Control of P1 plasmid replication by iterons

Ann L. Abeles, Lucretia D. Reaves, Brenda Youngren-Grimes and Stuart J. Austin*  
Laboratory of Chromosome Biology, ABL-Basic Research Program, NCI-Frederick Cancer Research and Development Center, Frederick, Maryland 21702–1201, USA.

Summary
The incA locus of plasmid P1 controls plasmid copy number by inhibiting the replication origin, oriR. Both loci contain repeat sequences (iterons) that bind the P1 RepA protein. Regulation appears to occur by contact of incA and oriR loci of daughter plasmids mediated by RepA-bound iterons. Synthetic incA iteron arrays were constructed with altered numbers, sequences or spacing of iterons. Using these in in vitro and in vivo assays, we examined two models: (i) that the origin and incA loci form a stable 1:1 complex in which multiple iterons of each locus are paired with those of the other, and (ii) that individual incA iterons act as freely diffusing nucleoprotein units that contact origin iterons in a random and dynamic fashion. The data presented here strongly favour the latter case. The origin, with its five iterons, acts as a target but not as an effector of regulation. We present a model for replication control based on random, dynamic contacts between incA iterons and the origin. This system would display randomness with respect to choice of templates and timing of initiation if multiple replicon copies were present, but would tend to act in a machine-like fashion in concert with the cell cycle if just two copies were present in a dividing cell.

Introduction
A variety of different plasmids contain tandem sequence repeats (iterons) within their basic replicons. The iterons bind initiator proteins, but are also implicated in negative control of initiation. This control is illustrated by observations in plasmids such as F, pSC101, RK2 and P1. Additional copies of iterons, when supplied in trans to these parent plasmids, can shut down replication in vivo (Tsutsui et al., 1983; Miller and Cohen, 1993) and in vitro (Kittle and Helinski, 1991; Abeles and Austin, 1991).

The basic replicon of the P1 plasmid prophage consists of an origin of replication, oriR, followed by the gene for the RepA initiator protein and the downstream copy-control locus, incA. The incA locus is not essential for P1 plasmid replication (Chattoraj et al., 1984), but acts as a negative control element for initiation. The copy number of the replicon in the presence of incA can be as low as one or two copies per cell but increases some eight- to 10-fold if incA is deleted (Pal et al., 1986). The incA locus consists of nine repeats (iterons) of a 19 bp consensus sequence that binds the P1 RepA protein (Fig. 1). Five additional repeats of this sequence are present in the P1 origin of replication (Fig. 1). Binding of RepA to the origin repeats is essential for origin initiation (Chattoraj et al., 1985; Abeles et al., 1989). Early models for incA activity were based on the notion that incA titrates RepA, limiting its supply to the origin (Pal et al., 1986). However, this can be discounted as the primary function of incA, because the element retains much of its ability to limit copy number when RepA is supplied in excess, both in vivo (Pal and Chattoraj, 1988) and in vitro (Abeles and Austin, 1991). This observation also rules out the possibility that incA acts principally by controlling the rate of repA synthesis (Chattoraj et al., 1988). Pal and Chattoraj (1988) have presented a model in which the RepA-bound incA locus interacts directly with the replication origin by DNA looping. Physical evidence for the ability of RepA to bring two iterons into contact has been forthcoming from electron microscope studies (Pal and Chattoraj, 1988). In such models, the RepA-bound incA locus would exert its negative effect by steric hinderance of the origin. If the equivalent trans-contacts between daughter plasmids can also occur, replication would be self-limiting as the number of plasmids increases (Pal and Chattoraj, 1988; Abeles and Austin, 1991). This basic principle is now accepted as the likely basis for regulation of P1 and similar iteron-regulated plasmids (Nordström, 1990), and has given rise to some more specific suggestions as to how this principle can be applied (Chattoraj et al., 1988; Pal and Chattoraj, 1988; Kittell and Helinski, 1991; Abeles and Austin, 1991; Papp et al., 1994).

For the purposes of this study, we will consider two extreme cases illustrated in Fig. 2. In Model A (a simplified restatement of the proposal of Pal and Chattoraj, 1988), each RepA-bound iteron in the cell (whether in incA or in the origin) is capable of contacting any other...
repeat, even those on the same molecule, in a reversible fashion. Some of these contacts involve origin iterons and, thus, inhibit replication in a concentration-dependent fashion. When the plasmid concentration exceeds a predetermined level, all origins will tend to form contacts with other iterons and be turned off, so that replication is self-limiting. In Model B (Fig. 2; Abeles and Austin, 1991), the two daughter plasmids produced by replication pair with each other in a specific and ordered fashion, such that the origin of each plasmid is stably bound to the incA locus of the other. Both origins would be turned off until the daughters are pulled apart by the partition process that places one plasmid in one cell and one in the other. A new round of initiation would then proceed for the next generation.

Model A has support from the observation that even single incA iterons can promote looping in vitro and can exert negative effects on replication in vivo under some circumstances (Pal and Chattoraj, 1988; Papp et al., 1994). Also, physical contacts between all combinations of origins and incA loci can be observed by electron microscopy using purified components (Chattoraj et al., 1988). Model B is attractive, however, because it provides an explanation of why the incA iterons are arranged in an orderly fashion with all nine iterons centred on approximately the same face of the helix (Fig. 1). It can also provide the machine-like accuracy needed for proper plasmid maintenance at the very low copy numbers claimed for P1, and would automatically integrate the P1 initiation cycle into the host cell cycle (Abeles and Austin, 1991). Here, we evaluate these models by exploiting the fact that incA...
control of replication can be reproduced in an in vitro replication system. Our results support a modified version of Model A (i.e. Model C, Fig. 2). We also describe in vivo experiments that are consistent with this model.

Results

Construction of an artificial incA array

Although the wild-type incA locus is a relatively orderly array of nine iterons, the iterons themselves vary in orientation and precise sequence and they are separated by spacers of more divergent sequence (Fig. 1). This makes construction of mutant incA loci with rational alterations in organization difficult. We therefore constructed a synthetic incA locus by end-to-end ligation of identical iteron-spacer oligonucleotides (Fig. 1) where the iteron sequence corresponds to the consensus sequence for all P1 iterons. In this construct, the sequence, orientation and associated spacer for all nine iterons are identical. A vector plasmid carrying this array of iterons is effective in displacing a mini-P1 plasmid in incompatibility tests and therefore appears to exert the same type of negative regulation as the wild-type incA locus (Table 1). By using modified oligonucleotides and various array lengths, it was then possible to alter the iteron number, spacing or sequence in a rational fashion.

Effects of iteron organization on in vitro replication

The in vitro replication system replicates P1 double-stranded DNA circles containing the P1 origin when purified P1 RepA protein is supplied (Fig. 3). When DNA circles containing the natural incA locus are added in increasing concentrations, replication is progressively blocked (Abeles and Austin, 1991; Fig. 3). Circles without incA have no significant effect. An artificial incA array with nine iterons (the same number as are present in the natural locus) is effective in regulating the origin, maximal inhibition occurring with slightly more DNA than when the natural sequence was used (Fig. 3). We have previously shown that the inhibitory effect of the wild-type locus is independent of increased RepA concentration. The RepA concentration used for Fig. 3 was eight times greater than that required to saturate the system (data not shown). Similar results were obtained when eightfold less RepA was used in the assays (data not shown). Thus, like the effect of the natural incA sequence, the inhibitory effect of the synthetic sequence is independent of increased RepA concentration, showing that inhibition is not due to titration of RepA.

In Model B, the grouping of incA iterons in regularly spaced arrays should be critical to their function whereas, in Model A, the concentration of iterons should be the only important feature, and their grouping in arrays should not be necessary. We constructed arrays with one, three, six or nine iterons inserted in an M13 vector and tested them as inhibitors in the in vitro system. Each of these proved to be effective inhibitors (Fig. 4A), whereas the vector DNA is not inhibitory (Fig. 3). However, arrays with fewer iterons required more DNA to be effective. In Fig. 4B, these data are replotted as a function of the amount of iterons added (i.e. the data points in Fig. 4A were multiplied by the number of iterons present on each DNA molecule). At a first approximation, the total amount of iterons added seems to be the critical factor rather than the number of iterons present in a particular array. Thus, three units of DNA containing three iterons (M13-IT-#109, Fig. 4B) are as effective as one unit of DNA containing nine iterons (M13-IT-#111, Fig. 4B). Other array lengths (with a possible small deviation in the case of DNA with a single iteron) show the same pattern. Thus, as predicted by Model A, the inhibitory effect in vitro is dependent upon iteron concentration rather than

Table 1. Incompatibility exerted by natural and synthetic incA loci.

| Incoming plasmid | Iteron array | Retention of resident P1 mini-plasmid during 25 generations (%) |
|------------------|-------------|---------------------------------------------------------------|
| pAL671           | None        | >98                                                           |
| pAL749           | Natural incA 9-mer | <1                                                             |
| pAL782           | Synthetic 9-mer | <1                                                             |

Incompatibility tests were carried out as described in the Experimental procedures. Cells containing the P1 mini-plasmid λc857-P1:5RKm were transformed with the plasmids shown.

© 1995 Blackwell Science Ltd, Molecular Microbiology, 18, 903–912

Fig. 3. Inhibition of in vitro replication by 9-mer iteron arrays. Each data point was generated using the complete in vitro replication system with 20 fmol (0.1 μg) of P1 replicative-form DNA containing the P1 origin as a template and 0.4 μg of purified RepA protein. The graph shows the response to the addition of increasing amounts of DNA: filled triangles, M13mp19 vector; open squares, M13-P1-#91 containing the wild-type incA locus with its nine iterons (Fig. 1); filled squares, M13-IT-#111 containing an array of nine iterons constructed from the 31 bp oligonucleotides shown in Fig. 1. The RepA concentration used is approximately eight times that required to saturate the system. Very similar results were obtained when 0.05 μg or 0.6 μg of RepA was used (data not shown).
incA function is proportional to the number of iterons present in vivo

If Model A applies to the behaviour of the system in vivo, the inhibition of origin activity in vivo should depend upon the concentration of incA iterons, but not upon any particular mode of organization into arrays. Figure 5 shows the results of incompatibility tests using arrays containing various numbers of iterons. Such tests measure the ability of an iteron array to down-regulate the origin function of a mini-P1 plasmid in trans. The experiments were carried out with the incA-containing plasmids at high (c. 30 plasmid copies per average cell) or low (c. 5 plasmid copies per average cell) copy number (see Fig. 5 legend). The artificial arrays of iterons were effective inhibitors in vivo. The plasmid with nine perfect repeats of the iteron consensus (pALA782) was indistinguishable from the wild-type incA-containing plasmid (pALA749) in both types of test (Table 1, Fig. 5). At high copy number, all of the constructs with iterons of normal sequence and spacing were effective in displacing a resident mini-P1 plasmid, except that with only a single iteron (pALA689). The latter had a partial effect. Previously, Papp et al. (1994) showed that a very high-copy-number plasmid containing a single iteron can be effective in incompatibility tests. At low copy number, a graded response was seen in which the more iterons present, the greater the incompatibility effect (Fig. 5). We conclude that no fixed number of iterons need be present in a given array in order to down-regulate the origin of replication. Rather, as was shown in the in vitro system, the total concentration of incA iterons seems to be the controlling factor.

incA function in vivo is sensitive to the sequence but insensitive to the spacing of the iterons

We constructed a number of arrays with mutated iterons or with altered spacer lengths. As a positive control, we chose to use an array with five iterons of normal sequence and spacing, because such an array should lie approximately at the midpoint of the response curve for such arrays in the low-copy-number incompatibility test (Fig. 5); assay of this 5-mer confirmed this (pALA731, Table 2). Plasmids pALA679 and pALA2433 have five-iteron arrays with altered spacer lengths (−1 bp and +5 bp, respectively). These changes in spacing had only minor effects upon the way in which they are organized with respect to each other.
that of any other effective five-iteron array (Table 2).

However, the points obtained deviate from the straight line expected for a strictly proportional relationship in vivo and in vitro but does not affect the integrity of iterons themselves (Austin et al., 1985; Abeles and Austin, 1991). This construct is substantially defective as an inhibitor, exerting incompatibility at high copy number but very little at low copy number (Table 2). The mutant origin is also defective as an inhibitor in the in vitro system (Abeles and Austin, 1991). We think it unlikely that the lack of inhibition is due to some special property of the rep30 mutation in this origin construct. Rather, we propose that some structural feature of the origin, wild-type or mutant, prevents it from being an effective inhibitor. This proposal is strongly supported by the observation that elevated origin concentrations in the in vitro system are not self-inhibitory: increasing origin concentration gave a proportionate increase in replication activity over a wide range under conditions where equivalent concentrations of incA iterons were completely inimicable to replication (Abeles and Austin, 1991). It seems probable that the involvement of the origin in an initiation complex prevents the origin iterons from acting as effective inhibitors. Thus, the origin, although a target for inhibition, is not itself an efficient effector of inhibition. A modified version of Model A is indicated in which inhibitory contacts between one origin and another are precluded, or are not relevant to origin activity (Model C, Fig. 2).

The relationship between mini-P1 copy number and growth rate

Model C incorporates the assumption that any contact between a RepA-bound incA iteron and the origin will inhibit replication. As incA iterons are in excess over origins, the inhibition can be considered as a pseudo first-order reaction. Thus, initiation rate should be governed by the concentration of the RepA-bound incA iterons. As a first approximation, the number of plasmids per cell volume should be constant. As average cell volume increases with increasing growth rate (Donachie, 1968), the average plasmid copy number should also increase. It has been shown that the copy number of the intact P1 prophage increases with growth rate (Preniki et al., 1977). However, the intact P1 has at least two replication systems (Sternberg and Austin, 1983) and it is not known if oriR is solely responsible for replication in this context. We therefore measured the copy number at several growth rates of the P1 mini-plasmid λ-P1:5RCm, which is driven by the oriR replicon (Sternberg and Austin, 1983). The number of plasmids per average cell in the population increased markedly with growth rate such that larger cells had a greater plasmid content (Fig. 6). This behaviour is what might be expected from Model C (or Model A), where increasing cell volume should give more replication. However, the points obtained deviate from the straight line expected for a strictly proportional relationship
between cell volume and copy number (Fig. 6). This may reflect experimental variation, or that the assumptions made in the models are too simplistic.

Plasmids with two origins and one incA locus replicate at low copy number

As Model C predicts that the inhibitory effect of incA iterons is a pseudo first-order reaction, the copy number of a mini-P1 plasmid should depend only on the concentration of the incA iterons. If this is the case, a plasmid with two origins but only one incA locus should achieve the same copy number as the normal mini-P1 plasmid with only one origin. In marked contrast, models involving stable pairing of daughter plasmids, such as Model B (Fig. 2), predict that a double-origin plasmid should over-replicate as the additional origin should remain unpaired. This over-replication should give the same eightfold increase in copy number seen with single-origin plasmids deleted for incA (Pal et al., 1988). We have constructed such plasmids (Fig. 7; Hayes et al., 1993) and measured their copy number in log-phase cells (Fig. 7). The copy numbers were similar, the double-origin plasmid giving the lower value (Fig. 7). This observation is consistent with Model C (or Model A) rather than Model B.

Discussion

The orderly arrangement of the nine iterons of the P1 incA locus appears to be a non-essential feature. The spacing, distribution of the iterons on a single face of the helix, and linking of multiple iterons on a single DNA molecule are all unnecessary for function as negative regulators. We cannot rule out the possibility that the orderly organization imparts some minor advantage to the function of the wild-type region by, for example, increasing effective local concentrations. However, the requirement for a stable association of multiple iterons, such as that illustrated in Model B, can be ruled out. Rather, the individual incA iterons appear to act like freely diffusing repressors that make random and dynamic contacts with the origin of replication in order to limit initiation. As such, contacts would frequently occur in trans between daughter plasmids. This system would limit plasmid copy number by measuring iteron concentration. As the origin itself does not appear to be capable of acting as an efficient inhibitor, it is the incA iterons that govern replication in the wild-type plasmid. The stable maintenance of incA-deleted plasmids at an eightfold higher copy number (Pal et al., 1986) may be due to some residual negative activity of the origin iterons, or to limitation of some other factor needed for initiation.

Model C predicts that replication is controlled such that incA iteron concentration is kept constant. The average copy number of a wild-type P1 miniplasmid in L-broth cultures is 5 to 6 (Austin and Eichorn, 1992). Thus, replication is regulated such that the average broth cell has c. 50 incA iterons. Introduction of 25 iterons in trans to the wild-type plasmid should approximately halve the plasmid copy number, whereas introduction of about 40 or so should block plasmid maintenance altogether (less than one mini-P1 per cell would not allow maintenance). Under high-copy-number conditions (copy number = c. 30), pBR322 constructs should block mini-P1 replication completely if they contain two or more iterons. Under low-
copy-number conditions (copy number = c. 4) the same pBR322 constructs should block mini-P1 maintenance completely if they contain about 10 iterons in each copy. The data in Fig. 5 are in good agreement with these predictions.

The presence in plasmids such as P1 and F (Murotsu et al., 1981) of a large array of iterons in a control locus separate from the origin can be regarded as an adaptation to maintenance at the very low copy number maintained in small, slow-growing cells. It is difficult to envisage a stochastic mechanism that distinguishes a cell with one plasmid from a cell with two by responding to plasmid concentration. However, distinguishing between 9 and 18 iterons by concentration is inherently more reasonable. At high growth rates, where the cells are large, there are more copies present (6–7 per average cell in the case of mini-P1). Under these conditions, the multiple copies would be associated in a dynamic net (Fig. 2C). Replication of a given origin will occur randomly throughout the cell cycle as volume increases and decreasing probability of contacts within the net leave an origin free to initiate. A net structure should prevent free diffusion of the plasmid copies to daughter cells during cell division. Plasmids such as P1 have acquired active partition systems to ensure distribution to daughter cells (Nordström and Austin, 1989). We assume that these act by pulling the net in two. In the absence of the partition system (Par−), proper distribution should only occur when, by chance, the net resolves itself and one or more plasmids are freed from it (Fig. 2A). Thus, distribution of Par− plasmids should be considerably worse than that predicted by random distribution of the plasmid copies present. We have previously shown this to be the case for mini-P1 plasmids (Austin and Eichorn, 1992).

In view of Fig. 2C, partition of the net into two roughly equal portions should not have much effect on the overall initiation rate, as the iteron concentrations remain roughly the same. The breaking of some of the incA-origin trans contacts should produce a small increase in the probability of replication at this time. However, at low growth rates, where there are only two copies of the plasmid, partition would have a major effect on the probability of initiation. In this case, all of the trans contacts would be broken by partition and the initiation probability would increase sharply at whatever time in the cell cycle partition occurs. This would tend to couple initiation to the cell cycle and encourage a single round of replication in each cell generation. A similar phenomenon should occur in cells at high growth rates if, by chance, partition were inequitable and one cell received only one plasmid. Again, because the single plasmid lacks trans contacts, it would have an enhanced probability of initiation. Thus, the system would enhance replication just at the time when the likelihood of generating a plasmid-free cell was highest.

We propose that this property of the system is the key to ensuring accurate plasmid maintenance.

**Experimental procedures**

**Bacteriophage and plasmids**

The P1 origin-bearing phage f1-P1#1 was as previously described (Abeles and Austin, 1991). The λ-P1 miniplasmid λc1857-P1:5Rkm was derived from λc1857-P1:5R (Stenberg and Austin, 1983) as previously described (Austin and Eichorn, 1992). λc1857-P1:5Rkm was derived by insertion of the Sell fragment of the Kanamycin Resistance GeneBlock (Pharmacia) into the unique SalI site of λc1857-P1:5R. λc-P1:5RΔ1005::pALA1716 was formed by recombination between a deletion mutant of λc-P1:5R and mini-P1 plasmid pALA1716 (Hayes et al., 1994). To facilitate cloning of the synthetic repeats, an MfuI adaptor fragment was introduced into the BamHI site of pUC19 (Yanisch-Perron et al., 1985) to form plasmid pAL670. The adapter was made by annealing the oligonucleotides 5'-GATCCACGCGTG-3' and 5'-GATC-ACGCCTG-3'. Plasmid pALA671 was made by replacing the corresponding fragment of pAL670 containing the modified multiple cloning sites, M13mp19 and pUC18 (Yanisch-Perron et al., 1985) and pBR322 (Bolivar, 1977) have been described. Plasmid pALA271 was as described (Abeles et al., 1985).

**E. coli strains**

N100 (Das et al., 1976), C600 (Appleyard, 1954), BR27 (Abeles et al., 1990), DH5x (Woodcock et al., 1989) and CC2004 (Austin and Eichorn, 1992) were as described. CC3937 was derived by introducing the recJ248::Tn10 marker into BR27 by P1 transduction (Sambrook et al., 1989) from strain JC12166 recJ::Tn10 (kindly supplied by J. Sawitzke and F. Stahl).

**Construction of synthetic iteron arrays**

The synthetic iteron arrays were produced by an adaption of the repeat ligation method of Rosenfeld and Kelly (1986). The arrays consisted of a series of perfect iteron-spacer repeats made by ligation of multiple copies of an iteron-spacer oligonucleotide (Fig. 1). The iteron sequence was the consensus derived from the 14 iterons found in the P1 incA locus (Fig. 1). The double-stranded oligonucleotide was designed with complementary 5' single-stranded extensions that serve to join multiple oligonucleotides together in tandem arrays (see the legend to Fig. 1). The overhanging ends of the fragments are complementary to those of the M13 site in pALA671. The oligonucleotides were synthesized, purified, kinase-treated, and annealed as described earlier (Brendler et al., 1991). The double-stranded oligonucleotides (Fig. 1) were then ligated with T4 ligase in the presence of BssHII and M13. Ligation of units head-to-head or tail-to-tail produces intervening BssHII or M13 sites, whereas head-to-tail ligation gives a hybrid site that is
resistant to both enzymes. A typical 100 µl ligation reaction contained 0.8 µg of oligonucleotide in 2–4 mM ATP, 50 mM Tris-HCl, 10 mM MgCl₂, 100 mM NaCl, 1 mM dithiothreitol, pH 8, 5 U ligase, 15–20 U BssHII and 30–40 U Mulu. The reaction was incubated at 37°C for 3h. Additional units of BssHII, ATP and ligase were added hourly during the incubation. Finally, the mixture was heated to 65°C for 5 min, cooled to 37°C, 16 U BssHII and 20 U Mulu added, and the digestion continued at 37°C for 1 h. The reaction mix was then phenol- and phenol–chloroform-extracted and purified over a Sephadex G25SF column. The products corresponding to the desired number of repeats were separated by PAGE and purified as previously described (Abeles, 1986).

**Plasmids and M13 replicative forms containing iterons**

The purified DNA fragments were ligated into the Mulu site of pALA671 and the mixtures used to transform competent N100 or DH5α cells as described (Sambrook et al., 1984). Plasmid clones containing inserts with the desired number of repeats were identified by restriction digestion and their structures confirmed by DNA sequencing as described (Abeles et al., 1980). The plasmids constructed with these synthetic oligonucleotides are described in Table 2 and Fig. 4. Plasmid pALA689 contains a single consensus iteron. Plasmid pALA687 contains five repeats of a mutant iteron in which the highly conserved 15th iteron base is changed from G to T in all five repeats. It also has the -1 bp spacer. Plasmid pALA2436 was constructed by replacing the small EcoRI to HindIII region of pBR322 with the EcoRI to HindIII region (containing the mutant Plor) from M13-Plor-#91 (Abeles and Austin, 1987). Plasmid pALA7, containing the natural IncA, was described earlier (Abeles et al., 1984). Plasmid pALA749 contains the EcoRI to HindIII region of M13-P1-#91 (Abeles and Austin, 1991), encompassing the normal P1 incA, cloned into the same sites of pBR322. Plasmid pALA850 was constructed by cloning the EcoRI to BamHI fragment of pALA145 (Abeles et al., 1984) containing the Plori 19 bp repeats into pUC18 and then moving the HindIII fragment containing these ori repeats into the HindIII site of pBR322. M13 replicative forms used in the *in vitro* replication tests were made by excising the EcoRI to HindIII regions of the pBR322-based plasmids containing the repeat sequences and ligating the fragments into the EcoRI to HindIII region of M13mp19 to form M13-IT-#108 (one repeat), M13-IT-#109 (three repeats), M13-IT-#110 (six repeats) and M13-IT-#111 (nine repeats).

**General methods, media, enzymes, reagents and buffers**

Unless otherwise mentioned, all general Experimental procedures were as described previously (Abeles and Austin, 1987; Sambrook et al., 1989).

**In vitro replication assay**

The *in vitro* replication using the P1 origin-containing f1-P1#1 DNA as a template was carried out essentially as described (Abeles and Austin, 1987; 1991) using additional DnaA protein. *Escherichia coli* fraction II extract, f1 and M13-based replicative form DNA and P1 RepA protein were prepared as described (Abeles, 1986; Abeles and Austin, 1987; 1991). DnaA protein was purified as described by Diederich et al. (1994). In the absence of any inhibitor DNA, the 25 µl system was saturated at about 0.05 µg of RepA protein (data not shown). All reactions presented here contained 0.4 µg of RepA to ensure that inhibitory effects were not due to titration of the available RepA protein. Inhibitor DNA concentration was normalized to that of the template DNA when necessary by diluting the inhibitor slightly until the two DNA concentrations appeared equal on ethidium bromide staining of the bands formed on gel electrophoresis (Sambrook et al., 1989). The mean value of two repeat assays carried out using identical components was used. Other data sets (not shown) with different batches of FII extract and other components gave essentially the same results, although the maximum and background incorporations varied from experiment to experiment over a twofold range.

**Incompatibility tests to measure replication inhibition by iterons in vivo**

Strains N100 and CC3937 were lysogenized by λcl857-P1:5RKm selecting for kanamycin resistance. Competent cells of these lysogens were prepared using the calcium chloride method (Sambrook et al., 1989). For high-copy-number incompatibility tests, N100 (λcl857-P1:5RKm) was transformed with pBR322 derivatives containing the iteron region to be tested, selecting for ampicillin resistance on L-ampicillin plates containing 20 mM sodium citrate at 30°C. Eight of the resulting colonies were harvested when they reached 2 mm in diameter, and were streaked for single colonies on the same medium. After overnight growth at 30°C, approximately 12 colonies from each streak (96 colonies per test) were picked with wooden toothpicks and stabbed sequentially onto sodium citrate agar containing ampicillin (50 µg ml⁻¹) or kanamycin (15 µg ml⁻¹). The proportion of colonies retaining both ampicillin and kanamycin resistance was taken as a measure of the proportion of the cells retaining λcl857-P1:5RKm during the approximately 25 generations of growth required to form the colonies on the transformation plates. Low-copy-number incompatibility tests were carried out in the same way, except that CC3937 was used as the host. This strain has a polAts mutation. Strains with this mutation have a partially defective DNA polymerase I at 30°C and maintain pBR322 derivatives at a copy number of approximately five per average cell in complete medium (S. J. Austin, unpublished).

**Copy-number determinations**

Absolute copy numbers of the λ-P1:5RKm in strain CC2004 at different growth rates were determined by the method of Austin and Eichorn (1992) with the following modifications: cells to be tested were grown in L-broth or in M63 minimal medium with 0.4% glucose; 0.4% casamino acids plus 0.4% glucose; 0.8% glycerol or 0.4% succinate at 30°C. Cells were harvested at OD₆0₀ = 0.2, chilled, and the number of cells per unit volume of culture for both test and reference
cultures determined under the microscope using a Petroff-Hauser counting chamber. Chilled cells (40 ml) were mixed with an equal volume of the reference standard consisting of cells of strain C600 containing a wild-type P1 plasmid grown at 37°C in M63 minimal medium with 0.2% glucose and harvested at OD600 = 0.2. After extraction, the plasmid DNA was digested with BamHI and the fragments separated by electrophoresis in a 0.3% agarose gel in 1 x TBE buffer. The visualized gel bands were scanned with a densitometer and the molar ratios of P1 and test plasmid bands determined. P1 has a copy number of approximately 1.8 per average cell grown under the conditions used for the reference culture (Prentki et al., 1977; Bremer and Dennis, 1987). Using the molar ratios of the P1 and test bands, and the number of each type of cells in the cultures, the copy number per average cell of the test plasmid could be determined. As the volume of the average cell goes up, the number of cells in a fixed OD600 of culture goes down. Thus, the relative cell volumes in different cultures could be estimated from the OD600 divided by the number of cells per unit culture volume. The cell volume of the smallest class of cells (those with the lowest growth rate) was arbitrarily set at 1.0.

The relative copy numbers of \( \lambda \cdot P1:5RCm \) and \( \lambda \cdot 5RA1005:5pALA1716 \) were determined in strain N100 containing plasmid pALA271 grown in L-broth (6 \( \mu \)g/ml chloramphenicol) to OD600 = 0.25 at 30°C. In this case, no external reference was used. Rather, the BamHI band derived from the resident plasmid was used as a reference standard. The molar ratios of pALA271 to \( \lambda \cdot P1:5RCm \) or \( \lambda \cdot 5RA1005:5pALA1716 \) bands were determined. The ratio of the two values was taken as indicating the relative copy numbers of the two \( \lambda \cdot P1 \) plasmids with that of \( \lambda \cdot P1:5RCm \) set arbitrarily at 1.0.

Acknowledgements

We thank Walter Messer for his kind gift of the DnaA overproducing strains and his specific instructions for the purification of the protein. We also thank Marilyn Powers and the Oligonucleotide Synthesis Laboratory, SAIC for synthesis of the oligonucleotides used in this study and Julie Ratliff for preparation of the manuscript. We also acknowledge the help of three undergraduate summer students: Jennifer Wu (1991), lan Shenlock (1992) and Jeannette Liu (1993). Research was sponsored by the National Cancer Institute, DHHS, under contract with ABL. The contents of this publication do not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products or organizations imply endorsement by the US Government.

References

Abeles, A.L. and Austin, S.J. (1985) Critical sequences in the core of the P1 plasmid replication origin. J Bacteriol 173: 3935–3942.

Abeles, A., Snyder, K., and Chatteraj, D. (1984) P1 plasmid replication: negative control by repeated DNA sequences. Proc Natl Acad Sci USA 81: 6456–6460.

Chatteraj, D.K., Snyder, K.M., and Abeles, A. (1985) P1 plasmid replication: multiple functions of HepA protein at the origin. Proc Natl Acad Sci USA 82: 2588–2592.

Chatteraj, D.K., Mason, R.J., and Wickner, S.H. (1988) Mini-P1 plasmid replication: the autoregulation-sequestration paradox. Cell 52: 551–557.

Das, A., Court, D., and Adhya, S. (1976) Isolation and characterization of conditional lethal mutants of Escherichia coli defective in transcription and termination of factor rho. Proc Natl Acad Sci USA 73: 1959–1963.

Diedrich, L., Roth, A., and Messer, W. (1994) A versatile plasmid vector system for regulated expression of genes in Escherichia coli. BioTechniques 16: 916–923.

Donnachie, W. (1968) Relationships between cell size and time of initiation of DNA replication. Nature 219: 1077–1079.

Hayes, F., Davis, M.A., and Austin, S.J. (1993) Fine-structure analysis of the P7 plasmid partition site. J Bacteriol 175: 3443–3451.

Hayes, F., Radnedge, L., Davis, M.A., and Austin, S.J. (1994)
The homologous operons for P1 and P7 plasmid partition are autoregulated from dissimilar operator sites. Mol Microbiol 11: 249–260.

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.

Miller, C.A., and Cohen, S.N. (1989) The partition (par) locus of pSC101 is an enhancer of plasmid incompatibility. Mol Microbiol 9: 695–702.

Murotsu, T., Matsubara, K., Sugisaki, H., and Takanami, M. (1981) Nine unique repeating sequences in a region essential for replication and incompatibility of the mini-F plasmid. Gene 15: 257–271.

Nordström, K. (1990) Control of plasmid replication: how do iterons set the replication frequency? Cell 63: 1121–1124.

Nordström, K., and Austin, S.J. (1989) Mechanisms that contribute to the stable segregation of plasmids. Annu Rev Genet 23: 37–69.

Pal, S., and Chattoraj, D. (1988) P1 plasmid replication: initiator sequestration is inadequate to explain control by initiator-binding sites. J Bacteriol 170: 3554–3560.

Pal, S., Mason, R.J., and Chattoraj, D.K. (1986) Role of initiator titration in copy number control. J Mol Biol 192: 275–285.

Papp, P.P., Mukhopadhyay, G., and Chattoraj, D.K. (1994) Negative control of plasmid DNA by iterons. J Biol Chem 269: 23563–23568.

Frentki, P., Chandler, M., and Caro, L. (1977) Replication of the prophage P1 during the cell cycle of Escherichia coli. Mol Gen Genet 152: 71–76.

Rosenfeld, J.P., and Kelly, T.J. (1986) Purification of nuclear factor I by recognition site affinity chromatography. J Biol Chem 261: 1398–1408.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn. Cold Spring Harbor, New York; Cold Spring Harbor Laboratory Press.

Nordström, K., and Austin, S. (1993) Isolation and characterization of P1 minireplicons, λ-P1:5R and λ-P1:5L. J Bacteriol 153: 800–812.

Tsutsui, H., Fujiyama, A., Murotsu, T., and Matsubara, K. (1988) Role of nine repeating sequences of the mini-F genome for expression of F-specific incompatibility phenotype and copy number control. J Bacteriol 156: 337–344.

Woodcock, D.M., Crowther, P.J., Doherty, J., Jefferson, S., Decruz, E., Noyer-Weidner, M., Smith, S.S., Michael, M.Z., and Graham, M.W. (1989) Quantitative evaluation of Escherichia coli host strains for tolerance to cytosine methylation in plasmid and phage recombinants. Nucl Acids Res 17: 3469–3478.

Yanisch-Perron, C., Vieira, J., and Messing, J. (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33: 103–119.