

## MicroReview

# Plasmid copy number control: an ever-growing story

Gloria del Solar\* and Manuel Espinosa

Centro de Investigaciones Biológicas, CSIC, Velázquez, 144, E-28006 Madrid, Spain.

### Summary

Bacterial plasmids maintain their number of copies by negative regulatory systems that adjust the rate of replication per plasmid copy in response to fluctuations in the copy number. Three general classes of regulatory mechanisms have been studied in depth, namely those that involve directly repeated sequences (iterons), those that use only antisense RNAs and those that use a mechanism involving an antisense RNA in combination with a protein. The first class of control mechanism will not be discussed here. Within the second class (the most 'classical' one), exciting insights have been obtained on the molecular basis of the inhibition mechanism that prevents the formation of a long-range RNA structure (pseudoknot), which is an example of an elegant solution reached by some replicons to control their copy number. Among the third class, it is possible to distinguish between (i) cases in which proteins play an auxiliary role; and (ii) cases in which transcriptional repressor proteins play a real regulatory role. This latter type of regulation is relatively new and seems to be widespread among plasmids from Gram-positive bacteria, at least for the rolling circle-replicating plasmids of the pMV158 family and the theta-replicating plasmids of the Inc18 streptococcal family.

### Introduction

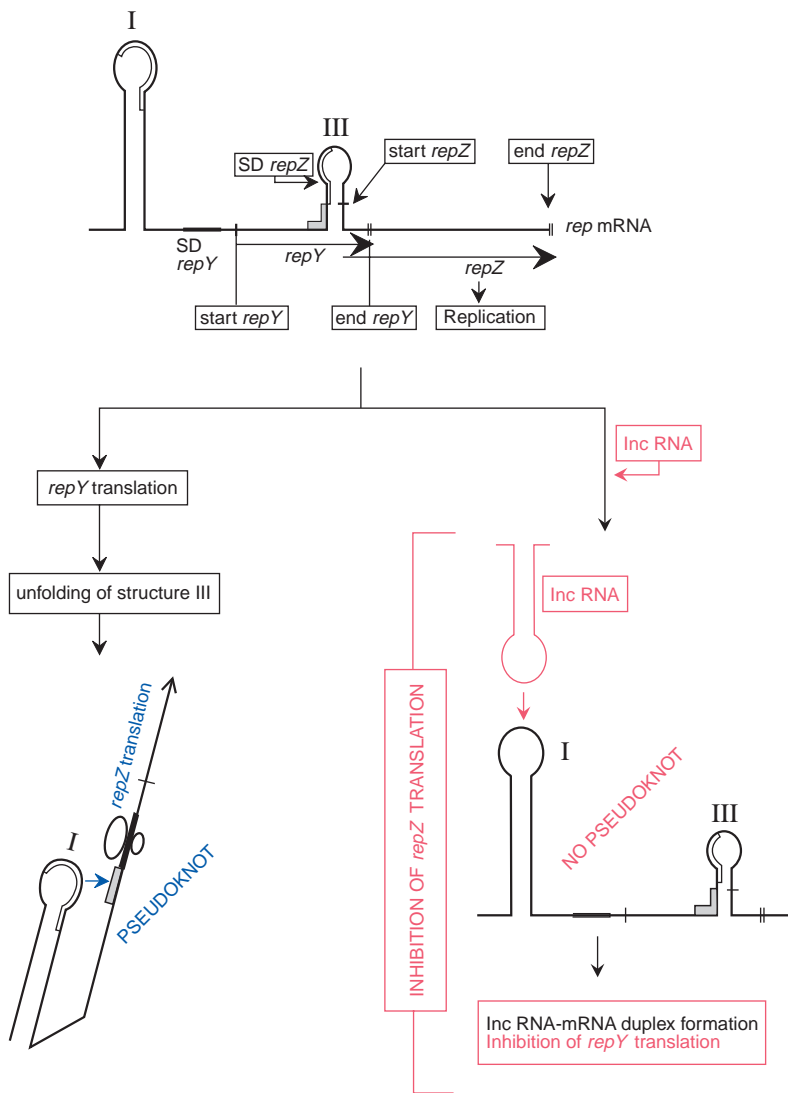
Bacterial plasmids are extrachromosomal genomes that replicate autonomously and in a controlled manner. Many plasmids are self-transmissible or mobilizable by other replicons, thus having the ability to colonize new bacterial species. Plasmids may provide the host with valuable functions, such as drug resistance(s) or metabolic pathways useful under certain environmental conditions,

although they are likely to constitute a slight metabolic burden to the host. To co-exist stably with their hosts and to minimize the metabolic load, plasmids must control their replication, so that the copy number ( $N$ ) of a given plasmid is usually fixed within a given host and under defined cell growth conditions.

It is convenient to distinguish two different stages of the plasmid life cycle. The first, termed establishment, occurs when a plasmid copy enters a new permissive host. A successful establishment may depend upon the plasmid's ability to replicate rapidly before the host divides (Highlander and Novick, 1987). This may result in an overshoot in  $N$  before it reaches the characteristic value ( $N_{av}$ ). In the second stage, the replicon enters into the steady state in which  $N_{av}$  is maintained because, on average, there is one replicative event per plasmid copy and cell cycle (Nordström and Wagner, 1994). To maintain this average rate, plasmids use self-encoded negative control systems that are able to 'sense' and correct up and down fluctuations from the  $N_{av}$  in individual cells (Summers, 1996). These control systems adjust the rate of replication per plasmid copy, so that it becomes higher or lower than 1, depending on whether there has been a decrease or an increase, respectively, in copy number with respect to the  $N_{av}$  in a given cell. Thus, the  $N$ -values from individual cells of a population should follow a narrow Gaussian distribution when the regulatory circuits are functioning under optimal conditions. This would not be the case for a replicon lacking specific control functions, such as the *oriC* minichromosomes (the cloned origin of replication of *Escherichia coli*). Narrow distribution of  $N$  in several plasmids and broad deviations in *oriC* minichromosomes have been measured by a combination of flow cytometry and plasmid-driven expression of the green fluorescent protein (Løbner-Olesen, 1999).

There are three general types of plasmid copy number control systems, depending on the type of negative control element used: (i) directly repeated sequences (iterons) that complex with cognate replication (Rep) initiator proteins; (ii) antisense RNAs that hybridize to a complementary region of an essential RNA, therefore termed countertranscribed (ct) RNAs; and (iii) ctRNA and a protein. Within this last group, there are two categories. In one of them, the ctRNA plays the main regulatory role, whereas the protein has been proposed as only an

Accepted 9 May, 2000. \*For correspondence. E-mail gdsolar@cib.csic.es; Tel. (+34) 91 561 1800; Fax (+34) 91 562 7518.



**Fig. 1.** Control of replication by pseudoknot formation in Collb-P9. Genes *repY* (for leader peptide) and *repZ* (for initiator of replication) are translationally coupled. On the mRNA, the Shine-Dalgarno sequence (SD) of *repY* is exposed, whereas the SD and the initiation codon of *repZ* can be occluded within structure III. If there is no interaction with Inc RNA (left), *repY* translation takes place. Stalled ribosomes at the end of *repY* unfold structure III, allowing the formation of the pseudoknot between the region located on the loop of structure I (double lines) and its complementary region (stippled rectangle). This facilitates binding of the ribosomes (ellipses) to the *repZ* SD sequences, followed by translation of *repZ*. The antisense Inc RNA (right) would hinder pseudoknot formation and translation of *repY*, leading to inhibition of *repZ* translation. Events and elements acting positively in replication are indicated in blue, whereas negative ones are depicted in red.

auxiliary element. Within the second category, both elements, acting on different targets, could correct fluctuations in the  $N$ -value at the steady state (del Solar *et al.*, 1995; 1998). Replication control by iterons will be covered in the accompanying review by Chattoraj, this issue pp. 467–476. Here, we will consider the other two types of mechanisms of replication control, focusing on cases in which significant advances have recently been achieved. The first case, including plasmids that use only ctRNA as a control element, involves the inhibition of the formation of a long-range RNA structure (the pseudoknot), which actively enhances translation of an essential replication initiator gene. The second focus will be on those systems that include proteins playing an auxiliary regulatory role. Next, we will pay attention to those replicons with two plasmid-encoded control elements. There are few reports on plasmids using this last control mechanism but, considering their efficiency, it is likely that

they will be found to be more widespread. Finally, a single instance of a new control mechanism, involving only the Rep protein in plasmids lacking iterons, will be discussed briefly (Burian *et al.*, 1999).

### Control by ctRNAs

Control systems using ctRNAs (Wagner and Brantl, 1998) are widespread within plasmids replicating by different mechanisms, but sharing a similar genetic structure in the control region: two oppositely oriented promoters direct, respectively, the synthesis of an RNA essential for replication and of the inhibitor ctRNA. The ctRNAs are complementary to a region (the target) near the 5' end of the essential RNA. An important feature of this kind of control system is that the rate of synthesis of the inhibitor ctRNA is much higher than that of the essential RNA. In addition, the ctRNAs are synthesized from a constitutive

promoter and have a short half-life, so that their intracellular concentration stays nearly proportional to *N*. Replication is inhibited by RNA–ctRNA pairing and abolition of the essential RNA activity. Differences between various replicons regulated by ctRNAs are found based on how the inhibition occurs and can be exemplified as follows: (i) inhibition of maturation of the primer essential for replication, as in plasmid ColE1 (Tomizawa and Itoh, 1981); (ii) premature termination of the synthesis of the essential *rep*-mRNA (pT181; Novick *et al.*, 1989); and (iii) inhibition of *rep* translation. This last method of ctRNA-dependent copy number control can be achieved by three mechanisms, namely inhibition of translation of a leader peptide (plasmid R1; Blomberg *et al.*, 1992), inhibition of both translation of a leader peptide and formation of an activator pseudoknot (Collb-P9; Asano *et al.*, 1991; Asano and Mizobuchi, 1998a) and direct inhibition of translation of the essential *rep* gene. A direct inhibition mechanism has been proposed for plasmid ColE2, in which the formation of a stable complex between the ctRNA and the complementary region in the *rep* mRNA would be able to inhibit *rep* expression, even though the ctRNA does not overlap the putative translation initiation region of the essential gene (T. Itoh, personal communication).

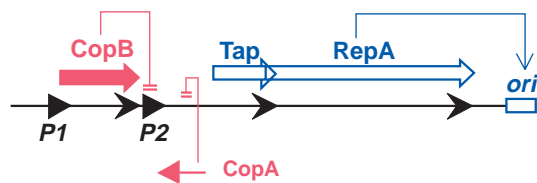
For a number of plasmids, studies on the kinetics of interaction between ctRNAs and their RNA targets have shown that stable duplex formation is not required for inhibition of *rep* expression, as this inhibition takes place faster than stable binding (reviewed by Wagner and Brantl, 1998). In contrast, a recent report on the ctRNA–*rep* mRNA interactions of the staphylococcal plasmid pT181 shows that the rate constant of stable complex formation is similar to the inhibition rate constant (Brantl and Wagner, 2000).

New findings on the molecular and biochemical bases for the control mechanism involving an intramolecular RNA–RNA interaction within the leader region of the *rep* mRNA have been provided for the Inc $\alpha$  Collb-P9 and the IncL/M pMU604 plasmids (Asano and Mizobuchi, 1998a; 2000; Athanasopoulos *et al.*, 1999). Replication depends on the expression of the essential *rep* gene, which requires coupled translation of a gene encoding a leader peptide and the formation of a pseudoknot by intramolecular pairing of two complementary sequences of the *rep* mRNA (Asano *et al.*, 1991; Wilson *et al.*, 1993).

The model for the control of replication of Collb-P9 (reviewed by Wagner and Simons, 1994) involves two stem–loop structures in the *repZ* mRNA that have been mapped *in vitro* (Asano and Mizobuchi, 1998b) and are located upstream (structure I) and in the middle (structure III) of the leader *repY* gene (Fig. 1). Structure III occludes both the translation initiation sequences of the essential *repZ* gene and a short sequence complementary to a

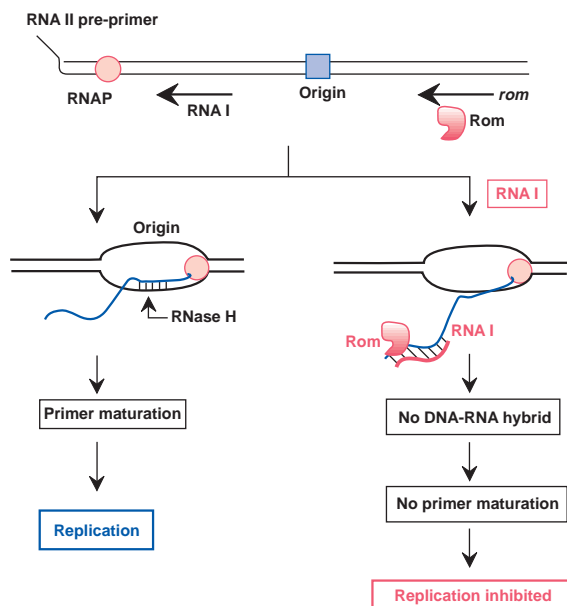
region in the loop of structure I. Appropriate termination of translation of the leader *repY* gene unfolds structure III, thus inducing the formation of the pseudoknot by intramolecular pairing of loop I with its complementary sequence. Pseudoknot formation facilitates the binding of the ribosomes to the Shine–Dalgarno sequence of the essential *repZ* gene. Mutations causing disruption of structure III have allowed the mapping of the pseudoknot *in vitro* (Asano and Mizobuchi, 1998b). Translation of *repY* and pseudoknot formation are inhibited by the interaction of the ctRNA (Inc RNA) with the complementary region of the *rep* mRNA. Generation of the duplex Inc RNA–target mRNA occludes the *repY* ribosome binding site, leading to an indirect inhibition of *repZ* translation. In addition, pairing between the single-stranded loops on the complementary structures of *rep* mRNA (structure I) and Inc RNA is sufficient to block generation of the pseudoknot, so that Inc RNA can repress *repZ* translation at the level of a transient interaction with its target before a stable duplex is detected. The same region in the loop of structure I of the *rep* mRNA is involved in the initial interactions that take place in pseudoknot formation and in Inc RNA binding. These initial interactions are stimulated by the presence of a hexanucleotide (conserved in various antisense systems; Asano and Mizobuchi, 1998a), which presumably supports the element termed U-turn and includes the structure I sequence involved in the initial interactions. The U-turn loop structures are general binding rate enhancers that facilitate rapid RNA–RNA interactions (reviewed by Franch and Gerdes, 2000). As the early stages in pseudoknot formation and in the binding of the inhibitory RNA are similar, an explanation as to how these two processes can compete with each other is easily deduced (Asano and Mizobuchi, 1998a). Although Inc RNA represses the translation of *repY* and *repZ* genes, repression of the former is much less efficient than that of the latter. Repression of *repZ* and *repY* expression are accomplished at different stages during the pairing between Inc RNA and *rep* mRNA (Asano and Mizobuchi, 2000). This differential repression allows the Inc RNA to keep the total level of *repZ* expression constant. A constant total rate of synthesis of the initiator was demonstrated early for IncFII plasmids (Nielsen and Molin, 1984). As the level of *repZ* expression is rate-limiting for replication, the Inc RNA-based regulation mechanism maintains a constant *N*-value.

A similar genetic structure and control mechanism were shown to exist in the IncB plasmid pMU720, closely related to the Inc $\alpha$ -Collb-P9 (Siemerling *et al.*, 1994; Wilson *et al.*, 1994). However, some differences are found in plasmid pMU604, belonging to the IncL/M group (Athanasopoulos *et al.*, 1999). In this case, replication control also involves translational coupling of a leader



**Fig. 2.** Auxiliary elements controlling plasmid copy number: the R1 paradigm. The initiator RepA protein acts on the origin of replication (*ori*) located downstream of the *repA* gene. Expression of *repA* is translationally coupled to that of *tap* (encoding a small leader peptide). CopB protein represses transcription from promoter *P2*, so that this promoter is normally silent. Transcription of the *copB*–*tap*–*repA* mRNA is mainly directed by the constitutive weak promoter *P1*. The antisense RNA, CopA, acts by blocking the translation of *tap*. Negatively acting elements are depicted in red; those acting positively are shown in blue.

gene and the formation of a pseudoknot, although, unlike Collb-P9, the positioning of sequences involved in the expression of the essential gene *repA* is different. The most important variation is the spacer between the pseudoknot and the translation initiation region of the essential *repA* gene, which seems to be suboptimal in pMU604. Mutational analyses showed that the requirement for pseudoknot formation in pMU604 could be partially replaced by improving the initiation of translation signals of the essential gene *repA*. Interestingly,



**Fig. 3.** Copy number control in ColE1. Synthesis of the preprimer RNA II by RNAP (stippled circle) is essential for replication. In the absence of interaction with the RNA I (left), the RNA II forms a stable hybrid with the template DNA at the origin of replication. This hybrid is cleaved by RNase H to generate the 3'-OH end of the RNA primer, from which replication starts. Interaction between the inhibitor RNA I and the complementary region in the RNA II preprimer (right) is aided by Rom protein (ellipse). RNA I–RNA II interaction inhibits the formation of the DNA–RNA II hybrid at the origin region, preventing maturation of the RNA II into the replication primer. Colours as in the legends to Figs 1 and 2.

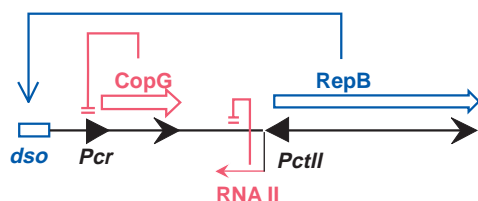
pseudoknot formation inhibited translation of the leader gene in pMU604 (Athanasopoulos *et al.*, 1999), a feature that has not been reported to exist in Collb-P9. The existence of such complex regulatory circuits in other plasmids awaits further investigation.

### Accessory proteins as inhibitory elements

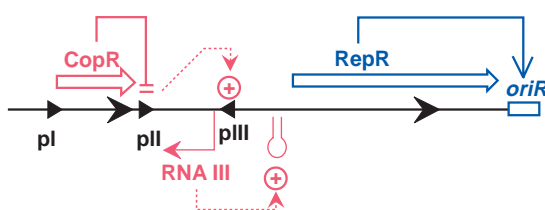
Plasmids R1 and ColE1, which control their *N*-values by ctRNAs, also encode a protein as a second inhibitory element that, however, plays only an auxiliary role. In the case of R1, expression of the essential gene *repA* requires the translation of the leader gene *tap*, which is translationally coupled to *repA* (Blomberg *et al.*, 1992). The main replication control element is the ctRNA, CopA, which inhibits translation of *tap* and, indirectly, that of *repA*. The second inhibitory element of R1 is the product of the *copB* gene, which is co-transcribed with *tap* and *repA* from promoter *P1* (Fig. 2). CopB protein is a transcriptional repressor of a second promoter, *P2*, located downstream of *copB*, which directs the synthesis of a *tap*–*repA* mRNA. At steady state, CopB is present at saturating concentrations, blocking transcription from *P2*, so that *repA* is expressed almost exclusively from *P1*. Deletion of the entire *copB* gene (including promoter *P1*) results in plasmids with an eightfold increase in *N* (Riise *et al.*, 1982). The CopB regulatory loop has been suggested to serve as a rescue mechanism that prevents plasmid loss in newborn cells harbouring very few plasmids. However, computer simulation of mini-R1 plasmid replication indicated that the CopB regulatory circuit contributes little to the stability of these replicons (Rosenfeld and Grover, 1993).

The second instance of plasmids with auxiliary proteins is ColE1 (Fig. 3). In this case, replication is mediated by the synthesis of a preprimer RNA (RNA II) by the host RNA polymerase (RNAP) and the formation of a DNA–RNA hybrid between the RNA II and the template DNA strand at the origin region. This hybrid is cleaved by RNase H, generating a 3'-OH end, which is used by DNA polymerase I to initiate leading strand synthesis. The availability of the primer 3'-OH end is rate-limiting for initiation, and it is modulated by the ctRNA I control element. Interaction between ctRNA I and its complementary region in the preprimer alters the secondary structure of the latter, leading to the inhibition of stable DNA–RNA hybrid formation. This, in turn, leads to inhibition of replication. The second element of this circuit is protein Rom (Rop), which enhances the rate of formation of a stable complex between the ctRNA I and the preprimer RNA. Rom does not seem to be an essential component of the ColE1 control system. Deletion of the *rom* gene leads to a two- to threefold increase in *N* in slowly growing cells, but it has no

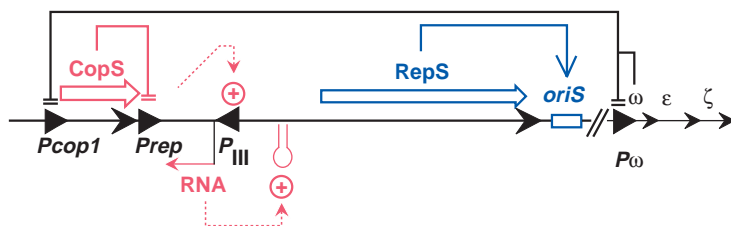
## pMV158



## pIP501



## pSM19035



phenotypic consequences on the  $N$ -value in fast-growing bacteria (Atlung *et al.*, 1999). When cloned on a compatible multicopy plasmid, the *rom* gene is able to complement a *rom*<sup>-</sup> derivative, although it has no further effect upon the replication of a co-resident ColE1. The absence of incompatibility caused by extra copies of *rom* shows that Rom is not a primary inhibitor of ColE1 replication, as it exerts its maximum effect at the wild-type concentration (Summers, 1996). Mathematical models of the dynamic features of copy number control in ColE1 and the experimental observations about the small effect caused by variations in the dosage of the *rom* gene have left open the question of why there is a Rom protein (Paulson *et al.*, 1998). At least three theoretical proposals have been made to account for an important role of Rom in the dynamics of ColE1 copy number control (Paulson *et al.*, 1998). First, Rom concentration would be proportional to the  $N$ -value, so that the response in replication frequency to variations in the  $N$ -value would be sharper than if RNA I acted alone. This hypothesis requires that Rom is rapidly degraded, which has not been tested

experimentally. Secondly, Rom would act by making the probability of plasmid replication very close to zero at high ctRNA I concentration because, in the absence of Rom, the intrinsic rate of ctRNA I–RNA II duplex formation would be too slow to ensure total inhibition of replication. Thus, Rom would ensure an efficient copy number control system. Thirdly, Rom could act as a back-up system when  $N$  (and, subsequently, Rom concentration) is greatly reduced: under normal conditions, the replication frequency would not depend on small deviations in Rom concentration but, if this concentration decreases greatly (as a result of a large reduction in  $N$ ), inhibition of primer formation would decrease, thus leading to an increase in the replication frequency. However, and as far as we are aware, no experiments have been performed to clarify these hypotheses. On the other hand, experimental evidence has shown that the presence of ColE1 derivatives lacking *rom* reduced bacterial growth in medium impoverished in carbon sources, whereas *rom*<sup>+</sup> derivatives did not show an adverse effect on cell growth (Atlung *et al.*, 1999). This is thought to be related to the

**Fig. 4.** Dual regulation of plasmid copy number, as exemplified by pMV158 (top) and by two members of the Inc18 plasmid family (middle and bottom). In pMV158, the initiator protein, RepB, acts on the leading strand origin, *dso*. The CopG repressor protein blocks transcription from promoter  $P_{cr}$ , which directs synthesis of the *copG*–*repB* mRNA, so that CopG autoregulates its own synthesis. From promoter  $P_{ctII}$ , the ctRNA II is synthesized, which, in turn, blocks translation of *repB*. In the case of pIP501, the initiator RepR protein acts on the origin (*oriR*) located downstream of *repR*. The transcriptional repressor CopR regulates transcription from *pII*, which is the only promoter that directs the expression of *repR*. CopR is synthesized from promoter *pl*, which would be constitutive. From promoter *pIII*, the long-lived inhibitor ctRNA III is synthesized. Interaction of ctRNA III with its target in the leader region of the *repR* mRNA induces ( $\oplus$ ) generation of an mRNA secondary structure, which acts as a transcriptional attenuator, avoiding *repR* expression. CopR-dependent inhibition of *pII* increases transcription from *pIII* ( $\oplus$ ) by alleviating the effect of convergent transcription. The replication and control regions of pSM19035 share similarities in the genes, promoters and regulatory circuits to those of pIP501. The *segB* region is shown as composed of three genes, their expression being controlled by a single promoter ( $P_{\omega}$ ). The product of gene  $\omega$  regulates this operon and also the promoter of gene *copS*. The main regulatory circuits would be the same as in pIP501 (Brantl *et al.*, 1990). Colours as in the legends to previous figures.

amplification of ColE1-type plasmids in slowly growing cells, which is higher in plasmids lacking *rom*. From these observations, a key role for Rom protein has been envisaged, that is to prevent ColE1-type plasmids from representing a metabolic burden to their hosts in natural habitats, in which cells grow slowly because of nutrient limitation.

In conclusion, CopB and Rom are inhibitory elements that limit plasmid replication, as their inactivation leads to an increase in the *N*-value of R1 or ColE1 respectively. However, the genes encoding these proteins do not constitute an incompatibility determinant against the wild-type plasmid, so that both proteins would be auxiliary negative elements unable to correct up-fluctuations in *N*. However, the influence of these proteins on the dynamics of plasmid copy number control remains to be solved.

### Dual regulation by ctRNA and inhibitory protein

A novel mode of regulation of replication, involving a ctRNA and a transcriptional repressor protein, has been shown for two plasmid families represented by pMV158 (del Solar and Espinosa, 1992) and pIP501 (Brantl, 1994). Unlike plasmids controlling their replication only by ctRNAs, the promoter directing the expression of the essential *rep* gene in these plasmids is not constitutive, but regulated by a Cop protein. Although the dual circuits of control of *N* in these two plasmids share similarities, significant differences exist with respect to the features and inhibitory mechanisms of both regulatory elements.

In the case of pMV158, characterization of mutations that result in increased *N*-values and definition of DNA regions (*inc* determinants) that affect plasmid replication *in trans* showed a third general mechanism of replication control involving the transcriptional repressor protein CopG and the ctRNA (RNA II), both involved in regulation of synthesis of the initiator RepB protein (del Solar *et al.*, 1995). Gene *copG* is co-transcribed with *repB* from a single promoter ( $P_{cr}$ ), and the *copG* product binds to a DNA region that includes promoter  $P_{cr}$ , thus inhibiting transcription of the *cop-rep* operon (Fig. 4). Competition between purified CopG and RNAP proteins indicates that CopG impairs the binding of RNAP to promoter  $P_{cr}$  (our unpublished observations). CopG protein is very stable, at least when overproduced in *Escherichia coli*. Promoter  $P_{ctII}$  directs the synthesis of RNA II, which has a short half-life ( $\approx 2$  min, G. del Solar, unpublished) and seems to inhibit *repB* expression by direct pairing with the translation initiation signals of *repB*. Both CopG and RNA II are able to sense and correct up-fluctuations in the *N*-value at the steady state, and together constitute an *inc* determinant much more powerful than each element alone. This synergistic effect argues in favour of a primary role for both elements in pMV158 control of *N* and allowed us to

suggest the importance of derepression of the CopG-regulated  $P_{cr}$  promoter to overcome severe decreases in *N* (del Solar *et al.*, 1995). Owing to its autorepressor capacity, CopG would keep the level of synthesis of the *cop-rep* mRNA within narrow limits. Variable concentrations of RNA II (the concentration depending upon the *N*-value because of the constitutive synthesis of RNA II and of its short half-life) would act on these practically constant levels of mRNA. A similar genetic structure exists in all plasmids from the pMV158 family.

Three closely related streptococcal plasmids belonging to the Inc18 family (pIP501, pAM $\beta$ 1 and pSM19035) have been studied. In the case of pIP501, control of *N* involves the transcriptional repressor CopR and the antisense RNA III (Fig. 4). Promoter  $pI$  directs the expression of gene *copR*, whose product inhibits transcription from promoter  $pII$  located downstream of  $pI$  (Brantl, 1994). CopR-mediated repression does not completely inhibit transcription from  $pII$ , which is the only promoter that directs the expression of gene *repR*, essential for plasmid replication. A third promoter,  $pIII$ , directs the synthesis of RNA III, a ctRNA that inhibits the expression of *repR* by a mechanism of transcriptional attenuation (Brantl *et al.*, 1993), similar to that postulated for plasmids pAM $\beta$ 1 (Le Chatelier *et al.*, 1996) and pT181 (Novick *et al.*, 1989), so that interaction between *repR* mRNA and RNA III leads to premature termination of the mRNA synthesis. Interestingly, RNA III has an unusually long half-life of about 30 min, which would make it unable to correct down-fluctuations in *N* rapidly. To explain how the control system of pIP501 copes with a reduction in *N*, a dual function has been assigned to CopR (Brantl and Wagner, 1997). A decrease in *N* would result in a decrease in CopR concentration, which would lead to derepression of promoter  $pII$  and to the concomitant increase in the expression of *repR*. In addition, transcription from promoter  $pIII$  is inhibited by the increased convergent transcription from  $pII$ , so that the slow reduction in the amounts of intracellular stable RNA III inhibitor could be slightly accelerated.

In the case of plasmid pAM $\beta$ 1, the situation may be more complex. The repressor CopF (equivalent to CopR) not only inhibits RepF (equivalent to RepR) synthesis, but it may also decrease primer formation, as the activating transcription fork that passes through the origin of replication originates from the *repF* promoter (L. Janni re in Espinosa *et al.*, 2000). A further degree of complexity arises from new findings on plasmid pSM19035. In this plasmid, part of the *segB* region (involved in better-than-random segregation) contains genes  $\omega$ ,  $\varepsilon$  and  $\zeta$  (Fig. 4). These genes are organized in an operon controlled by the product of gene  $\omega$ . Purified Omega protein not only binds to its promoter region, but also to a DNA fragment containing the promoter region of gene *copS* (the

homologue of pIP501-*copR* and pAM $\beta$ 1-*copF*). Consequently, it would appear that plasmid partition and plasmid copy number control are under a common pathway of regulation (de la Hoz *et al.*, 2000).

There is a fundamental difference between the control mechanisms governed only by ctRNA and those governed by dual regulators. In the former, the rate of synthesis per plasmid copy of the essential RNA needed for replication is constant but rather low compared with that of the ctRNA. In the latter, expression of the *rep* gene is directed by a strong and Cop-regulated promoter so that, when the regulatory protein does not operate, there is a high rate of *rep* transcription. This seems to be at least the case for the pMV158-*P<sub>cr</sub>* promoter, which is the strongest promoter of the plasmid (our unpublished results). A high potential to transcribe the essential *rep* gene would represent an advantage for these plasmids during the establishment stage, as they would replicate rapidly to reach  $N_{av}$ , thus decreasing the frequency of appearance of plasmid-free cells from newly colonized bacteria. We have preliminary evidence suggesting that the efficiency of establishment of pMV158 derivatives is influenced by the activity of the promoter directing the expression of the *repB* gene. It is worth pointing out that plasmids from the Inc18 family and several replicons of the pMV158 family are mobilizable, and that these replicons have a very broad host range. Plasmids with auxiliary proteins involved in their control may also share the same advantage during the establishment stage (Summers, 1996).

#### Non-iteron non-ctRNA plasmid control?

It seems that a new mechanism for copy number control independent of antisense RNA and iterons operates in small cryptic plasmids of *E. coli*, with the Rep protein being the only plasmid-encoded factor involved in initiation and repression of replication (Burian *et al.*, 1999). The copy number control (*cop*) region, which also constitutes an *inc* determinant, includes the *rep* promoter and two Rep binding sites (BD1 and BD2). BD1 is close to the replication initiation region and seems to bind preferentially monomers/dimers of the Rep protein, whereas BD2 is adjacent to the *rep* promoter region and would appear to bind Rep oligomers preferentially. Initiation of replication would require binding of the Rep protein to both binding sites. Increases in  $N$ , and subsequent elevation of the Rep concentration, would favour the formation of Rep oligomers, displacing Rep monomers/dimers from BD2, thereby autorepressing *rep* transcription. Thus, control of replication is proposed to be exerted by the monomer-multimer Rep equilibrium and, unlike the iteron-based control systems, incompatibility in these replicons would easily be over-ridden by an excess of Rep protein.

Whether this mechanism is widespread among small plasmids of enterobacteria remains to be determined.

#### Perspectives

The recently reported new mechanisms controlling plasmid copy number show that these processes are more complex than previously envisaged. Pseudoknot formation, known for a number of years, has only recently been characterized in some depth. Mechanisms controlling the replication of newly described replicons, especially those isolated from extremophiles, are mostly unknown, and a novel control mechanism (still to be explored in some depth), independent of iterons and ctRNAs, seems to exist in small cryptic plasmids of *E. coli*. New and exciting findings will be derived from a general mechanism for interactions between antisense and target RNAs in prokaryotes. This mechanism involves rapid interaction between both RNAs mediated by a motif (Pyr-U-N-Pur, the so-called U-turn) that is conserved in RNA recognition loops in either the antisense or the target RNAs in many antisense RNA-regulated gene systems (Franch *et al.*, 1999; Franch and Gerdes, 2000). However, and in spite of the knowledge accumulated during recent years, there is a lack of structural studies on the elements involved in the regulation of replication, with a few exceptions. The structure of pMV158-CopG repressor has been solved both alone and in complex with its target DNA, and it has been shown that the CopG dimer has a ribbon-helix-helix structure, resembling that of the Arc repressor of phage P22 (Gomis-Rüth *et al.*, 1998). Modelling of the first 62 residues of the pIP501-CopR repressor indicates that it belongs to the helix-turn-helix family of DNA-binding proteins (Steinmetzer *et al.*, 2000), and preliminary X-ray diffraction data are available from the pSM19035-Omega repressor (Murayama *et al.*, 1999). In addition, a three-dimensional model of the antisense RNA of plasmid R1, together with *in vitro* structural determinations, have revealed that the antisense-mRNA binding product is not a full duplex, but a complicated extended complex that is stabilized by distal basepairings (Kolb *et al.*, 2000). Determination of the crystal structure of this antisense RNA may throw new light on the nature of the contacts between the two RNA species. Finally, and in spite of the early characterization of auxiliary proteins such as CopB or Rom, there seems not to be enough information on the precise role of these proteins in replication control at the steady state and during the plasmid establishment stage.

#### Acknowledgements

Thanks are due to D. Chatteraj for his critical reading of the manuscript, and T. Itoh for communicating his unpublished

results. The authors' laboratories were financed by CICYT (grants BIO97-0347 and 2FD97-0518, to M.E.) and by Comunidad Autónoma de Madrid (grant 07B/0049/1999 to G.d.S.).

## References

- Asano, K., and Mizobuchi, K. (1998a) Copy number control of IncI $\alpha$  plasmid Collb-P9 by competition between pseudoknot formation and antisense RNA binding at a specific RNA site. *EMBO J* **17**: 5201–5213.
- Asano, K., and Mizobuchi, K. (1998b) An RNA pseudoknot as the molecular switch for translation of the *repZ* gene encoding the replication initiator of IncI $\alpha$  plasmid Collb-P9. *J Biol Chem* **273**: 11815–11825.
- Asano, K., and Mizobuchi, K. (2000) Structural analysis of late intermediate complex formed between plasmid Collb-P9 Inc RNA and its target RNA. How does a single antisense RNA repress translation of two genes at different rates? *J Biol Chem* **275**: 1269–1274.
- Asano, K., Moriwaki, H., and Mizobuchi, K. (1991) Positive and negative regulations of plasmid Collb-P9 *repZ* gene expression at the translational level. *J Biol Chem* **266**: 3774–3781.
- Athanasopoulos, V., Praszkiel, J., and Pittard, A.J. (1999) Analysis of elements involved in pseudoknot-dependent expression and regulation of the *repA* gene of an IncL/M plasmid. *J Bacteriol* **181**: 1811–1819.
- Atlung, T., Christensen, B.B., and Hansen, F.G. (1999) Role of the Rom protein in copy number control of plasmid pBR322 at different growth rates in *Escherichia coli* K-12. *Plasmid* **41**: 110–119.
- Blomberg, P., Nordstrom, K., and Wagner, E.G.H. (1992) Replication control of plasmid R1: RepA synthesis is regulated by CopA RNA through inhibition of leader peptide translation. *EMBO J* **11**: 2675–2683.
- Brantl, S. (1994) The copR gene product of plasmid pIP501 acts as a transcriptional repressor at the essential *repR* promoter. *Mol Microbiol* **14**: 473–483.
- Brantl, S., and Wagner, E.G.H. (1997) Dual function of the copR gene product of plasmid pIP501. *J Bacteriol* **179**: 7016–7024.
- Brantl, S., and Wagner, E.G.H. (2000) Antisense RNA-mediated transcriptional attenuation: an *in vitro* study of plasmid pT181. *Mol Microbiol* **35**: 1469–1482.
- Brantl, S., Behnke, D., and Alonso, J.C. (1990) Molecular analysis of the replication region of the conjugative *Streptococcus agalactiae* plasmid pIP501 in *Bacillus subtilis*. Comparison with plasmids pAM $\beta$ 1 and pSM19035. *Nucleic Acids Res* **18**: 4783–4790.
- Brantl, S., Birch-Hirschfeld, E., and Behnke, D. (1993) RepR protein expression on plasmid pIP501 is controlled by an antisense RNA-mediated transcription attenuation mechanism. *J Bacteriol* **175**: 4052–4061.
- Burian, J., Stuchlík, S., and Kay, W.W. (1999) Replication control of a small cryptic plasmid of *Escherichia coli*. *J Mol Biol* **294**: 49–65.
- Espinosa, M., Cohen, S.N., Couturier, M., del Solar, G., Díaz-Orejás, R., Giraldo, R., *et al.* (2000) Plasmid replication and copy number control. In *The Horizontal Gene Pool: Bacterial Plasmids and Gene Spread*. Thomas, C.M. (ed.). Amsterdam: Harwood Academic Publishers, pp. 1–47.
- Franch, T., Petersen, M., Wagner, E.G.H., Jacobsen, J.P., and Gerdes, K. (1999) Antisense RNA regulation in prokaryotes: rapid RNA/RNA interaction facilitated by a general U-turn loop structure. *J Mol Biol* **294**: 1115–1125.
- Franch, T., and Gerdes, K. (2000) U-turns and regulatory RNAs. *Curr Opin Microbiol* **3**: 159–164.
- Gomis-Rüth, F.X., Solá, M., Acebo, P., Párraga, A., Guasch, A., Eritja, R., *et al.* (1998) The structure of plasmid-encoded transcriptional repressor CopG unliganded and bound to its operator. *EMBO J* **17**: 7404–7415.
- Highlander, S.K., and Novick, R.P. (1987) Plasmid repopulation kinetics in *Staphylococcus aureus*. *Plasmid* **17**: 210–221.
- de la Hoz, A.B., Ayora, S., Sitkiewicz, I., Pankiewicz, R., Alonso, J.C., and Ceglowski, P. (2000) Plasmid copy-number control and better-than-random segregation genes of pSM19035 share a common regulator. *Proc Natl Acad Sci USA* **97**: 728–733.
- Kolb, F.A., Malmgren, C., Westhof, E., Ehresmann, C., Ehresmann, B., Wagner, E.G.H., *et al.* (2000) An unusual structure formed by antisense-target RNA binding involves an extended kissing complex with a four-way junction and a side-by-side helical alignment. *RNA* **6**: 311–324.
- Le Chatelier, E., Ehrlich, S.D., and Jannière, L. (1996) Countertranscript-driven attenuation system of the pAM $\beta$ 1 *repE* gene. *Mol Microbiol* **20**: 1099–1112.
- Løbner-Olesen, A. (1999) Distribution of minichromosomes in individual *Escherichia coli* cells: implications for replication control. *EMBO J* **18**: 1712–1721.
- Murayama, K., de la Hoz, A.B., Alings, C., López, G., Orth, P., Alonso, J.C., *et al.* (1999) Crystallization and preliminary X-ray diffraction studies of *Streptococcus pyogenes* plasmid pSM19035-encoded  $\omega$  transcriptional repressor. *Acta Crystallog D* **55**: 2041–2042.
- Nielsen, P.F., and Molin, S. (1984) How the R1 replication control system responds to copy number deviations. *Plasmid* **11**: 264–267.
- Nordström, K., and Wagner, E.G.H. (1994) Kinetic aspect of control of plasmid replication by antisense RNA. *Trends Biol Sci* **19**: 294–300.
- Novick, R.P., Iordanescu, S., Projan, S.J., Kornblum, J., and Edelman, I. (1989) pT181 plasmid replication is regulated by a countertranscript-driven transcriptional attenuator. *Cell* **59**: 395–404.
- Paulson, J., Nordström, K., and Ehrenberg, M. (1998) Requirements for rapid plasmid ColE1 copy number adjustments: a mathematical model of inhibition modes and RNA turnover rates. *Plasmid* **39**: 215–234.
- Riise, E., Stougaard, P., Bindsvlev, B., Nordström, K., and Molin, S. (1982) Molecular cloning and functional characterization of a copy number control gene (*copB*) of plasmid R1. *J Bacteriol* **151**: 1136–1145.
- Rosenfeld, R., and Grover, N.B. (1993) Control of mini-R1 plasmid replication: a computer simulation. *Plasmid* **29**: 94–116.
- Siemering, K.R., Praszkiel, J., and Pittard, A.J. (1994) Mechanisms of binding of the antisense and target RNAs involved in the regulation of IncB plasmid replication. *J Bacteriol* **176**: 2677–2688.
- del Solar, G., and Espinosa, M. (1992) The copy number of

- plasmid pLS1 is regulated by two *trans*-acting plasmid products: the antisense RNA II and the repressor protein, RepA. *Mol Microbiol* **6**: 83–94.
- del Solar, G., Acebo, P., and Espinosa, M. (1995) Replication control of plasmid pLS1: efficient regulation of plasmid copy number is exerted by the combined action of two plasmid components, CopG and RNA II. *Mol Microbiol* **18**: 913–924.
- del Solar, G., Giraldo, R., Ruiz-Echevarria, M.J., Espinosa, M., and Diaz-Orejas, R. (1998) Replication and control of circular bacterial plasmids. *Microbiol Mol Biol Rev* **62**: 434–464.
- Steinmetzer, K., Hillisch, A., Behlke, J., and Brantl, S. (2000) Transcriptional repressor CopR: structure model based localization of the DNA binding motif. *Proteins* **38**: 393–406.
- Summers, D.K. (1996). *The Biology of Plasmids*. Oxford: Blackwell Science.
- Tomizawa, J., and Itoh, T. (1981) Plasmid ColE1 incompatibility determined by interaction of RNA1 with primer transcript. *Proc Natl Acad Sci USA* **78**: 6096–6100.
- Wagner, E.G.H., and Brantl, S. (1998) Kissing and RNA stability in antisense control of plasmid replication. *Trends Biochem Sci* **23**: 451–454.
- Wagner, E.G.H., and Simons, R.W. (1994) Antisense RNA control in bacteria, phages, and plasmids. *Annu Rev Microbiol* **48**: 713–742.
- Wilson, I.W., Praszquier, J., and Pittard, A.J. (1993) Mutations affecting pseudoknot control of the replication of B group plasmids. *J Bacteriol* **175**: 6476–6483.
- Wilson, I.W., Praszquier, J., and Pittard, A.J. (1994) Molecular analysis of RNAI control *repB* translation in IncB plasmids. *J Bacteriol* **176**: 6497–6508.