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The Iteron Regions Necessary for the RepE–Iteron Interaction *In Vivo* in Mini-F Plasmids of *Escherichia coli*[1]

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We have determined the nucleotide positions of an *incC* iteron essential for RepE binding by analyzing mutated *incC* iterons defective in exerting incompatibility towards mini-F plasmids. The mutations affecting this incompatibility occurred mostly at two positions within the *incC* iteron, *i.e.* an iteron conserved position and a mini-F specific position. Most of the iterons with a base-change at either of these two positions had lost the binding affinity for RepE. This agrees with the crystallographic structure of the RepE-iteron complex which showed that the N and C terminal domains of RepE interact with the two major grooves on one face of the iteron DNA. These grooves contain the iteron conserved and mini-F specific positions necessary for RepE binding. Thus the binding mode may be common to in the case of mini-F like plasmids.

Key words: crystallographic structure of RepE–iteron complex, incompatibility defective mutants, iteron-RepE interaction, mini-F plasmid.

The F plasmid is stably maintained in *Escherichia coli* at a copy number of one or two per host chromosome. Mini-F (*1*) and its shorter derivative, pKV713 (*2*), possess all the necessary functions for the maintenance of F plasmids such as DNA replication, copy number control, incompatibility, and partitioning. The structure of mini-F plasmids is shown in Fig. 1. The replication origin (*ori2*) consists of two DnaA boxes recognized by the DnaA protein, an AT-rich region, a 13-mer sequence homologous to that of *oriC* (the chromosomal origin), and four direct repeats of a 19 bp sequence (iterons) to which RepE binds. The *repE* gene encodes the replication initiator protein RepE of mini-F plasmids (Fig. 1A).

The cellular amount of the RepE protein is regulated autogenously at the transcription level (*1*, *3*, *4*). The RepE protein usually exists as a homo-dimer that is active in autogenous repression but inactive in replication initiation (*5*). RepE dimers are converted to the monomer form (active initiator form) by a group of molecular chaperones (DnaK, DnaJ, and GrpE) (Fig. 1B; Wada *et al.*, unpublished data). Thus, the activation of the RepE protein in a cell is important for the initiation of DNA replication of mini-F plasmids.

The *incC* region contains five directly repeated iterons that share the consensus sequence (TGAGGGTT/AG/ATTTGTCACAG) with *ori2* iterons, although they are oriented in opposite directions (Fig. 1B). The RepE monomer protein binds to the *incC* iterons as well as to the *ori2* iterons (*5–8*). Earlier reports and our studies have indicated that iterons in the *incC* region are responsible for both incompatibility and control of the copy number (*1*, *8–13*). We found that extra rounds of DNA replication initiation of the mini-F plasmid are inhibited by the formation of a nucleoprotein complex consisting of *ori2* iterons and *incC* iterons bound by RepE (*8*), and that the dimerization domain of RepE (*14*) might be responsible for the formation of this complex (*8*). It is known that the replication of mini-F like plasmids such as P1, R6K, and RK2 is also controlled through the interaction between iteron-Rep complexes, as proposed in the Hand-cuffing model (*15–20*). The N terminal domain (residues 33–88) and C terminal domain (residues 168–242) of RepE are essential for binding to iterons (*21*, *22*). The crystallographic structure of the RepE–iteron complex clearly indicated the iteron region interacted with RepE (*21*). However, the loci on the iteron required for the interaction with RepE *in vivo* have remained unclear. We have now determined these loci using incompatibility defective mutations in the *incC* region.

Isolation and Localization of incC-Defective Mutations—As described in an earlier report, we previously isolated twenty-six independent incompatibility defective mutants by PCR mutagenesis (*23*) and determined the mutation sites in the *incC* region (Table I; *8*). Among these mutants, we characterized four that were defective in incompatibility and copy number control by *incC*. As expected, three of the four mutants were found to have lost their binding affinity for RepE (*8*). This indicates that the mutated sites within the *incC* iteron are important for binding to RepE. The consensus sequence of the five iterons of the *incC* region is TGAGGGTT/AG/ATTTGTCACAG (Fig. 2A). All the mutations that occurred within iterons of the mutated *incC* region were summarized on the consensus iteron in a histogram (Fig. 2B). The iteron sequences exhibiting high mutation frequencies (3-A:T, 5-G:C, 6-G:C, 7-T:A, 8-T/A:A/T, 12-T:A, 14-T:A, 15-C:G, and 16-A:T) probably contribute directly to the iteron–RepE interaction. On the other hand, the sequences exhibiting low mutation frequencies (1-T:A, 2-

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G:C, 4-G:C, 9-G/A:C/T, 10-T:A, 11-T:A, 13-G:C, 17-C:G, 18-A:T, and 19-G:C) may not contribute directly to binding to RepE. The sequences exhibiting high mutation frequencies were mostly in two regions, at iteron conserved and mini-F specific positions (Fig. 2B). The iteron conserved positions (5'-TGAGGG-3') are common to the replicons (*E. coli oriC*, λ, mini-F, P1, Rts1, R6K, and RK2 plasmid; *24*) whose DNA replication is controlled through the binding of initiator proteins to iterons. We define this position as the iteron conserved position. The mini-F specific positions (5'-TTGTCACA-3') are shared by the inverted repeat of the *repE* operator and the *ori2* iterons (Fig. 1B).

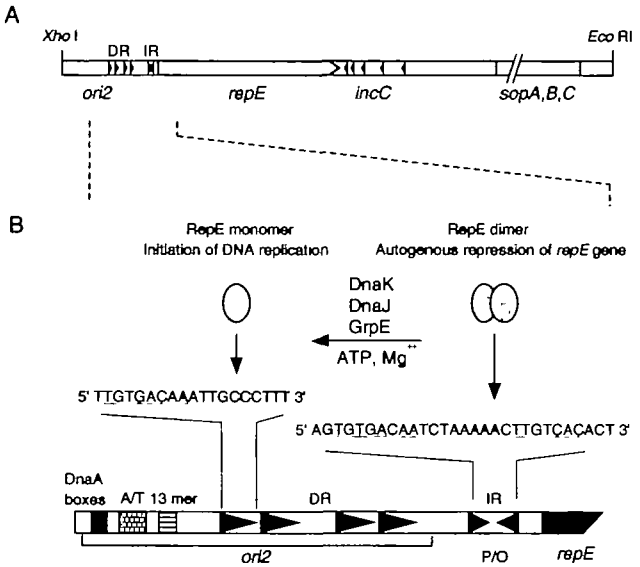


Fig. 1. **The structure and function of mini-F.** (A) The genes of the *ori2* mini-F plasmid. *ori2*, *repE*, *incC*, and *sopA,B,C* denote the genes for the replication origin, initiator protein, copy control, and partitioning, respectively. (B) The *ori2* region contains a DnaA box, an A/T rich region, a 13-mer region and four direct repeats (DR) to which the RepE monomers bind. The RepE dimers bind to the promoter and operator (P/O) region [the inverted repeat (IR) containing 8 base-pairs, indicated by underlining has the same sequence as the 8 base-pairs of the origin iterons], and thereby repress *repE* transcription. RepE dimers are converted to monomers *in vitro* by DnaK, DnaJ and GrpE in the presence of ATP.

The Binding Affinity of RepE to the Mutated Iteron 7 DNA—Iteron 7, which possesses the *incC* iteron consensus sequence, was taken to be representative of the other four iterons. Various iteron 7 fragments with the same base-pair change at positions where the mutations occurred at high frequency (Figs. 2B and 3C) were constructed by annealing two synthetic complementary polynucleotide strands (*Eco*RI site-mutated iteron–*Hin*cII site), and then the fragments were cloned between the *Eco*RI and *Hin*cII sites of the pHSG299 plasmid (pUC19 derivative; *8*, *25*). The plasmid carrying iteron 7-A3G [showing that the third A (Adenine) of iteron 7 was changed to G (Guanine)] exerted normal incompatibility towards mini-F plasmids, but the plasmids carrying mutated iterons 7-G5A, 7-T (Thymine)7A, 7-G13A, 7-T14C (Cytosine), and 7-A16G failed to exert incompatibility towards mini-F plasmids (data not shown). The possibility that the mutated iterons had lost their binding affinity for RepE was examined by means of a gel retardation assay. All of the iteron mutants, except 7-A3G, which exerted the same level of incompatibility as the wild type iteron, had lost their binding affinity for RepE (Fig. 3, B and C).

Iteron 9 Has no Binding Affinity for RepE—In contrast to iteron 7, the pHSG299 plasmid carrying iteron 9 did not exert incompatibility towards mini-F plasmids (data not shown). Iteron 9 exhibited a lower mutation frequency than the other *incC* iterons (Table I). These results suggest that iteron 9 may be defective in RepE binding. We examined the RepE binding to iteron 9 and, as expected, found that it was poor (Fig. 3, A, B, and C). When 13-A of iteron 9 was

TABLE I. **Locations of base changes in incompatibility defective mutants.**

Mutant	Number of mutations			Base changes within iterons				
	Total	within iterons	others	5 TGAGGGTTATCTGTCACAG	6 TGAGGGTGGTTCGTCACAT	7 TGAGGGTAATTTGTCACAG	8 TGAGGGCAGTTTGTCACAG	9 TGAGGGTTGATTATCACAG
incC1	10	6	4	----------T----	----T-A----------	----------G---	----------C-----	----------A----
incC3	12	7	5	----A----------	C-------A----C-----	----------C-----	----------C-A-----	----------
incC5	9	8	1	G-G----------	-A----------	----A----------	-------T------T----	---T----------G-
incC6	11	7	4	----------C-----	----------A--C-----	----------G---	C-T---------A-------	----------
incC7	2+Δ	1+Δ	1+Δ	----------C-----	----------	----------	----------	----------
incC8	8	5	3	----------A-----	------A----------	--G----------	----------C---T-	----------
incC9	8	3+Δ	4	------ ----------	------A----------	----------G---	------A----------	----------
incC10	8	7	1	--G---------G----	------A----------	---------C-A-----	----------A------	--G----------
incC14	10	6+Δ	3	----------A-----	----------A--C-----	--T----------T-	----------A ----	----------
incC16	9	6	3	----T----------	----------G-----	------A----------	--T----T----------	----------G---
incC17	10	7	3	----T----------	----------G-----	------A----------	--T----T----------	----A---------G---
incC20	5	5	0	----------C---	----------C-----	----------C-----	---------C-----G---	----------
incC21	11	7+Δ	3	------ ----C-------	----T---------AG---	----------C-----	----------C-----	----------G-
incC22	8	5	3	----------G-----	--G----------	----------G---	----C----------	-------C---------
incC25	9	5+Δ	3	----------G---	----C----------	------ ----------	--T-A----------	--------G---------
incC27	3+Δ	2+Δ	1+Δ	-----			--G---------C-------	----------
incC28	7+Δ	5+Δ	2+Δ		--G----------	-A----------C-----	-----A---------T---	----------
incC33	11	7	4	-------C---A-------	--G----------	----------C-----	----------C---G---	----------T---
incC34	5	3+Δ	1	----------C-----	----------A-----	---------- -------	-----T----------	----------
incC43	9	5+Δ	3	----------C-----	----------T-C-----	----------C-----	---------- ---G---	----------
incC44	4	4	0	----------C---	----------G---	-----A----------	-----A----------	----------
incC45	8	4	4	----------C-----	----------C-----	-----T----------	----------C-----	----------
incC61	4+Δ	2+Δ	2+Δ	----C----------	----------A			----------
incC64	3+Δ	1+Δ	2+Δ	----------G---	----------			----------
incC65	5	3+Δ	1	----------G---	------- ----------	----------G-----	----------T----	----------
incC94	1+Δ	1+Δ	0+Δ	----------C-----				----------

Number of mutations indicates the total number of mutations that occurred in *incC* mutants. Δ indicates deletion. The bases (A, T, G and C) between the broken lines indicate the changed bases. The interrupted portions of the broken lines indicate the deleted regions.

changed to 13-G, the mutated iteron 9, which now possessed the consensus sequence of the *incC* iterons, exhibited a similar level of binding affinity for RepE to that of iteron 7 (Fig. 3, B and C). The wild type pHSG299 carrying the mutated iteron 9-A13G also exerted normal incompatibility towards the mini-F plasmids (data not shown). The role played in the *incC* function by wild type iteron 9, which binds so poorly to RepE, remains unclear. These results suggest that the consensus iteron sequences, 5-G:C, 7-C:G, 13-G:C, 14-T:A, and 16-A:T, are important for interactions with RepE *in vivo* (Figs. 2B and 4).

Comparison with the Crystallographic Structure of the RepE–Iteron Complex—The crystallographic structure of RepE complexed with an *ori2* iteron has been determined (*21*). The *ori2* iterons possess the same consensus sequence as that of *incC* iterons except for 7-C:G (7-T:A in *incC* iterons; Fig. 4). It is clear that 13-G, 15-C:G, 16-T, and 17-G of the consensus iteron directly interact with the C-terminal domain of RepE (Fig. 4). The N-terminal domain of RepE interacts directly with 2-G, 5-G, and 6-G but not with 3-A of the consensus iteron (Fig. 4). Furthermore, the phosphate backbone also interacts with the N-terminal and C-terminal domains (Fig. 4). The 14-T mutation caused a reduction in RepE binding, but the direct interaction between iteron and RepE was not observed in the crystallographic structure. This base (methyl group of 14-thymine) may maintain a conformation important for the interaction between the C-terminal domain of RepE and the F-plasmid specific position of the iteron. These results are consistent with results obtained on genetic analysis of *incC* mutants.

2:G, 13:G, or 17:G directly interacts with RepE, but the mutation frequencies of these bases were lower than expected. This could have been caused by the PCR mutagenesis method used to isolate the incompatibility defective mutants. The method used was based on the low fidelity of Taq DNA polymerase under reaction conditions involving $MnCl_2$. In earlier experiments involving this method, we observed a marked preference of Taq DNA polymerase for certain base substitutions: the frequencies of the base changes were 72% for thymine (T), 23% for adenine (A), 5% for guanine (G), and <5% for cytosine (C) (*22*). This indicates that the mutation frequency of G or C is lower than

A

incC iteron	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
5	T	G	A	G	G	G	T	T	A	T	C	T	G	T	C	A	C	A	G
6	T	G	A	G	G	G	T	G	G	T	T	C	G	T	C	A	C	A	T
7	T	G	A	G	G	G	T	A	A	T	T	T	G	T	C	A	C	A	G
8	T	G	A	G	G	G	C	A	G	T	T	T	G	T	C	A	C	A	G
9	T	G	A	G	G	G	T	T	G	A	T	T	A	T	C	A	C	A	G
Consensus	T	G	A	G	G	G	T	T/A	G/A	T	T	T	G	T	C	A	C	A	G

B

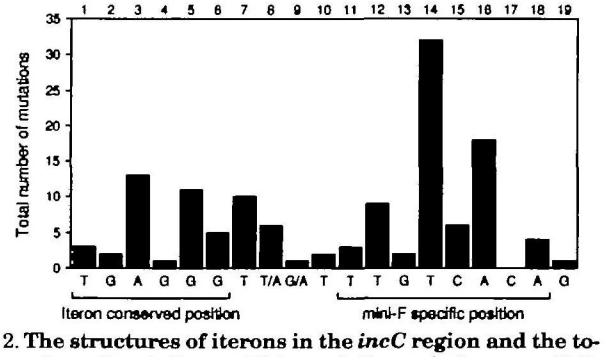
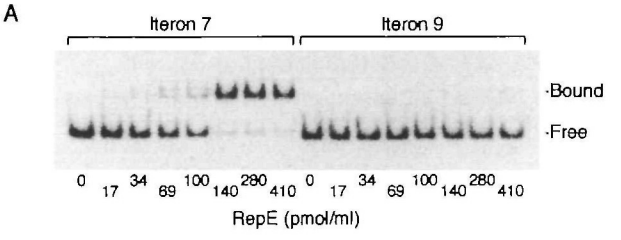
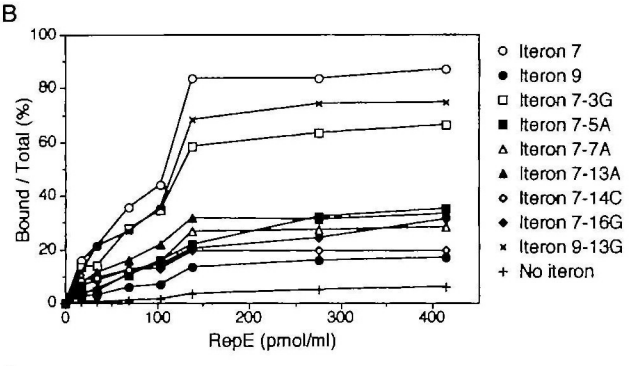


Fig. 2. **The structures of iterons in the *incC* region and the total number of mutations within each iteron in incompatibility defective mutants.** (A) The DNA sequences of the five iterons in the *incC* region and their consensus sequence are shown. (B) The mutations that occurred in the iterons of the incompatibility defective mutants were taken from Table I, and the total numbers of mutations at individual bases were plotted as a bar graph on a consensus iteron.

A



B



C

Iteron	Base change of iteron	Binding affinity of RepE (%)
7	**TGAGGGTAATTTGTCACAG**	87
7-3G	**--G**----------	66
7-5A	**----A**----------	35
7-7A	**------A**----------	28
7-13A	----------**A**------	33
7-14C	----------**C**------	19
7-16G	----------**G**---	31
9	**TGAGGGTTGATTATCACAG**	17
9-13G	----------**G**------	75

Fig. 3. **Gel retardation assay for RepE binding to mutated iterons.** (A) The assays were performed essentially as described previously (*28*). The reaction mixture (20 μl) contained 20 mM Tris-HCl, pH 7.5, 40 mM NaCl, 40 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 0.1 mg/ml bovine serum albumin (BSA), 10 μg/ml poly (dI-dC), 5 fmol of probe DNA end-labeled with [γ-^{32}P]ATP, and RepE, which was added last. The *Pvu*II fragment of the pHSG299 plasmid containing iteron 7 (iteron 9) or mutated iteron 7 (iteron 9) (326 bp) was used as a DNA probe. The mixture was incubated at 30°C for 30 min and then electrophoresed on a 6% polyacrylamide gel. (B) The gels were dried and DNA bands were quantified with a Fujix bioimaging analyzer BAS2000 (Fuji, Tokyo). The ratios of bound DNA to total DNA were plotted as a function of the concentration of RepE. (C) The base changes of iteron 7 or 9 are shown. The bases between the broken lines indicate the changed bases. The binding affinity for RepE (%) is shown as the maximum amount of iteron fragment bound by RepE obtained from Fig. 3B.

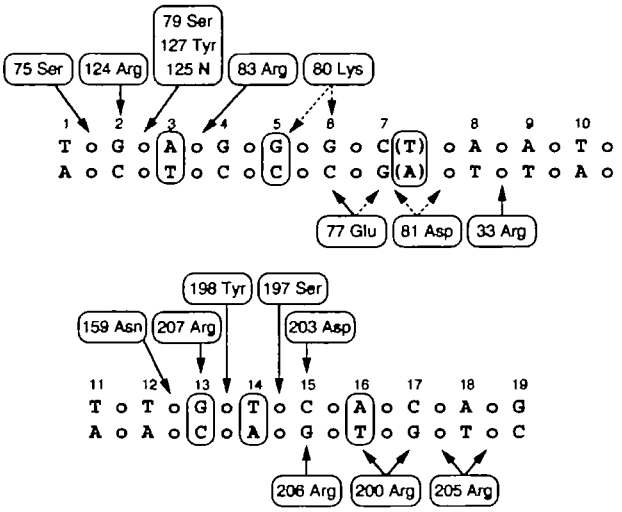


Fig. 4. **The interaction between the iteron and RepE, as determined by crystallographic structural analysis.** "O" indicates the phosphate backbone of an iteron. The arrows indicate the interactions between the bases (A, G, T, or C) or phosphate backbone of an iteron and the interacting amino acid residue of RepE (251 residues). The numbers 1–19 indicate the sequence numbers of an iteron. The broken lines from Lys 80 show that RepE binding through these residues would have a high B-factor (*21*). The broken lines from 77 Glu and 81 Asp show RepE binding to iterons through water or Mg^{2+}. The enclosed base pairs indicate the locations of the mutations of the mutated iterons that were constructed synthetically and analyzed by a genetical method (see text). The consensus sequence of the *incC* iterons is the same as that of the *ori2* iterons except for position 7 (the corresponding base pair of the *incC* iteron is shown in parenthesis).

that of T or A. A similar tendency was observed in the experiments described above. Thus, the low mutation frequency of 2:G, 13:G, or 17:C at the two positions probably reflects the base preference of Taq DNA polymerase under these reaction conditions.

The C- and N-terminal domains of RepE interact with the two major grooves on one face of the iteron DNA (*21*), where the functionally defined areas of the iteron conserved and mini-F specific positions required for RepE binding are located (Figs. 2B and 4). This indicates that the crystallographic structure of the RepE-iteron complex is probably reflected in the mode of binding of the iteron to RepE *in vivo*. Such an iteron-initiator protein interaction was observed for the pPS10 (*26*) and P1 (*27*) plasmids. This mode of interaction between RepE and iterons may be common to mini-F like plasmids.

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REFERENCES

1. Kline, B.C. (1985) A review of mini-F plasmid maintenance. *Plasmid* **14**, 1–16
2. Kawasaki, Y., Wada, C., and Yura, T. (1990) Roles of *Escherichia coli* heat shock proteins DnaK, DnaJ and GrpE in mini-F plasmid replication. *Mol. Gen. Genet.* **220**, 277–282
3. Muraiso, K., Tokino, T., Murotsu, T., and Matsubara, K. (1987) Replication of mini-F plasmid *in vitro* promoted by purified E protein. *Mol. Gen. Genet.* **206**, 519–521
4. Wada, C., Imai, M., and Yura, T. (1987) Host control of plasmid replication: requirement for the sigma factor sigma 32 in transcription of mini-F replication initiator gene. *Proc. Natl. Acad. Sci. USA* **84**, 8849–8853
5. Ishiai, M., Wada, C., Kawasaki, Y., and Yura, T. (1994) Replication initiator protein RepE of mini-F plasmid: functional differentiation between monomers (initiator) and dimers (autogenous repressor). *Proc. Natl. Acad. Sci. USA* **91**, 3839–3843
6. Masson, L. and Ray, D.S. (1986) Mechanism of autonomous control of the *Escherichia coli* F plasmid: different complexes of the initiator/repressor protein are bound to its operator and to an F plasmid replication origin. *Nucleic Acids Res.* **14**, 5693–5711
7. Tokino, T., Murotsu, T., and Matsubara, K. (1986) Purification and properties of the mini-F plasmid-encoded E protein needed for autonomous replication control of the plasmid. *Proc. Natl. Acad. Sci. USA* **83**, 4109–4113
8. Uga, H., Matsunaga, F., and Wada, C. (1999) Regulation of DNA replication by iterons: an interaction between the *ori2* and *incC* regions mediated by RepE-bound iterons inhibits DNA replication of mini-F plasmid in *Escherichia coli EMBO J.* **18**, 3856–3867
9. Bergquist, P.L., Downard, R.A., Caughey, P.A., Gardner, R.C., and Lane, H.E. (1981) Analysis of mini-F plasmid replication by transposition mutagenesis. *J. Bacteriol.* **147**, 888–899
10. Kline, B.C. and Trawick, J. (1983) Identification and characterization of a second copy number control gene in mini-F plasmids. *Mol. Gen. Genet.* **192**, 408–415
11. Manis, J.J. and Kline, B.C. (1978) F plasmid incompatibility and copy number genes. Their map locations and interactions. *Plasmid* **1**, 492–502
12. Tsutsui, H., Fujiyama, A., Murotsu, T., and Matsubara, K. (1983) Role of nine repeating sequences of the mini-F genome for expression of F-specific incompatibility phenotype and copy number control. *J. Bacteriol.* **155**, 337–344
13. Wada, C. and Yura, T. (1984) Control of F plasmid replication by a host gene: evidence for interaction of the *mafA* gene product of *Escherichia coli* with the mini-F *incC* region. *J. Bacteriol.* **160**, 1130–1136
14. Matsunaga, F., Ishiai, M., Kobayashi, G., Uga, H., Yura, T., and Wada, C. (1997) The central region of RepE initiator protein of mini-F plasmid plays a crucial role in dimerization required for negative replication control. *J. Mol. Biol.* **274**, 27–38
15. Blasina, A., Kittell, B.L., Toukdarian, A.E. and Helinski, D.R. (1996) Copy-up mutants of the plasmid RK2 replication initiation protein are defective in coupling RK2 replication origins. *Proc. Natl. Acad. Sci. USA* **93**, 3559–3564
16. Chattoraj, D.K., Mason, R.J., and Wickner, S.H. (1988) Mini-P1 plasmid replication: the autoregulation-sequestration paradox. *Cell* **52**, 551–557
17. McEachern, M.J., Bott, M.A., Tooker, P.A., and Helinski, D.R. (1989) Negative control of plasmid R6K replication: possible role of intermolecular coupling of replication origins. *Proc. Natl. Acad. Sci. USA* **86**, 7942–7946
18. Miron, A., Mukherjee, S., and Bastia, D. (1992) Activation of distant replication origins *in vivo* by DNA looping as revealed by a novel mutant form of an initiator protein defective in cooperativity at a distance. *EMBO J.* **11**, 1205–1216
19. Mukhopadhyay, G., Sozhamannan, S., and Chattoraj, D.K. (1994) Relaxation of replication control in chaperone-independent initiator mutants of plasmid P1. *EMBO J.* **13**, 2089–2096
20. Papp, P.P., Mukhopadhyay, G., and Chattoraj, D.K. (1994) Negative control of plasmid DNA replication by iterons. Correlation with initiator binding affinity. *J. Biol. Chem.* **269**, 23563–23568
21. Komori, H., Matsunaga, F., Higuchi, Y., Ishiai, M., Wada, C., and Miki, K. (1999) Crystal structure of the procaryotic replication initiator protein bound to DNA at 2.6 Å resolution. *EMBO J.* **18**, 4597–4607
22. Matsunaga, F., Kawasaki, Y., Ishiai, M., Nishikawa, K., Yura, T., and Wada, C. (1995) DNA-binding domain of the RepE initiator protein of mini-F plasmid: involvement of the carboxyl-terminal region. *J. Bacteriol.* **177**, 1994–2001

23. Leung, D.W., Chen, E., and Goeddel, D.V. (1989) A method for random mutagenesis of a defined DNA segment using a modified polymerase chain reaction. *Technique* **1**, 11–15
24. Tolun, A. and Helinski, D.R. (1981) Direct repeats of the F plasmid *incC* region express F incompatibility. *Cell* **24**, 687–694
25. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: a Laboratory Manual* (2nd ed.). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York
26. Giraldo, R., Andreu, J.M., and Diaz-Orejas, R. (1998) Protein domains and conformational changes in the activation of RepA, a DNA replication initiator. *EMBO J.* **17**, 4511–4526
27. Papp, P.P., Chattoraj, D.K., and Schneider, T.D. (1993) Information analysis of sequences that bind the replication initiator RepA. *J. Mol. Biol.* **233**, 219–230
28. Kawasaki, Y., Wada, C., and Yura, T. (1992) Binding of RepE initiator protein to mini-F DNA origin (*ori2*). Enhancing effects of *repE* mutations and DnaJ heat shock protein. *J. Biol. Chem.* **267**, 11520–11524