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Regulated expresssion of a gene important for replication of plasmid F in *E. coli*

Liselotte Søgaard-Andersen[1], Luis A. Rokeach[2] and Søren Molin[1,3]*

[1]Department of Molecular Biology, Odense University, Campusvej 55, DK-5230 Odense M, Denmark, and [2]Laboratory of Genetics, Department of Molecular Biology, Université Libre de Bruxelles, 67, rue des Chevaux, B-1640 Rhode St. Genese, Belgium

[3]Present address: Applied Microbial Genetics, The Technical University of Denmark, Building 221, DK-2800 Lyngby, Denmark
*To whom reprint requests should be sent
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Fusions between the gene encoding the E protein of the IncFI plasmid F and the *lac* genes were constructed. Analysis of the expression of β-galactosidase from these fusions shows that the promoter for the E protein gene is located between the *incB* region and the structural gene for the E protein. Near this promoter is a regulatory site on which a negative control element acts. Most likely the E protein itself acts as a repressor of *E* gene expression and thus autoregulates its own expression. No other gene products seem to affect the expression of the E protein gene.
Key words: plasmid F/E protein/gene expression/*E. coli*

Introduction

Plasmid F in *Escherichia coli* is a 94.5-kb plasmid belonging to the IncFI incompatibility group. All genes required for stable maintenance of the plasmid are located on one 9-kb *Eco*RI fragment (coordinates: 40.3–49.3) – the so-called f5 fragment (Lovett and Helinski, 1976; Timmis *et al.*, 1975). The copy number of F and of miniplasmids carrying the f5 fragment is 0.8–1.2 per chromosome equivalent (Collins and Pritchard, 1973).

The f5 fragment codes for at least nine proteins, it carries two origins of replication, *oriV* and *oriS*, and three incompatibility loci, *incB, incC* and *incD* (see Figure 1). The smallest autonomously replicating fragment, the minimal replicon, derived from the f5 fragment contains *oriS, incB, incC* and the gene coding for a 29-kd protein, the E protein (Murotsu *et al.*, 1981; Bex *et al.*, 1981; Komai *et al.*, 1982) (see Figure 2). The gene coding for the E protein is denoted the *E* gene in the following. The E protein was proposed to be a specific replication factor (Watson *et al.*, 1982; Tolun and Helinski, 1982). In addition, mutations giving rise to an increased plasmid copy number have been mapped to the *E* gene region (Seelke *et al.*, 1982). Copy number mutations have been found to change the primary structure of the E protein and to increase its level of synthesis (F. Bex, personal communication). Moreover, synthesis of the E protein is negatively regulated by an effector encoded by the 42.8–46.2 region (Bex *et al.*, 1982). Based on these observations it seems likely that the *E* gene region plays a central role for controlled replication of plasmid F. It is therefore of interest to assess the role of this protein in the replication control system of plasmid F, since the expression of the *E* gene could be rate limiting for initiation of replication of F in a manner like that previously described for the *repA* gene of plasmid R1 (Light and Molin, 1981, 1983).

In an attempt to analyse in more detail the role of the E protein we present an investigation of its expression with emphasis on possible regulatory loops involved. Since the nucleotide sequence of the *E* gene and the surrounding regions is known (Murotsu *et al.*, 1981) it was possible to construct both transcriptional and translational gene fusions between the *E* gene and the *lac* genes and probe these fusions for gene expression and its control.

Results

Location of the promoter for the E gene

To map the *E* gene promoter, several translational fusions between the *E* gene and the *lacZ* gene were constructed using the *lac* fusion vector pMC1403 (Casadaban *et al.*, 1980) (cf. Figure 2). Plasmids used are listed in Table I.

In all these fusions the *Sma*I site (45.348 in F coordinates, cf. Figures 1 and 2) at base pair number 96 in the promoter proximal end of the *E* gene was ligated to the *Bam*HI site (deleted of its 5′-protruding ends with S1 nuclease) in pMC1403, thus creating an in-frame translational fusion between the *E* gene and the *lacZ* gene (see Materials and methods and Figure 2). The inserted fragments in pF1403-30, pF1403-20 and pF1403-10 extend from the *Pst*I site at 44.100, the *Stu*I site 31 bp upstream of *incB* and the *Alu*I site 7 bp downstream of *incB*, respectively, to the *Sma*I site. The *incB* region is defined as the sequence between the first base pair in the first direct repeat to the last base pair in the fourth direct repeat (cf. Figures 1 and 2). The three fusion plasmids have the same copy number, as indicated by their identical levels of single cell resistance to ampicillin (data not shown), and they all mediate a Lac$^+$ phenotype as shown on McConkey lactose plates.

Table II shows that the specific activities of β-galactosidase expressed from the three plasmids are the same. Since the fused E-β-galactosidase polypeptides are identical in the three cases (the same internal restriction site used in the *E* gene for the constructions), and the gene dosages are also identical, we conclude that all information necessary for *E-lacZ* expression is located between the *Alu*I site (45.161) and the NH_2 end of the structural *E* gene. This is in accordance with the published sequence of this region, since a promoter-like sequence can be found between this *Alu*I site and the start of the structural *E* gene (cf. Figure 1).

Control of E-lacZ expression

To study the regulation of *E-lacZ* expression, we introduced f5 subfragments, cloned in plasmids compatible with F and with the fusion vectors, into CSH50 harbouring one of the fusions. By monitoring the activity of β-galactosidase with different plasmids *in trans, trans*-acting factors affecting *E-lacZ* expression could be detected.

Two sets of fusions were used: (i) pF1403-10, pF1403-20

Table I. Relevant data about the plasmids employed

Plasmid	Parent replicon	Resistance phenotype[a]	Restriction site used in vector	Coordinates of cloned F sequence[b]	Lac phenotype and type of fusion[c]	Reference/source
pML31	F	Km				Lovett and Helinski (1976)
pMC1403	pBR322	Ap			Lac$^-$	Casadaban *et al.* (1980)
pOU122	R1	Ap			Lac$^-$	Larsen (unpublished)
pJL260	pGA39	Cm			Lac$^-$	Light and Molin (unpublished)
pBR322	pMB1	Ap,Tc				Bolivar *et al.* (1977)
pBR325	pBR322	Ap,Cm,Tc				Bolivar (1978)
pF1403-10	pMC1403	Ap	*Bam*HI	45.161 – 45.348	Lac$^+$, trl	This paper
pF1403-20	pMC1403	Ap	*Bam*HI	45.029 – 45.348	Lac$^+$, trl	This paper
pF1403-30	pMC1403	Ap	*Bam*HI	44.100 – 45.348	Lac$^+$, trl	This paper
pF122-10	pOU122	Ap	*Eco*RI-*Cla*I	45.161 – 45.348	Lac$^+$, trl	This paper
pF122-20	pOU122	Ap	*Eco*RI-*Cla*I	45.029 – 45.348	Lac$^+$, trl	This paper
pF260-30	pJL260	Cm	*Pst*I-*Sma*I	44.100 – 45.348	Lac$^+$, trs	This paper
pBK50	pSC101	Km,Tc				Manis and Kline (1978)
pBK53	pBK50	Ap,Km	*Bam*HI	40.3 – 40.45,46.19 – 49.3		Manis and Kline (1978)
pBK55	pBK50	Km	*Bam*HI	40.45 – 42.85		Manis and Kline (1978)
pBK57	pBK50	Km	*Bam*HI	42.85 – 46.19		Manis and Kline (1978)
pF325-31	pBR325	Ap,Tc	*Eco*RI	40.3 – 49.3		This paper
pF325-8	pBR325	Cm,Tc	*Pst*I	44.10 – 45.88[d]		This paper
pIF41	pBR322	Tc	*Pst*I	44.10 – 45.88[d]		Kahn *et al.* (1979)
pF322-6	pBR322	Tc	*Pst*I	45.8 – 47.3		This paper
pF325-7	pBR325	Cm,Tc	*Pst*I	40.3 – 45.8,47.3 – 49.3		This paper
pF325-4	pBR325	Cm,Tc	*Pst*I	44.1 – 47.3		This paper
pF325-9	pBR325	Tc	*Eco*RI-*Pst*I	40.3 – 43.7		This paper
pF325-10	pBR325	Tc	*Eco*RI-*Pst*I	47.3 – 49.3		This paper

[a]Abbreviations used to indicate to which antibiotics the plasmids confer resistance. Ap: ampicillin, Cm: chloramphenicol, Km: kanamycin, Tc: tetracycline.
[b]Coordinates are in kb, cf. Figure 1.
[c]The Lac phenotype is scored on McConkey lactose plates and is only mentioned for plasmids carrying the *lac* genes. The type of fusion is indicated by trl: translational fusion, and trs: transcriptional fusion, and is only mentioned for plasmids with a fragment inserted upstream of the *lac* genes.
[d]In pF325-8 and pIF41 the cloned fragment is orientated in opposite directions: in pF325-8 the *Pst*I site at 45.88 is next to the *bla* gene promoter, and in pIF41 the *Pst*I site at 44.10 is next to this promoter.

and pF1403-30 (all pBR322 high copy number replicons) and (ii) pF122-10 and pF122-20 (both R1 low copy number replicons). pF122-10 and pF122-20 contain the same F-sequences as pF1403-10 and pF1403-20, respectively. (For the construction see Materials and methods.) The specific activities of β-galactosidase are shown in Table II, and they show that the rate of *E-lacZ* expression is gene dosage-dependent and independent of the presence of *incB* upstream of the *E* gene promoter.

Addition of pSC101 hybrids carrying different parts of the f5 fragment had similar effects on all the five fusions: only the central fragment cloned in pBK57 (harbouring the genes for the H1, H2, G1, G2, D and E proteins, and *incB* and *incC*) expresses a function(s) inhibiting *E-lacZ* expression. No stimulation of *E-lacZ* expression was observed with any of the hybrids.

By employing the pF322 and pF325 hybrid plasmids carrying much smaller f5 subfragments cloned in pBR322/pBR325 this inhibitory effect could be mapped to a fragment only containing the *E* gene, *incB, incC* and *oriS* (pF325-4). Plasmids containing either 80% or the NH_2 end of the *E* gene, *incB* and *oriS* (pIF41 and pF325-8), or 20% of the COOH end of the *E* gene and *incC* (pF322-6) do not affect *E-lacZ* expression. It should be noted that *incB* and *incC per se* do not have any effect on *E-lacZ* expression, and that all the four *E-lacZ* fusions respond similarly to repression independent of the presence of *incB* upstream of the *E* gene promoter.

The degree of repression observed with the various combinations depends on the relative dosages of the fusion plasmid and the effector (E$^+$ plasmid) (cf. Table II), in accordance with results reported by Bex *et al.* (1982) using the λ-u.v. infection system.

To distinguish between control exerted at the transcriptional level and the translational level, a transcriptional fusion between the *E* gene and the *lac* genes was constructed (see Materials and methods). This plasmid, pF260-30, contains the *Pst*I-*Sma*I fragment 44.10 – 45.348 with the *Sma*I site closest to the *lacZ* gene, and it mediates a Lac$^+$ phenotype on McConkey lactose plates. Expression of β-galactosidase from this hybrid is only affected by the plasmids which also affect the E-β-galactosidase expression from the translational fusions. With pBK57 and pF325-4, *in trans* expression of β-galactosidase is repressed to the same extent as observed for

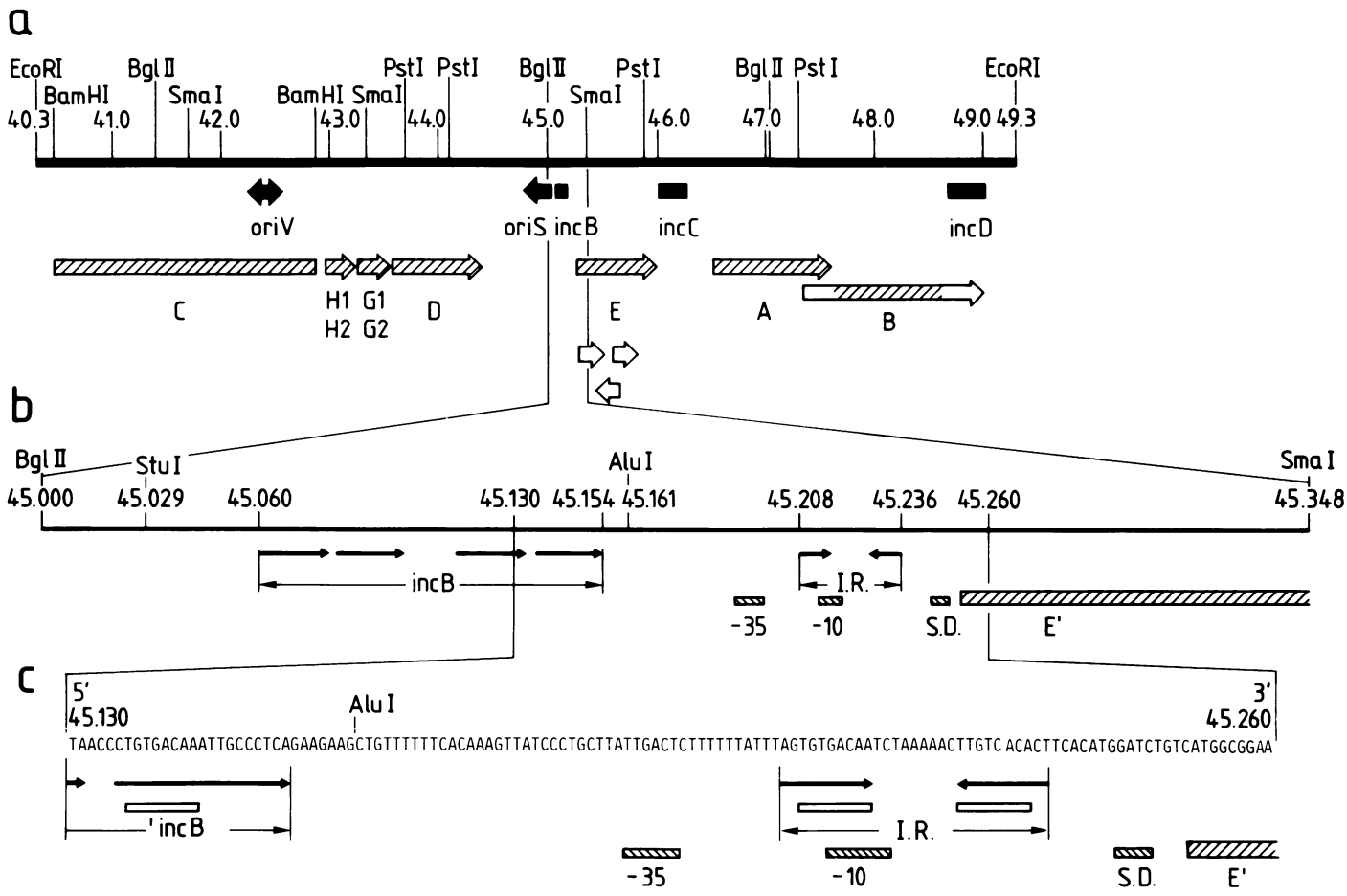

Fig. 1. **(A)** Physical and genetic map of the f5 fragment. Coordinates (40.3 – 49.3) are in kb (Sharp *et al.*, 1972), and the positions of relevant restriction sites are shown. *oriV* and *oriS* are the origins of replication (Eichenlaub *et al.*, 1977; Bergquist *et al.*, 1981) from which replication is initiated in a direction indicated by the fully shaded arrows. *incB, incC* and *incD* are the three loci involved in incompatibilty between F plasmids (Seelke *et al.*, 1982; Tolun and Helinski, 1981; Gardner *et al.*, 1982). The hatched arrows/box labelled C, H1, H2, G1, G2, D, E, A and B indicate the coding regions and the direction of transcription for the nine known proteins encoded by the f5 fragment; for the B protein the exact coding region is unknown – the arrow indicates the maximal size of the coding region and the hatched area the expected coding region; for the C protein the exact coding region and the direction of transcription is unknown – the hatched box indicates the maximal size of the coding region (Bex *et al.*, 1983; Komai *et al.*, 1981; Wehlmann and Eichenlaub, 1980). The small open arrows overlapping the coding region for the E protein indicate the location of three open reading frames as shown from the sequence (Murotsu *et al.*, 1981). **(B)** Expansion of the region between coordinates 45.000 and 45.348. The map shows the positions of *incB*, the start of the *E* gene and the region between these, with relevant restriction sites and coordinates. The hatched boxes labelled –35, –10, S.D. and E′ represent the putative promoter (–35 and –10) and ribosomal binding site (S.D.) for the *E* gene, and the NH_2 end of the *E* gene deduced from the nucleotide sequence (Murotsu *et al.*, 1981). *incB* is defined as the region between the first base pair in the first repeat and the last base pair in the fourth repeat (45.060 – 45.154) indicated by the four shaded arrows. The inverted repeats overlapping the putative promoter for the *E* gene and showing homology with the *incB* and *incC* direct repeats are indicated by the shaded arrows (cf. sequence below). **(C)** The nucleotide sequence from the last two base pairs in the third *incB* repeat to the third codon in the *E* gene. Only the sequence corresponding to the non-coding strand in the *E* gene is shown with the 5′ end to the left and the 3′ end to the right. The fourth repeat of *incB*, the promoter, the ribosome binding site and the NH_2 end of the *E* gene are indicated together with the inverted repeat (I.R.) overlapping the –10 sequence of the *E* gene promoter. The open bars overlapping the fourth *incB* repeat and the inverted repeats show the regions of homology.

the pF1403-10 and the pF122-10 translational fusion plasmids (data not shown).

We therefore conclude that *E* gene expression, most likely at the transcriptional level, is negatively controlled by a *trans*-acting factor encoded by the *E* gene region.

Discussion

The aim of the present investigation has been to define the elements involved in the expression of the *E* gene of the plasmid F by studying the expression of β-galactosidase from *E-lac* gene fusions.

The *E* gene, the production of which is a 29-kd protein (Bex *et al.*, 1981; Komai *et al.*, 1982), seems to play a central role in the replication of mini-F replicons carrying *oriS* and deleted of *oriV* (Watson *et al.*, 1982). However, it should be emphasized that the significance of this gene for the wild-type F replicon, in which the replication is normally initiated from *oriV*, has never been clearly demonstrated. The requirement for the intact *E* gene for *oriS* replication makes its expression a potential target for replication control functions, and since the F-specific replication control functions seem to include *incB* and *incC* it was thought possible that some interaction between these genes (or gene products) could take place. Our data show that the *E* gene promoter is located between *incB* and the start of the structural *E* gene. The *E* gene expression is negatively regulated by a *trans*-acting factor. No stimulatory effect on the *E* gene expression was observed.

The inhibitor of *E* gene expression is encoded by the region coding for the E protein. Since plasmids carrying part of the *E* gene and either of the sequences flanking the *E* gene do not express any inhibitory activity, the entire *E* gene seems to be

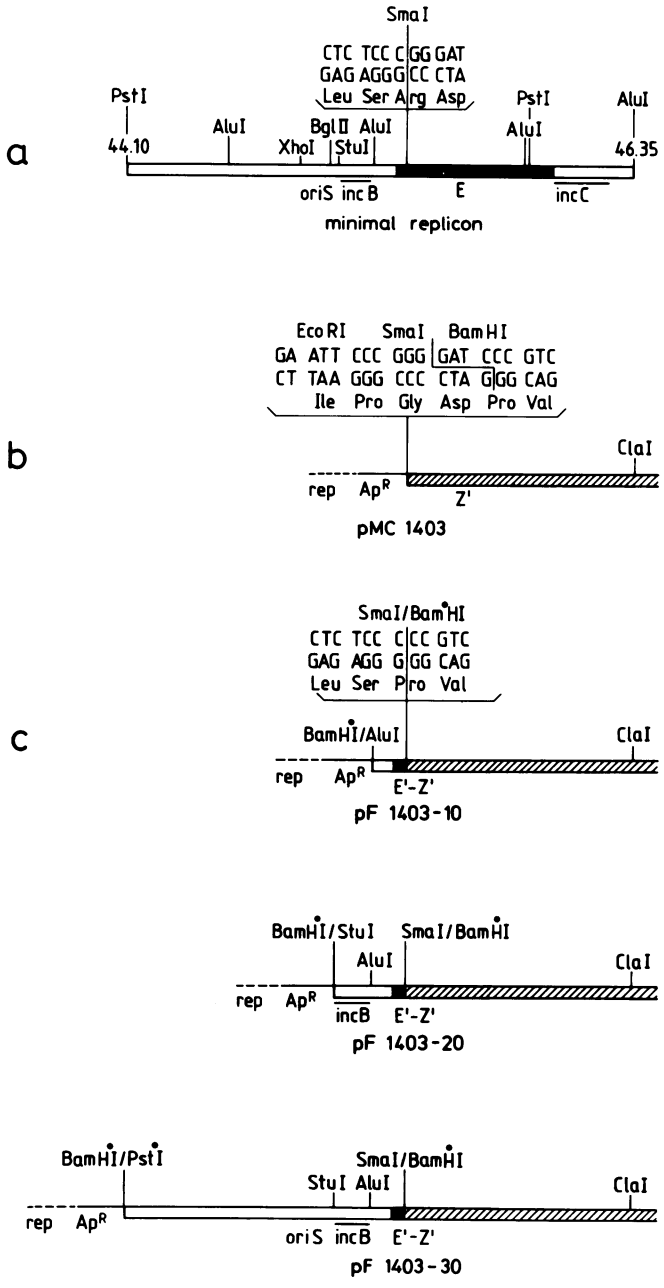

Fig. 2. Construction of translational fusions between the *E* gene and the *lacZ* gene. (**A**) Physical and genetic map of the minimal replicon of F (Murotsu *et al.*, 1981). Coordinates in kb and relevant restriction sites are shown. The nucleotide and amino acid sequences (Murotsu *et al.*, 1981) of the region around the *SmaI* site in the *E* gene is shown. In all fusion plasmids the part of the *E* gene to the right of the *SmaI* site was replaced with the *lac* genes from pMC1403. The origin of replication, *oriS*, of the minimal replicon is indicated together with the incompatibility loci, *incB* and *incC*, and the structural gene for the E protein (shaded area). (**B**) Relevant parts of the translational fusion vector pMC1403 (Casadaban *et al.*, 1980) in which the *lacZ* gene is deleted of its promoter, translational start signal and first seven 1/3 codons. Th nucleotide sequence around the *Bam*HI site used in the constructions is shown together with the amino acid sequence of the NH_2 end of the deleted β-galactosidase polypeptide. (**C**) Relevant parts of three translational fusion plasmids, pF1403-10, pF1403-20 and pF1403-30. DNA of pMC1403 was restricted with *Bam*HI and digested with S1 nuclease to remove the 5'-protruding ends. The *AluI-SmaI* (45.161–45.348) fragment, the *StuI-SmaI* (45.029–45.348) fragment and the *PstI-SmaI* (44.100–45.348) fragment were inserted to construct the three *E-lacZ* fusions. The nucleotide and amino acid sequences around the *SmaI/Bam*HI* ligation site which fuses the *E* gene and the *lacZ* gene are shown for plasmid pF1403-10. Restriction sites labelled * indicate that the single-stranded protruding ends have been digested away with S1 nuclease.

required for expression of the inhibitor. From the published sequence it is known that overlapping the *E* gene are three open reading frames which could give rise to three 9–11 kd proteins (cf. Figure 1). These three reading frames are contained within the *PstI* fragment extending from 44.10–45.88. This fragment cloned in either orientation in the *PstI* site in the *bla* gene in a pBR322/pBR325 vector (pIF41 and pF325-8) has no effect on *E-lacZ* expression from any of the fusions. Therefore, these putative proteins do not seem to have any effect on *E* gene expression, leading to the suggestion that the inhibitor is the E protein itself; thus *E* gene expression most likely is autogenously regulated.

Since a transcriptional fusion between the *E* gene and the *lacZ* gene responds in a quantitatively similar way to the translational fusions, the control of *E* gene expression is most likely exerted at the transcriptional level. The operator for the *E* gene is thus located downstream of *incB*. Although the repeat sequences in *incB* therefore do not contribute to the operator function it is interesting that overlapping the putative *E* gene promoter is a 10-bp inverted repeat separated by 9-bp, and that eight out of the 10 base pairs in the inverted repeat are identical to eight consecutive base pairs in the repeat sequences found in *incB* and *incC* (see Figure 1). If this region of dyad symmetry constitutes the *E* gene operator it may indicate that the E protein can also bind to *incB* and *incC*.

From experiments designed to define the smallest autonomously replicating subfragment from the f5 fragment (Kahn *et al.*, 1979; Ebbers and Eichenlaub, 1981) it has been concluded that the *PstI* fragment from 44.10–45.88 is not self-replicating. Since this fragment contains both *oriS* and the three open reading frames for the three 9–11 kd proteins, none of these three putative proteins is the *trans*-acting factor found by Tolun and Helinski (1982) to be necessary for the replication of *oriS*. We therefore suggest that the *E* gene region gives rise to only one gene product important for replication, and this product has two functions: one as an autorepressor and one as a *trans*-acting factor necessary for *oriS* replication (initiator function). This gene product is the E protein.

Although it is premature to postulate any specific involvement of the control loop described here for the expression of the *E* gene in the overall control system operating for replication of *oriS* (plasmid F), predictions based on the two proposed activities of the E protein would be: assuming that *E* gene expression is rate limiting for initiation of replication, mutations affecting the autorepressor function should show up as copy number mutants and mutations affecting the initiator function should show up as replication-deficient mutants. The fact that mutations giving rise to an increased copy number of *oriS* replicons (Seelke *et al.*, 1982; F. Bex, personal communication), and mutations conferring a non-conditional replication-deficient phenotype on *oriS* replicons (Watson *et al.* 1982) have been mapped to the *E* gene region gives support to the suggested importance of this particular control circuit.

Materials and methods

Bacterial strains and plasmids

The *E. coli* K-12 strain CSH50 (*△pro-lac, rpsL)* (Miller, 1972) was used throughout. The cells were grown in LB medium (Bertani, 1951), and growth was followed using a Gilford Stasar II spectrophotometer at a wavelength of 450 nm.

LA plates contain LB medium with 1.5% agar. McConkey lactose plates

Table II. Specific activities of β-galactosidase[a] expressed from *E-lacZ* fusions

Plasmids added	Genes and incompatibilty loci in the cloned fragments[b]	pF1403-10	pF1403-20	pF1403-30	pF122-10	pF122-20
None		100 (741.0)	100 (751.0)	100 (747.2)	100 (80.9)	100 (76.0)
pBK53	*A, B, incD*	101	98	105	103	106
pBK55	*C*	95	100	94	104	102
pBK57	*H1,H2,G1,G2,D,E,incB,incC*	38	39	43	20	21
pBK50		102	98	99	101	97
pIF41	*D',E',incB*				103	104
pF325-8	*D',E',incB*				98	104
pF322-6	*A',E',incC*				99	93
pF325-7	*A',B,C,D,H1,H2,G1,G2,E',incB*				103	97
pF325-9	*C,H1,H2,G1,G2,D'*				99	94
pF325-10	*A',B,incD*				100	96
pF325-4	*A',D',E,incB,incC*				12	11
pF325-31	The whole f5 fragment				13	13
pBR322					94	97

[a]Specific activities of β-galactosidase are expressed as OD_{420}/OD_{450}/ml/min x 10^{-3}. In parentheses absolute specific activities are given for the five fusions in the absence of any added plasmid. These absolute activities are converted to 100. The specific activities obtained with different plasmids added are given in percentages of the specific activities obtained without any plasmid added. With pF1403-10, pF1403-20 and pF1403-30 the cultures were grown at 37°C; with pF122-10 and pF122-20 the cultures were grown at 34°C.

[b]*A, B, C, D, E, H1, H2, G1* and *G2* are the genes coding for the A-, B-, C-, D-, E-, H1-, H2-, G1- and G2- proteins, respectively.
' indicates that only part of the gene is cloned.

were prepared as described by the manufacturer (Difco). Antibiotics were dissolved in the plate media at the following concentrations: ampicillin 50 μg/ml, kanamycin 50 μg/ml, chloramphenicol 50 μg/ml, tetracycline 10 μg/ml.

Transfer of plasmids to bacteria

Plasmids were transferred by transformation (Cohen *et al.*, 1972).

Preparation of plasmid DNA

Plasmid DNA was prepared as described by Stougaard and Molin (1981).

Enzyme treatment

Restriction enzyme digestion, ligation with T4 polynucleotide ligase and S1 exonuclease treatment was done according to the manufacturers instructions.

Isolation of DNA fragments

Restriction enzyme fragments were isolated from agarose gels according to Yang *et al.* (1979).

β-Galactosidase activity measurements

Measurements of β-galactosidase expressed from fusion plasmids were done as described by Light and Molin (1981).

Construction of E-lacZ fusions (see Figure 2)

pF1403-10. The *Xho*I-*Pst*I fragment (44.87 – 45.88) was restricted with *Alu*I and *Sma*I. pMC1403 was digested with *Bam*HI and subjected to S1 nuclease treatment to remove the protruding 5' ends. After inactivation of the enzymes the DNA was ligated.

Selection after transformation was for Ap^r and screening for Lac^+ phenotype on McConkey lactose plates. Verification that the fragment had been inserted in the correct orientation was done by mapping the plasmid with *Sma*I, *Pvu*II, *Dde*I and *Hin*fI.

pF1403-20. The *Stu*I-*Sma*I fragment (45.03 – 45.35) was cloned in the *Bam*HI site in pMC1403, treated as in the pF1403-10 cloning. Selection was for Ap^r and screening for Lac^+ on McConkey lactose plates and expression of incompatibility against pML31. Verification that the fragment had been cloned in the correct orientation was done as described for pF1403-10.

pF1403-30. The *Pst*I-*Sma*I fragment (44.10 – 45.35) was digested with S1 nuclease to remove the 3'-protruding end at the *Pst*I site and cloned in the *Bam*HI site in pMC1403, treated as in the pF1403-10 cloning. Selection was for Ap^r and screening for Lac^+ on McConkey lactose plates and expression of incompatibility against pML31. Verification that the fragment had been cloned in the correct orientation was done by mapping the plasmid with *Sma*I, *Bgl*II, *Eco*RI and *Pvu*II.

pF260-30. The *Pst*I-*Sma*I fragment (44.10 – 45.35) was cloned in the corresponding sites in the promoter cloning vector pJL260. Selection was for Cm^r and screening for Lac^+ on McConkey lactose plates and expression of incompatibility against pML31. Verification that the fragment had been cloned in the correct orientation was done by mapping the plasmid with *Pst*I, *Hind*III and *Sma*I.

pJL260 is a derivative of plasmid pGA39 (An and Friesen, 1979) carrying the *Hind*III-*Bgl*II fragment from pMC81 (Casadaban and Cohen, 1980) which contains the *lac* genes deleted of the *lac* promoter.

pF122-10 and pF122-20. The *Eco*RI-*Cla*I fragments from pF1403-10 and pF1403-20 were cloned in the corresponding sites in pOU122. Selection was for Ap^r and λ-immunity and screening for a temperature-dependent Lac expression on McConkey lactose plates. Plasmid pOU122 is a derivative of plasmid R1 mediating resistance to ampicillin and immunity to phage λ. It carries the *lac* genes from pMC1403 and, at tempertures below 38°C, the copy number is the same as for the R1 plasmid, i.e., one per chromosome. At temperatures above 38°C the copy number is increased due to an insertion of the λp_R promoter and the cI_{857} allele of phage λ near the replication genes, and at 42°C pOU122 exhibits run-away replication phenotype (Larsen, Gerdes, Light and Molin, in preparation).

Construction of pF322 and pF325 hybrids

Plasmid pML31 was digested partially with *Pst*I alone or with both *Pst*I and *Eco*RI; the fragments were cloned in pBR322 restricted with *Pst*I, or in pBR325 restricted with *Pst*I alone or with *Pst*I and *Eco*RI together. Selection was for Tc^r, and screening for Ap^s in the *Pst*I clonings and Ap^s and Cm^s in the *Pst*I and *Eco*RI clonings.

Incompatibility test

The presence of an IncFI incompatibility region on a plasmid containing an f5 subfragment was detected by transforming the plasmid to CSH50 harbouring pML31; after phenotypic expression for 1 h at 37°C the cells were spread on plates selecting only for the incoming plasmid. After incubation at 37°C for ~12 h, 100 colonies were tested for the presence of pML31 (Km^r). Loss of pML31 in a majority (usually 100% loss) of the colonies tested was taken as evidence that the incoming plasmid contained an IncFI incompatibility region.

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