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*

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In bacteria, plasmids and some DNA viruses, DNA replication is initiated and regulated by binding of initiator proteins to repetitive sequences. To understand the control mechanism we used the plasmid mini-F, whose copy number is stringently maintained in *Escherichia coli*, mainly by its initiator protein RepE and the *incC* region. The monomers of RepE protein bound to *incC* iterons, which exert incompatibility *in trans* and control the copy number of mini-F plasmid *in cis*. Many incompatibility defective mutants carrying mutations in their *incC* iterons had lost the affinity to bind to RepE, while one mutant retained high level binding affinity. The mutated *incC* mini-F plasmids lost the function to control the copy number. The copy number of the wild-type mini-F plasmid did not increase in the presence of excess RepE. These results suggested that the control of replication by *incC* iterons does not rely on their capacity to titrate RepE protein. Using a ligation assay, we found that RepE proteins mediated a cross-link structure between *ori2* and *incC*, for which the dimerization domain of RepE and the structure of *incC* seem to be important. The structure probably causes inhibition of extra rounds of DNA replication initiation on mini-F plasmids, thereby keeping mini-F plasmid at a low copy number.

Keywords: copy number control/cross-link structure/incompatibility/RepE protein/replication control

Introduction

F-factor plasmid is stably maintained in *Escherichia coli* at a copy number of one or two per host chromosome. The 9 kbp *EcoRI* fragment of F factor replicates autonomously, and has been termed mini-F (reviewed by Kline, 1985). Mini-F and its shorten derivative, pKV713 (Kawasaki *et al.*, 1990), possess all of the necessary functions for the maintenance of F plasmids such as DNA replication, copy number control, incompatibility and partition. The replication origin (*ori2*) consists of two DnaA boxes recognized by DnaA protein, an AT-rich region, a 13mer sequence homologous to those of *oriC* (the chromosomal origin) and four direct repeats of a 19 bp sequence (iterons) to which RepE binds specifically. Along with the *incC* and the *sopA*, *B* and *C* genes, the *repE* gene is located next to *ori2* and encodes the replica-

tion initiator protein RepE which is specific to mini-F plasmids (Figure 1).

RepE monomers bind to four iterons in *ori2* and cause bending of this region, which in turn induces the localized melting of the duplex DNA from the 13mer to the AT-rich region with the assistance of HU and DnaA proteins (Kawasaki *et al.*, 1996). This step is essential for the initiation of mini-F plasmid DNA replication.

The cellular amount of RepE protein is regulated autogenously by RepE at the transcriptional level (Søgaard-Andersen *et al.*, 1984; Trawick and Kline 1985; Muraiso *et al.*, 1987; Wada *et al.*, 1987). RepE protein usually exists as homodimers which are inactive for replication initiation but active for autogenous repression (Ishiai *et al.*, 1994). RepE dimers are converted to the monomer form (active initiator form) by a set of molecular chaperones (DnaK, DnaJ and GrpE) (Figure 1) (C.Wada, F.Matsunaga and G.Kobayashi, unpublished data). Thus, it is thought that the amount of active RepE protein in a cell is critical for determining the initiation frequency of DNA replication.

The *incC* region as well as the amount of active RepE in a cell are important for the stringent control of mini-F copy number. The *incC* region contains five directly repeated iterons which share with *ori2* iterons the consensus sequence (TGAGGGTXG/ATTTGTCACAG), although oriented in the opposite direction (Figure 1). It is known that two plasmids of the same incompatibility group cannot be stably maintained in the same cell (Kline, 1979; Seelke *et al.*, 1982; Kline and Trawick, 1983). This is also the case for a mini-F plasmid and multicopy plasmids carrying the *incC* region (Tolun and Helinski, 1981; Tsutsui *et al.*, 1983). Mutations of *incC* cause the loss of incompatibility and an increase in the copy number of mini-F plasmids (Kline, 1985). An insertion of the transposon Tn3 into an *incC* iteron also increases the copy number, whereas insertions into the other regions of *incC* do not (Bergquist *et al.*, 1981; Kline and Trawick, 1983; Tsutsui *et al.*, 1983; Wada and Yura, 1984). These reports indicate that iterons in *incC* are responsible for both incompatibility and the control of copy number. The strength of incompatibility and/or the stringent control of the copy number seem to be closely related to the number of iterons in *incC* (Tolun and Helinsky, 1981; Kline and Trawick, 1983; Tsutsui *et al.*, 1983). It is also known that RepE protein binds to the *incC* iterons as well as to the *ori2* iterons (Masson and Ray, 1986; Tokino *et al.*, 1986). On the basis of these observations, the titration model was proposed for mini-F replication control by *incC*. According to this model, the binding of RepE to the *incC* iterons represses the initiation of DNA replication by limiting the supply of RepE to the replication origin (Tsutsui *et al.*, 1983).

A similar control mechanism had been proposed for

P1 plasmid replication control (Chattoraj *et al.*, 1984). However, further experiments with P1 plasmid indicated that the replication frequency of P1 does not increase even in the presence of excess initiator protein (RepA) both *in vivo* (Pal and Chattoraj, 1988) and *in vitro* (Abeles and Austin, 1991). This is contradictory to the titration model. In addition, physical evidence for the ability of the initiator protein to come into contact with the iterons of two regions [*oriR* (replication origin) and *incA* (a copy control region similar to *incC*)] was obtained by electron microscopy studies (Chattoraj *et al.*, 1988; Pal and Chattoraj, 1988). It was proposed that RepA-bound *incA* interacts directly with *oriR*-RepA complex, so that the initiation of replication is repressed (termed the 'hand-cuffing model'; Pal and Chattoraj, 1988; Abeles and Austin, 1991). In R6K and RK2 plasmids which carry iterons bound by their respective initiator protein, a similar regulatory mechanism involving iterons has been proposed (McEachern *et al.*, 1989; Kittell and Helinski, 1991).

On the other hand, studies on the mechanism of mini-F DNA replication control by *incC* have been suspended for ~15 years since the proposal of the titration model. We attempted a detailed analysis of mini-F plasmid replication in order to obtain deeper insights into the control mechanism of DNA replication by iterons. In this study, we isolated and characterized *incC* mutants expressing no incompatibility towards mini-F plasmids. The analysis of these mutants indicated that the initiation of mini-F DNA replication was inhibited by the formation of a nucleoprotein complex consisting of *ori2* iterons and *incC* iterons bound by RepE. Furthermore, we present evidence that the dimerization domain of RepE may be responsible for the formation of this complex.

Results

The low copy number of mini-F plasmid is maintained even in the presence of an excess amount of RepE

To elucidate the mechanism of copy number control of mini-F plasmid by the *incC* region, the copy number of mini-F was measured at different concentrations of RepE. The *E. coli* strain KY1461 carrying mini-F plasmid (pKV713 or pKV7304, see Figure 1B) and pKV7301 (compatible with mini-F plasmid, carrying the *P_{BAD}-repE* gene), were grown in L-broth with ampicillin and chloramphenicol. The copy number of the mini-F plasmids and the amount of RepE in the cell were measured as described in Materials and methods. The amount of intracellular RepE was regulated by changing the L-arabinose concentration in the medium. All plasmids used in this work are listed in Table I. If mini-F replication were controlled by titration of RepE (Tsutsui *et al.*, 1983), the copy number would be expected to increase when RepE is supplied beyond the titration capacity of *incC*. However, the copy number of the mini-F plasmid (pKV713) did not increase despite the presence of excess RepE (Figure 2). This result clearly indicates that copy number control does not rely solely on the titration of RepE.

The copy number of a mini-F plasmid lacking the *incC* region (pKV7304) was greater than that of the wild-type, even at the basal level of RepE in the cell

Table I. Plasmids used in this study

	Relevant genotype	Reference/source
Mini-F		
pKV5110	<i>ori2 repE bla</i>	Kawasaki <i>et al.</i> (1991)
pKV713	<i>ori2 repE incC sopA,B,C bla</i>	Kawasaki <i>et al.</i> (1990)
pKV7304	<i>ori2 repE ΔincC sopA,B,C bla</i>	this work
Others		
Mini-R6K	<i>ori_{R6K-γ} bla</i>	Inuzuka and Wada (1985)
pHT282	<i>ori_{pBR322} tet incC</i>	Tsutsui <i>et al.</i> (1983)
pKV7190	<i>ori_{pBR322} cat trpR repE</i>	Kawasaki <i>et al.</i> (1991)
pKV7310	<i>ori_{pBR322} tet</i>	this work
pKV7311	<i>ori_{pBR322} tet incC^{la}</i>	this work
pKV7312	<i>ori_{pBR322} tet incC^{ra}</i>	this work
pKV7313	<i>ori_{pBR322} tet incC^{ld}</i>	this work
pHSG299	<i>ori_{pUC19} km</i>	Takara Shuzo Co.
pKV7305	<i>ori_{pUC19} km incC</i>	this work
pACYC184	<i>ori_{p15A} cat tet</i>	Chang and Cohen (1978)
pACYC177	<i>ori_{p15A} bla tet</i>	Chang and Cohen (1978)
pSTV29	<i>ori_{p15A} cat</i>	Takara Shuzo Co.
pKV7320	<i>ori_{p15A} km</i>	this work
pKV7321	<i>ori_{p15A} km incC</i>	this work
pMW219	<i>ori_{pSC101} km</i>	Nippon Gene
pKV7330	<i>ori_{pSC101} km</i>	this work
pKV7331	<i>ori_{pSC101} km incC</i>	this work
pBAD18-Cm	<i>ori_{pBR322} cat P_{BAD}</i>	Guzman <i>et al.</i> (1995)
pKV7300	<i>ori_{pSC101} cat P_{BAD}</i>	this work
pKV7301	<i>ori_{pSC101} cat P_{BAD}-repE</i>	this work
pKV7302	<i>ori_{pSC101} cat P_{BAD}-repE54</i>	this work

^a*incC^l* and *incC^r*, single *incC* fragments in the opposite orientation; *incC^{ld}*, tandem *incC* fragments.

(no induction). Furthermore, the copy number increased as the amount of RepE increased, and reached a plateau when the amount of RepE exceeded a certain level (Figure 2). The same result was also observed using pKV5110 which lacks the *incC* and *sopA,B,C* genes present on pKV713 (data not shown). Thus, the copy number of mini-F is not lowered by the *sopA,B,C* region but by the *incC* region. The results also show that the copy number of pKV713 does not increase despite the presence of an amount of active RepE sufficient to maintain the high copy number of *ΔincC* mini-F plasmids (pKV7304 or pKV5110). These results indicate that copy number control of mini-F plasmids is expressed mainly by the *incC* region but not solely by the capacity of the *incC* region to titrate intracellular RepE.

Plasmids containing the *incC* region express incompatibility towards mini-F plasmids

When multicopy plasmids carrying *incC* are transferred to a host cell containing a mini-F plasmid, the mini-F plasmid is lost and the plasmids carrying *incC* are retained (Tolun and Helinski, 1981; Tsutsui *et al.*, 1983). This is termed incompatibility. Incompatibility could be caused by DNA replication inhibition or competition for the partition apparatus (reviewed by Austin and Nordström, 1990; Nordström, 1990). It is known that incompatibility by *incC* iterons is related to the replication process (reviewed by Nordström, 1990).

Fig. 1. Genetic organization of mini-F plasmid and replication control by RepE and *incC*. (A) The RepE monomer binds to the four *ori2* iterons [direct repeats (DR)]. Binding of RepE to *ori2* iterons is the first step in the initiation of DNA replication. The *incC* region regulates the copy number of mini-F plasmids and expresses incompatibility towards mini-F plasmids. The *sopA,B,C* genes concern with the partition of mini-F plasmid. The RepE dimers bind to the inverted repeat (IR) of the *repE* promoter–operator (P/O) to repress *repE* transcription. RepE dimers are inactive initiators and are converted to monomers *in vitro* by DnaK, DnaJ and GrpE (molecular chaperones) in the presence of ATP. The wavy line indicates the *repE* mRNA. (B) The mini-F plasmids (pKV713, pKV7304 and pKV5110) used in this study are shown as the region corresponding to (A). MCS indicates a multicloning site.

Fig. 2. Effect of the amount of RepE on the copy number of mini-F. KY1461 containing mini-F (pKV713 or pKV7304) and pKV7301 (pSC101 *P_{BAD}-repE*) was grown until a Klett unit of 70 (equivalent to an OD₆₀₀ of ~1.0) in L-broth containing appropriate concentrations of L-arabinose, ampicillin and chloramphenicol at 30°C, and cells were collected. Plasmid DNA and RepE protein from the same sample were prepared separately to determine the copy number of the mini-F plasmid and the amounts of RepE. Other details are described in Materials and methods. The intracellular amounts of RepE are shown as a function of that of pKV713 which was taken to be equivalent to 1, and the copy numbers of mini-F plasmids are shown as a function of that of pACYC184 which was taken to be equivalent to 1. Open circles indicate pKV713 and closed circle indicates pKV7304 (pKV713 lacking the *incC* region).

We established a system in which mini-F replication was inhibited by *incC* supplied *in trans* and assayed the degree of incompatibility. The strains harboring the mini-F plasmid (pKV713 or pKV5110; Figure 1) were transformed with the various multicopy plasmids carrying *incC* and transformation frequencies were measured. Irrespective of the type of replicon, the orientation of the *incC* or

Table II. Plasmids with *incC* express incompatibility toward mini-F

Incoming plasmid (<i>incC</i> plasmid)			Transformants containing the resident plasmid		
Plasmid	<i>ori</i>	<i>incC</i>	Mini-F		Mini-RK ^b
			pKV5110	pKV713	
pKV7310	pBR322	– ^a	2.8×10 ^{5b}	8.5×10 ⁵	8.4×10 ⁵
pKV7311		+	<1.2×10 ³	<1.2×10 ³	1.3×10 ⁶
pKV7312		+	<1.2×10 ³	<1.2×10 ³	1.1×10 ⁶
pKV7313		++	<1.2×10 ³	<1.2×10 ³	1.5×10 ⁶
pHSG299	pUC19	–	2.5×10 ⁶	5.7×10 ⁶	3.8×10 ⁶
pKV7305		+	<1.2×10 ³	<1.2×10 ³	3.5×10 ⁶
pKV7321	pACYC184	+	NT	<1.2×10 ⁴	NT
pKV7331	pSC101	+	NT	<1.2×10 ⁴	NT

pKV7311 and pKV7312 contain single *incC* fragments in the opposite orientation.

^a–, without *incC*; +, single *incC* fragment; ++, tandem *incC* fragments.

^bValues indicate transformation frequencies (transformants per µg of incoming plasmid DNA).

NT, not tested.

the number of iterons, hardly any transformants were obtained (Table II). The control plasmids (pKV7310 and pHSG299) lacking *incC* were able to transform the same strain harboring pKV713 (or pKV5110) at high frequencies. The results of earlier studies (Tolun and Helinski, 1981; Tsutsui *et al.*, 1983) were confirmed in our mini-F plasmid system.

The *incC* plasmid could transform efficiently the strain carrying a mini-R6K plasmid, whose origin contained directly repeated iterons for π initiator protein binding (Table II). This result indicates that the *incC* region expresses incompatibility specifically towards the mini-F

Fig. 3. Histogram of *incC* mutants and the location of the mutation sites. The total number of mutated bases in 26 *incC* mutants is shown for a 261 bp *incC* fragment. The screening procedure used for the isolation of mutated *incCs* is described in Materials and methods. The arrows with a number indicate the iteron number, the position and the direction. The mutated positions of four representative mutants used in this study are indicated as vertical solid bars (the upper is an intact base and the lower is a mutated base). The interrupted portions of the horizontal lines indicate the deleted regions.

origin as shown previously (Manis and Kline, 1978; Kline, 1979). This suggests that incompatibility exerted by *incC* targets events during the initiation step of mini-F plasmid DNA replication rather than those of the elongation step, as these would be shared by compatible plasmids such as R6K plasmid.

Isolation of *incC* mutants defective in incompatibility and localization of the mutation sites

Earlier reports demonstrated that the mutation of iterons within *incC* caused an increase in the copy number and loss of incompatibility (reviewed by Kline, 1985). To elucidate the *incC* control mechanism, we isolated and characterized *incC* mutants which did not express incompatibility towards the mini-F plasmid (pKV713). When *incC* plasmids constructed by inserting the *incC* region into multicopy plasmid are transferred into a host cell carrying a mini-F plasmid, the mini-F plasmid is expelled. Thus, two plasmids of the same incompatibility group are unable to reside within the same cell, but the mutated *incC* plasmids are able to co-exist stably with mini-F in a host cell. We isolated many mutated *incC* plasmids by inserting randomly mutated *incC* fragments resulting from PCR mutagenesis into multicopy plasmids (pHSG299, Km^R). After transformation of a strain carrying a mini-F plasmid (pKV713, Amp^R), stable double (Km^R and Amp^R) transformants were selected. In this way, we succeeded in isolating 26 mutated *incC* plasmids.

The *incC* mutants obtained were sequenced to determine the mutation sites. On average, seven mutations occurred per *incC* fragment (~270 bp) (Figure 3). Stable *incC* mutant transformants could only be obtained under conditions that permitted multiple mutations within the *incC* region. No transformants were observed under the weak PCR mutagenesis conditions which gave a mutation frequency of ~0.3 bases per *incC* fragment (Matsunaga *et al.*, 1995). Mutations occurred with high frequency within the iterons, and appeared mostly in the iterons 5, 6, 7 and 8 (Figure

3). Some mutants had mutations also in iteron 9 which was not bound efficiently by RepE (data not shown). Although many mutants had a few base changes outside of the iterons, the two mutants *incC20* and *incC44* had base changes only in the iterons (Figure 3). These results indicate that the iterons in *incC* are essential for incompatibility.

The RepE-binding capacity of *incC* does not always correlate with the degree of incompatibility

Since the mutation sites of *incC* mutants were concentrated within the iterons of *incC*, it seemed reasonable to assume that the loss of incompatibility was caused by the inability of RepE to bind to *incC*. In order to evaluate this possibility, the binding activities of the *incC* mutants to RepE were analyzed by gel retardation assay.

In the case of the wild-type RepE protein (RepE⁺, mainly dimers), *incC*⁺-RepE⁺ complexes appeared as multi-bands, not as the five discrete bands expected (Figure 4A, left). However, in the case of the *ori2*-RepE⁺ complex, four discrete bands appeared which corresponded to the stoichiometry of RepE monomer per iteron (Ishiai *et al.*, 1994). The amount of RepE⁺ required to bind 50% of the *incC*⁺ probe was 20 pmol/ml, which was nearly equivalent to the amount of RepE⁺ required to bind the iterons of the *ori2* region (Ishiai *et al.*, 1994; confirmed also by the present study). The mobility of the *incC*⁺-RepE⁺ complexes during the gel shift assay suggests that the final higher-order structures of *incC*⁺-RepE⁺ complexes are different from those of *ori2*-RepE⁺. This is probably caused by the differences in the structures of *ori2* and *incC* such as discrepancies in the number, sequences and spacing of iterons.

The binding analysis was carried out with four (*incC7*, *incC20*, *incC44* and *incC64*) of the isolated *incC* mutants (Figures 3, and 4B and C). As we expected, all the representative *incC* mutants except for *incC44* showed decreased binding affinity for RepE. However, the follow-

Fig. 4. Gel retardation analyses for RepE and RepE54 binding to wild-type and mutated *incC* DNA. The gel retardation assay was carried out as described in Materials and methods. (A) RepE⁺ or RepE54 binding to *incC*⁺ DNA. (B) RepE⁺ binding to mutated *incC* DNAs. (A) and (B) are autoradiographs of DNA bands containing *incC*–RepE DNA complex. The reaction mixture (20 μ l) contained 5 fmol of ³²P-labeled (wild-type or mutated) *incC* DNA and the indicated concentrations of RepE⁺ or RepE54. (C) Quantification of the assay: the autoradiographs of the gels were analyzed using a bioimaging analyzer, and the ratios of bound DNA to total DNA were plotted as a function of the concentration of RepE.

ing observation indicates that the relationship between the affinity of the *incC* region for RepE and incompatibility is not straightforward. First, the mutants *incC7* and *incC64* showed very little difference in their binding properties (Figure 4C), while the mutant *incC7* expressed weak but significant incompatibility and *incC64* expressed virtually no incompatibility (Figure 5A and B). Secondly, both *incC44* and *incC20* displayed no incompatibility, whereas *incC44* showed much higher binding affinity (50% decrease in free DNA is 67 pmol/ml) for RepE than did *incC20* (>410 pmol/ml) (Figures 4C and 5A). We also observed that a single iteron with a mutation which had a lower binding affinity than that of *incC44*, expressed strong incompatibility (data not shown). Thus, incompatibility cannot be explained solely on the basis of intracellular titration of RepE by *incC*.

At the low concentration of RepE, the *incC44*–RepE⁺ complex appeared as a band with a mobility lower than

that of *incC*⁺–RepE⁺ complex (Figure 4B). This may reflect a conformational difference between the *incC44*–RepE⁺ and *incC*⁺–RepE⁺ complexes. This may be responsible for the loss of incompatibility, despite the relatively high RepE-binding capacity of *incC44*.

Monomer form of RepE binds to iterons within the *incC* region

Using the RepE54 mutant, we examined if monomers of RepE bind to *incC* iterons. RepE54, a mutant RepE defective in dimerization, exists solely in monomer form and has very high binding affinity for iterons of *ori2* (Ishiai *et al.*, 1994; Matsunaga *et al.*, 1997). The complex of *incC*⁺ and RepE54 was detected as five discrete bands with decreasing mobility (Figure 4A, right). Furthermore, We examined whether the monomer form of RepE⁺ binds to the iterons of *incC*⁺. The dimer form of RepE⁺ can be changed to the monomer form by guanidine hydrochloride

Fig. 5. (A) Relationship between binding affinity for RepE and incompatibility of the *incC* mutants toward mini-F (pKV713). The binding affinity for RepE was estimated in two ways. The maximum amount of *incC* fragment bound by RepE and the RepE concentration that gave a 50% decrease in the amount of free DNA were obtained from Figure 4B. The incompatibility of each plasmid containing the *incC* fragment (wild-type, *incC44*, *incC7*, *incC64* and *incC20*) toward pKV713 was examined as described in Materials and methods. + = $<1.2 \times 10^3$ transformants; — = $>2.9 \times 10^6$ transformants; ± = 5.5×10^6 transformants (very small colonies, see below). Transformation frequencies are expressed as the number of colonies (Amp^R and Km^R) per µg of donor plasmid DNA. ○, intact iteron; ●, mutated iteron; ▲, deleted iteron. (B) Photographs of Amp^R and Km^R transformants. Left and right panels are the photographs of transformants harboring pKV713 (Amp^R) and pKV7305-*incC44* (Km^R) and transformants harboring pKV713 and pKV7305-*incC7*, respectively. The photographs of transformants harboring pKV713 and pKV7305-*incC20* or pKV7305-*incC64* were similar to those in the left panel.

or chaperone treatments (Ishiai *et al.*, 1994; C.Wada, F.Matsunaga and G.Kobayashi, unpublished data). We tested whether the binding of *incC*⁺ to RepE⁺ was stimulated by these treatments. RepE⁺ binding to iterons increased 1.5-fold with 4 M guanidine hydrochloride treatments of RepE⁺ and 2-fold after treatment with 800 ng of DnaK, 200 ng of DnaJ and 1 mM ATP (both incubations were at 30°C for 30 min) (data not shown). These results indicate that the monomer form of RepE⁺ binds to the iterons of *incC*⁺.

The *incC*⁺-RepE54 complex showed a gel retardation pattern similar to that of the *ori2*-RepE54 complex. However, the binding affinity of RepE54 for *incC*⁺ was three times lower than that for *ori2* (data not shown). The *incC*⁺-RepE54 complex did not show the same pattern as *incC*⁺-RepE⁺ complex in the gel shift assay (Figure 4A, compare left and right). These results may indicate that the higher-order conformation of the *incC*⁺-RepE54 complex is different from that of the *incC*⁺-RepE⁺ complex and the *ori2*-RepE54 complex.

A mini-F plasmid containing a mutated *incC* region exhibited a copy-up phenotype which was accentuated by an increase in the amount of intracellular RepE

We next examined whether mini-F plasmids carrying a mutated *incC* lost the copy number control function. The four mutant *incC* mini-F plasmids were constructed by

substituting the native *incC* with a mutated *incC*. All four mini-F plasmids containing the mutated *incC* regions showed a higher copy number than the wild-type mini-F plasmid (Figure 6A). The copy number of pKV7304 (deleted *incC*; $\Delta incC$) was higher than that of mini-F plasmids containing a mutated *incC* region. This suggests that these *incC* mutants still retain weak copy number control. However, these mini-F plasmids increased their copy number in the presence of an excess amount of RepE⁺, whereas the wild-type mini-F maintained its low copy number (Figure 6A, induced). These results indicate that in addition to the defect in incompatibility, these mutated *incC* regions also lose the function responsible for copy number control.

When RepE54, which binds to iterons efficiently, was supplied *in trans*, the wild-type mini-F plasmid (pKV713), $\Delta incC$ (pKV7304) and the mutated *incC* mini-F plasmids displayed a remarkable increase in their copy number (compare Figure 6A and B). When RepE54 protein was induced, the copy number of *incC20* and *incC44* mini-F plasmid was not so high as those of the other *incC* mutants and wild-type mini-F plasmid. The significance is unclear at present. Rather, we emphasize that the copy number of these all *incC* mutants increase remarkably in the presence of RepE54.

In the case of RepE54, copy number control by *incC* iterons seems to be defective despite the high binding affinity of RepE54 (50% of the *incC*⁺ probe was bound

Fig. 6. Effect of an excess amount of RepE⁺ or RepE54 on the copy number of mini-F plasmids containing a mutated *incC*. Strain KY1461 harboring both mini-F (carrying wild-type or mutated *incC*) and pKV7301 (P_{BAD}-*repE*) or pKV7302 (P_{BAD}-*repE54*) was grown overnight in L-broth with (induced) or without (no induction) 0.2% L-arabinose at 30°C. The mini-F plasmids were prepared and their copy numbers were determined. Wild-type mini-F (pKV713), *incC*-deleted mini-F (pKV7304) and *incC* mutants were used. *incC7*, *incC20*, *incC44* and *incC64* indicate pKV7304 carrying an *incC7*, *incC20*, *incC44* or *incC64* fragment at the MCS, respectively. The relative copy numbers of mutant mini-F plasmids in the presence of excess RepE⁺ (A) or RepE54 (B) are shown. The details of the assay are described in Materials and methods. The copy numbers were normalized to that of the wild-type mini-F.

at a concentration of 0.98 pmol/ml of RepE54). As RepE54 is defective in dimerization, the replication control elicited by the *incC* region may be related to the dimerization domain of RepE.

An interaction between *incC*-RepE and *ori2*-RepE complexes promoted by the dimerization domain of RepE controls the copy number of mini-F plasmids

The results described above do not support the titration model for the control of mini-F plasmid DNA replication (Tsutsui *et al.*, 1983). The DNA replication control of mini-F-like plasmids, such as P1, RK2 and R6K, has been explained by the hand-cuffing model (Pal and Chatteraj, 1988; McEachem *et al.*, 1989; Abeles and Austin, 1991; Kittell and Helinski, 1991). In this model, a cross-link structure is formed either between the origin and replica-

Fig. 7. Interaction between *ori2* and *incC* mediated by RepE. The intermolecular ligation assay was done using 634 bp *incC* (60 ng) and 801 bp *ori2* (60 ng) fragments. The *FspI*-*SmaI* fragment (608 bp) from pHSG299 was used as a control for non-specific DNA ligation. All the DNA fragments were blunt-ended. The reaction mixture containing both the DNA fragments and RepEs was pre-incubated for 30 min at 30°C, then 100 U of T4 DNA ligase was added and the reaction mixture was incubated further for 1 h at 30°C. The reaction products were purified and examined using 1.5% agarose gel electrophoresis. Details of the reaction are described in Materials and methods.

tion control regions (equivalent to a mini-F *incC* region) or between the origin regions: the initiator proteins mediate two regions, obstructing the formation of an active initiation complex and preventing DNA replication.

We used a ligation assay to test the interaction between *ori2* and *incC* in the presence of RepE *in vitro*. If the interaction occurs between these two regions in this assay, DNA fragments carrying *ori2* will be drawn closer to DNA fragments carrying *incC*, and the efficiency of ligation between both fragments will increase. An *ori2* fragment of 801 bp and an *incC* fragment of 634 bp were incubated in the presence of T4 DNA ligase and purified RepE⁺ or RepE54, and the products of ligation were subjected to agarose gel electrophoresis. In this assay, the amount of RepE was adjusted to be equivalent to that of RepE in a cell containing pKV713 (60–100 RepE monomer molecules per cell; data not shown). The ligation efficiency increased in the presence of RepE and T4 DNA ligase (Figure 7, lane 3, compare lanes 1 and 2), and the ligation products contained the three expected bands which correspond to the ligation products of *ori2*-*ori2* (1.6 kbp), *ori2*-*incC* (1.45 kbp) and *incC*-*incC* (1.3 kbp, faint band) product (Figure 7, lane 3). The ligation products were not observed for non-specific DNA fragments in the presence of RepE (Figure 7, lane 7), indicating that the increase in ligation efficiency was sequence specific. These results indicate that in mini-F plasmid the cross-link structures is formed by a RepE-mediated interaction between *ori2*-*ori2*, *ori2*-*incC* and, rarely, *incC*-*incC* *in vitro*.

Ligation products larger than dimers were also observed (Figure 7, lane 3). From the size of the four bands, they seem to be trimers corresponding to *ori2*-*ori2*-*ori2* (2.4 kbp), 2(*ori2*)-*incC* (2.2 kbp), 2(*incC*)-*ori2* (2.1 kbp) and *incC*-*incC*-*incC* (1.9 kbp, very faint band). When the *ori2* or *incC* fragment was incubated with RepE and T4 DNA ligase, trimer products of 2.4 (3*ori2*) or 1.9 kbp (3*incC*), respectively, were observed, in addition to products of 1.6 (*ori2*-*ori2*) or 1.3 kbp (*incC*-*incC*) (data not shown). Trimers are probably produced as a result of an interaction between dimer and monomer fragments mediated by

RepE, suggesting that the complex of dimers mediated by RepE can be resolved spontaneously *in vitro*. Such multimer products were also obtained during analyses of P1, R6K and RK2 plasmids by the ligation assay (Miron *et al.*, 1992; Mukhopadhyay *et al.*, 1994; Papp *et al.*, 1994; Blasina *et al.*, 1996).

In the case of *incC20* and *incC44*, *ori2-ori2* ligation products were easily observed whereas *ori2-incC* and *incC-incC* products could hardly be detected (Figure 7, lanes 5 and 6). As these mutants had lost copy number control and showed the copy-up phenotype (Figure 6), the *ori2-incC* complex, and not the *ori2-ori2* complex, may be important for copy number control *in vivo*. In the presence of *ori2* and *incC* fragments, *ori2-ori2* and *ori2-incC* ligation products were detected mainly after incubation with RepE (Figure 7, lane 3). We therefore speculate that the *ori2-incC* interaction dominates the replication control of mini-F *in vivo*.

Ligation products were barely detected in the presence of RepE54 (Figure 7, lane 4). This suggests that the loss of copy number control by RepE54 is due to its inability to form this kind of cross-link structure. Thus, the dimerization domain of RepE is probably critical for the establishment of the cross-link structure. We propose that the interaction between *ori2* and *incC* mediated by the dimer domain of RepE would impede the formation of the initiation complex and inhibit the initiation of a new round of replication from the origin. This cross-link structure probably serves to regulate negatively the DNA replication of mini-F plasmids.

Discussion

To understand how the *incC* iteron controls the replication of mini-F plasmids, we isolated and characterized a large number of *incC* mutants defective in incompatibility. The mutation sites of the *incC* mutants were found exclusively within iterons of the *incC* region and these mutants simultaneously had also lost stringency of copy number control. The results indicate that the *incC* iterons were responsible for the both functions, i.e. the control of plasmid copy number *in cis* and the exertion of incompatibility *in trans*, as suggested in earlier reports (Tolun and Helinsky, 1981; Kline and Trawick, 1983; Tsutsui *et al.*, 1983). The results also suggested that these functions of *incC* iterons are regulated by a common mechanism.

The binding of RepE proteins to iterons of *incC* is essential for incompatibility, because many *incC* mutants displayed decreased binding affinity for RepE. However, one of the *incC* mutants retained a relatively high RepE-binding affinity in spite of the loss of incompatibility. Moreover, the copy number of mini-F did not increase even in the presence of excess amounts of RepE *in vivo* (Figure 2). These results are inconsistent with the titration model (Tsutsui *et al.*, 1983). As the level of free RepE in a cell is controlled by autoregulation (Søgaard-Andersen *et al.*, 1984; Trawick and Kline 1985; Muraiso *et al.*, 1987; Wada *et al.*, 1987), all the RepE-binding sites will be filled before the synthesis of RepE is shut down. Any titration will tend to be compensated for by increased synthesis, as was indicated with the plasmid P1 (Chattoraj *et al.*, 1988). Thus, RepE may never become limiting within a cell. These results suggest that some other

mechanism besides the titration of RepE is involved in the iteron control of mini-F plasmid DNA replication.

The negative regulation of the DNA replication of other iteron-containing plasmids such as P1, R6K and RK2 has been explained by the hand-cuffing model. According to this model, origin function is hindered by an initiator protein-mediated interaction either between the *ori* and regulatory regions of the plasmid (equivalent to *incC* for F plasmid) or between origins. This model was supported by the results of electron microscopy and ligation assays which provided evidence for the interaction (Chattoraj *et al.*, 1988; Pal and Chattoraj, 1988; McEachern *et al.*, 1989; Miron *et al.*, 1992; Mukhopadhyay *et al.*, 1994; Papp *et al.*, 1994; Blasina *et al.*, 1996).

We used the ligation assay to demonstrate a RepE-promoted interaction between the *incC* and *ori2* regions (Figure 7). We propose that the copy number of mini-F plasmids is regulated by a cross-link structure (hand-cuffing model). In Figure 8, we present a model for *incC* iteron control of mini-F DNA replication. The following *in vivo* and *in vitro* results indicate that the dimerization domain of RepE is responsible for the cross-link structure. In contrast to wild-type RepE, the copy number of the mini-F plasmid increased considerably in the presence of excess RepE54 (Figure 6), which is unable to form a homodimer of RepE (Ishiai *et al.*, 1994; Matsunaga *et al.*, 1997). In the ligation assay, the *ori2-incC* product was not observed in the case of RepE54 (Figure 7, lane 4). The dimerization domain may be involved in the formation of higher order structures made up of nucleoprotein complexes by RepE and iterons at *incC*. Otherwise, it could be involved directly in the interaction between the *ori2* complex and the *incC* complex. Although it is not clear which is the case, it appears reasonable to assume that the formation of the cross-link structure is carried out by the dimerization domain of RepE. This would occur even if RepE were bound to *ori2* and *incC* iterons, as the dimerization domain of RepE is distinct from the DNA-binding domain (Matsunaga *et al.*, 1995, 1997) and exposed on the surface of RepE, as indicated by the crystal structure of the RepE-iteron complex (H.Komori, F.Matsunaga, Y.Higuchi, M.Ishiai, C.Wada and K.Miki, unpublished data).

The monomer form of RepE or RepE54 bound to *ori2* ~1.5–3 times more efficiently than to *incC* (data not shown). Though the iterons of both regions share the consensus sequence, the number of iterons, their sequences and the distances between them are different in both cases. These differences may explain the distinct conformations of the nucleoprotein complexes at *ori2* and *incC*. Interestingly, the sequence and spacing of iterons are important for origin activity at *oriR* of plasmid P1, and a specific conformation of initiator–origin complex is needed for the origin activity (Brendler *et al.*, 1997). The pattern of *incC*-shifted bands observed during gel retardation analysis was different from that of *ori2*, and the pattern of *incC44*-shifted bands was also different from that of wild-type *incC* (Figure 4). This indicates that nucleoprotein complexes with different conformations are formed at *ori2* and *incC*, and that this difference reflects the distinct functions of *ori2* (initiation of replication) and *incC* (regulation of replication). Thus, an appropriate nucleoprotein conformation may be needed for *ori2* activity and

Fig. 8. Model of the mechanism controlling the initiation of mini-F DNA replication. The monomer forms of RepEs bind to *incC* iterons (arrows), except iteron 9 (solid arrow, see text), and change the conformation of the *incC* region (B). A cross-link structure mediated by RepE is formed between *ori2* and *incC* and inhibits the initiation of mini-F DNA replication. The intermolecular interaction or intramolecular interaction between *ori2* and *incC* (C) mediated by RepE would inhibit the initiation of mini-F DNA replication. The similar mechanism would result in incompatibility and the stringent control of the copy number. The dimerization domain of RepE is involved in the formation of the cross-link structure between *ori2* and *incC* shown in (C). The *ori2* and *incC* mediated by RepE54 cannot form such a cross-link structure due to the defects in the dimerization domain and, therefore, cannot lead to inhibition of initiation of DNA replication (E). The mutated *incC* (solid and shaded arrows) is able to bind RepE but cannot adopt the correct conformation to form the cross-link structure (F). The inactive *ori2* may be activated by a putative host factor 'X' or by plasmid partition (D). Details are discussed in the text.

the function of *incC*. Also, *incC* mutants which alter the conformation of such nucleoprotein complexes may suffer loss of function, even if they bind RepE with nearly normal efficiency.

The copy number of the mini-F plasmid is controlled stringently at 1–2 per chromosome. In this study, we have presented evidence to indicate that the initiation frequency of DNA replication of mini-F plasmid is regulated negatively by *ori2*–*incC* interaction mediated by RepE. The *ori2*–*incC* interaction mediated by RepE could occur in two possible ways; as a *trans* interaction (intermolecular) between replicated plasmids or as a *cis* interaction (intramolecular). In the case of an intermolecular interaction, the newly replicated plasmids may be tethered and then separated by the plasmid-partitioning apparatus (products of the *sop* genes). In the case of an intramolecular interaction, on the other hand, some other mechanism that resolves the *ori2*–*incC* interaction would be required to initiate another cycle of mini-F plasmid replication. Mini-F plasmid is reported to be localized either at the center of the cell or between the center and the cell

extremities (Gordon *et al.*, 1997; Niki and Hiraga, 1997). Host factor(s) that mediates the resolution of the cross-link complexes may exist at these positions. Our previous work found that the host factor *mafA* is required for mini-F plasmid replication (Wada and Yura, 1984). An isolation of the suppressor mutants of *mafA*[−] revealed that this gene interacts genetically with the *incC* region. This host factor may be the hypothetical factor that resolves intramolecular interaction for the next round of replication. In the case of P1 plasmid, the random interaction between *oriR* and *incA* mediated by RepA (initiator protein) occurs irrespective of inter- or intramolecular interaction. When *oriR* is released from this *trans/cis* network, the next cycle of DNA replication starts (Abeles *et al.*, 1995). In the ligation assays of *incC* and *ori2* fragments of mini-F, trimer ligation products were observed (Figure 7, lane 3), indicating that spontaneous dissociation of cross-link structures can occur. Thus, we do not exclude the possibility that the spontaneous dissociation of the cross-link structure determines the rate of initiation.

The intracellular RepE concentration of mini-F

(pKV713) was found to be ~60–100 molecules/cell (data not shown). This is similar to the concentration of RepA reported for P1 plasmid (50 molecules/cell) (Swack *et al.*, 1987). RepE is activated by molecular chaperones (DnaK, DnaJ and GrpE) (Kawasaki *et al.*, 1992; C.Wada, F.Matsunaga and G.Kobayashi, unpublished data). The saturation of the copy number of pKV7304 in the presence of excess wild-type RepE may be due to limitations in the molecular chaperone capacity. Otherwise, the *ori2*–*ori2* cross-link structure mediated by RepE, which was seen in the ligation assay (Figure 7), may be formed to inhibit *ori2* activity. The copy number of pKV7304 was not saturated by an increase in the amount of RepE54 (Figure 6). This is not surprising, as RepE54 already exists as a monomer without chaperones and could not form any cross-link structure (Figure 7).

The simple titration model is insufficient to explain the copy number control. Our data propose that the copy number of mini-F plasmids is controlled by the cross-link structure of the *ori2*–*incC* complex mediated by RepE. However, the control mechanism of initiation of mini-F plasmid DNA replication *in vivo* contains a number of unsolved issues that will be the target of future analyses.

Materials and methods

Bacterial strain, plasmids and media

Bacterial strain KY1461 (Matsunaga *et al.*, 1995) derived from *E. coli* K12 was used in this study. Plasmids used are listed in Table I. The mini-F plasmids (pKV5110 and pKV713) were described previously (Kawasaki *et al.*, 1990, 1991). Plasmid pKV7310 was constructed by deletion of the *ScaI*–*SspI* region from pBR322 and was sensitive to ampicillin. The plasmids pKV7320 and pKV7330 were constructed by replacing the *cat* gene (*NaeI*–*NheI* fragment) of pSTV29 and the *km* gene (blunted *Clal*–*NcoI* fragment) of pMW219, respectively, with the *km* gene (blunted *BstEII*–*NheI* fragment) of pACYC177. The plasmids pKV7311, pKV7312 and pKV7313 were constructed by cloning the *incC* fragment isolated by *PstI* digestion of pHT282 into the *PstI* site of pKV7310. pKV7305, pKV7321 and pKV7331 were constructed by inserting an *incC* fragment into the *PstI* site within the multicloning site of pHSG299, pKV7320 and pKV7330, respectively. pKV7300 was constructed by replacing the origin region (blunted *Clal*–*AseI* fragment) of pBAD18-Cm (Guzman *et al.*, 1995) with the origin region (blunted *EcoNI*–*Clal* fragment) of pMW219 (pSC101 derivative). The fragment (blunted *Clal*–*HpaI* fragment) from pKV7190 or pKV7190–*repE54* was cloned in the *SmaI* site of pKV7300 to place the *repE* and *repE54* gene under the control of the arabinose promoter (P_{BAD}) to control the expression finely (pKV7301 and pKV7302, respectively). pKV7305 was used for the isolation of *incC* mutants. pKV7304 was constructed by replacing the *incC* region (*EcoRV*–*Eco4VII*) of pKV713 with the synthetic multicloning site (MCS) (5'-GGGATATCACTTCCATGACAGGATAGGTCCGCATGCGAATTCAGCTAGCGCTGG-3') (Figure 1). The mutated *incC* fragments were inserted into the multicloning site of pKV7304 to construct mini-F plasmids carrying the mutated *incC*.

Bacterial cells were cultured in L-broth (Kawasaki *et al.*, 1991). For the selection of transformants, 50 µg/ml ampicillin, 20 µg/ml chloramphenicol or 25 µg/ml kanamycin was added to the plates.

Incompatibility tests

KY1461 harboring a mini-F plasmid was transformed with an *incC*-containing plasmid, and transformants carrying both plasmids were selected on L-agar containing appropriate antibiotics. After overnight incubation at 37°C, the colonies were counted and the number of transformants per microgram of incoming plasmid DNA was calculated.

Isolation of *incC* mutants

Base substitution mutations of *incC* were obtained by PCR mutagenesis using *Taq* DNA polymerase (Leung *et al.*, 1989; Matsunaga *et al.*, 1995). The reaction mixture (100 µl) contained 10 mM Tris–HCl pH 8.8, 50 mM KCl, 1.5 mM MgCl₂, 0.3–0.5 mM MnCl₂, 0.1% Triton X-100, 200 µM each of dATP, dTTP, dGTP and dCTP and 4 U of *Taq* DNA

polymerase (Wako Chemical Co.). DNA amplification was carried out via 25 cycles of treatment at 94°C for 30 s, 50°C for 1 min and 70°C for 2 min. The mutagenized *incC* DNA fragments obtained were digested with *SphI* and *EcoRI*, and inserted into the parental plasmid (pHSG299) cut with the same enzymes.

The plasmids carrying the mutagenized *incC* fragment were used to transform KY1461 cells harboring pKV713. After overnight incubation at 37°C on L-agar containing 50 µg/ml ampicillin and 25 µg/ml kanamycin, the transformants were replica plated on the same selective plates. After further overnight incubation at 37°C, colonies, apparently expressing no incompatibility, were picked. The plasmid DNAs prepared from the cells carrying both pKV713 and the mutagenized *incC* plasmid were confirmed to be separate entities by agarose gel electrophoresis.

Purification of RepE protein

RepE and RepE54 proteins were purified essentially as described previously (Matsunaga *et al.*, 1997).

Gel retardation assay

The DNA-binding activities of isolated *incC* mutants were assayed with purified RepE or RepE54. The assay conditions were essentially as described previously (Kawasaki *et al.*, 1992). The reaction mixture (20 µl) contained 20 mM Tris–HCl pH 7.5, 40 mM NaCl, 40 mM KCl, 10 mM MgCl₂, 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 -³²P]ATP, and RepE or RepE54, which were added last. The wild-type or mutated *incC* fragments (312 bp) were used as DNA probes. The mixture was incubated at 30°C for 30 min and then electrophoresed on a 6% polyacrylamide gel. The gels were dried and DNA bands were quantified with a Fujix bioimaging analyzer BAS2000 (Fuji, Tokyo, Japan).

Determination of plasmid copy number

KY1461 cells harboring the wild-type or mutant mini-F plasmids and pKV7301 (or pKV7302) were grown overnight or until mid-log phase, in L-broth containing 25 µg/ml ampicillin, 20 µg/ml chloramphenicol and an appropriate amount of L-arabinose (0.0005–0.2%) at 30°C. One OD₆₀₀ equivalent of cells was mixed with a fixed volume of a separately grown culture of cells carrying pACYC184. Plasmid DNAs were then prepared, linearized by digestion with *EcoRI* and examined by 1% agarose gel electrophoresis. DNA bands were scanned and analyzed by a densitometer (Personal Densitometer PD-110, Molecular Dynamics Co.). The density of mini-F DNA was normalized to that of pACYC184 and corrected for plasmid-free segregants appearing in the cultures.

Quantification of RepE protein

RepE protein was quantified using Western blotting essentially in accordance with the procedure of Durland and Helinski (1990). KY1461 cells harboring the wild-type or mutant mini-F plasmids and pKV7301 (or pKV7302) were grown to mid-log phase in L-broth containing 25 µg/ml ampicillin, 20 µg/ml chloramphenicol and an appropriate amount of L-arabinose (0.0005–0.2%) at 30°C. One OD₆₀₀ equivalent of cells was mixed with a fixed volume of a separately grown culture of cells expressing the deletion mutant of RepE (RepEΔC57; Matsunaga *et al.*, 1995) for normalization. The cells were centrifuged and the cell pellets were suspended in sample loading buffer (125 mM Tris–HCl pH 6.8, 0.65% SDS, 15% glycerol, 0.005% bromophenol blue). Equivalent amounts of plasmid-free *E. coli* lysates were mixed with known amounts of purified RepE as a standard. Samples and standards were electrophoresed in a 12.5% SDS–polyacrylamide gel. Polypeptides in the gel were electrotransferred to a PVDF membrane (Millipore Co.). After incubation of anti-RepE serum followed by horseradish peroxidase-conjugated anti-rabbit antibody (Amersham Pharmacia Biotech Co.), immunoreactive proteins were detected by an ECL-Western blotting system (Amersham Pharmacia Biotech Co.). The exposed films (Amersham Pharmacia Biotech Co. or Kodak Co.) were scanned and analyzed by a densitometer (Personal Densitometer PD-110, Molecular Dynamics Co.). The amounts of RepE protein were estimated from a standard curve prepared with known samples and the data were normalized to that of the deletion mutant RepE. RepE molecules per cell were calculated on the basis of 1 OD₆₀₀ corresponding to 5.0×10^8 cells/ml. This value was determined by counting the number of colonies of the same culture growing on L-agar containing 25 µg/ml ampicillin and 20 µg/ml chloramphenicol.

Ligation assay

The assay of intermolecular ligation was done using a 634 bp *incC* fragment and a 801 bp *ori2* fragment. A 608 bp *FspI*–*SmaI* fragment

from pHSG299 was used as a control for non-specific DNA. The DNA fragments used were blunt-ended. The reaction was performed in 250 µl vols of a solution containing 20 mM Tris-HCl pH 7.5, 40 mM NaCl, 40 mM KCl, 10 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, 0.1 mg/ml BSA, 1 mM ATP and 2% polyvinyl alcohol, and pBR322 DNA at 0.5 µg per reaction was added as competitor. The reaction mixture containing both the DNA fragments (150 fmol for *incC*, 120 fmol for *ori2*) and RepEs (17.2 pmol for RepE or RepE54) was pre-incubated for 30 min at 30°C, 100 U of T4 DNA ligase was added and the reaction mixture was incubated further for 1 h at 30°C. The sample DNAs were recovered by precipitation with ethanol and analyzed using 1.5% agarose gel electrophoresis.

Other general methods

DNA manipulations and SDS-PAGE were performed according to the standard procedures (Sambrook *et al.*, 1989). Nucleotide sequencing was performed with a dye terminator cycle sequencing kit and an ABI 373A sequencer (PE Biosystems Co.).

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References

- Abeles, A.L. and Austin, S.J. (1991) Antiparallel plasmid-plasmid pairing may control P1 plasmid replication. *Proc. Natl Acad. Sci. USA*, **88**, 9011–9015.
- Abeles, A.L., Reaves, L.D., Youngren-Grimes, B. and Austin, S.J. (1995) Control of P1 plasmid replication by iterons. *Mol. Microbiol.*, **18**, 903–912.
- Austin, S. and Nordström, K. (1990) Partition-mediated incompatibility of bacterial plasmids. *Cell*, **60**, 351–354.
- 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.
- 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.
- Brendler, T.G., Abeles, A.L., Reaves, L.D. and Austin, S.J. (1997) The iteron bases and spacers of the P1 replication origin contain information that specifies the formation of a complex structure involved in initiation. *Mol. Microbiol.*, **23**, 559–567.
- Chang, A.C. and Cohen, S.N. (1978) Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A miniplasmid. *J. Bacteriol.*, **134**, 1141–1156.
- Chattoraj, D.K., Cordes, K. and Abeles, A.L. (1984) Plasmid P1 replication: negative control by repeated DNA sequences. *Proc. Natl Acad. Sci. USA*, **81**, 6456–6460.
- Chattoraj, D.K., Mason, R.J. and Wickner, S.H. (1988) Mini-P1 plasmid replication: the autoregulation-sequestration paradox. *Cell*, **52**, 551–557.
- Durland, R.H. and Helinski, D.R. (1990) Replication of the broad-host-range plasmid RK2: direct measurement of intracellular concentrations of the essential TrfA replication proteins and their effect on plasmid copy number. *J. Bacteriol.*, **172**, 3849–3858.
- Gordon, G.S., Sitnikov, D., Webb, C.D., Teleman, A., Straight, A., Losick, R., Murry, A.W. and Wright, A. (1997) Chromosome and low copy plasmid segregation in *E.coli*: visual evidence for distinct mechanisms. *Cell*, **90**, 1113–1121.
- Guzman, L.M., Belin, D., Carson, M.J. and Beckwith, J. (1995) Tight regulation, modulation, and high-level expression by vectors containing the arabinose P_{BAD} promoter. *J. Bacteriol.*, **177**, 4121–4130.
- Inuzuka, M. and Wada, Y. (1985) A single amino acid alteration in the initiation protein is responsible for the DNA overproduction phenotype of copy number mutants of plasmid R6K. *EMBO J.*, **4**, 2301–2307.
- 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.
- 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.
- Kawasaki, Y., Wada, C. and Yura, T. (1991) Mini-F plasmid mutants able to replicate in the absence of sigma 32: mutations in the *repE* coding region producing hyperactive initiator protein. *J. Bacteriol.*, **173**, 1064–1072.
- 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.
- Kawasaki, Y., Matsunaga, F., Kano, Y., Yura, T. and Wada, C. (1996) The localized melting of mini-F origin by the combined action of the mini-F initiator protein (RepE) and HU and DnaA of *Escherichia coli*. *Mol. Gen. Genet.*, **253**, 42–49.
- Kittell, B.L. and Helinski, D.R. (1991) Iteron inhibition of plasmid RK2 replication *in vitro*: evidence for intermolecular coupling of replication origins as a mechanism for RK2 replication control. *Proc. Natl Acad. Sci. USA*, **88**, 1389–1393.
- Kline, B.C. (1979) Incompatibility between Flac, R386 and F:psc101 recombinant plasmids: the specificity of F incompatibility genes. *Plasmid*, **2**, 437–445.
- Kline, B.C. (1985) A review of mini-F plasmid maintenance. *Plasmid*, **14**, 1–16.
- 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.
- 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.
- Manis, J.J. and Kline, B.C. (1978) F plasmid incompatibility and copy number genes. Their map locations and interactions. *Plasmid*, **1**, 492–502.
- 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.
- 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.
- 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.
- 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.
- 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.
- 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.
- 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.
- Niki, H. and Hiraga, S. (1997) Subcellular distribution of actively partitioning F plasmid during the cell division cycle in *E.coli*. *Cell*, **90**, 951–957.
- Nordström, K. (1990) Control of plasmid replication: how do DNA iterons set the replication frequency? *Cell*, **63**, 1121–1124.
- Pal, S.K. and Chattoraj, D.K. (1988) P1 plasmid replication: initiator sequestration is inadequate to explain control by initiator-binding sites. *J. Bacteriol.*, **170**, 3554–3560.
- 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.
- Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*. 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Seelke, R.W., Kline, B.C., Trawick, J.D. and Ritts, G.D. (1982) Genetic

- studies of F plasmid maintenance genes involved in copy number control, incompatibility, and partitioning. *Plasmid*, **7**, 163–179.
- Søgaard-Andersen, L., Rokeach, L.A. and Molin, S. (1984) Regulated expression of a gene important for replication of plasmid F in *E. coli*. *EMBO J.*, **3**, 257–262.
- Swack, J.A., Pal, S.K., Mason, R.J., Abeles, A.L. and Chatteraj, D.K. (1987) P1 plasmid replication: measurement of initiator protein concentration *in vivo*. *J. Bacteriol.*, **169**, 3737–3742.
- 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.
- Tolun, A. and Helinski, D.R. (1981) Direct repeats of the F plasmid *incC* region express F incompatibility. *Cell*, **24**, 687–694.
- Trawick, J.D. and Kline, B.C. (1985) A two-stage molecular model for control of mini-F replication. *Plasmid*, **13**, 59–69.
- 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.
- 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.
- 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.

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