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Purification and properties of the mini-F plasmid-encoded E protein needed for autonomous replication control of the plasmid

(initiator protein/DNA replication/DNA binding protein/incompatibility/autorepression)

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ABSTRACT **Mini-F plasmid encodes a protein, E protein, that is indispensable for its autonomous replication. We have constructed a plasmid that overproduces the E protein and have purified the protein to apparent homogeneity. Using nitrocellulose filter binding and nuclease digestion assays, we demonstrated that the E protein binds to three unique regions of the mini-F DNA sequence: (*i*) the replication origin (*ori2*) and an incompatibility locus (*incB*), (*ii*) another incompatibility locus (*incC*), and (*iii*) the promoter for the *E* gene. These binding sites have a common 8-base-pair sequence. These findings suggest the direct role of the E protein in initiation of mini-F replication and copy number control. They are also in line with the *in vivo* evidence that (*i*) the incompatibility phenotype caused by *incB* and *incC* DNA is due to titration of a factor(s) indispensable for replication and (*ii*) that the production of the E initiator protein of the mini-F plasmid is under autoregulatory control.**

F plasmid, which is 94.5 kilobase pairs (kb) in size and belongs to the FI incompatibility group, replicates under a stringent control, maintaining a copy number of 1 to 2 per host chromosome. It is regarded as a model system for understanding the control systems that act in replication of more complex cellular chromosomes.

A 9-kb autonomously replicating DNA fragment called mini-F plasmid has been obtained by digesting the F plasmid with *Eco*RI. The mini-F plasmid shows incompatibility phenotype, copy number control, and stability identical with the parental F plasmid (1, 2). We have obtained even smaller autonomously replicating derivatives of mini-F (3) consisting of (*i*) an origin for initiation of replication (*ori2*), (*ii*) a gene that is assumed to code for an initiator protein (4–7) called 29-kDa protein (3), F4 (4), or E protein (8), and (*iii*) nine repeats of a 19-base-pair (bp) sequence that determines incompatibility (*incB* and *incC*; ref. 9).

The E protein was suggested to be a replication initiator (5–7), and its synthesis, to be autogeneously regulated (10). As to the mechanism of expression of the FI-type incompatibility phenotype, Tsutsui *et al.* (11) have provided evidence that the *incB* and *incC* regions, which carry four and five of the 19-bp repeats, respectively, titrate the initiator, and hence the phenomenon is tightly connected with the copy-number control. Thus, E protein and the repeats seem to play a central role in replication control of F plasmid.

In this paper, we report purification of the E protein and show that it binds to these repeating sequences as well as to two 8-bp sequences located at its own promoter region. These observations suggest possible roles of the E protein, the repeating units, and the promoter in replication control of F plasmid.

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MATERIALS AND METHODS

Bacterial Strains and Plasmids. *Escherichia coli* K-12 strains Km723 (F⁻ *rspL his recA1* Δ*gel sup*^{0}) (12) and N100 (*rspL galK2 recA3*) (13) were used as the hosts for plasmids. Plasmids pBR322-Xa7 (14) and pMY12-6 (15) are pBR322-based plasmids, carrying a DNA segment from mini-F plasmid and phage λ, respectively. pTA14 is a plasmid constructed for overproduction of E protein (see *Result* section).

Nitrocellulose Filter Binding Assay. The assay was performed as described by Tsurimoto and Matsubara (16). The ^{32}P-end-labeled DNA was prepared by digesting with *Hin*fI followed by labeling with [γ-^{32}P]ATP (5000 Ci/mmol, Amersham; 1 Ci = 37 GBq) by use of T4 polynucleotide kinase (Takara Shuzo, Kyoto, Japan). The E protein (60 ng) was admixed with 0.5 ml of buffer B [10 mM Tris·HCl (pH 7.4)/10 mM $MgCl_2$/0.1 M KCl/0.1 mM EDTA/6 mM 2-mercaptoethanol/50 μg of bovine serum albumin per ml] containing the ^{32}P-end-labeled DNA (4 fmol). The mixture was kept at 0°C for 20 min, then filtered through a nitrocellulose membrane filter (25 mm, Sartorius SM 1103) and washed with 1 ml of buffer B without the bovine serum albumin, and the filtrate was collected. The retained DNA was recovered from the filter by washing with 0.5 ml of elution buffer (0.1% $NaDodSO_4$/5 mM EDTA). To each sample was added calf thymus DNA (5 μg), and the DNA was precipitated with 0.3 M sodium acetate and ethanol. The DNA fragments in each sample were separated by 5% polyacrylamide gel electrophoresis and autoradiographed.

DNase I Protection Assay. This was performed as described by Galas and Schmitz (17). The ^{32}P-end-labeled DNA fragment (0.05 pmol) was incubated with the E protein (0–0.4 μg) in 100 μl of buffer B at 20°C for 20 min and then digested with 0.3 μg of DNase I (Takara Shuzo) for 2.5 min. The reaction was stopped by adding 30 μl of phenol/chloroform, 1:1, (vol/vol). The aqueous phase was saved, and calf thymus DNA (5 μg) was added as a carrier, which was then precipitated with 0.3 M sodium acetate and ethanol at −70°C for 15 min and centrifuged. The pellet was washed with 75% ethanol, resuspended in loading dye (0.1% bromophenol blue and 0.1% xylene cyanol in 95% deionized formamide), electrophoresed in a 6% polyacrylamide gel containing 8 M urea, and autoradiographed.

Exonuclease III Digestion Assay. This was performed as described by Shalloway *et al.* (18). The ^{32}P-end-labeled DNA fragment (4 fmol) was incubated with E protein (0–16 ng) in 50 μl of buffer B at 37°C for 30 min and then digested with 30 units of exonuclease III (New England Biolabs) for 2.5 min. The reaction was stopped by addition of 20 μl of phenol/chloroform, 1:1, and the sample was subjected to polyacrylamide gel electrophoresis as described for DNase I protection assay.

Abbreviations: bp, base pair(s); kb, kilobase pair(s).

RESULTS

Construction of a Strain That Overproduces E Protein. We have constructed a pBR322-based plasmid that is designed to overproduce the E protein. This plasmid, referred to as pTA14 plasmid (Fig. 1*A*), carries the E protein-encoding region under transcriptional control of the tandemly arranged phage λ promoters, P_R-P_L, which are regulated by a thermosensitive repressor, *c*I857. The *E* gene is transcribed by incubating the carrier cells at 42°C. Since the *E* gene carried a Shine–Dalgarno ribosome binding site sequence different from a typical sequence needed for efficient translation, we replaced this region by the Shine–Dalgarno sequence of phage λ *cro(tof)* as described by Shirakawa *et al.* (19). The *E* gene with the phage λ *cro* Shine–Dalgarno sequence was expressed 5-fold more efficiently than with its own Shine–Dalgarno sequence (data not shown). In addition, we replaced the ampicillin-resistance gene with a chloramphenicol-resistance gene to facilitate identification of the E protein, which is poorly separated from β-lactamase, the product of the ampicillin-resistance gene, on polyacrylamide gel electrophoresis.

Purification of the E Protein. *E. coli* N100 (pTA14) was grown in 10 liters of PBB medium (11) at 30°C to OD_{660} = 0.7, 0.33 volume of PBB medium preheated to 65°C was added, and the mixture was incubated at 42°C for 2–3 hr. The cells (about 20 g of wet cells) were harvested by centrifugation and used for preparation of E protein. The purification was monitored by polyacrylamide gel electrophoresis, and all procedures were carried out at 2°C.

The cells (20 g) were suspended in 20 m of buffer D (50 mM Tris·HCl, pH 7.5/1 mM EDTA/0.3 M KCl/14 mM 2-mercaptoethanol), lysed by sonication, and centrifuged at

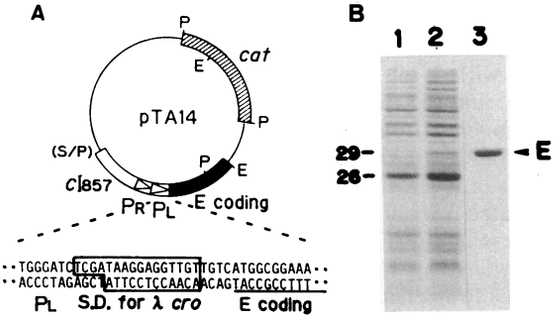

FIG. 1. (*A*) The structure of plasmid pTA14, which overproduces E protein. This plasmid consists of five DNA segments: *i*, an *Eco*RI–*Sal* I segment of pBR322 (—); *ii*, a 2-kb segment carrying the chloramphenicol acetyl transferase gene, *cat* (▨) (19) inserted at the *Pst* I site of pBR322 DNA; *iii*, a 1.4-kb *Pst* I–*Bam*HI segment of phage λ carrying the P_R–P_L promoter along with *c*I857 from plasmid pMY12-6 (▭) (14); *iv*, a 1.1-kb mini-F segment that covers a region of nucleotide numbers 1148–2248 (3) carrying the *E* coding sequence but not its promoter (■); and *v*, a chemically synthesized fragment equivalent to the phage λ *cro* Shine–Dalgarno sequence, inserted between the *E* coding sequence and the phage λ promoters (expanded at the bottom of the figure). The direction of transcription by P_R–P_L is shown by the arrowheads. E and P represent restriction sites for *Eco*RI and *Pst* I, respectively. (S/P) represents the junction between the filled-in ends of the *Sal* I site in pBR322 and the *Pst* I site in the phage λ segment. (*B*) Electropherograms of the E protein. The extract of N100 (pTA14) cells before (lane 1) and after (lane 2) heat induction are also shown. Cells were grown to OD_{660} = 0.4 in PBB medium at 30°C, then incubated at 42°C for 4 hr. Aliquots (0.1 ml) of the cultures were taken, and cells were collected by centrifugation and then suspended in 10 μl of sample buffer (62.5 mM Tris·HCl, pH 6.8/2% $NaDodSO_4$/0.07 M 2-mercaptoethanol/0.5 mg of bromphenol blue per ml/10% glycerol) and used by heating at 100°C for 2 min. The dense 26-kDa band in lanes 1 and 2 is chloramphenicol acetyltransferase (19). Electrophoresis was in a 0.1% $NaDodSO_4$/15% polyacrylamide gel stained with Coomassie blue. The arrow indicates the position of the purified E protein (3 μg, lane 3) as calculated from its nucleotide sequence (3). Molecular sizes in kDa are shown.

100,000 × *g* for 30 min. The supernatant was applied to a DEAE-cellulose column (Whatman, DE 52; 2.0 × 7 cm) equilibrated with buffer D, and the column was washed with 60 ml of buffer D. The unadsorbed fraction was collected, and to it was added 3 vol of buffer C (50 mM potassium phosphate, pH 6.9/0.1 mM EDTA/14 mM 2-mercaptoethanol) containing 0.3 M KCl. The mixture was applied to a CM-Sephadex C-50 column (Pharmacia; 1.4 × 13 cm) equilibrated with buffer C containing 0.3 M KCl, followed by elution with 120 ml of linear KCl gradient (0.3–1.0 M) in buffer C. The 0.5–0.6 M KCl fraction was pooled, diluted to 0.2 M with buffer C, and applied to a phosphocellulose column (Whatman, P 11: 1.0 × 4 cm) equilibrated with buffer C containing 0.2 M KCl. The column was developed with 30 ml of 0.2–1.0 M linear KCl gradient in buffer C, and the peak fractions of A_{280} eluted at 0.5–0.6 M KCl were pooled, dialyzed against buffer H (0.01 M KPO_4, pH 6.9/0.1 dithiothreitol) and applied to a hydroxyapatite column (Bio-Rad; 1.0 × 4 cm) equilibrated with buffer H, followed by elution with 40 ml of 0.01–0.1 M linear potassium phosphate linear gradient in buffer H. A total of 0.4 mg of the purified E protein was obtained. It was stored in liquid nitrogen for more than 6 months without loss of activity. Fig. 1*B* shows an electropherogram of the purified protein.

Amino Acid Sequence of the E Protein. Amino and carboxyl termini of E protein were determined respectively by Edman degradation and carboxypeptidase A digestion. The first 13 amino acids from the amino terminus were Ala-Glu-Thr-Ala-Val-Ile-Asn-His-Lys-Lys-Arg-Lys-Asn, in full agreement with the arrangement predicted from the nucleotide sequence (3), except that the first formylmethionine was eliminated. The carboxyl terminus was glycine, again in agreement with that predicted from the nucleotide sequence.

DNA Binding Activity. We then studied DNA binding activity of the isolated E protein, using [^{3}H]DNAs of pBR322 and pBR322-Xa7 as binding probes. The latter plasmid carries the *Xho* I (position 768)–*Alu* I (position 2246) segment of mini-F plasmid inserted at the *Eco*RI–*Bam*HI site of pBR322 (Fig. 2). This region has been shown to be essential for autonomous replication for mini-F plasmid (14). The E protein bound to pBR322-Xa7 but not to pBR322 (data not shown). To localize the binding sites, we isolated the mini-F DNA segment from pBR322-Xa7, digested it with *Hin*fI, and obtained six fragments, designated a–f (see Fig. 2). The mixture of fragments was end-labeled and incubated with the E protein and then filtered through a nitrocellulose membrane. Fragments to which protein binds were trapped on the membrane. The unbound DNA and the retained DNA, which was recovered by subsequent elution, were electrophoresed and autoradiographed. The results (Fig. 3) show that the E protein bound only to the fragments a, b, and f.

To narrow down the binding sites, we isolated the a and f fragments and digested them with *Bgl* II and *Bst* NI, respectively. Fragment a generated segments a_1 (positions 768–903) and a_2 (positions 903–1094), and fragment F generated segments f_1 (position 1858–2198) and f_2 (positions 2198–2246), each of which was end-labeled and subjected to binding assays. The results, not shown here, revealed that the E protein binds to the DNA fragments that determine the *incB* and *incC* regions (9).

As Tsutsui *et al.* pointed out (11), fragments a_2 and f_1 contain, respectively, four and five 19-bp repeats. Fragment b contains the putative *E* gene promoter (10), where 10-bp inverted repeats are located. An 8-bp sequence, T-G-T-G-A-C-A-A, in the 10-bp inverted repeats is identical to a sequence in the 19-bp repeats in the *incB–ori2* and *incC* regions (see Fig. 6).

DNase I Protection Assay. To determine the E protein binding sites in the fragments a_2, b, and f_1 in greater detail, we performed DNase I protection experiments (17), using the

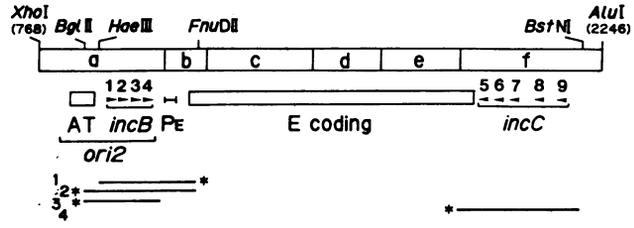

FIG. 2. Genetic and physical map of a mini-F region capable of autonomous replication and the fragments used for binding studies. The *Xho* I (768)–*Alu* I (2246) DNA segment of mini-F plasmid carried by plasmid pBR322-Xa7 is shown. This region allows autonomous replication (13). Numbers in parentheses denote the mini-F plasmid nucleotide number taken from ref. 3. The six fragments (a through f) generated by *Hin*fI digestion have molecular sizes, respectively, of 328, 111, 281, 173, 209, and 384 bp. Several restriction sites are shown, but *Bst*NI (789), *Hae* III (1847), and *Fnu*DII (1419, 1879, 2182) sites are omitted. *ori2*, AT, P_E, and "*E* coding" represent, respectively, the origin of replication of mini-F plasmid (13, 20), an A+T-rich cluster, and the promoter and coding region for the E protein. Arrowheads with numbers indicate the locations and directions of the 19-bp repeating sequences (3); *incB* and *incC* denote the loci that determine FI incompatibility (3, 10, 21). The 5′-end-labeled DNA fragments 1 through 4 used for DNase I protection and exonuclease III digestion assays are shown at the bottom of the figure and have the following sizes: 1, 270 bp; 2, 298 bp; 3, 195 bp; and 4, 343 bp. The labeled 5′ ends are represented by asterisks.

three 5′-end-labeled DNA fragments shown in Fig. 2. Various amounts of the E protein were mixed with the fragments and then digested with DNase I. This cuts on the average once per DNA molecule, generating a ladder of labeled fragments of various sizes. After the digestion, the sample was electrophoresed and autoradiographed.

The results (Fig. 4) show that with increasing amounts of the E protein, the protected regions become discernible. These protected regions match exactly with the inverted repeats in the promoter and the nine 19-bp repeats, though they seem to have order of preference in binding: at the lowest protein concentration, the E protein binds to its own promoter (Fig. 4*A*); at intermediate concentrations, it also binds to repeats 1 through 4 in the *incB–ori2* region (Fig. 4 *A* and *B*) and to repeats 7 and 8 in the *incC* region (Fig. 4*C*); at the highest concentration, the protein binds also to repeats 5, 6, and 9 in the *incC* region.

In addition to protection, the binding of E protein created hypersensitive sites to DNase I action in some of the repeats, suggesting a conformational change due to the protein–DNA interaction. Since we do not know the structure of the E protein–DNA complex, it is difficult to speculate how these hypersensitive sites are made.

Exonuclease III Digestion Assay. To determine the boundaries of the binding sites and the order of protein in more detail, we performed exonuclease III assays, again using 5′-end-labeled DNA fragments (Fig. 5). This experiment relies on the fact that a DNA interacting with a sequence-specific DNA binding protein is protected from the processive digestion by exonuclease III at the boundary of the complex. Because the exonuclease III digestion proceeds from the 3′ side of a strand in duplex DNA, we can define the 3′-side protection border (18).

Without addition of the E protein (Fig. 5, lane 3), the exonuclease III digestion terminated nearly at the center of the DNA molecule, producing "half-cut molecules." With increasing amounts of the E protein, these bands disappeared and unique protection bands appeared (Fig. 5, lanes 4–6). We can see, again, that the protected boundaries coincide with the 3′ borders of the direct repeats and the inverted repeats.

Even with the lowest E protein concentration, the protection at the border of the inverted repeats in the *E* gene promoter region was complete, though other repeats were not protected (Fig. 5*B*). With increasing amounts of the E protein, protection bands appeared at the 3′ border of repeats 1 through 4 in the *incB–ori2* region (Fig. 5*A*) and of repeats 7 and 8 in the *incC* region (Fig. 5*C*). Even at the highest E protein concentration, protected boundaries of repeats 5, 6, and 9 in the *incC* region were not clear, in agreement with the result obtained by DNase I protection assay. These results are summarized in Fig. 6.

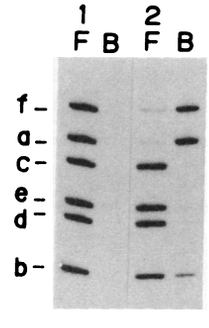

FIG. 3. Binding of the E protein to unique fragments of mini-F DNA. The *Xho* I–*Alu* I 1.5-kb fragment in mini-F plasmid was digested with *Hin*fI, producing the six fragments a–f (see Fig. 2). The mixture was 5′-end-labeled, incubated with (lane 2) or without (lane 1) the E protein and then filtered through a nitrocellulose membrane. Lanes B and F contain the membrane-entrapped and nonentrapped DNA, respectively. Each sample was electrophoresed and autoradiographed.

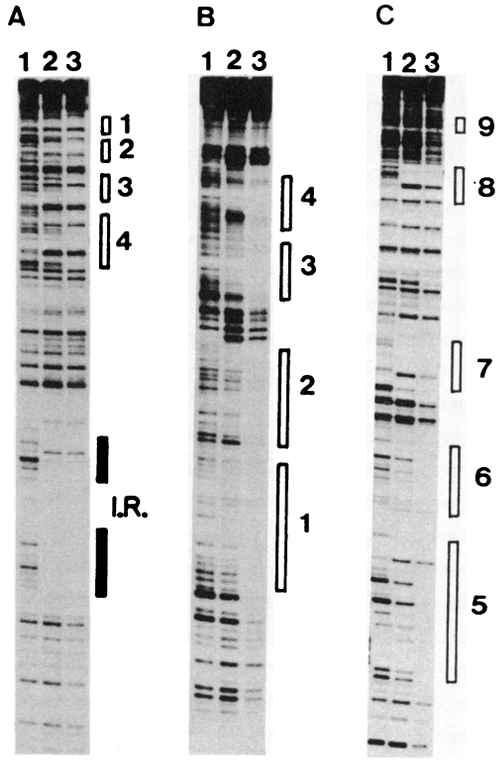

FIG. 4. DNase I protection assay. Three 5′-end-labeled DNA fragments 1 (*A*), 2 (*B*), and 3 (*C*) shown in Fig. 2 were incubated with various amounts of the E protein, digested with DNase I, and electrophoresed. Lanes 1 through 3 contain DNA protected by 0, 0.1, and 0.2 μg of the E protein, respectively. Positions of the 19-bp repeats and the 10-bp inverted repeats (I.R.) in the *E* gene promoter region are shown.

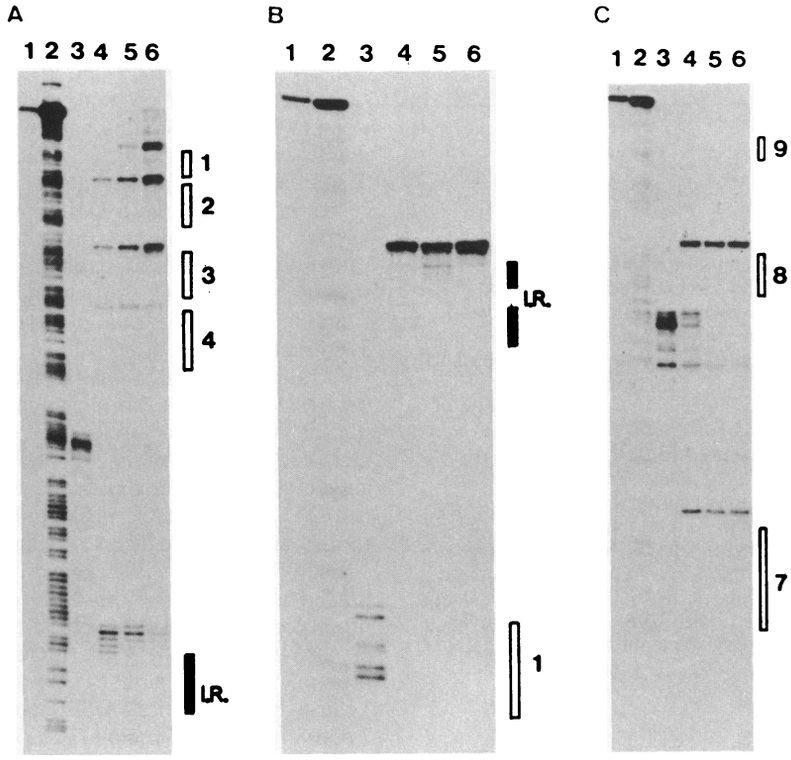

FIG. 5. Protection of mini-F DNA fragments against exonuclease III digestion. Three 5'-end-labeled DNA fragments shown in Fig. 2, 1 (*A*), 2 (*B*), and 4 (*C*), were each incubated with various amounts of the E protein, digested with sufficient amounts of exonuclease III, and electrophoresed. Lanes: 1 and 2, undigested DNA and Maxam–Gilbert "A > G" cleavage reaction products of the same fragment, respectively; 3–6, samples with respectively 0, 4, 8, and 16 ng of the E protein added. Positions of the 19-bp repeats and the 10-bp inverted repeats (I.R.) in the *E* gene promoter are shown.

DISCUSSION

The mini-F plasmid-encoded E protein is the only protein indispensable for replication that is encoded by a region within the minimal autonomously replicating mini-F plasmid (3). This protein has been assumed to act as an initiator and to participate in copy-number control and incompatibility, but the mechanisms of its action is not understood at the molecular level. Our demonstration that the E protein binds to unique sites on mini-F DNA provides some clues in considering these problems.

That the E protein's role is indispensable in F plasmid

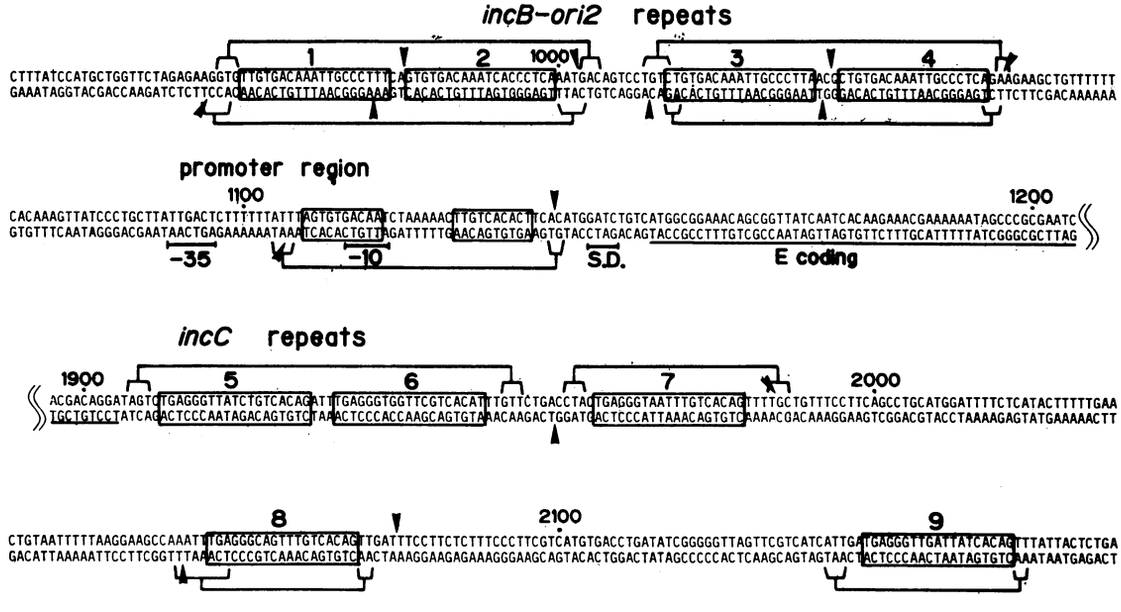

FIG. 6. Nucleotide sequence of mini-F DNA protected by E protein against digestion by DNase I or exonuclease III. The nine 19-bp repeats in *incB–ori2* and *incC* along with the inverted 10-bp repeats in the *E* gene promoter region are boxed. These repeats have the 8-bp consensus sequence T-G-T-G-A-C-A-A. Brackets show regions that were protected against DNase I digestion. Arrowheads show the 3' borders of the regions protected against exonuclease III digestion in the upper or lower strand. The −35, −10, and Shine–Dalgarno (S.D.) sequences in the promoter region and the coding region of the *E* gene are indicated. Nucleotide numbers are adopted from Murotsu *et al.* (3).

replication has been shown by analyzing transposon insertion mutants and by constructing an amber mutant (5, 7). Murotsu *et al.* (3) have provided evidence that the *incB* region, carrying four of the nine 19-bp repeats, is the likeliest site of the replication origin (*ori2*). The structure of this region—namely, four repeats along with an A+T-rich region—is very similar to that of the replication origin region in phage λ and plasmid λdv (22, 23). The unique binding of the E protein to the repeats parallels the unique binding of phage λ initiator protein, O, to the four λ repeats (24). Since the binding induces replication of phage λ DNA, it is highly likely that a similar molecular interaction in the F plasmid constitutes a step in the replication initiation of this plasmid DNA. Similar protein–DNA interactions that lead to replication initiation have been shown with R6K, pSC101, and P1 plasmids (25–27).

Tsutsui *et al.* (11) provided evidence that FI incompatibility is mostly determined not by a repressor action but by the titration of an indispensable protein that acts upon the *incB* and *incC* regions where the 19-bp repeats are located. The DNase I and exonuclease III assays proved unambiguously that the 19-bp repeating sequences in the *incB* and *incC* regions bind to the E protein. It may not be unreasonable to assume that this interaction plays an important part in the expression of IncB and IncC phenotype. According to Tsutsui *et al.* (11), this interaction should also control the plasmid copy number. Our observations are completely in line with this notion: when the plasmid DNA is overproduced, it will titrate the initiator E protein and restrict further replication until the increase of cell mass lowers the relative concentration of the plasmid DNA.

We observed that the binding order (or affinity) of the nine repeats in the *incB* and *incC* regions differs. The E protein prefers repeats 1 through 4 in the *incB* region and repeats 7 and 8 in the *incC* region. Repeats 5, 6, and 9 are less preferred. The *incC* region is not indispensable for mini-F replication (14), and mini-F plasmid with the *incC* region deleted becomes a high-copy-number mutant (11, 28). These results also agree well with the notion that the *incC* region acts as a titration site of the E protein. Though we have not been able to quantitate the binding order, the lower affinity of repeats 5 and 6 may account for the weaker intensity of incompatibility phenotype of these repeats than of the others (11).

The *E* gene is located between the *incB* and *incC* regions, with the promoter located close to *ori2* (3). This region carries 10-bp inverted repeats and an 8-bp sequence, T-G-T-G-A-C-A-A, which also appears in the 19-bp repeats to the *incB–ori2* and *incC* regions (see Fig. 6). Our results show that the inverted repeats strongly bind to the E protein. Søgaard-Andersen *et al.* (10) have shown that the expression of the *E* gene is negatively controlled by the E protein itself and proposed an autoregulation circuit for the expression of this gene. Thus, the *in vivo* and *in vitro* observations are now in full agreement.

These observations suggest the basic features of the replication control system acting in the stringently controlled F replicon. At steady state, the E protein binds to the 10-bp inverted repeats in its own promoter and autogenously regulates the level of its own expression. When a perturbation occurs, such as increase in cellular mass, production of the E protein starts and continues until it is repressed again. The E protein produced will bind to the *incB–ori2* region, forming a replication complex, along with some host proteins, leading to replication initiation. One can also imagine that the repeats in the *incB* region "take up" the E protein without initiating replication until all four sites are saturated. Any excess of E protein will be titrated by binding to the *incC* region. Any overreplicated plasmid DNA in the cell will help to take up the protein until its concentration is sufficiently lowered. Hence, a new steady state will be established. A similar autoregulatory circuit (namely, autogenous control of the expression of an operon that codes for the initiator, activation of the origin by the initiator binding, and titration of the initiator by an excessive origin DNA sequence) has been shown with the λdv plasmid (29). In this case, however, the plasmid genome bears a region equivalent to *incB–ori2* but not one equivalent to *incC*, and the repressor and initiator are separate entities.

P1 codes for a protein, repA, that binds to repeats, autoregulates its expression, and initiates replication. Thus, the overall control circuit is likely to be similar to that of mini-F plasmid. An attractive diagram to illustrate the control system has been presented by Chattoraj *et al.* (27).

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