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# Control of P1 plasmid replication by iterons

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## 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

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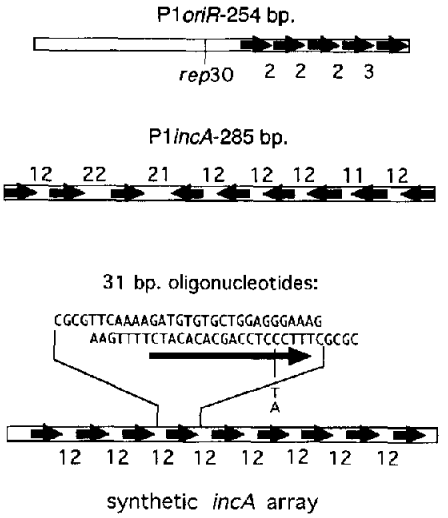


**Fig. 1.** Arrangement of iterons in the P1 replication region and construction of synthetic iteron arrays. The origin (P1*oriR*) and *incA* loci lie some 1.2 kb apart and are separated by the *repA* gene. The position of the P1*oriRrep30* mutation in M13-P1*ori*-#88 and pALA2436 is shown. The origin and *incA* each contain multiple repeats (iterons, black arrows) of a sequence that varies somewhat from repeat to repeat but which form the consensus shown (underlined by large arrow). The sequences of the spacer regions between iterons vary more widely. The numbers below the spacer regions indicate their length in base pairs. Spacer lengths are much shorter in the origin than in the *incA* locus, but each is such that all iterons are approximately centred on the same face of the DNA helix.

The sequence of the 31 bp oligonucleotide used to construct synthetic *incA* arrays is shown. It corresponds to the consensus iteron sequence (large arrow) plus a consensus spacer derived from the *incA* sequence. By ligating these double-stranded oligonucleotides together, arrays of perfect repeats are obtained as shown (*Experimental procedures*). By changing the length of the spacer region to the left of the iteron in the oligonucleotides, variant arrays in which every spacer was altered in length were produced. Omission of the sequence TCAAA that lies to the left of the marked iteron region gave a −5 bp spacer change, and omission of one of the A bases from the same sequence gave a −1 bp spacer change. By substituting TCAGTTGAAA for TCAAA, a +5 spacer change was made. The marked 15th iteron base pair (G/C) is conserved in all of the natural iterons. By changing this to T/A in the oligonucleotides, arrays with five mutated iterons were produced.

The *incA* sequence appears to have been derived from an ancestral simple tandem array. Insertion of 1 bp, deletion of 10 bp, and a simple inversion event can give rise to a tandem array with 12 bp spacers (not shown). In this hypothetical form, the iterons and the spacer sequences conform to the consensus shown. By ligation of iteron-spacer oligonucleotides corresponding to this consensus, a perfectly repeated synthetic version of this sequence was constructed (lower map).

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 be in contact 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*

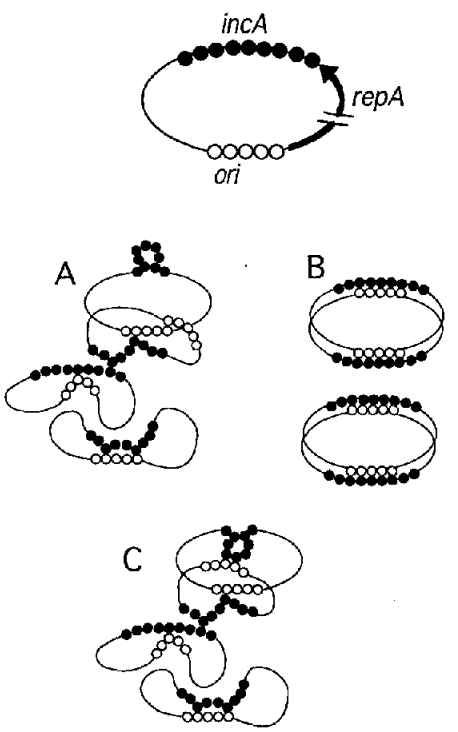


**Fig. 2.** Models for iteron action. The diagram above depicts a plasmid containing the P1 replication region. Iterons with RepA protein bound are represented as closed (*incA*) or open (origin) circles. A to C represent possible configurations of four sibling plasmids in the origin control models described in the text. Note that C differs from A only in that origin–origin contacts are not present. The *repA* gene is omitted for clarity.

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 f1 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.

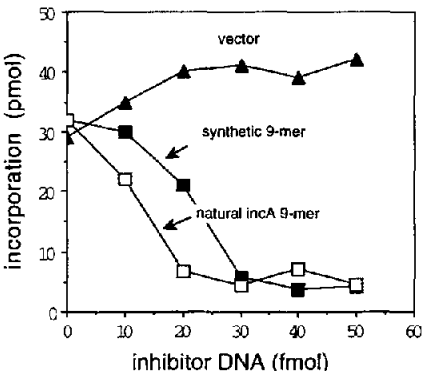


**Fig. 3.** Inhibition of *in vitro* replication by 9-mer iteron arrays. Each data point was generated using the complete *in vitro* replication system with 20 fmol (0.1 μg) of f1 replicative-form DNA containing the P1 origin as a template and 0.4 μg of purified RepA protein. The graph shows the response 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).

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 (%) |
|---|---|---|
| pALA671 | None | >98 |
| pALA749 | Natural *incA* 9-mer | <1 |
| pALA782 | Synthetic 9-mer | <1 |

Incompatibility tests were carried out as described in the *Experimental procedures*. Cells containing the P1 mini-plasmid λ*cI*857-P1:5RKm were transformed with the plasmids shown.

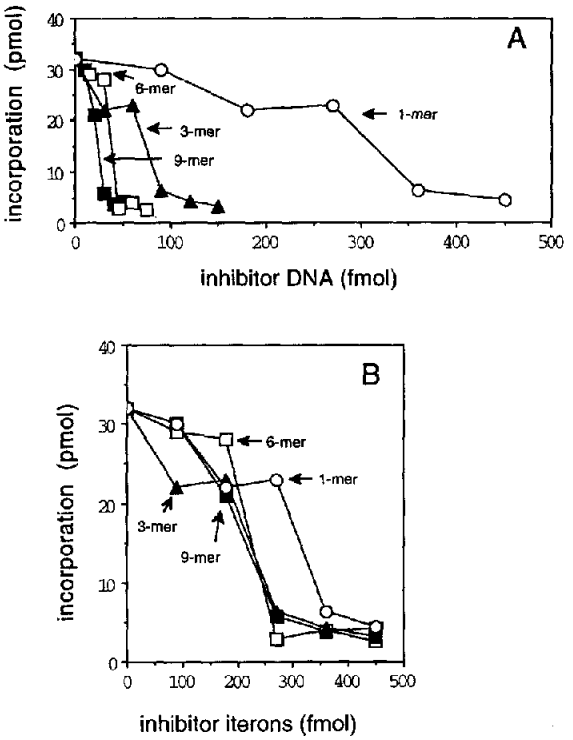


**Fig. 4.** Inhibition of *in vitro* replication by iteron arrays with varying numbers of repeats. Conditions used were as in Fig. 3, with RepA protein in excess. The indicated amounts of the following M13 derivatives were used: open circles, M13-IT-#108 (1-mer, contains a single consensus iteron); triangles, M13-IT-#109 (3-mer); open squares, M13-IT-#110 (6-mer); filled squares, M13-IT-#111 (9-mer).
A. Data plotted as a function of amount of added M13 iteron-containing DNA (fmol per assay).
B. Data replotted as a function of total amount of added iterons per origin template (fmol of iterons per assay).
The inhibition seen upon addition of increasing amounts of iteron-containing DNA is due to the iterons because vector DNA is not inhibitory (Fig. 3).

upon the way in which they are organized with respect to each other.

### 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

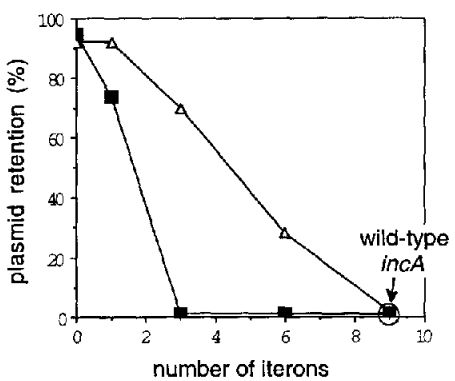


**Fig. 5.** Loss of a resident mini-P1 plasmid in response to the introduction of plasmids carrying iteron arrays. Cells carrying the P1 mini-plasmid λ*cI*857-P1:5RKm were transformed with pBR322 derivatives carrying iteron arrays with one, three, six or nine iterons. The retention of the resident plasmid was determined after 25 generations of growth selecting only for the incoming plasmid. Squares: data from assays done in strain N100 where the incoming plasmid replicates at high copy number. Triangles: data from strain CC3937 (*polAts*) grown at 30°C, where the incoming plasmid replicates at low copy number. The open circle represents the data obtained with an incoming plasmid that contains the wild-type P1*incA* locus with its nine iterons. Both high- and low-copy-number assays gave the same value (<2% retention) with this construct.

**Table 2.** Incompatibility exerted by mutated iteron arrays.

| Incoming plasmid | Iteron array | Retention of resident P1 miniplasmid during 25 generations (%). low copy no. | high copy no. |
|---|---|---|---|
| pALA731 | 5-mer, normal spacer | 59 | <2 |
| pALA679 | 5-mer, −1 bp spacer | 55 | <2 |
| pALA2433 | 5-mer, −5 bp spacer | 40 | <2 |
| pALA2435 | 6-mer, −5 bp spacer | 40 | <2 |
| pALA697 | 5-mer, −1 bp spacer, mutant iterons | 98 | 98 |
| pALA750 | Natural iteron region from P1*oriR* (5-mer) | 50 | <2 |
| pALA2436 | Entire P1*oriRrep30* | 94 | <2 |

Cells containing the P1 mini-plasmid λ*cI*857-P1:5RKm were transformed with the plasmids shown. Incompatibility tests were carried out with the derivatives of plasmid pBR322 containing 5- or 6-iteron arrays replicating at either low or high copy number (see the *Experimental procedures*). The low-copy-number data are taken from a single experiment. Repeat assays showed that pALA697 and pALA2436 gave little variation, whereas the other plasmids gave values that varied randomly in the range of 40–60%.

on their function *in vivo* (Table 2), which, because of the variability of the assay (Table 2 legend), we do not believe to be significant. We did not obtain a 5-mer with a −5 bp spacer change, but a 6-mer with this change (pALA2435, Table 2) gave a result similar to that with a 6-mer with normal spacing (pALA780, Fig. 5). We conclude that it is not important for activity that all iterons are normally spaced or aligned on the same face of the helix. In contrast, the sequence of the iterons themselves is critical. A construct that has a −1 bp spacer change (pALA679) has wild-type activity, but plasmid pALA697, which is identical to pALA679 except that the highly conserved 15th iteron base is changed from G to T in all five iterons (Fig. 1), is inactive (Table 2).

### *The origin itself is not very effective as a negative regulator*

We have provided evidence that, despite having an array of iterons, the origin region does not itself act as an efficient inhibitor (Abeles and Austin, 1991). To investigate this further, we constructed pBR322-based plasmids that contain the natural iteron array from the origin and used them in the *in vivo* incompatibility test. Construct pALA750 contains the five iterons from the origin, but lacks other origin sequences. It has an effect similar to that of any other effective five-iteron array (Table 2). Thus, the close spacing and other novel sequence features of the iterons in the origin do not prevent them from being effective inhibitors. A second construct (pALA2436) has a complete origin region with a point mutation (*rep30*) which blocks its own origin activity *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 (Prentki *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 miniplasmid 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

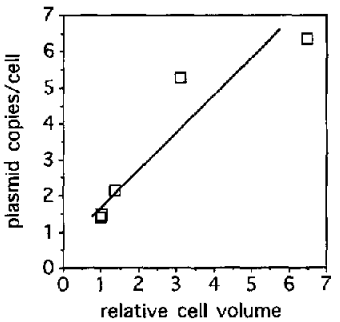


**Fig. 6.** Copy number of mini-P1 as a function of cell volume. The copy number of P1 mini-plasmid λ*cI*857-P1:5RCm per average cell was determined in logarithmic growth in various growth media (see the *Experimental procedures*). The average cell volume was estimated as described (see the *Experimental procedures*). The units used for cell size were set arbitrarily so that the cells at the slowest growth rate had an average volume of 1.0.

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.*, 1986). 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-

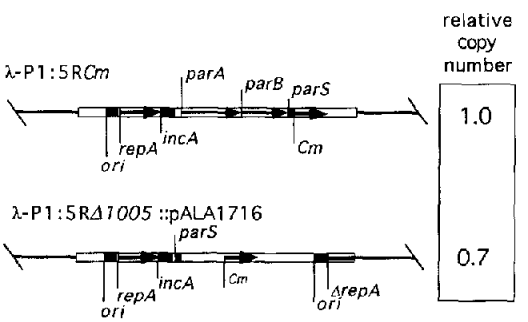


**Fig. 7.** Copy number of a plasmid with two origins and one *incA* locus. The P1 mini-plasmid λ-P1:5R*Cm* consists of the plasmid-maintenance region inserted into a λΔ*att* phage vector. It can be propagated as a lambda phage, but forms lysogens as a stable plasmid under P1 control. It has one P1 origin and one *incA* locus. Below, λ-P1:5RΔ*1005*::pALA1716 is formed by recombination between a deletion mutant of λ-P1:5R and a mini-P1 plasmid pALA1716 (Hayes and Austin, 1993). It has a similar structure to λ-P1:5R*Cm* except that it has two P1 replication origins and one *incA* locus, and lacks the *parA* and *parB* genes required for plasmid partition. It lysogenizes the host to form a stable plasmid when the partition proteins ParA and ParB are supplied *in trans*. The relative plasmid copy numbers were determined (as described in the *Experimental procedures*) in log-phase cells that produce P1 ParA and ParB. The boxed regions are P1 or mini-P1 sequences. The truncated solid lines extending from the ends of these sequences are parts of the lambda vector DNA.

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 λ*cI*857-P1:5RCm was derived from λ*cI*857-P1:5R (Sternberg and Austin, 1983) as previously described (Austin and Eichorn, 1992). λ*cI*857-P1:5RKm was derived by insertion of the *Sal*I fragment of the Kanamycin Resistance GenBlock (Pharmacia) into the unique *Sal*I site of λ*cI*857-P1:5R. λ-P1:5RΔ*1005*::pALA1716 was formed by recombination between a deletion mutant of λ-P1:5R and mini-P1 plasmid pALA1716 (Hayes *et al.*, 1994). To facilitate cloning of the synthetic repeats, an *Mlu*I adaptor fragment was introduced into the *Bam*HI site of pUC19 (Yanisch-Perron *et al.*, 1985) to form plasmid pALA670. The adapter was made by annealing the oligonucleotides 5′-GATCCACGCGTG-3′ and 5′-GATCCACGCGTG-3′. Plasmid pALA671 was made by replacing the smaller *Eco*RI–*Hin*dIII fragment of pBR322 with the corresponding fragment of pALA670 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), BR827 (Abeles *et al.*, 1990), DH5α (Woodcock *et al.*, 1989) and CC2004 (Austin and Eichorn, 1992) were as described. CC3937 was derived by introducing the *recJ248*::Tn*10* marker into BR827 by P1 transduction (Sambrook *et al.*, 1989) from strain JC12166 *recJ*::Tn*10* (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* and *oriR* loci (Fig. 1). The spacer sequence was based on a weakly conserved consensus of the spacer bases associated with the *incA* iterons (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 *Mlu*I 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 *Bss*HII and *Mlu*I. Ligation of units head-to-head or tail-to-tail produces intervening *Bss*HII or *Mlu*I 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 $MgCl_2$, 100 mM NaCl, 1 mM dithiothreitol, pH 8, 5 U ligase, 15–20 U *Bss*HII and 30–40 U *Mlu*I. The reaction was incubated at 37°C for 3 h. Additional units of *Bss*HII, 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 *Bss*HII and 20 U *Mlu*I 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 *Mlu*I 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.*, 1990). The plasmids constructed with these synthetic oligonucleotides are described in Table 2 and Fig. 4. Plasmid pALA689 contains a single consensus iteron. Plasmid pALA697 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 *Eco*RI to *Hin*dIII region of pBR322 with the *Eco*RI to *Hin*dIII region (containing the mutant P1*ori*) from M13-P*ori*-#88 (Abeles and Austin, 1987). Plasmid pALA18, containing the natural *incA*, was described earlier (Abeles *et al.*, 1984). Plasmid pALA749 contains the *Eco*RI to *Hin*dIII region of M13-P1-#91 (Abeles and Austin, 1991), encompassing the normal P1 *incA*, cloned into the same sites of pBR322. Plasmid pALA750 was constructed by cloning the *Eco*RI to *Bam*HI fragment of pALA145 (Abeles *et al.*, 1984) containing the P1*ori* 19 bp repeats into pUC18 and then moving the *Hin*dIII fragment containing these *ori* repeats into the *Hin*dIII site of pBR322. M13 replicative forms used in the *in vitro* replication tests were made by excising the *Eco*RI to *Hin*dIII regions of the pBR322-based plasmids containing the repeat sequences and ligating the fragments into the *Eco*RI to *Hin*dIII 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 λ*cI*857-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 (λ*cI*857-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 λ*cI*857-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 *polA*ts 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-P1:5RCm 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 $OD_{600} = 0.2$, 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 $OD_{600} = 0.2$. After extraction, the plasmid DNA was digested with *Bam*HI and the fragments separated by electrophoresis in a 0.3% agarose gel in 1 × 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 $OD_{600}$ of culture goes down. Thus, the relative cell volumes in different cultures could be estimated from the $OD_{600}$ 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 λ-P1:5RCm and λ:5RΔ*1005*::pALA1716 were determined in strain N100 containing plasmid pALA271 grown in L-broth (6 μg ml$^{-1}$ chloramphenicol) to $OD_{600} = 0.25$ at 30°C. In this case, no external reference was used. Rather, the *Bam*HI band derived from the resident pALA271 was used as a reference standard. The molar ratios of pALA271 to λ-P1:5RCm or λ:5RΔ*1005*::pALA1716 bands were determined. The ratio of the two values was taken as indicating the relative copy numbers of the two λ-P1 plasmids with that of λ-P1:5RCm set arbitrarily at 1.0.

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