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Genetics

Antiparallel plasmid–plasmid pairing may control P1 plasmid replication

(*in vitro* replication/plasmid incompatibility/repeated sequences/origin control)

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ABSTRACT **The copy number of the P1 plasmid replicon is stringently controlled, giving only one or two copies per newborn cell. Control is achieved by the action of the copy-control locus *incA*, which contains nine repeats of the 19-base-pair binding site for the plasmid-encoded initiator protein RepA. A set of five similar repeats are present in the replication origin where RepA acts to trigger initiation. Using an *in vitro* replication system consisting of an *Escherichia coli* extract, the P1 origin as a template, and purified RepA protein, we show that supercoiled DNA circles containing the *incA* locus block origin function in trans. Shutdown becomes complete at a 1:1 ratio of origin to *incA* sequences. This is not due to titration of the RepA protein, as an excess of RepA can be added without restoring activity. Rather, the *incA* sequences appear to block the origin by direct contact in a plasmid–plasmid pairing event. When both the origin and the *incA* locus are present on one plasmid, trans contacts with daughter molecules appear to predominate over cis looping. The results are consistent with a model for replication control where daughter plasmids block their own replication by a pairing in which each origin is in contact with the *incA* locus of its partner.**

The low-copy-number plasmid replicon (*repR*) (1) from bacteriophage P1 is illustrated in Fig. 1. It consists of the origin of replication, the gene for the synthesis of the RepA initiator protein, and the copy-control locus *incA* (2). The origin is sufficient to promote plasmid replication when the RepA protein is supplied in trans (3, 4). However, constructs lacking the *incA* locus are maintained at an elevated copy number (5). Additional copies of *incA*, either on a second plasmid or in the host chromosome, block P1 miniplasmid replication (6). The origin and *incA* both contain series of 19-base-pair (bp) imperfect repeats that are binding sites for the RepA protein (7). This suggested that *incA* might control copy number by titrating RepA, limiting its availability to the origin for initiation (5). However, this model is hard to reconcile with the autoregulation of RepA synthesis (4, 8) and the demonstration that P1 copy number does not increase significantly in cells with elevated RepA concentrations (9). An alternative type of model invokes negative control by contact of one DNA locus by another mediated by the bound proteins (9, 10). Contacts might limit replication by repressing the RepA gene (9, 10) or by direct action on the origin (9). Cis contacts of the origin and *incA* loci (DNA looping) could set the maximal level of origin function (10). However, true copy-number regulation might be achieved if trans contacts between daughter plasmids contribute, so that the more plasmids produced, the lower the frequency of initiation (9).

The concept of control by plasmid–plasmid contacts ("DNA handcuffing") was originated independently for plasmids R6K (11) and RK2 (12, 13). As these plasmids lack an equivalent to the P1 *incA* locus, origin–origin contacts are invoked (11). The origin repeats of RK2 inhibit the origin in trans in an *in vitro* replication system (12). This inhibition is independent of initiator protein concentration over a wide range (12). In the P1 and R6K cases, physical evidence for the formation of relevant DNA–DNA contacts mediated by the appropriate initiator proteins has been presented (10, 11).

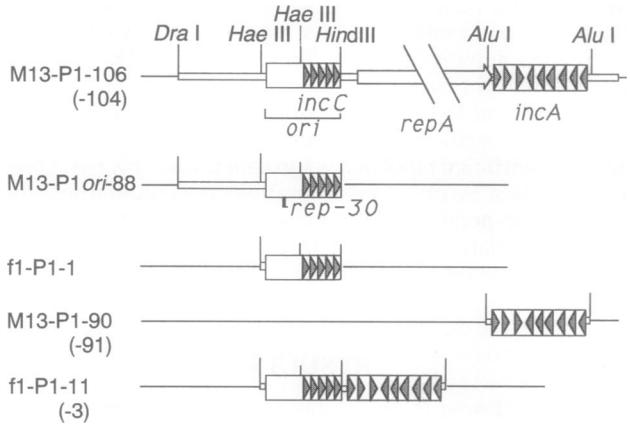

FIG. 1. Construct containing the wild-type P1*repR* replicon is shown above. The 19-bp RepA binding repeats are shown as shaded triangles whose direction indicates the repeat orientation. Relevant portions of maps of other M13 or f1 phage derivatives are shown below; all sequences are from P1 except for M13 or f1 sequences (horizontal lines). Numbers in parentheses indicate modified versions: M13-P1-104 has the *rep-30* mutation (cf. M13-P1*ori*-88); M13-P1-91 and M13-P1-3 have *incA* inverted.

MATERIALS AND METHODS

Strains, Bacteriophage, and Plasmids. *Escherichia coli* fraction II extract was from strain C600 (14). M13- and f1-based DNA were prepared by using JM103 (15) and JJ119 (16), respectively. Vectors were M13mp10, M13mp11 (17), M13mp18 (18), and $f1h_0$ (16). The *Hin*dIII end of the *Hin*dIII/*Eco*RI fragment encompassing the wild-type P1 origin in M13-P1*ori*-49 (19) was trimmed with BAL-31 to P1 bp 373 and ligated to an *Eco*RI linker; the resulting *Eco*RI fragment was inserted into $f1h_0$ to give f1-P1-1 (Fig. 1). M13-P1*ori*-88 (19) has the *rep-30* origin mutation. Constructs f1-P1-3 and f1-P1-11 contain the *Bam*HI *incA* fragment of pALA18 (2) in the *Bam*HI site of f1-P1-1 in opposite orientations (Fig. 1). M13-P1-90 and -91 have the same fragment, in opposite orientations, in the *Bam*HI site of M13mp18 (Fig. 1). M13-P1-88 was cut at *Hin*dIII and the *Hin*dIII *repA incA* fragment of pALA136 (2) was inserted to give M13-P1-104 (Fig. 1). M13-P1-106 has the intact replicon from pALA136 (2) as a *Dra* I/*Hin*dIII fragment in M13mp10. The molecular weights of the origin template and the competitor DNA are similar,

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≈7 kilobases (kb), so the weight ratios and molar ratios are the same.

General Methods, Media, Enzymes, Reagents, and Buffers. Unless otherwise mentioned, all general methods and materials were as described (19, 20).

***In Vitro* Replication Assay.** *In vitro* replication was essentially as described (19, 21) except that the reactions were incubated at 37°C for 20 min and were not enriched with additional DnaA protein. Origin and regulatory DNAs were mixed before addition of other components. RepA protein was purified as described (7) and modified as follows: the extract was batch adsorbed to Affi-Gel blue, washed extensively with 0.2 M NaCl-RPB [RepA purification buffer (7)], and eluted from a column with a gradient of 0.2–2 M NaCl-RPB. Pooled peak fractions were dialyzed against 0.25 M NaCl-RPB and purified without precipitation by chromatography over heparin/agarose, Sephacryl S-300HR, and Pharmacia Mono S columns.

Treatment of Data. The mean value of a number of repeat assays was used. The standard deviations from the mean ranged from 0–2.0 pmol at background levels to 0.5–4.5 pmol at the highest levels of incorporation. Background incorporation corresponding to the mean level of control reactions lacking template DNA or RepA (4–7 pmol of dTTP incorporated) was subtracted from the values, except for those in Table 1.

RESULTS

The *incA* Locus Is an Inhibitor of the P1 Origin *in Vitro*. Using an *in vitro* replication system for the P1 plasmid origin (19), incorporation of nucleotide precursors is dependent on DNA containing the origin and addition of the P1 RepA initiator protein. Table 1 confirms this and shows that incorporation can be modulated by altering the composition of the supercoiled replicative-form circles used for all input DNA. When the P1 plasmid origin was the sole P1 sequence present (f1-P1-1), incorporation was maximal. However, if both the origin and the *incA* copy control locus were present in their normal context (M13-P1-106; Fig. 1), incorporation was minimal (Table 1). [S. Wickner (personal communication) has also observed no *in vitro* replication when *incA* sequences are present in cis to an origin template.] This inhibitory effect of the *incA* sequence also works in trans: when the same individual concentrations of origin and *incA* sequences were used, but on separate molecules (f1-P1-1 plus M13-P1-90; Fig. 1), no incorporation was seen (Table 1). When M13 vector DNA was substituted for the *incA* DNA, origin function was fully restored. Thus, the *incA* sequences have a strong inhibitory effect on origin function both in cis and in trans under the conditions used.

Table 1. Cis and trans effects of *incA* sequences on P1 replication *in vitro*

	DNA	dTTP incorporated, pmol	
		− RepA	+ RepA
Vector	M13mp11	5	5
	$f1h_0$	6	7
P1*ori* alone	f1-P1-1	6	45
P1*ori* + *incA*	M13-P1-106	4	7
in cis	f1-P1-3	5	7
	f1-P1-11	5	7
incA alone	M13-P1-90	6	6
	M13-P1-91	8	6
P1*ori* + *incA*	f1-P1-1 + M13-P1-90	7	5
in trans	f1-P1-1 + M13-P1-91	6	8
P1*ori* + vector			
in trans	f1-P1-1 + M13mp11	8	41

The *in vitro* replication was performed as described. Each reaction mixture contained 0.1 μg (20 fmol) of template DNA and 0.1 μg (3 pmol) of purified RepA. For reactions with two plasmids, the second was present at 0.1 μg.

***incA* Inhibition Is Insensitive to Increasing RepA Concentration.** Fig. 2 shows the effects of varying RepA concentration on *incA* inhibition in trans. At a fixed concentration of f1-P1-1 template, incorporation was saturated at the lowest concentration of RepA used (0.1 μg or 3 pmol of RepA per assay). Increasing the RepA concentration over a 10-fold range caused a significant but gradual decline in incorporation to approximately one-half the optimum level. This experiment was repeated by using the same concentration of template plus an equal concentration of DNA carrying the *incA* locus. No incorporation was seen, and inhibition was not relieved significantly by adding up to 10-fold additional RepA (Fig. 2). Thus, the inhibitory effect of the *incA* sequences is largely independent of RepA concentration and persists at concentrations well in excess of those normally required to saturate the origin.

Inhibition by *incA* Becomes Maximal at a 1:1 Ratio to the Origin, Independent of RepA Concentration. Fig. 3 shows the effect of increasing the concentration of *incA* sequences in trans to a fixed concentration of origin-containing template at different RepA concentrations. Inhibition by *incA* was maximal at the lowest RepA concentration used (0.1 μg) and was somewhat relieved by a doubling of RepA. However, when RepA concentration was increased stepwise a further 5-fold, the response to *incA* was virtually unaltered. The half-maximal inhibition was achieved at about the same *incA* concentration, irrespective of how much RepA was present. Thus, *incA* is not acting by RepA titration except at the lowest RepA concentration used. If it was, the inhibition curves would be displaced from each other by a factor of up to 5 on the *incA* concentration scale. When RepA is in excess, full inhibition is achieved at an *incA*/origin ratio close to 1:1.

Origin Repeat Sequences Have Little Effect in Trans. The *incA* locus and the origin both contain multiple repeats of the 19-bp RepA binding sequence (Fig. 1). The origin cluster (termed *incC*) has an inhibitory effect *in vivo* but only when cloned into a high-copy-number vector (2). When these sequences (M13-P1*ori*-88; Fig. 1) were added in trans to an active origin, no effect was seen until the added DNA was in considerable excess (Fig. 4*B*). M13-P1*ori*-88 has a complete origin including the *incC* origin cluster. To inactivate the origin, it has the *rep-30* mutation, a single base change well outside the *incC* repeats (Fig. 1) that blocks origin function

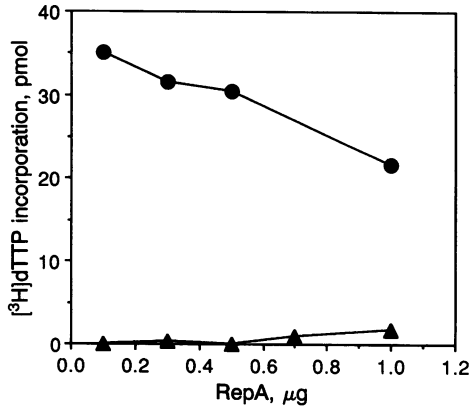

FIG. 2. Replication of P1*ori* (0.1 μg per reaction mixture; f1-P1-1) in the presence of no other DNA (circles), or with 0.1 μg of *incA* DNA (M13-P1-90; triangles) in trans. The reactions were performed as described. Incorporation of [³H]dTTP (pmol) is plotted against the amount of RepA protein (μg) per reaction mixture; 0.1 μg of each DNA corresponds to ≈20 fmol; 0.1 μg of RepA is ≈3 pmol.

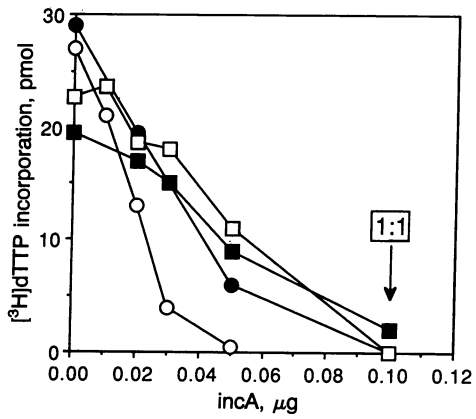

FIG. 3. Replication, shown as incorporation (pmol) of [^{3}H]dTTP, is plotted for 0.1 μg of P1*ori* DNA in the presence of increasing amounts of *incA* DNA (μg per reaction mixture) for four different amounts of RepA: open circles, 0.1 μg; solid circles, 0.2 μg; open squares, 0.5 μg; solid squares, 1.0 μg of RepA added per reaction mixture. The point at which the ratio of *incA* to origin sequences is 1:1 is shown.

both *in vivo* and *in vitro* (3, 19). The wild-type origin is not self-inhibitory either, as incorporation in the presence of excess RepA increases in proportion to DNA concentration (Fig. 4*A*). We conclude that, unlike *incA*, the origin region with its five RepA binding repeats is not an effective origin inhibitor. This is consistent with the observation that origin–origin contacts are formed less readily than origin–*incA* contacts, as demonstrated by electron microscopy (10). However, origin–origin contacts might serve to prevent "runaway" replication in plasmids where *incA* is deleted.

Cis Contacts Are Unlikely to Account for Origin Inhibition on the Wild-Type Template. It has been proposed that the inhibitory effect of *incA* on replication of the intact P1 replicon is due to cis contacts (DNA looping) between the origin and *incA*, promoted by the binding of RepA to both loci (10). These sites are normally separated by ≈1 kb. In construct fl-P1-3 (Fig. 1), the two loci were brought within 15 bp of each other. Table 1 shows that, like the normally spaced wild-type (M13-P1-106), the closely spaced construct has no measurable template activity *in vitro*. A similar construct with *incA* in the opposite orientation (fl-P1-11; Fig. 1) was also inactive. Thus, an *incA* locus very close to the origin (which should disfavor DNA looping) still appears to be inhibitory. Note that if the close spacing of *incA* to the origin in constructs like M13-P1-3 caused some direct damage to the origin, this conclusion would be invalid. However, this is unlikely because the construct is functional *in vivo* (data not shown).

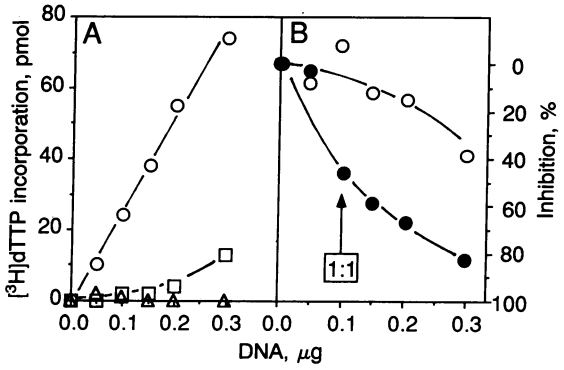

FIG. 4. (*A*) Replication, shown as incorporation (pmol) of [^{3}H]dTTP in the presence of 0.5 μg of RepA, is plotted against increasing DNA concentrations (μg per reaction mixture) of fl-P1-1 (functional origin; circles); M13-P1*ori*-88 (defective origin; squares); M13-P1-104 (*incA*+ defective origin; triangles). (*B*) Inhibition of replication (compared with activity without inhibitor DNA) was measured in the presence of 0.5 μg of RepA and a fixed concentration (0.1 μg) of fl-P1-1 DNA. Inhibition is plotted against increasing concentrations of competing DNAs: M13-P1*ori*-88 (open circles) or M13-P1-104 (solid circles). The point at which the ratio of competitor DNA to active origin reaches 1:1 is shown.

The Presence of an Origin in Cis to *incA* Does Not Block the Effect of *incA* in Trans. Construct M13-P1-104 has the *incA* sequence in cis, spaced normally from a defective origin containing the *rep-30* mutation. It has no activity as a template (Fig. 4*A*). Fig. 4*B* shows the effect of increasing the concentration of this DNA in trans to a fixed concentration of active origin-containing template. RepA was present in excess. In contrast to M13-P1-88, which has the same mutant origin but no *incA* sequence, M13-P1-104 DNA is an effective inhibitor in trans. Thus, DNA looping in M13-P1-104, if it occurs at all, does not prevent the *incA* locus from being available to inhibit an active origin in trans. Note that the origin of M13-P1-104 differs from the wild-type by only 1 bp, distal from the RepA binding repeats (Fig. 1). We think it unlikely that the *rep-30* mutation itself prevents looping. Comparison of the inhibitory effect of M13-P1-104 (Fig. 4*B*) with that seen with *incA* alone (M13-P1-90; Fig. 3) shows that more M13-P1-104 than M13-P1-90 DNA is needed to give a comparable level of inhibition of an active origin in trans. This is expected if inhibition is due to trans contacts, because M13-P1-104 can make contacts with itself as well as with the active origin DNA (see *Discussion*).

DISCUSSION

The P1 *incA* locus is a powerful inhibitor of origin initiation, both in cis and in trans. Although it consists only of a series of RepA binding repeats similar to those found in the origin, it does not act by titrating available RepA because replication inhibition is independent of increased RepA concentration. Rather, *incA* appears to block the origin by some sort of direct interaction with it.

Chattoraj *et al.* (9, 10) showed by microscopy that RepA protein is capable of bringing origin and *incA* sequences together in complexes. The intact *rep* region DNA formed loops (cis contacts) and DNA complexes in which two DNA molecules are brought together (trans contacts; ref. 10). These contacts were proposed to be the basis of origin regulation, either by regulation of RepA synthesis by occlusion of the *repA* promoter (9, 10) or by a direct interference with origin function ("steric hindrance"; ref. 9).

Here we tested the potential of direct origin interference by trans contacts *in vitro*. We eliminated any contribution of regulation of RepA synthesis or RepA titration by supplying all the RepA exogenously and in excess. Any contribution of cis contacts (looping) was eliminated by having the target sequence (the origin) and the effector sequence (*incA*) on different molecules. This simplified system exhibited a remarkably powerful regulation. When a 1:1 ratio of separate DNAs carrying the origin and *incA* were mixed in the presence of excess RepA, none of the origins could fire. If this effect is due to DNA contacts, they must form very efficiently in trans and result in a stable block to origin function. Parallel tests for origin–origin contacts showed that they are much less effective.

The wild-type replicon has the origin and *incA* in cis. In this case, *incA* origin contacts could control replication by either cis contacts (DNA looping) or trans contacts between two daughter molecules (DNA handcuffing). Which predominates? Our results suggest that DNA handcuffing does. First, *incA* still inhibits the origin when brought very close to it. It seems unlikely that a spacing of 15 bp is sufficient to allow specific contact of the two regions by DNA looping. That

such a short range contact could be made with *incA* in either orientation seems even less likely. Second, if DNA looping were to predominate, an origin placed on the same DNA as *incA* should prevent it working in trans. No such effect was seen using the defective origin–*incA* construct M13-P1-104. Moreover, the stoichiometry of inhibition with M13-P1-104 is consistent with all contacts occurring in trans. In this case, M13-P1-104 should give 50% inhibition in trans at a 1:1 molar ratio with active origin DNA. At a 2:1 ratio, the inhibition should be 66.6%; at 3:1, it should be 75%. This follows from a simple calculation assuming that the *incA* sequence of M13-P1-104 contacts the origin of its daughter molecules in trans as easily as it can contact the active origin-containing DNA. Our results fit this prediction well (Fig. 4*B*). The result would not obtain if M13-P1-104 predominantly formed loops: in this case, no inhibition would be seen. The result is also inconsistent with the trivial explanation that the *incA* region of M13-P1-104 is free because the *rep-30* mutation blocks origin–*incA* contacts: in this case, the DNA would behave like that with *incA* alone (M13-P1-90; Fig. 3) and give complete inhibition at a 1:1 ratio to an active origin.

Two observations qualify the interpretation of these data. First, the *rep-30* mutant origin has some template activity of its own at high DNA concentration (M13-P1-88; Fig. 4*A*). This would tend to decrease the inhibition seen with M13-P1-104. Second, the same mutant origin is itself somewhat inhibitory at high DNA concentration (M13-P1-88; Fig. 4*B*), which would tend to increase inhibition slightly. Thus, the match of the M13-P1-104 inhibition data to theoretical predictions for trans contacts can only be said to be approximate. However, we can conclude that if looping does occur, it does not predominate. Perhaps cis looping is less stable than handcuffing between two DNAs so that it does not compete successfully.

The wild-type plasmid has the origin and *incA* placed in cis. However, after replication, these sequences are in trans to daughter molecules. We envision that the daughter molecules would pair with each other as shown in Fig. 5. The pairing is shown in the antiparallel configuration (with their origins in contact with the *incA* loci of their partners) because, like this,

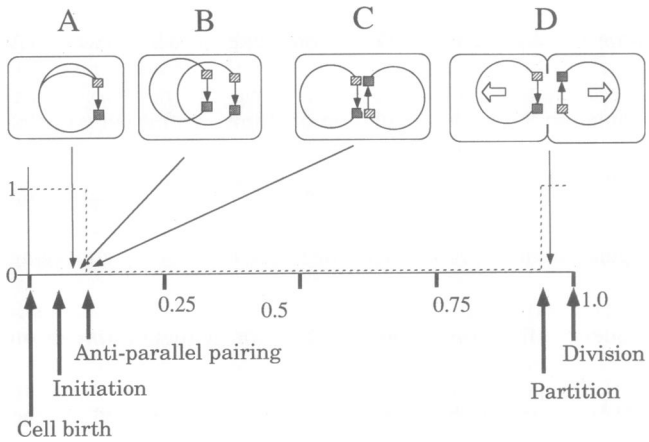

FIG. 5. Model for P1 plasmid replication control is illustrated above. A single copy of the plasmid initiates replication rapidly (*A*). When two copies are produced (*B*), they rapidly pair in an antiparallel configuration (*C*), blocking origin function of both until partition (*D*) resets the cycle. A typical cell cycle is illustrated below with the abscissa representing fractional divisions of the cell cycle and the ordinate representing the probability that initiation will occur. Only one initiation event can occur per cell cycle, as replication is self-blocking and is only reset by partition. Initiation could occur at different times in different cells, but the result at the time of cell division would always be the same. The *incA* locus and the origin are shown by solid and hatched boxes, respectively. Vertical arrows show the orientation of the *repA* gene.

the favored *incA*–origin contacts are established. Our data imply that such contacts can form readily without undue interference from cis looping. The complex would be inactive, with both origins occluded. This suggests a simplified model for replication control illustrated in Fig. 5.

When a single copy of the plasmid is present in a cell, the probability of initiation is maximal. The origin is functional and all necessary components (including RepA) are present in sufficient amounts to ensure initiation (Fig. 5*A*). After replication, the two daughter plasmids bind to each other via RepA protein to form a stable complex, with the *incA* sequence of each contacting the origin of the other (the antiparallel configuration; Fig. 5*B*). This blocks both origins and the probability of initiation is minimal. No further initiation occurs until a plasmid-partitioning event (22, 23) pulls the pair apart and puts each plasmid into a new daughter cell (Fig. 5*C*). Reinitiation can then occur.

In this simple model, control of RepA synthesis is not a key factor. Replication control is achieved solely by direct inhibition of the origin by contact with another plasmid copy. Inhibition by *incA* is independent of RepA concentration because the DNA itself is the critical inhibitor. Maximal *incA* inhibition is achieved at a 1:1 ratio of *incA* to origin because one *incA* sequence inhibits one origin by binding to it.

The model has some advantages in explaining the remarkable accuracy of control observed *in vivo*. It predicts a machine-like, 1-to-2-to-1 cycling of the copy number with each cell generation (Fig. 5). It satisfies the important requirement that the probability of replication within a cell generation is high, and yet overreplication is minimal, thus explaining the high efficiency of P1 plasmid maintenance at very low copy number. The model describes a true control system because it has a powerful negative feedback loop. If, by error, a second initiation event occurs in a cell, three plasmids will be present at cell division. One daughter cell will receive two. These copies will pair immediately, shutting off plasmid replication during the next cell cycle, thus restoring the copy number to one in the following generation.

A number of plasmids found in prokaryotes as well as some eukaryotic viruses such as simian virus 40 (24), Epstein–Barr virus (25), and bovine papilloma virus (26) also have repeat sequences in or near their origins of replication. It is conceivable that a similar model for replication control applies in these cases. In the case of the prokaryotic plasmid RK2, strong evidence for a role for trans contacts in replication control has been presented (12). It has also been proposed that the origin of newly replicated simian virus 40–bovine papilloma virus is blocked by trans contacts (27).

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