

MicroReview

Control of plasmid DNA replication by iterons: no longer paradoxical

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Summary

Replication origins of a family of bacterial plasmids have multiple sites, called iterons, for binding a plasmid-specific replication initiator protein. The iteron–initiator interactions are essential for plasmid replication as well as for inhibition of plasmid over-replication. The inhibition increases with plasmid copy number and eventually shuts plasmid replication off completely. The mechanism of inhibition appears to be handcuffing, the coupling of origins via iteron-bound initiators that block origin function. The probability of a *trans*-reaction such as handcuffing is expected to increase with plasmid copy number and diminish with increases in cell volume, explaining how the copy number can be maintained in a growing cell. Control is also exerted at the level of initiator synthesis and activation by chaperones. We propose that increases in active initiators promote initiation by overcoming handcuffing, but handcuffing dominates when the copy number reaches a threshold. Handcuffing should be ultrasensitive to copy number, as the negative control by iterons can be stringent (switch-like).

Introduction

Plasmids are extrachromosomal genetic elements that are maintained on the limited resources of the host. So that they are not lost, plasmids ensure that their replication rate is co-ordinated with the cell growth rate. A slower replication rate will lead to plasmid-free cells. Moreover, plasmid-free cells often grow faster and can outnumber plasmid-carrying cells in the population (Proctor, 1994). A faster plasmid replication rate is also undesirable. Accumulation of plasmid copies adds to the metabolic burden of the host. This slows cell growth and, taken to the

limit, causes cell death. Plasmid-free cells can accumulate rapidly if the plasmid mutates to higher copy numbers (Matsunaga *et al.*, 1997). It is therefore imperative that the plasmid concentration be maintained, preferably at a low level. This is achieved by an autoregulatory control mechanism: plasmid DNA concentration itself determines the rate at which new plasmid copies are generated. In fact, the concentration of a small part of the plasmid replicon, about 100–300 bp, called the control or *inc* locus, serves as the primary sensor for plasmid concentration and determinant of the replication rate. The rest of the replicon can be viewed merely as a vehicle for maintaining the control locus. Plasmid copy number control has been the subject of recent reviews (Helinski *et al.*, 1996; Chattoraj and Schneider, 1997; del Solar *et al.*, 1998; Filutowicz and Rakowski, 1998). Here, I have tried to update information about how multiple iterons comprising one type of control locus function in plasmid replication. Plasmid P1 has generally been used here as the prototype of iteron-mediated control primarily because of my familiarity with the system. However, the goal has been to emphasize features that are likely to be common in the iteron family of plasmids.

Replicon structure

Plasmid replicons under the control of iterons are organized rather similarly. A few examples, referred to frequently in this article, are shown in Fig. 1. There are two essential functional units: an origin and an adjacent gene that codes for a replication initiator protein, usually called Rep protein. About half the origin sequences are taken up by an array of ≈ 20 bp repeats, called iterons. The iteron sequences are characteristic for each plasmid, and they specifically bind the cognate Rep protein. Some plasmids that have low copy numbers, such as P1, F and RK2, carry a second set of iterons outside the origin. These, I believe, only increase the stringency of control, i.e. lower the mean copy number as well as lower deviations from the mean.

Iterons belonging to the same plasmid show a high degree of sequence conservation (Chattoraj and Schneider, 1997). In fact, the conservation appears excessive, more

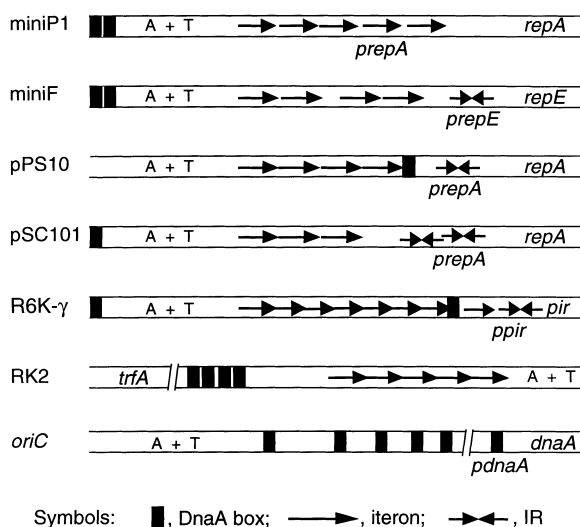


Fig. 1. Origin region of some representative iteron-carrying plasmids (maps not drawn to scale). Iterons are about 20 bp each, and they cover about half the origin. The inverted repeat (IR) sequences are outside, but adjacent to, the origin and serve as operators for the downstream initiator genes (*repA*, etc.). In miniP1, there is no IR. The origin iterons also serve as operators for the *repA* gene. The initiators are thus autorepressed except for RK2.

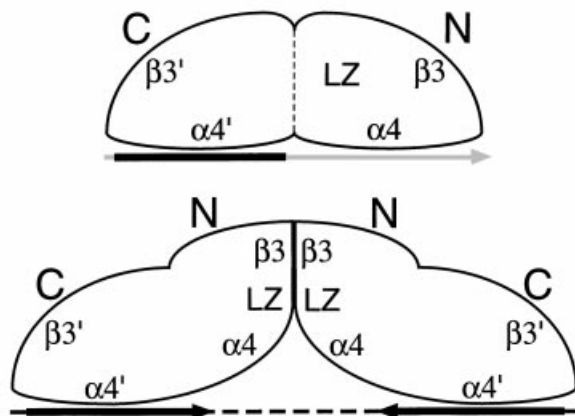


Fig. 2. Model of conformation change in the N-terminal domain of Rep of F plasmid that allows operator binding as a dimer (adapted from Komori *et al.*, 1999). Top: A schematic of the crystal structure of a Rep monomer bound to an iteron showing that the protein consists of two topologically similar N- and C-terminal domains. The twofold axis of symmetry is shown by the dotted line. A non-canonical leucine-zipper (LZ) motif is hidden in the hydrophobic core of the N-terminal domain. The $\alpha 4'$ and $\alpha 4$ helices contact two consecutive major grooves on the same face of an iteron (arrow). The black bar on the iteron shows the region present as IR (arrows in the bottom diagram) in the *repE* operator. Bottom: A model of RepE dimer bound to the *repE* operator. The $\alpha 4'$ helix of the C-terminal domain contacts the sequences present in the IR. Modelling shows that, in order for the two C-terminal domains to contact the IR, the N-terminal domains must flip out of DNA so that the $\alpha 4$ helices are no longer in contact with DNA. The conformational change may allow the LZ motif to be available on the surface of the protein that can aid in dimerization. The dimerization energy is believed to be contributed primarily by the $\beta 3$ strands of the N-terminal domain (represented here by $\beta 3$ only for simplicity), as the dimerization-defective mutations map mostly in the coding sequences of these strands.

than a protein should need to recognize iterons specifically over a vast background of non-specific sites. It appears that some of the conservation is there for reasons other than initiator binding. There is no experimental support for this assertion at present (Papp *et al.*, 1994), although other protein binding sites have been found to overlap the array of iterons: DnaA boxes in pSC101 iterons (Stenzel *et al.*, 1991) and FIS binding sites in R6K iterons (Filutowicz and Rakowski, 1998). Comparison of iterons belonging to different plasmids shows patches of homology, indicating that the iterons could be evolutionarily related (Chattoraj and Schneider, 1997). The patches of homology may suggest that gene conversion events are taking place in cells in which the plasmids are co-resident. By analogy, intraplasmid gene conversion would be expected to maintain the relatedness of sets of iterons on the same plasmid, perhaps explaining the excessive conservation (D. Summers, personal communication). The initiator proteins also share sequence similarities (25–35% in pairwise alignments) (del Solar *et al.*, 1998).

Initiator proteins: requirement for conformation change

Understanding the functioning of Rep proteins is the primary problem in deciphering the mechanism of iteron-mediated control. One complexity of the system comes from an apparently simple fact that Rep exists in both monomer and dimer forms. Monomers bind to iterons specifically and serve as initiators (Wickner *et al.*, 1991; Manen *et al.*, 1992; Toukdarian *et al.*, 1996; Giraldo *et al.*, 1998; Komori *et al.*, 1999). A high-resolution structure of a monomer–iteron complex of the F plasmid has been obtained recently. Two independent globular domains of the monomer were found to bind to two halves of the iteron (Komori *et al.*, 1999; Fig. 2). However, purified Rep proteins are found largely as dimers that are inactive in iteron binding. The dimers can be converted to monomers by a conformational change that can be mediated by chaperones. The dimers can bind DNA sequences that specifically serve as operators for *rep* genes of some plasmids (Manen *et al.*, 1992; Ishiai *et al.*, 1994; Urh *et al.*, 1998). In these operators, half-iteron sequences are present as an inverted repeat (IR; Fig. 1; Germino and Bastia, 1983; Vocke and Bastia, 1983). Note that the P1 origin lacks an IR, Fig. 1. The iterons are also the operators in P1; Sozhamannan and Chattoraj, 1993). In addition to iteron and IR binding, the Rep proteins are also involved in coupling Rep–iteron complexes *in trans*, a reaction called handcuffing that is believed to inhibit plasmid over-replication (Pal and Chattoraj, 1988; McEachern *et al.*, 1989). Thus, although preformed dimers are inactive in iteron binding, iteron-bound monomers can still

handcuff. Therefore, the protein–protein interaction interfaces involved in dimerization and in handcuffing cannot be identical. However, there is genetic evidence that the two interfaces could be related (Giraldo *et al.*, 1998; Uga *et al.*, 1999). Most probably, one is derived from the other by a conformational change (discussed in the next section).

Why dimers do not bind iterons can be explained from the crystal structure of the Rep–iteron complex of the F plasmid (Komori *et al.*, 1999). When two monomers were modelled on the operator (IR) DNA of F, a large steric hindrance was created by the N-terminal domain of the monomers. This implies that a marked conformational change in Rep is required to accommodate its dimeric form on the operator DNA. The N-terminal domain probably contains the dimerization surface of Rep of F, as the changes in dimerization-defective Rep mutants are located primarily in this region (amino acid residues 93–161, represented as $\beta 3$ in Fig. 2 for simplicity) (Matsunaga *et al.*, 1997). The region also buries the conserved leucines of a non-canonical leucine-zipper (LZ) motif to the inside of the protein. The motif commonly serves as a dimerization interface and is present in a few other initiators belonging to the iteron family (Garcia de Viedma *et al.*, 1996). The importance of the motif in dimerization has also been shown in two cases (Garcia de Viedma *et al.*, 1996; Wu *et al.*, 1997). Therefore, it was speculated that the conformational change in the N-terminal domain upon dimerization could also uncover the hydrophobic cluster of the leucines, allowing them to join the dimer interface and stabilize dimers. When these movements were allowed in the model, the N-terminal $\alpha 4$ DNA-binding helix could no longer contact DNA. The contact of the $\alpha 4'$ helix to half an iteron is expected to be weak, explaining why dimer binding to iterons has gone largely undetected. An exception has been the Rep protein of R6K, which can bind an iteron either as a monomer or as a dimer (Urh *et al.*, 1998). In the dimer, only one subunit makes contact with DNA. The relative orientation of the two DNA-binding domains in a monomer apparently does not change significantly upon dimerization in this plasmid.

Experimental evidence in favour of dimerization-induced conformational change has been obtained in the case of Rep of pPS10 (Giraldo *et al.*, 1998). The proposed structure of this protein from biochemical, spectroscopic and hydrodynamic studies has turned out to be quite similar to the crystallographic structure of Rep of F (Komori *et al.*, 1999). However, unlike the situation in F, in which the N-terminal LZ domain is not canonical and perhaps plays a secondary role in dimerization, the N-terminal LZ domain in the case of pPS10 is clearly the primary dimerization domain (Garcia de Viedma *et al.*, 1996). Deletion of the domain leads to preparations of largely monomeric initiators. These contain two globular

domains that bind the two halves of the iteron sequences much like the Rep of F. The binding is equally efficient to the two halves, although in the intact protein, DNA binding by the N-terminal globular domain is inefficient. The experiments support the view that dimerization compacts the N-terminal DNA-binding domain and thereby interferes with its DNA-binding activity. Thus, both modelling and experimental studies indicate that the DNA-binding domain containing or adjacent to the dimerization interface undergoes drastic change upon dimerization and thereby fails to bind iterons. Establishing the precise change upon dimerization would require determination of the dimer structure at high resolution.

How do chaperones activate dimers for iteron binding? *In vitro* studies strongly favour the view that chaperones, such as DnaJ-DnaK-GrpE (Wickner *et al.*, 1991), ClpA (Pak and Wickner, 1997) or ClpX (Konieczny and Helinski, 1997), dissociate dimers to monomers and thereby make more monomers available for iteron binding. Some biophysical studies have shown that dissociation can be obtained without chaperones, which has led to the suggestion that reduced dimerization was a consequence of chaperone-mediated change in the protein conformation (Ingmer *et al.*, 1995; Chatteraj *et al.*, 1996; Garcia de Viedma *et al.*, 1996; Dibbens *et al.*, 1997). This was also evident from the properties of some chaperone-independent mutants. For example, Rep⁺ monomers of F purified after dissociation of dimers with guanidine hydrochloride bind to iterons an order of magnitude less well than dimerization-defective RepE54 monomers (Ishiai *et al.*, 1994), although the change in RepE54 is far removed from the DNA-binding domain (Komori *et al.*, 1999). A mutant Rep of P1 (m5) that favours the monomeric state showed a significant increase in iteron binding when the purified monomers were treated with chaperones (Mukhopadhyay *et al.*, 1994). Thus, monomer initiators could also be substrates for chaperones. Most probably, chaperones can recognize both forms of the protein and help them to refold in the activate form (Dibbens *et al.*, 1997).

Why dimerization?

As monomers serve as initiators, the purpose of dimers is not obvious. Autoregulation cannot be their primary function as, in P1, the origin iterons also serve as operators, and monomer binding to the origin achieves both initiation and repression (Sozhamannan and Chatteraj, 1993). Yet P1 Rep forms dimers and requires chaperones for iteron binding just like initiators that bind to operator DNA as dimers. We can only speculate on the purpose of dimerization. It may be a mechanism to limit initiator availability and thereby delay initiation. Dimerization may assist in protein folding, as is the case with bZip

proteins (Thompson *et al.*, 1993). The role of chaperones may be minor in the overall protein folding scheme. One cycle of chaperone binding and release suffices to activate P1 Rep (Pak and Wickner, 1997). It is also noteworthy that, although the presence of the two forms of the protein is seen without exception, not all initiators in the iteron family of plasmids seem to require chaperones (namely Rep of R6K, pSC101, pPS10).

My best guess is that the dimerization potential was selected primarily for handcuffing, and other features of dimers, operator binding and chaperone requirement, were later elaborations. There is considerable overlap in the positions of changes in Rep of F that confer a dimerization defect and increase plasmid copy number (i.e. handcuffing defect?) (Uga *et al.*, 1999). In pSC101 Rep, changes close to the LZ motif can promote initiator activity (Ingmer and Cohen, 1993). Copy-up mutants in R6K Rep also have changes that map in a region that covers one of its putative dimerization domains (Urh *et al.*, 1998). This plasmid has multiple origins, and coupling interactions between iterons are involved in the activation of one origin by another. An amino acid change (P113S) that affects this interaction between initiators also occurs in the region implicated in dimerization (Miron *et al.*, 1994). Interfaces involved in the dimerization of free monomers and in interactions between iteron-bound monomers (handcuffing) could thus have overlaps. The observation that changes in the same region can also confer chaperone independence implies that dimerization is the basis for the chaperone requirement. Thus, dimer dissociation and the required conformational change seem to contribute to control in two crucial ways: in regulating initiator supply and in effecting handcuffing.

The dimers have also been postulated to participate in handcuffing without undergoing conformational change. In R6K, in which dimers can bind to iterons using one subunit, the other can be used for handcuffing (Urh *et al.*, 1998). In other words, the dimerization and handcuffing domains are one and the same in R6K. In RK2, dimers may bridge two iteron-bound monomers so that there can be a tetrameric bridge in handcuffing (Toukdarian and Helinski, 1998). The results in RK2 not only suggest a direct role for so-called inactive dimers in handcuffing, but also imply that the dimerization and handcuffing domains can be distinct. In R6K also, a coupling-defective mutant has been found that is apparently intact in dimerization, suggesting that handcuffing and dimerization domains are not identical (Miron *et al.*, 1992).

Initiation

Iteron-carrying plasmids replicate by generating theta-form intermediates. The stages of replication in this mode are basically understood from the pioneering work on *oriC*

plasmids (Bramhill and Kornberg, 1988). This programme is apparently modified by the presence of iterons and Rep proteins, as plasmid replication needs to be independent from the controls that operate on the host chromosome. Plasmid copy number with respect to the host chromosome can vary at birth in different cells (because of either random distribution of plasmid copies or replication error in the mother cell). Adjustment for this fluctuation in copy number by varying the rate of plasmid replication in different cells is crucial for plasmid maintenance. In other words, plasmids cannot afford to replicate in synchrony with the host chromosome. Below, we discuss how Rep proteins could be helping to make plasmid replication independent of host controls.

For *oriC*, the key effector of initiation is DnaA. It binds to the origin at multiple sites, called DnaA boxes, and organizes the DNA so that it can melt with energy supplied by ATP hydrolysis and negative superhelicity. DnaA uses its own ATP binding and hydrolysing activities for *oriC* melting. For all well-studied, iteron-carrying plasmids, DnaA also serves as an initiator, but the requirement for ATP is not obligatory (Lu *et al.*, 1998; Skovgaard *et al.*, 1998). Instead, origin melting requires Rep binding to iterons (Kawasaki *et al.*, 1996; Doran *et al.*, 1998; Park *et al.*, 1998). The Rep proteins are not ATPases themselves and most probably help by organizing the origin DNA so that it can melt. The involvement of Rep implies that plasmid replication initiation cannot be solely under the control of host initiators.

DnaA not only initiates *oriC* melting, it interacts with DnaB helicase and directs it to the origin for more extensive and rapid unwinding (Marszalek *et al.*, 1996). The DnaB in turn loads the primase DnaG that lays primers for new DNA synthesis on the melted strands of template DNA (Lu *et al.*, 1996; Fang *et al.*, 1999). The DnaA boxes in plasmid origins were therefore thought to provide the platform for loading DnaB and, subsequently, DnaG to the plasmid origin (Seufert and Messer, 1987). The observation that Rep can interact with DnaA indicated that plasmids have direct control of these important steps of initiation (Lu *et al.*, 1998). This view is further supported by the finding that Rep interacts directly with both DnaB (Ratnakar *et al.*, 1996; Datta *et al.*, 1999) and DnaG (D. Bastia, unpublished). These observations not only add to the centrality of Rep proteins in initiation, but also help us to understand how plasmids use host resources in competition with the host.

Initiation control

As discussed in the *Introduction*, the control of plasmid copy number is such that the initiation rate per plasmid copy is a decreasing function of plasmid concentration (autoregulation or negative feedback regulation). Iterons

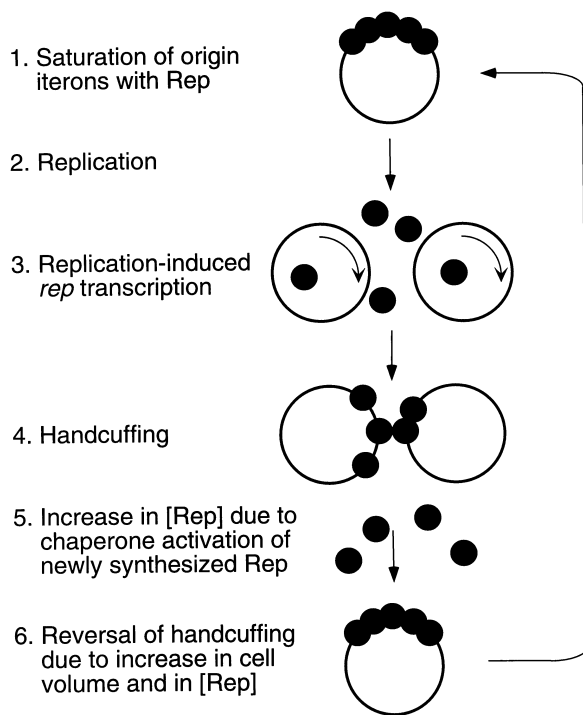


Fig. 3. A model showing the role of Rep protein in the replication cycle of plasmid P1. In this model, replication is activated by increase in [Rep] and inhibited by handcuffing. 1. Rep saturation of origin iterons not only effects initiation but also represses the *rep* promoter that maps within the origin iterons (autorepression). 2 and 3. Replication activates the promoter by cleaning the iterons of bound Rep. Newly made Rep molecules perhaps associate readily to form dimers (not shown) that are inactive in iteron binding. 4. The pre-existing active Rep gets titrated by daughter origins, which allows handcuffing and promoter repression. 5 and 6. Handcuffing is effectively reversed by increases in cell volume and in active [Rep]. Dilution may also help in Rep activation, as the active Rep gets inactivated upon concentration *in vitro* (Wickner *et al.*, 1991). Requirement for Rep activation could be a mechanism to delay initiation. Additionally, Rep saturation could be slow in view of steric hindrance from handcuffing (Mukhopadhyay *et al.*, 1994). Artificial increase in [Rep] only shows a modest increase in copy number, apparently because even a modest increase in copy number beyond the steady-state value increases the strength of handcuffing too much for Rep to undo (stringent or switch-like control). In this model, Rep acts as a positive regulator only at or below the steady-state concentration of iterons.

are clearly the negative regulators. Copy numbers decrease without exception when the iteron concentration is increased *in cis* or *in trans* (Helinski *et al.*, 1996). This implies that it is the increase in iteron concentration with increase in copy number that eventually turns replication off. As iterons function by binding Rep proteins, it was initially thought that iterons titrate initiators and thereby make them limiting for replication (Uga *et al.*, 1999). However, increasing Rep beyond the physiological concentration was of little consequence to the copy number in several different plasmids (Pal and Chattoraj, 1988; Durland and Helinski, 1990; Garcia de Viedma *et al.*,

1996; Uga *et al.*, 1999). Handcuffing now appears to be a more reasonable model for negative control (Fig. 3). Copy-up mutants of Rep have a coupling defect *in vitro* in several plasmid systems (Miron *et al.*, 1994; Mukhopadhyay *et al.*, 1994; Blasina *et al.*, 1996; Uga *et al.*, 1999).

Recently, we have compared copy numbers in isogenic cells carrying either plasmid monomers or plasmid dimers. The origin concentration in dimer-only cells was one-fifth of that present in monomer-only cells. This difference could be eliminated using copy-up mutants of Rep. Physical evidence for coupling of origins could also be obtained *in vivo* (K. Park, E. Han and D. K. Chattoraj, unpublished). Apparently, dimers allowed coupling of origins more easily because of their higher local concentration and/or helped in the stability of the coupled complex. Thus, handcuffing can be a mechanism for negative control.

How handcuffing blocks initiation is not known. As organizing the origin DNA with bound initiators into an orderly complex is widely viewed as a requirement for strand melting, handcuffing could be causing steric hindrance to the complex formation. We have also proposed that handcuffing prevents induction of the autorepressed *rep* gene of P1, and this could limit Rep availability (Chattoraj *et al.*, 1988). Additionally, handcuffing could interfere with Rep binding to iterons (Mukhopadhyay *et al.*, 1994).

Is handcuffing the only mode of negative control? As discussed above, so far, only a limited number of copy-up mutants has been tested for handcuffing defects *in vitro*, and most are found to be deficient in handcuffing. This correlation underscores the importance of handcuffing in negative control. How is handcuffing reversed to effect initiation? This, I believe, occurs as the result of an increase in cell volume, and is controlled at the level of initiator synthesis and post-translational initiator conformation, as discussed below.

How is initiation ensured (positive control)?

This is a concern if initiation factors are limiting. Otherwise, with increasing cell volume, dissociated products of handcuffing (plasmid monomers) should accumulate following the law of mass action, and this ought to suffice for initiation. Alternatively, build-up of initiator concentration may be required to saturate the origin iterons (positive control), even if the dissociation of handcuffed complexes is spontaneous. It is also possible that the initiator concentration needs to be increased to dissociate the complexes actively by favouring intramolecular folding of the origin. There is no particular reason to doubt that initiation factors are limiting. The two initiators, Rep and DnaA, that could possibly have positive control activities are both autoregulated proteins; their concentration

should be nearly constant during cell growth. Moreover, as discussed earlier, while ATP-DnaA is the initiator for *oriC* replication, ADP-DnaA suffices for plasmid replication, and increasing Rep concentration does little to increase plasmid copy number under normal conditions. However, positive regulatory roles of initiators are seen in situations that reduce replication. Itron-mediated reduction in copy number can sometimes be overcome by increasing Rep protein concentrations (Kim and Meyer, 1985; Park *et al.*, 1998). In other words, Rep can provide the positive force to counter handcuffing.

The positive regulatory role of initiators has also been seen in somewhat more artificial situations that reduce replication. Although a limited increase in Rep supply helps in replication, a surfeit of Rep supply can even stop replication in many plasmids (Filutowicz *et al.*, 1986; Muraiso *et al.*, 1990; Miller and Cohen, 1999). A proposed mechanism for this inhibition, which is an extension of the handcuffing model, is that origin-bound initiators become covered with a second layer of initiators, not themselves bound to iterons (McEachern *et al.*, 1989). This layering is likely to pose steric problems for Rep interaction with host factors such as DnaA, thus blocking replication (D. Bastia, personal communication). Another mechanism suggested from studies on pSC101 is that plasmid copies clump into a network mediated by handcuffing at multiple iteron sites (Miller and Cohen, 1999). In other words, at abnormally high concentration, Rep exacerbates handcuffing rather than reversing it. An interesting finding in this connection is that increased production of DnaA and DnaB can reverse replication inhibition and plasmid instability resulting from Rep overproduction (Miller and Cohen, 1999). The initiation factors apparently help by committing origins to the initiation mode that precludes handcuffing. A relevant finding in P1 replication *in vitro* is that origins fail to act as inhibitors (*inc* elements), although *in vivo* intact origins are as good as iterons in blocking replication *in trans* (Abeles and Austin, 1991; Park *et al.*, 1998). Similarly, when RK2 plasmids contain additional iterons outside the origin, the plasmid replication *in vitro* requires additional DnaA (Kittell and Helinski, 1991). These results suggest that it is possible to over-ride the inhibitory effect of iterons by spiking the *in vitro* systems with initiation factors.

Increased negative superhelicity can be another mechanism for promoting initiation and negating handcuffing. These insights have come from studies on the pSC101 *par* locus (Miller and Cohen, 1993). The locus, a gyrase binding site, increases Rep–iteron interaction by increasing negative superhelicity. It also helps in plasmid distribution (hence the name *par*), believed to be by reducing handcuffing. In other words, one way to reduce handcuffing could be to improve conditions that help initiator binding.

The suggestion that replication is controlled by a competition between positive and negative forces, with positive winning once per cell cycle on average per plasmid, appears more reasonable than control by a single negative feedback loop (von Meyenburg and Hansen, 1987; Manen and Caro, 1991; Boye *et al.*, 1996). The existence of positive control can also be argued in the extreme case in which the copy number at birth can be as low as one per cell. When there is one plasmid per cell, there cannot be copy number-dependent negative feedback to prevent replication, yet replication is found to be spread out all over the cell cycle (Helmstetter *et al.*, 1997). Availability of positive factors most probably dictates the timing of replication, as is currently believed to be the case for the timing of *oriC* replication (Løbner-Olesen, 1999).

In summary, I would like to call P1 Rep a positive regulator of copy number. This view is based on the fact that the copy number of P1 goes down when Rep is reduced below the physiological level. This is more clearly seen in miniP1 derivatives deleted for the extra iterons outside the origin. These plasmids replicate with a copy number eightfold higher than the wild-type plasmid, but also make about three times more protein than the wild-type plasmid. The extra protein is required to sustain the higher copy number (Pal and Chattoraj, 1988). In other words, physiological Rep may not be limiting, but it is not in excess either. Similar results have been obtained recently for miniF (Uga *et al.*, 1999). Whether a protein is a regulator or not can sometimes be judged only by reducing its concentration from the physiological value, as was the case with ColE1 Rom and R1 CopB negative regulators (Summers, 1996).

Economy of control

The efficiency of antisense control has been discussed in terms of its cost to the host (Paulsson *et al.*, 1998). Rapid correction of fluctuation in copy number requires, among other things, rapid turnover of the antisense RNAs, which is a costly business. Without a rapid turnover, the concentration of the RNA no longer remains proportional to the plasmid copy number and causes the plasmid copy number to oscillate (Summers, 1996). Iterons do not make any product; their copy number is always proportional to the plasmid copy number. In this sense, the control system cannot get any more economical.

Economy is also evident in other plasmid operations. Origin iterons have dual roles: they serve in initiation and in its negative control. The initiator genes are auto-repressed. We have found that replication induces transcription of the P1 *rep* gene (S. Mukhopadhyay and D. K. Chattoraj, 2000). Thus, both repression and

induction of P1 *rep* are accomplished by exploiting the required steps of initiation.

We have already described how, because of handcuffing, the copy number in cells with dimer plasmids is much less than that in cells with monomer plasmids. The handcuffing mechanism can therefore avoid drifting of the plasmid population towards dimeric and higher oligomeric forms by specifically reducing their replication rates compared with monomers. In ColE1, this process requires the synthesis of a new gene product (Hodgman *et al.*, 1998).

A common theme among copy number control systems

A critical concentration of DnaA-ATP triggers the initiation of *oriC* replication. Reinitiation is prevented by limiting new DnaA synthesis temporarily by sequestering the promoter of *dnaA*, titration of existing DnaA particularly by the high-affinity *dat* locus and post-initiation inactivation of DnaA-ATP by the β clamp of PolIII (Løbner-Olesen, 1999). The picture is similar in plasmids controlled by antisense RNA (del Solar and Espinosa, 2000). In these plasmids, initiators are also positive regulators: copy number increases with an increase in initiator concentration. The negative regulators (antisense RNA and, sometimes, a protein repressor) function by reducing initiator concentration. There is also post-initiation inactivation of initiators (Rasooly *et al.*, 1994).

Unlike the systems described above, negative regulation by iterons appears to be primarily by handcuffing, although initiator limitation is very much involved. Like the above systems, availability of active (monomeric) Rep is crucial for initiation, and the protein acts as a positive regulator by rescuing situations that reduce copy number. In fact, handcuffing could limit the initiator by preventing its synthesis through autorepression and preventing its binding to the origin by steric hindrance. Rep proteins are also inactivated upon dimerization. Although details may vary, limiting initiators appear to be common in negative regulation of plasmid copy number.

Concluding remarks

Recent progress in our understanding of iteron-mediated control has been significant. (i) Physical evidence for handcuffing *in vivo* has now been obtained with P1 Rep, as was the case *in vitro* for Rep from several plasmids. The primary mechanism that prevents plasmid over-replication appears to be handcuffing. (ii) The regulatory role of initiator concentration that confounded the field for years is now better understood. We believe from studies in P1 that extra Rep can overcome handcuffing as long as the iteron concentration is below or close to the steady-state value. Within this limit, Rep can be thought

of as a positive regulator that guarantees initiation. (iii) Rep proteins of R6K and pSC101 have now been shown to interact directly with host proteins, DnaA, DnaB and DnaG. This shows the importance of Rep proteins as effectors of initiation. (iv) The high-resolution structure of the Rep–iteron complex of F has not only revealed the details of Rep monomer binding to an iteron for the first time, but has also made obvious the need for a conformational change in protein dimerization. (v) The interfaces of dimerization and handcuffing appear to be largely overlapping in F Rep, although dimers cannot bind iterons unless activated by chaperones. Modulation of initiator conformation appears to be a dominant feature of iteron-mediated control.

Studies on λ repressor and its interactions with three operators illustrated how an epigenetic switch uses multiple sites on DNA (Gottesman, 1999). Origin iterons are also at the heart of an epigenetic switch that turns replication on and off, but how the multiple iterons are exploited in the switch remains to be understood. The presence of multiple iterons can perhaps be appreciated in situations in which the plasmid is maintained at one or two copies per cell. As statistical fluctuations are inherent in small numbers, the fluctuation would decrease if the control system counts 14–28 iterons (using the P1 example, which normally has 14 iterons) rather than one or two plasmids. Iterons can function individually in control and do not have to be presented as a set (Abeles *et al.*, 1995). They thus appear to be suited for a multistep mode of negative control. As has been discussed for antisense control, in a multistep mode of control, multiple opportunities for interactions between the control element and its target make the switch more sensitive to fluctuation in copy number than single-step inhibitory mechanisms (Koshland, 1998; Paulsson *et al.*, 1998). Multiple iterons may have been selected to reduce fluctuation in plasmid copy number distribution and thereby increase plasmid stability. Measuring the relationship of copy number distribution to varying iteron numbers is now a realizable goal (Løbner-Olesen, 1999).

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