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

RepE monomers bind to four iterons in ori2 and cause binging 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 Helinski, 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 Helsinki, 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 control mechanism of DNA replication by iterons. 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 Helsinki, 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 LB 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 (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 presence of excess RepE, despite the presence of an amount of active RepE sufficient to maintain the high copy number of Delta incC 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 Helsinki, 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).

<table>
<thead>
<tr>
<th>Table I. Plasmids used in this study</th>
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<tbody>
<tr>
<td>Relevant genotype</td>
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<tr>
<td>Mini-F pKV5110</td>
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<tr>
<td>pKV713</td>
</tr>
<tr>
<td>pKV7304</td>
</tr>
<tr>
<td>Others</td>
</tr>
<tr>
<td>Mini-R6K</td>
</tr>
<tr>
<td>HTT282</td>
</tr>
<tr>
<td>pKV1900</td>
</tr>
<tr>
<td>pKV7310</td>
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<tr>
<td>pKV7311</td>
</tr>
<tr>
<td>pKV7312</td>
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<tr>
<td>pKV7313</td>
</tr>
<tr>
<td>pHS299</td>
</tr>
<tr>
<td>pKV7305</td>
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<tr>
<td>pACYC184</td>
</tr>
<tr>
<td>pACYC177</td>
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<td>pSTV29</td>
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<td>pKV7320</td>
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<td>pKV7321</td>
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<td>pMW219</td>
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<tr>
<td>pKV7330</td>
</tr>
<tr>
<td>pKV7331</td>
</tr>
<tr>
<td>pBAD18-Cm</td>
</tr>
<tr>
<td>pKV7300</td>
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<tr>
<td>pKV7301</td>
</tr>
<tr>
<td>pKV7302</td>
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*incCl and incCr, single incC fragments in the opposite orientation; incClΔ, tandem incC fragments.
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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 pBAD-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 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

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

<table>
<thead>
<tr>
<th>Incoming plasmid (incC plasmid)</th>
<th>Transformants containing the resident plasmid</th>
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<tbody>
<tr>
<td>Plasmid</td>
<td>ori</td>
</tr>
<tr>
<td>pKV7310</td>
<td>pBR322</td>
</tr>
<tr>
<td>pKV7311</td>
<td>+</td>
</tr>
<tr>
<td>pKV7312</td>
<td>+</td>
</tr>
<tr>
<td>pKV7313</td>
<td>++</td>
</tr>
<tr>
<td>pHSG299</td>
<td>pUC19</td>
</tr>
<tr>
<td>pKV7305</td>
<td>+</td>
</tr>
<tr>
<td>pKV7321</td>
<td>pACYC184</td>
</tr>
<tr>
<td>pKV7331</td>
<td>pSC101</td>
</tr>
</tbody>
</table>

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

-a, without incC; +, single incC fragment; ++, tandem incC fragments.

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

NT, not tested.
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 were 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, KmR). After transformation of a strain carrying a mini-F plasmid (pKV713, AmpR), stable double (KmR and AmpR) 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 μl) 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.

Our 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
DNA replication control by iterons

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. + = <1.2×10^3 transformants; − = >2.9×10^6 transformants; ± = 5.5×10^5 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. s, intact iteron; d, mutated iteron; m, 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.

<table>
<thead>
<tr>
<th>Mutants</th>
<th>incC region</th>
<th>Maximum bound (%)</th>
<th>RepE concentration at 50% decrease in the amount of free DNA (pmol/ml)</th>
<th>Incompatibility toward mini-F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>5 6 7 8 9</td>
<td>98</td>
<td>27</td>
<td>+</td>
</tr>
<tr>
<td>incC44</td>
<td>• • • • • •</td>
<td>72</td>
<td>67</td>
<td>−</td>
</tr>
<tr>
<td>incC7</td>
<td>• • • • • •</td>
<td>45</td>
<td>280</td>
<td>±</td>
</tr>
<tr>
<td>incC64</td>
<td>• • • • • •</td>
<td>44</td>
<td>410</td>
<td>−</td>
</tr>
<tr>
<td>incC20</td>
<td>• • • • • •</td>
<td>26</td>
<td>&gt;410</td>
<td>−</td>
</tr>
</tbody>
</table>

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, ΔincC) 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), ΔincC (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 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

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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 Chattoraj, 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 replication 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 kb), ori2–incC (1.45 kb) and incC–incC (1.3 kb, 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 kb), 2(ori2)–incC (2.2 kb), 2(incC)–ori2 (2.1 kb) and incC–incC (1.9 kb, very faint band). When the ori2 or incC fragment was incubated with RepE and T4 DNA ligase, trimer products of 2.4 (3ori2) or 1.9 kb (3incC), respectively, were observed, in addition to products of 1.6 (ori2–ori2) or 1.3 kb (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 (Sogaard-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, the newly replicated plasmids may 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 PI 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 Scal–SspI region from pBR322 and was sensitive to ampicillin. The plasmids pKV7320 and pKV7330 were constructed by replacing the cat gene (NruI–NheI fragment) of pBTV29 and the km gene (blunted ClaI–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 pH728 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 pHS2299, pKV7320 and pKV7330, respectively. pKV7300 was constructed by replacing the origin region (blunted ClaI–Asel fragment) of pBAD18-Cm (Guzman et al., 1995) with the origin region (blunted EcoNI–ClaI fragment) of pMW219 (pSCL101 derivative). The fragment (blunted ClaI–HpaI fragment) from pKV7190 or pKV7190-repE54 was cloned in the Smal site of pKV300 to place the repE and repE54 gene under the control of the arabinose promoter (PArn) to control the expression finely (pKV301 and pKV302, respectively). pKV7305 was used for the isolation of incC mutants. pKV7304 was constructed by replacing the incC region (Ecorv–Eco47III) of pKV713 with the synthetic multicloning site (MCS) (5′-GGGATATATCACTTCCATGACA-GGATGGATCCGCTCATCGAATCCGATACCCTCGAGA-GGATCCC-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., 1990, 1991). Plasmid pKV7310 was constructed by replacing the origin region (blunted EcoNI–ClaI fragment) of pMW219 (pSCL101 derivative). The fragment (blunted ClaI–HpaI fragment) from pKV7190 or pKV7190-repE54 was cloned in the Smal site of pKV300 to place the repE and repE54 gene under the control of the arabinose promoter (PArn) to control the expression finely (pKV301 and pKV302, respectively). pKV7305 was used for the isolation of incC mutants. pKV7304 was constructed by replacing the incC region (Ecorv–Eco47III) of pKV713 with the synthetic multicloning site (MCS) (5′-GGGATATATCACTTCCATGACA-GGATGGATCCGCTCATCGAATCCGATACCCTCGAGA-GGATCCC-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, dGTP, dTTP 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 mutated incC DNA fragments obtained were digested with SphI and EcoRI, and inserted into the parental plasmid (pPH5299) cut with the same enzymes.

The plasmids carrying the mutated 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 mutated 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 diithiothreitol (DTT), 0.1 mg/ml bovine serum albumin (BSA), 10 μg/ml poly(dI–dC), 5 fmol of probe DNA 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 Fuji Bio-imaging 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 t-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 t-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% bumphenol 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 5.0×107 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 of
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 of a solution containing 20 mM Tris–HCl pH 7.5, 40 mM NaCl, 10 mM KCl, 0.1 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, 0.1 μg/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 or2) and RepEs (17.2 pmol for RepE or RepE54) was pre-incubated for 30 min at 30°C, 100 μl 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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