) pBR322–mini-F#1 DNA. Arrows at fractions 10 and 14 represent the positions of the DNA molecules in closed circular and open circular forms, respectively. As pBR322–mini-F DNA is larger than pBR322 DNA, the former sedimented faster. Some ^{35}S-labeled proteins sedimented along with these two DNA molecules. Sedimentation is from right to left.

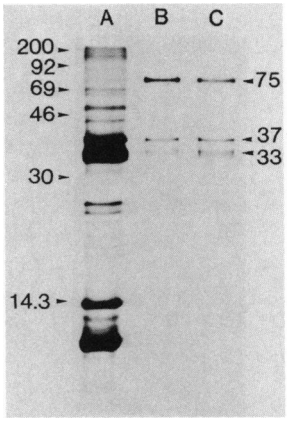

FIG. 3. Electrophoretic analyses of DNA-binding proteins. ^{35}S-labeled lysate from minicells carrying pBR322-A2^{del} was mixed with pBR322–mini-F DNA and centrifuged as described in the legend to Fig. 2, and peak fractions of closed circular and open circular molecules were separated. The fractions were denatured and electrophoresed in 0.1% sodium dodecyl sulfate–10% polyacrylamide gel and fluorographed. Numbers represent molecular sizes in kd. Lane A, Lysate before centrifugation; lanes B and C, proteins bound to pBR322–mini-F DNA in closed circular and open circular forms, respectively.

the same effect. Spontaneously arising cells carrying the deleted plasmid apparently have some growth advantages over those with the intact plasmid. Despite its instability, intact pBR322-A2 DNA was separated from the 50% mixture of molecular species prepared at 30 generations by agarose gel electrophoresis. No deletions were detected in the other recombinant plasmid DNAs exhibited in Fig. 1, including pBR322-A1, PBR322-D5, pBR322-B26, pBR322-BC2, and pBR322-C68.

The recombinant plasmids, including pBR322-A2^{del}, were each transformed into minicell producer Km1196, and the minicells were purified and labeled with [^{35}S]methionine. The [^{35}S]methionine-labeled extracts were assayed for DNA-binding activity. Extracts of minicells harboring either pBR322-BC2 or pBR322-A2^{del} were found to bind to pBR322–mini-F DNA (Fig. 2), while extracts from minicells harboring pBR322-A1, pBR322-B26, and pBR322-C68 lacked such activity (binding was found in less than 0.2% of the total labeled proteins). pBR322-D5 was not tested in this series of experiments. About 8% of the labeled proteins in the pBR322-A2^{del} extract bound to pBR322–mini-F DNA, but not to pBR322 DNA. Under the same conditions, only 0.5% of the labeled proteins in the pBR322-BC2 extract bound to pBR322–mini-F, suggesting strongly that the binding activity(ies) observed in pBR322-A2^{del} and the pBR322-BC extracts represents different entities. This paper focuses on the binding protein(s) produced from pBR322-A2^{del}. This protein did not bind to pBR322, λ, or λ *imm*^{21} dv DNAs (11).

Characterization of binding proteins and binding site. To characterize the protein(s) that bound to mini-F DNA, ^{35}S-labeled protein(s) synthesized in minicells carrying pBR322-A2^{del} was added to pBR322–mini-F DNA and centrifuged, and samples from the peak fractions of DNA-protein complex were taken, denatured, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 3). Three major proteins, with molecular weights of 75,000, 37,000, and 33,000, were detected. Of these, the 37-kd protein has the same molecular weight as F2 protein, whose gene has been assigned in the A2 segment of mini-F

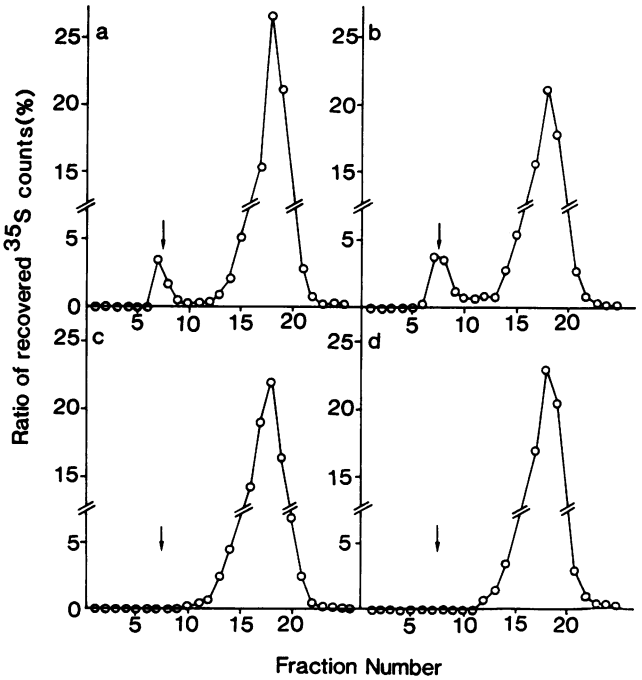

FIG. 4. Sepharose CL-4B column chromatography to detect interaction of mini-F coded protein with A2 DNA. The ^{35}S-labeled extract of minicells harboring pBR322-A2^{del} was mixed with (a) pBR322–mini-F DNA circular DNA, (b) purified linear A2 DNA, (c) purified linear A2^{del} DNA, and (d) pBR322–mini-F DNA digested with *Ava*II. The preparation of A2 and A2^{del} DNA fragments is described in the text. The protein-DNA mixture was applied to a Sepharose CL-4B column and fractionated. With this column, the circular and linear DNA molecules are eluted in the void fraction (fractions 7 to 9, shown by arrows). Unbound proteins are eluted at fractions around fractions 18. Total radioactivity of the ^{35}S-labeled proteins used for each assay was 6.3×10^4 to 7.2×10^4 cpm.

(7). The other two proteins were not assigned in the mini-F genome. It is likely that at least these two proteins are *E. coli* proteins which are somehow concentrated in the DNA-protein complex. The protein of 32 to 33 kd appeared as smear bands, and its content varied from experiment to experiment. ^{35}S-labeled proteins from minicells carrying pBR322–mini-F showed similar behavior. The NaCl concentration did not affect the binding in the range of 0.1 to 0.2 M. At a high concentration (0.6 M) of NaCl, however, the binding was eliminated, whereas at a low concentration (below 0.05 M), nonspecific binding to several mini-F-unrelated DNAs became predominant.

To locate the DNA site of interaction with the binding proteins, the extracts from minicells harboring pBR322-A2^{del} were mixed with pBR322–mini-F, pBR322-A1, pBR322-B26, pBR322-C68, pBR322-BC2, or pBR322-A2 DNA. The extract showed binding activity to pBR322–mini-F and pBR322-A2 DNAs to the same extent, but no binding activity to other DNAs. Therefore, the binding site must be located in the A2 segment of mini-F DNA.

Next, the effect of the deletion in the A2 region was examined. The intact A2 DNA fragment was prepared from pBR322–mini-F DNA by digestion with restriction enzymes *Eco*RI and *Pst*I. The corresponding DNA fragment was also prepared from pBR322-A2^{del}. The binding activity was assayed by Sepharose CL-4B column chromatography instead of by sucrose gradient centrifugation, because the linearized DNAs do not migrate fast enough on centrifugation. In this assay system, the DNA-protein complex is eluted in the void fraction irrespective of its molecular form, and the labeled free proteins are eluted in included fractions. The results in Fig. 4 demonstrate that labeled proteins were eluted in the void fraction in association with pBR322–mini-F and A2 DNAs (Fig. 4a and b), but not A2^{del} DNA (Fig. 4c). These results demonstrate that the proteins bind to A2 DNA, but not to A2^{del} DNA. Therefore, it is highly likely that the binding site is located at or very close to the deletion site in the A2^{del} DNA.

This conclusion was supported by the finding that the digestion of pBR322–mini-F DNA with *Ava*II abolishes the binding activity (compare Fig. 4a and d). A2^{del} covers the *Ava*II site(s); other regions of the plasmid DNA are not cleaved by this enzyme. Digestion with *Bam*HI, *Hin*dIII, *Kpn*I, *Sst*I, *Xho*I, or *Xma*I, restriction enzymes which do not cleave the A2 DNA, did not affect the activity (data not shown).

Deletion in the A2 region and incompatibility. An incompatibility locus defined by *incD* is located at kb 48.66 to 49.02 in the A2 region of the F plasmid (Fig. 1). To see the effect of the deletion on this activity, the ability to eliminate resident F′ 8*gal* plasmid was compared in pBR322-A2 and pBR322-A2^{del} plasmids. For this purpose, pBR322-A2 and pBR322-A2^{del} DNAs were separated from a mixture and purified by agarose gel electrophoresis and then by hydroxylapatite chromatography. Each DNA was then added to $CaCl_2$-treated cells harboring F′8*gal*. The transformants carrying the tetracycline-resistant marker of pBR322 vector were selected. The efficiency of transformation was the same for both DNAs and more than 95% of the transformants in both cases were cured of the resident plasmid F′8*gal*. This result indicates that the incompatibility is maintained despite the deletion.

DISCUSSION

This study has demonstrated that proteins with molecular weights of 75,000, 37,000, and 33,000 that can be labeled in the cells harboring pBR322–mini-F bind specifically to mini-F DNA. Such proteins are not detected in cells which do not carry the recombinant plasmid. According to Komai et al. (7), mini-F codes for six proteins whose size is greater than 12 kd. Among these proteins, F2 protein that is coded by the A2 segment has the same molecular weight as the 37,000 protein among the three binding proteins. Cells carrying the A2 segment, but not other segments of mini-F, produced the specific binding protein. These results strongly suggest that the 37-kd DNA-binding protein is F2, which is coded for by the A2 segment. The deletion in the A2^{del} genome spans 450 bp around 48.9 kb on the F coordinate map, but the deletion does not destroy the F2 coding capacity.

The other DNA-binding proteins of 75 and 33 kd are not detected in the absence of F2 protein. It is possible that they are coded for by pBR322–mini-F or pBR322-A2^{del} as artifacts, although no space for coding such proteins is left in the A2 segment (7). It is more likely that these proteins are *E. coli* proteins that form a DNA-binding protein complex in the presence of F2 protein.

The binding site is located at or very close to the *Ava*II site at kb 48.9 in the A2 segment on the F coordinate map, because the binding activity was abolished by digestion of mini-F DNA with the *Ava*II restriction enzyme or by spontaneous deletion covering the *Ava*II site(s). It is interesting that this spontaneous loss of the binding site occurs when the A2 segment alone is cloned into pBR322. T. Miki et al. (personal communication) have recently found that this

region has at least 11 tandemly arranged, 43-bp repeating sequences, each of which carries an *Ava*II site. It must be this repeat sequence that binds to the binding proteins and undergoes deletion. We have found that the deletion eliminates practically all of the repeat sequences, leading to the loss of all the *Ava*II sites (unpublished observation).

The reason for the loss of the binding site for the proteins is not clear, although the site is stably maintained when intact mini-F, rather than the A2 segment, is cloned into the same vector. A preliminary experiment has shown that the DNA of the C-A2 segment is also stably cloned in pBR322. Our studies have shown that the deleted plasmid occurs spontaneously and that the cells carrying the deletion have a growth advantage over cells carrying the intact plasmid. At present, unnatural regulation of transcription of either the "right" or "left" portion of the A2 segment due to a cloning artifact or, alternatively, the synthesis of a fused protein from truncated β-lactamase in pBR322 and truncated F1 protein in the A2 region seems to be the most likely trigger for selection of the deletion derivatives. The third explanation for the spontaneous deletion ($A2^{del}$) might be that the binding site takes up proteins required for the survival of the host in combination with excess F2 protein.

The A2 segment plays a role in the stability of mini-F plasmid. We have observed that the B-C segment of mini-F replicates autonomously, but is unstable. On the other hand, a plasmid carrying the B-C-A2 segment replicates stably (unpublished observation). Ogura and Hiraga (14) have observed that the A2 segment also stabilizes the unstable plasmid *oriC*. They analyzed the elements that act in the stabilization and found that two *trans*-acting genes, *sopA* and *sopB*, along with a *cis*-acting site which they call *sopC*, are involved. Comparison of the sites they assigned for these genes with the protein-coding regions assigned by Komai et al. (7) and the results reported here indicates that the *sopA* and *sopB* genes correspond to F1 and F2 proteins, respectively, and *sopC* corresponds to the site that carries the *Ava*II site(s) and undergoes deletion. Our observations strongly suggest that the F2 protein, along with other proteins possibly of *E. coli* origin, binds to the *Ava*II site and provides the molecular basis for the model of partition with Ogura and Hiraga proposed from their results of genetic analyses (15).

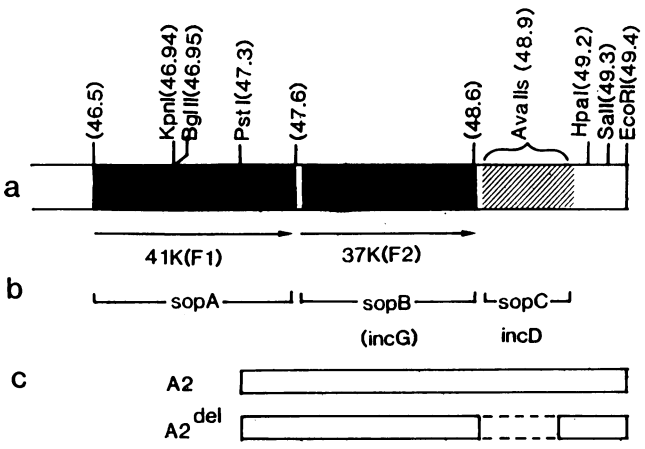

FIG. 5. Coding region of F2 protein and its binding site. (a) A portion of the mini-F map covering a fraction of segment C and complete A2 (Fig. 1). Numbers in parentheses indicate the kb coordinate. Filled boxes and the hatched box indicate the coding regions for F1 and F2 proteins proteins and the protein-binding site which contains *Ava*II sites, respectively (see the text). (b) Loci assigned to partition functions by Ogura and Hiraga (14). The *incD* locus was defined by Gardner et al. (5). (c) Fragments cloned in pBR322 plasmid in this study. Note that the 450-bp deletion shown by dashed lines does not influence the ability to code for F2 protein.

The deletion in the $A2^{del}$ segment did not abolish the incompatibility conferred by the A2 segment of the mini-F genome. Ogura and Hiraga (14) reported that the A2 segment codes for two incompatibility loci, *incD* and *incG*. According to their discussion, incG is a *trans*-acting entity which is ascribed to *sopB*, the gene for the F2 protein (Fig. 5). *incD*, originally defined within 47.5 to 49.4 kb on the F coordinate map (6, 16), is a *cis*-acting site and was relocated at kb 48.66 to 49.02 (5), a site which coincides with *sopC* (14). Therefore, the incompatibility by pBR322-$A2^{del}$ observed in this paper may be due to the expression of F2 or truncated F1 protein(s), corresponding to the IncG defined by Ogura and Hiraga (14).

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