Hypothesis: Hypersensitive Plasmid Copy Number Control for ColE1

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ABSTRACT Initiation of replication of the plasmid ColE1 is primed by the *cis*-acting RNA II. Copy numbers are regulated by inhibition of RNA II by the antisense RNA I, whose concentration is proportional to the plasmid concentration. This inhibition is enhanced by a protein, Rom, and takes place during a time set by the transcription of 250 bases of the gene for RNA II. When this transcription is dominated by several steps of about equal duration, the probability for RNA II to prime DNA replication is approximately determined by $e^{-\text{constant}[\text{RNA I}]}$. For large values of the "constant" small changes in [RNA I] give large variations in the priming probability. It is shown, first, that this type of mechanism can reduce the rate of plasmid loss and enable single copies of ColE1 to duplicate at a well-defined time in the cell cycle; second, that when the rate of initiation of transcription of RNA II increases, plasmid losses decrease and the distribution of single copy duplication times becomes narrower; third, that the action of Rom may further reduce plasmid losses and further narrow the distribution of duplication times in the single-copy case.

INTRODUCTION

Replication of the *E. coli* plasmid ColE1 is initiated by a *cis*-acting RNA (RNA II, R_{II}), which can form a duplex with a complementary stretch of DNA at the origin of plasmid replication. Subsequent cleavage of the hybridized part of R_{II} by RNase H creates a primer for DNA synthesis (Itoh and Tomizawa, 1980). Copy numbers are controlled by inhibition of R_{II} by the transacting RNA I (R_I) (Tomizawa and Itoh, 1981; Tomizawa, 1984; Masukata and Tomizawa, 1986), which is antisense to the 5′ region of R_{II} (Morita and Oka, 1979). The concentration of R_I is nearly proportional to the concentration of plasmid (Brenner and Tomizawa, 1991; Brendel and Perelson, 1993). Inhibition is enhanced by a protein, Rom, which binds to the initial complex between R_I and R_{II}, and increases the probability of duplex formation between the two RNAs (Cesareni et al., 1982; Lacatena et al., 1984; Tomizawa and Som, 1984; Eguchi et al., 1991; Eguchi and Tomizawa, 1991; Brendel and Perelson, 1993). The concentration of Rom is also nearly proportional to plasmid concentration (Brendel and Perelson, 1993). Recently, Brenner and Tomizawa (1991) (B and T) and Brendel and Perelson (1993) (B and P) presented quantitative models for ColE1 copy number control. According to B and T the steady-state plasmid concentration is proportional to the logarithm of the ratio between the initiation rate of transcription of R_{II} (k_{II}) and the growth rate (k_H) of the host cells. In contrast, B and P find a linear relation between the plasmid concentration and k_{II}/k_H. The two models were recently compared by Merlin and Polisky (1995). The comparison did not clarify, however, why the ratio k_{II}/k_H appears as a linear term in the B and P model, whereas it comes out in the argument of a natural logarithm in the B and T study. In the present work the difference between these two models is traced to different assumptions concerning how R_I inhibits R_{II}. B and T assume, with support from experiments (Tomizawa, 1986), that R_{II} can be attacked by R_I during a time window defined by the transcription of bases 110 to 360 of the gene for R_{II}. They assume, furthermore, that transcription proceeds in 250 steps of about equal duration. Thereby, the length of the time window has a narrow distribution, and inhibition of RNA II by RNA I occurs in multiple steps, where each contributes in the same proportion to the overall inhibition. Implicit in the model of B and P is, instead, that the inhibition time is dominated by a single step (cf. Keasling and Palsson, 1989a).

The present work compares a number of consequences of the B and P and B and T models, and it suggests experimental designs to discriminate between them. It also explores a number of general consequences of multiple step inhibition as suggested by B and T: when suitably tuned and combined with a mechanism for plasmid partitioning (Gerdes et al., 1985) the B and T model has remarkable properties. These may be of general interest because they reveal new options concerning replication control and, possibly, regulation of gene expression.

We discuss how plasmid duplications are distributed through the cell cycle and demonstrate several dramatic consequences of multiple-step inhibition of RNA II by RNA I according to the B and T model:

First, single copies of ColE1 in a cell can, in principle, precisely coordinate their duplication time with the cell cycle. In the multiple-copy number case the new mechanism may lead to small copy number fluctuations and extremely small plasmid loss rates.

Second, for single-copy plasmids the distribution of the duplication time becomes narrower as the ratio k_{II}/k_H increases, given a fixed rate of initiation of plasmid replication. When this ratio increases, the probability that R_{II}

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survives the time window where it can be attacked by R_I decreases. This means that the plasmids can accomplish an improved coordination with the cell cycle, at the cost of increased turnover rates of both R_I and R_{II}.

In the multiple-copy number case increasing k_{II}/k_H results in ever smaller plasmid losses.

Third, the action of Rom can further narrow the distribution of duplication times in the single-copy case and dramatically lower plasmid losses in the multiple-copy case.

The possibility for precise timing between cell and plasmid cycles has recently been discussed for the plasmid R1. Here, however, the mechanism of such coordination would be quite different (Ehrenberg and Sverredal, 1995).

When there are several ColE1 copies per cell, the present analysis suggests that their duplication times are spread in the cell cycle, in accordance with experiments (Leonard and Helmstetter, 1988). A similar behavior characterizes the plasmid R1, as shown by experiment (Nordström, 1983) and as suggested by theory (Ehrenberg and Sverredal, 1995).

MACROSCOPIC FEATURES OF THE ColE1 COPY NUMBER CONTROL

The inhibition of R_{II} priming of ColE1 replication is, first, governed by the concentrations of R_I and Rom in the cells ($[R_I]$ and [Rom]). Second, it is also determined by the second-order, effective association rate constant k_{12} for binding and duplex formation between R_I and R_{II}. The effective rate constant k_{12} is, in turn, determined by the association rate constant k_{aI} for initial complex formation between R_I and R_{II} multiplied by the probability P(duplex) that this initial encounter leads to duplex formation and breakdown of R_{II}:

$$k_{12} = k_{aI} \cdot P(\text{duplex}). \tag{1}$$

This simple form for the rate of inhibition, k_{12}, is derived in Appendix 1, and its range of validity is specified in terms of the underlying microscopic rate constants (see also below Eq. 13, where this point is further discussed).

The probability P(duplex) is enhanced by the action of Rom, according to the simplified scheme (Eguchi et al., 1991; Eguchi and Tomizawa, 1991; Brendel and Perelson, 1993):

$$\begin{array}{l} R_{II} \underset{k_{-aI}}{\overset{k_{aI}\cdot[R_I]}{\rightleftharpoons}} C_I \xrightarrow{k_{dI}} \\ \qquad\qquad \downarrow k_{aR}\cdot[\text{Rom}] \\ \qquad\qquad C_{II} \xrightarrow{k_{dII}} . \end{array} \tag{2}$$

R_{II} forms an initial complex C_I with R_I with the rate $k_{aI} \cdot [R_I]$. C_I may dissociate again, with the rate k_{-aI}. The complex C_I may be converted to a stable duplex, without any interaction with Rom, with the rate constant k_{dI}. Finally, C_I may bind Rom, with rate $k_{aR} \cdot$ [Rom], forming the complex C_{II}. Once C_{II} is formed RNA II is degraded with very high probability (Brendel and Perelson, 1993). According to Scheme 2 the probability, P(duplex), that C_I is converted to a stable duplex between R_I and R_{II} and that R_{II} becomes degraded is given by

$$P(\text{duplex}) = \frac{k_{dI} + k_{aR} \cdot [\text{Rom}]}{k_{-aI} + k_{dI} + k_{aR} \cdot [\text{Rom}]}. \tag{3}$$

Both R_I and Rom are constitutively expressed from ColE1 plasmids and both are rapidly degraded, so that their concentrations are nearly proportional to the plasmid concentration y (cf. Brenner and Tomizawa, 1991; Brendel and Perelson, 1993; Keasling and Palsson, 1989a,b; Bremer and Lin-Chao, 1986). Denoting the production rates per plasmid of R_I and Rom by k_I and k_R, respectively, and their respective degradation rate constants by ϵ_I and ϵ_R, their concentrations can be written as

$$[R_I] = \frac{y \cdot k_I}{\epsilon_I} \quad \text{and} \quad [\text{Rom}] = \frac{y \cdot k_R}{\epsilon_R}. \tag{4}$$

These relations are derived in Appendix 2, along with a discussion of their range of validity. The main point is that the larger the parameters k_I, ϵ_I, k_R and ϵ_R (Table 1) are, for given ratios k_I/ϵ_I and k_R/ϵ_R, the better are the approximations in Eq. 4 (cf. Ehrenberg and Sverredal, 1995). We note that this is one of several cases where increasing precision of the control system is bought at increasing energy cost (see Discussion).

The rate of change of the plasmid concentration y is governed by the differential equation (cf. Bremer and Lin-Chao, 1986; Keasling and Palsson, 1989a,b; Brenner and Tomizawa, 1991; Brendel and Perelson, 1993)

$$\frac{dy}{dt} = k_{II} \cdot Q_0 \cdot y - k_H \cdot y. \tag{5}$$

k_{II} is the rate constant of initiation of transcription of R_{II}. Q_0 is the probability that R_{II} is not inhibited by R_I, so that it can form a primer for intiation of plasmid replication. The parameter k_H is the (exponential) growth rate of the host cells, and the negative term to the right in Eq. 5 describes the dilution of plasmids by the exponential volume expansion with time (Ehrenberg and Kurland, 1984; Brendel and Perelson, 1993; Ehrenberg and Sverredal, 1995).

Inhibition of R_{II} can occur during transcription of bases 110–360 of its gene (Tomizawa, 1986), as illustrated by the following scheme (see Appendix 2):

$$\begin{array}{l} \overset{\{\text{base}\}}{\{110\}} \qquad\qquad\qquad\qquad\qquad\qquad \overset{\{\text{base}\}}{\{360\}} \\ \bullet \xrightarrow{n k \epsilon} \bullet \xrightarrow{n k \epsilon} \bullet \xrightarrow{n k \epsilon} \cdots \bullet \xrightarrow{n k \epsilon} \bullet \xrightarrow{n k \epsilon} \cdots \bullet \xrightarrow{n k \epsilon} \left\{ \begin{array}{c} \text{initiation} \\ \text{of} \\ \text{replication} \end{array} \right\} \\ \downarrow k_{12}[R_I] \downarrow k_{12}[R_I] \downarrow k_{12}[R_I] \quad \downarrow k_{12}[R_I] \downarrow k_{12}[R_I] \quad \downarrow k_{12}[R_I] \\ \{\text{inhibition of } R_{II} \text{ by } R_I\} \end{array} \tag{6}$$

No inhibition can occur during transcription of the first 110 bases, because the binding site for R_I on R_{II} is not yet formed. Therefore, the rate of transcription over bases 1–110 is irrelevant for the extent of inhibition.

TABLE 1 Parameters used in the model for ColE1 copy number control

$k_{12}(k_{12}^{+}, k_{12}^{-})$	Effective second-order rate constant for the inhibition of RNA II by RNA I in the presence (+) and absence (−) of Rom.
C_I, C_{II}	First encounter (kissing) complex between RNA I and RNA II in the absence of Rom (C_I) and after Rom association (C_{II}).
k_{aI}	Second-order rate constant for C_I formation
k_{-aI}	Rate constant for dissociation of C_I.
k_{dI}, k_{dII}	Rate constant for duplex formation from C_I and C_{II}, respectively.
k_{aR}	Second-order rate constant for binding of Rom to C_I to form C_{II}.
P(duplex)	Probability that a duplex is formed between RNA I and RNA II, given that C_I has been formed.
y, $\bar{y}$, $\bar{y}_\mu$	Cytoplasmic concentration of plasmids. y (current), $\bar{y}$ (macroscopic steady state average), and $\bar{y}_\mu$ (microscopic, exact steady-state average).
k_I, k_{II}, k_R	Rate of synthesis (per plasmid) of, respectively, RNA I, RNA II, and Rom.
ϵ_I, ϵ_R	Degradation rate constants for RNA I and Rom, respectively.
k_H, τ_H	Growth rate and generation time, respectively, for host cells ($k_H = \ln(2)/\tau_H$).
Q_0	Probability that an initiated RNA II is not inhibited by RNA I.
DR	Dynamic range of inhibition defined as the reciprocal of Q_0 at saturating concentrations of RNA I.
n	Effective number of transcription steps in the time window where RNA II can be inhibited by RNA I.
τ_ℓ, τ_ℓ/n, nk_ℓ	Respectively, expected time of inhibition window, expected time, and rate constant of single transcription step.
λ, $\lambda(M, t)$	Macroscopic respectively microscopic (for M plasmids in a cell at time t after division) values of $\lambda = k_{12} \cdot [\text{RNA I}] \cdot \tau_I$,
M, $\bar{M}$, $\bar{M}_\mu$, $\bar{M}_t$	Number of plasmids per cell. Current (M), macroscopic ($\bar{M}$) and microscopic ($\bar{M}_\mu$) averages, microscopic average at time t in cell cycle ($\bar{M}_t$).
$v(t)$, $\bar{v}$	Current and average cell volumes.
$P_I(M, t)$	Probability that there are M plasmids in a cell at time t, on the condition of I plasmids at $t = 0$.
$\bar{\alpha}_I$	Steady state probability of I plasmids in a cell just when its cycle begins.
$f_D(t)$	Plasmid duplication frequency at time t in the cell cycle.
P(loss)	Probability that a randomly chosen daughter cell lacks plasmid.

Scheme 6 shows the special case where all individual transcription steps have identical rates $nk_\ell \approx 50\ s^{-1}$, and where $n = 251$. The average transcription time τ_ℓ in the inhibition window is about 5 s ($\tau_\ell = n/nk_\ell = 251/50 = 5$). The scheme may, however, also illustrate cases where transcription through the inhibition window is dominated by one ($n = 1$) or a few ($n \approx 1$) pause sites.

The validity of Scheme 6 is specified in Appendix 1 and discussed in relation to Eq. 13 below.

The standard deviation (σ_τ) of the time spent in the inhibition window is inversely proportional to the square root of the number of dominating steps. For n identical steps σ_τ is given by

$$\sigma_\tau = \frac{\tau_\ell}{\sqrt{n}}. \tag{7}$$

Accordingly, the relative standard deviation is much smaller when transcription proceeds over 251 steps of the same rate ($\sigma_\tau = 0.06\tau_\ell$) than when transcription is dominated by a single pause site ($\sigma_\tau = \tau_\ell$).

The probability that no inhibition occurs during one transcription step is given by

$$\frac{nk_\ell}{nk_\ell + k_{12}[R_I]} = \frac{1}{1 + \lambda/n}, \tag{8}$$

where

$$\lambda = \frac{k_{12}[R_I]}{k_\ell}. \tag{9}$$

The probability Q_0 that there is no inhibition in any of the n steps is obtained by taking the single step probability in Eq. 8 to the nth power

$$Q_0 = \left(\frac{1}{1 + \lambda/n}\right)^n. \tag{10}$$

Expression 9 is constructed so that λ and the average time $\tau_\ell = 1/k_\ell$ that R_{II} spends in the inhibition window are unchanged by the number n of steps in Scheme 6. The advantage of this convention is that it facilitates comparisons between single-step inhibition, as in the B and P model (1993), and multiple-step inhibitions, as in the B and T model (1991).

Two special cases will now be considered. The first concerns rom$^-$ plasmids, which lack Rom proteins altogether. Here

$$k_{12} = k_{12}^{-} = k_{aI} \cdot \frac{1}{1 + k_{-aI}/k_{dI}}, \tag{11}$$

according to Eqs. 1 and 3.

In the second case the term $k_{aR} \cdot [\text{Rom}]$ is assumed to dominate the numerator but to be negligible in the denominator of Eq. 3. Here, instead

$$k_{12} = k_{aI} \cdot \frac{[\text{Rom}]}{K_R}. \tag{12}$$

According to Eqs. 1 and 3 the parameter K_R must be chosen as

$$K_R = \frac{k_{-aI} + k_{dI}}{k_{aR}}. \tag{13}$$

This latter, special case is chosen to highlight new properties of Rom function, as will be discussed below.

As shown in Appendix 1, the relevance of the idealized Scheme 6, which leads to Eqs. 11 and 12, requires first that the dynamic range, DR, of the mechanism is sufficiently large. DR is defined as the reciprocal of Q_0 at saturating inhibitor concentrations. A second requirement is that current values of $1/Q_0$ are much smaller than DR, allowing the degree of inhibition to change easily both up and down for small changes in the inhibitor concentrations.

Equation 4, the definition of λ in Eq. 9, and the definitions of k_{12} in Eqs. 11 and 12 imply that λ depends linearly on the plasmid concentration y in the rom$^-$ and quadratically on y in the rom$^+$ case:

$$\lambda = \frac{y \cdot k_{12}^- \cdot k_I}{k_\ell \cdot \epsilon_I} \quad \text{and} \quad \lambda = \frac{y^2 \cdot k_{aI} \cdot k_I \cdot k_R}{k_\ell \cdot \epsilon_I \cdot \epsilon_R \cdot K_R}. \tag{14}$$

When n in Eq. 10 goes to infinity, Q_0 takes the simple form

$$Q_0 = e^{-\lambda}. \tag{15a}$$

Such an expression is the starting point for the B and T model (1991). As long as $n \gg 1$ and $\lambda/n \ll 1$, the exponential form for Q_0 in Eq. 15a is an excellent approximation for the exact expression Eq. 10. This means, in particular, that if transcription through the inhibition window proceeds through 250 steps of about equal duration (without long pauses), then the B and T model is correct.

A remarkable feature of multiple-step inhibition is that small, relative variations in λ may lead to very large relative variations in the probability of initiation of plasmid duplication, Q_0! The condition is that Q_0 is small (λ is large). Assume, to give an example, that λ initially is 8 and then decreases by a factor of 2 to 4. According to Eq. 15a this twofold change in λ leads to about a 50-fold increase in Q_0. Indeed, by making λ sufficiently large this type of mechanism may, at least in principle, be made as sensitive as one wishes with respect to how small variations in plasmid concentration y affect the plasmid duplication frequency Q_0.

If, on the other hand, transcription is dominated by a single pause site, then inhibition is also accomplished in a single step and the B and P model (1993) is correct. Here, instead

$$Q_0 = \frac{1}{1+\lambda}, \tag{15b}$$

as follows from Eq. 10 with $n = 1$. A twofold reduction in λ, in this case, cannot lead to more than a twofold increase in Q_0.

The macroscopic steady-state concentration of plasmids in the bacterial population is obtained by putting the time derivative on the left side in Eq. 5 equal to zero, so that

$$k_{II} \cdot Q_0 = k_H. \tag{16}$$

This relation, together with the expression for Q_0 in Eq. 10, gives a steady-state value of $\bar{\lambda}$ as

$$\bar{\lambda} = \left(\left(\frac{k_{II}}{k_H}\right)^{1/n} - 1\right) \cdot n. \tag{17}$$

The steady-state plasmid concentrations in the rom$^-$ and rom$^+$ cases, expressed in the microscopic parameters of the plasmid copy number control system, can now be obtained from Eqs. 14 and 17

$$\bar{y} = \left(\left(\frac{k_{II}}{k_H}\right)^{1/n} - 1\right) \cdot n \cdot \frac{k_\ell \cdot \epsilon_I}{k_{12}^- \cdot k_I}$$

and (18)

$$\bar{y} = \left(\left(\frac{k_{II}}{k_H}\right)^{1/n} - 1\right)^{1/2} \cdot n^{1/2} \cdot \left(\frac{k_\ell \cdot \epsilon_I \cdot \epsilon_R \cdot K_R}{k_{aI} \cdot k_I \cdot k_R}\right)^{1/2}.$$

When n tends to infinity, the plasmid concentration in Eq. 18 becomes for the rom$^-$ case

$$\bar{y} = \ln\left(\frac{k_{II}}{k_H}\right) \frac{k_\ell \cdot \epsilon_I}{k_{12} \cdot k_I}. \tag{19a}$$

This corresponds to Eq. 2 in the B and T model (1991). When $n = 1$, the plasmid concentration for the same rom$^-$ case is

$$\bar{y} = \left(\frac{k_{II}}{k_H} - 1\right) \frac{k_\ell \cdot \epsilon_I}{k_{12} \cdot k_I}. \tag{19b}$$

This corresponds to Eq. 5 of the B and P model (1993).

It may be worth stressing at this point that the macroscopically calculated steady-state concentrations $\bar{y}$ in Eqs. 18 and 19 are approximate. The true, average concentrations $\bar{y}_\mu$ require a microscopic definition, and these are given in Eq. 29 below. The differences between $\bar{y}$ and $\bar{y}_\mu$ are normally negligible, but for copy numbers per cell between 1 and 2, they may be significant. The approximate nature of the macroscopic derivations comes from the assumption that the average of a function of a variable (e.g., y) is the same as the function of the averaged variable ($\bar{y}$).

COPY NUMBER CONTROL IN SINGLE CELLS

An approximate relation between the expected copy number per cell, the average cell volume, and the average plasmid concentration is (Ehrenberg and Sverredal, 1995)

$$\bar{M} = \bar{y} \cdot \bar{v} = \frac{\bar{y} \cdot v(0)}{\ln(2)}. \tag{20}$$

An exact, microscopic definition of the average copy number per cell, $\bar{M}_\mu$, is given in Eq. 30. The average microscopic cell volume $\bar{v}$ is given by

$$\bar{v} = \frac{1}{\tau_H} \int_0^{\tau_H} v(0) \cdot e^{k_H \cdot t}\, dt = \frac{v(0)}{\ln(2)}. \tag{21}$$

$v(0)$ is the volume of a single cell that has just divided, and $2v(0)$ is the volume of cells just about to divide. k_H (= (ln 2)/τ_H) is, as before, the growth rate of the host cells, and τ_H is their generation time.

The (microscopic) plasmid concentration $y = y(t)$ is the current number $M = M(t)$ of plasmids in a cell divided by its volume $v = v(t)$ (Ehrenberg and Sverredal, 1995):

$$y(t) = \frac{M}{v(t)} = \frac{M}{v(0) \cdot e^{k_H \cdot t}}. \tag{22}$$

From Eqs. 14 and 22 microscopic versions of the inhibition parameter λ in the rom$^-$ and rom$^+$ cases follow as

$$\lambda(M, t) = \frac{M \cdot e^{-k_H t}}{\bar{v} \cdot \ln(2)} \cdot \frac{k_{12}^- \cdot k_I}{k_\ell \cdot \epsilon_I}$$

and (23)

$$\lambda(M, t) = \left(\frac{M \cdot e^{-k_H t}}{\bar{v} \cdot \ln(2)}\right)^2 \cdot \frac{k_{aI} \cdot k_I \cdot k_R}{k_\ell \cdot \epsilon_I \cdot \epsilon_R \cdot K_R}.$$

These expressions can be used, together with Eq. 10, to derive time-dependent, microscopic formulations of $Q_0(t)$, here denoted $Q_0(M, t)$ to keep track of the number of plasmids in single cells. With the aid of Eqs. 18 and 20, the microscopic cell volume can be eliminated and $\lambda(M, t)$ related to the macroscopic average number of plasmids per cell:

$$\lambda(M, t) = \lambda(M, 0) \cdot e^{-\theta_H t}, \tag{24a}$$

where in the rom$^-$ case

$$\lambda(M, 0) = \frac{M}{\bar{M} \cdot \ln(2)} \cdot \left(\left(\frac{k_{II}}{k_H}\right)^{1/n} - 1\right) \cdot n \tag{24b}$$

$$\text{with} \quad \theta_H = k_H,$$

and for rom$^+$ plasmids

$$\lambda(M, 0) = \left(\frac{M}{\bar{M} \cdot \ln(2)}\right)^2 \cdot \left(\left(\frac{k_{II}}{k_H}\right)^{1/n} - 1\right) \cdot n \tag{24c}$$

$$\text{with} \quad \theta_H = 2k_H.$$

It follows from Eq. 24 that for a fixed copy number M of plasmids in a cell, $\lambda(M, t)$ decreases through the cell cycle by a factor of 2 in the rom$^-$ and by a factor of 4 in the rom$^+$ case.

When a newly formed cell starts out with I plasmids, the probabilities $P_I(M, t)$ that there are M plasmids at time t in the cell cycle are governed by the following differential equations

$$\frac{dP_I(I, t)}{dt} = -k_{II} \cdot I \cdot Q_0(I, t) \cdot P_I(I, t),$$

$$\frac{dP_I(M, t)}{dt} = -k_{II} \cdot M \cdot Q_0(M, t) \cdot P_I(M, t) \tag{25}$$

$$+ k_{II} \cdot (M - 1) \cdot Q_0(M - 1, t) \cdot P_I(M - 1, t),$$

$$M = I + 1, I + 2 \cdots$$

The initial conditions are that $P_I(M, 0) = 0$ for all $M > I$ and that $P_I(I, 0) = 1$. These differential equations follow from the law of mass action. Thus, the probability $P_I(t)$ that the original number I of plasmids remains unchanged over time decreases with a rate proportional to I (the number of plasmids that may duplicate) as well as to the rate of initiation of replication, which is $k_{II} \cdot Q_0(I, t)$. The probability $P_I(M, t)$ that there are M ($M > I$) plasmids in a cell at time t decreases with a term similar to that for $P_I(I, t)$. In addition, there is a positive "source" term tending to enhance $P_I(M, t)$. This takes into account how cells with M plasmids emerge from cells containing $M - 1$ plasmid copies.

The probability $Q_0(M, t)$ that an RNA II primer survives the inhibition window in a cell containing M plasmids at time t after cell division is given in Eq. 10, with $\lambda(M, t)$ from Eq. 24.

The differential equations in Eq. 25 can be integrated, and the result is

$$P_I(I, t) = e^{-I \cdot k_{II} \cdot \int_0^t Q_0(I, u)\, du}$$

$$P_I(M, t) = e^{-M \cdot k_{II} \int_0^t Q_0(M, u)\, du} \cdot (M - 1) \cdot k_{II} \tag{26}$$

$$\cdot \int_0^t Q_0(M - 1, v) \cdot P_I(M - 1, v) \cdot e^{M \cdot k_{II} \int_0^v Q_0(M, z)\, dz}\, dv.$$

$$M = I + 1, I + 2 \cdots.$$

Equation 26 can be used to obtain the probability distribution $P_I(M, t)$ for the number M of plasmids at different time points of the cell cycle for a given number I of plasmids at its start.

A case of particular relevance is when both host cell and plasmid populations are in a steady state. Here, one needs to know the steady-state probabilities $\bar{\alpha}_I$ that there are I plasmids in the beginnning of a cell cycle, and these can be obtained iteratively (Ehrenberg and Sverredal, 1995): first an initial set of α_I values is guessed. From this set the probability that there are M plasmids in the cell at the next cell divison at $t = \tau_H$ is given by $\sum_I \alpha_I \cdot P_I(M, \tau_H)$. A second set of α_I values now follows by application of the appropriate rule for plasmid partitioning upon cell divison (see below), and this second set can be used for new rounds of calculations until the α_I values stabilize. Convergence for these iterations requires that the α_I values are normalized, so that $\sum_{I=1}^{\infty} \alpha_I = 1$, at each round of calculation (Ehrenberg and Sverredal, 1995). This normalization is necessary, because otherwise the plasmid copy numbers decrease toward zero during the iterations, and no steady-state set is obtained.

The steady-state probability $P(M, t)$ that a cell contains M plasmids at time t after cell division is given by

$$P(M, t) = \sum_{I=1}^{\infty} \bar{\alpha}_I \cdot P_I(M, t). \tag{27}$$

The expected, steady-state copy number of plasmids at time t in the cell cycle then follows as

$$\bar{M}_t = \sum_{M=1}^{\infty} M \cdot P(M, t). \quad (28)$$

The exact, microscopic steady-state plasmid concentration is given by the time integral

$$\bar{y}_\mu = \frac{1}{\tau_H} \int_0^{\tau_H} dt \cdot \frac{\bar{M}_t}{v(t)}. \quad (29)$$

The microscopic, average plasmid copy number is obtained by integrating over the cell cycle

$$\bar{M}_\mu = \frac{1}{\tau_H} \int_0^{\tau_H} dt \cdot \bar{M}_t. \quad (30)$$

The expected number $j_D(t)$ of plasmid duplications that occur per time unit at a time t in the cell cycle follows from the law of mass action and is given by

$$j_D(t) = k_{II} \cdot \sum_{M=1}^{\infty} M \cdot Q_0(M, t) \cdot P(M, t). \quad (31)$$

$j_D(t)$ defines a plasmid duplication frequency distribution $f_D(t)$

$$f_D(t) = \frac{j_D(t)}{\int_0^{\tau_H} j_D(u)\, du}. \quad (32)$$

To complete the set of analytical tools needed to characterize the copy number control of ColE1, one also requires an expression for the probability, P(loss), of plasmid loss per cell cycle. Plasmid loss arises when one daughter cell fails to obtain one or several plasmids from the mother cell upon cell division. Here we will in particular consider the case when the plasmids have an active partitioning system (par$^+$), making sure that when there are two or more plasmids in a dividing mother cell, then at least one plasmid will go to each daughter cell. ColE1 is normally par$^-$, but can be made par$^+$ (Gerdes et al., 1985) and in the latter case the only way that a daughter cell can become void of a plasmid is that a dividing mother cell contains only one plasmid. When ColE1 is par$^+$ the predictions of the exponential inhibition suggested here and by Brenner and Tomizawa (1991) are particularly dramatic and therefore comparatively easy to check experimentally. We will therefore focus attention on this somewhat unusual situation for ColE1, but the analysis is easily modified to cover par$^-$ cases as well.

When ColE1 is par$^+$, the probability that there is only one plasmid in a mother cell at the end of its cycle can be obtained from Eq. 27 by putting $M = 1$ and $t = \tau_H$. Now, P(loss) is one-half of the probability that a mother cell has only one plasmid copy, so that

$$P(\text{loss}) = \frac{1}{2} P(1, \tau_H) = \frac{1}{2} \sum_{I=1}^{\infty} \bar{\alpha}_I \cdot P_I(1, \tau_H). \quad (33)$$

When the average number of plasmids per cell is between 1 and 2, a cell that has just divided normally contains one plasmid, and this must duplicate before the next cell division, because otherwise a plasmid-lacking daughter cell will be created. In these situations, with extremely low copy numbers, the difference between multiple-step inhibition, as in the B and T model, and single-step inhibition, as suggested by B and P, is dramatic. Also the influence of Rom on plasmid duplication is clearly seen under these extreme conditions. Fig. 1 illustrates the power of multiple-step inhibition in the case where the average plasmid copy

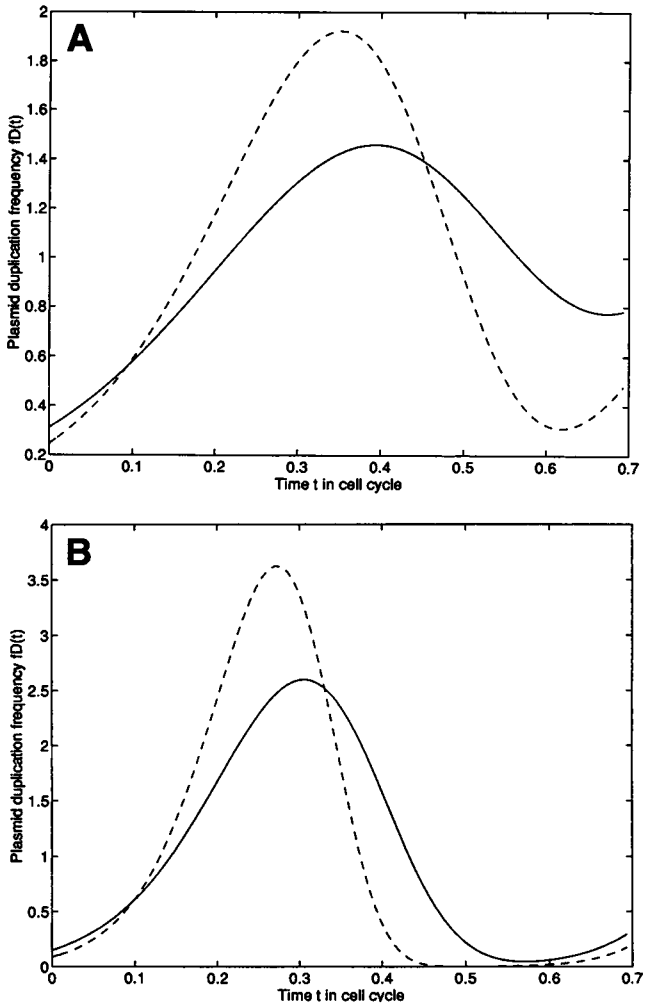


FIGURE 1 Frequency distribution, $f_D(t)$, of plasmid duplications in the cell cycle according to Eq. 32. The plasmids are par$^+$, and there is even partitioning. The average copy number, according to Eq. 30, is 1.5. The rom$^-$ (a) and rom$^+$ (b) cases are shown for k_{II}/k_H, defined in Eq. 5, equal to 700 (——) and 7000 (– – –). The growth rate k_H is set equal to 1, and the time t runs from zero (after first cell division) to the generation time τ_H (= (ln 2)/k_H ≈ 0.7).

number is 1.5. For the calculations here and below we assume that the plasmids are par$^+$ and that they are evenly distributed upon cell division: if the plasmid number is even in the mother cell, the daughter cells always receive the same number of plasmids. If the mother plasmid copy number is odd, one daughter cell gets one more plasmid than the other.

In Fig. 1 *a* the frequency of plasmid duplications $f_D(t)$, defined in Eq. 32, is plotted for different values of the ratio k_{II}/k_H in the absence of Rom. In Fig. 1 *b* the corresponding distributions are shown for the rom$^+$ case. It follows immediately from the figure that the duplication frequency f_D(t) gets narrower as k_{II}/k_H increases. Furthermore, for a given ratio between k_{II} and k_H, the presence of Rom makes the distribution of plasmid replication times in the cell cycle significantly narrower. A more detailed analysis reveals that the standard deviation, σ_D, of $f_D(t)$ to a good approximation is inversely proportional to the natural logarithm of k_{II}/k_H:

$$\sigma_D \propto \frac{1}{\ln(k_{II}/k_H)}.$$

Accordingly, multiple-step inhibition, as in Scheme 6, allows a precise timing of cell and plasmid cycles. This timing is bought, however, at the cost of an ever-increasing dissipation of free energy, associated with synthesis and breakdown of RNA II and RNA I. The logarithmic dependence of σ_D on k_{II}/k_H shows that there is a diminishing return for how much σ_D decreases for a given, relative increase in k_{II}/k_H and energy dissipation. From Fig. 1 it also follows that the presence of Rom reduces σ_D approximately twofold, for a fixed value of k_{II}/k_H. The reason why Rom has this strong influence on the timing between cell and plasmid cycles can be understood by inspecting Eq. 14 or 24. These show how the inhibition parameter λ depends linearly on plasmid concentration $y(t)$ in the rom$^-$ but quadratically on $y(t)$ in the rom$^+$ case. This means that, in cases where there is only one plasmid per cell, the presence of Rom makes λ inversely proportional to the square of the cell volume, rather than to the volume itself, as in the rom$^-$ case. Therefore, Rom can significantly increase the sensitivity of $Q_0(t)$ to the cell volume increase through the cell cycle.

If we assume that not more than one initiation of transcription of RNA II per second is possible, then k_{II}/k_H will have an upper bound, which also depends on the growth rate k_H of the host cell. Duplication frequencies $f_D(t)$ at such upper limits of k_{II}/k_H are of interest, because they represent the "best" timing between cell and plasmid cycle that the ColE1 copy number control system is capable of. In Fig. 1 the generation time is 80 min and the maximal k_{II}/k_H is in this particular case equal to 7000.

If the inhibition window in Scheme 6 is dominated by a single step ($n = 1$), the timing between cell and plasmid cycles will be poor, irrespective of the magnitude of the ratio k_{II}/k_H.

When the average plasmid copy number is increased to higher values the frequency distribution $f_D(t)$ becomes broad, even for large values of k_{II}/k_H, as illustrated in Fig. 2. Here, the rom$^-$ case is shown for $n = 250$ in Scheme 6 and with $k_{II}/k_H = 7000$. It is seen that when there are several plasmid copies per cell, the duplication times tend to be spread out in the cell cycle. This is because a duplication event that increases the plasmid concentration will reduce $Q_0(t)$, and thereby delay the next duplication, until the cell volume has increased enough so that $Q_0(t)$ has become sufficiently large to allow a new duplication event. The effects of multiple inhibition steps as part of copy number control are in this case quite subtle. In fact, a hypersensitive dependence of $Q_0(t)$ on plasmid concentration will tend to make the time between duplication events regular and reduce those fluctuations in the plasmid concentration that arise because of small copy numbers.

One important feature of multiple-step inhibition concerns how it influences plasmid maintenance stability. In Fig. 3 we show how plasmid losses, calculated from Eq. 33, depend on the ratio k_{II}/k_H. The average copy number per cell is 4. For single-step inhibition ($n = 1$ in Scheme 6) and in the rom$^-$ case plasmid losses go down to a plateau, when k_{II}/k_H increases, where P(loss) is about 1%. Interestingly, introduction of Rom leads to much smaller plasmid losses, also for single-step inhibition. In the presence of Rom a plateau of about 10^{-4} % loss per cell division is reached at high values of k_{II}/k_H. In multiple-step ($n = 250$ in Scheme 6) inhibition of RNA II by RNA I, plasmid losses are reduced to extremely low levels as k_{II}/k_H increases, and P(loss) is much lower in rom$^+$ than in rom$^-$ cases. For instance, at a moderate k_{II}/k_H value of 70, P(loss) is smaller than 10^{-5} % in rom$^-$ and smaller than 10^{-14} % (!) in rom$^+$ cases. These results strongly suggest that an important biological function of Rom is to enhance the maintenance stability of ColE1 plasmids. This conclusion is reinforced

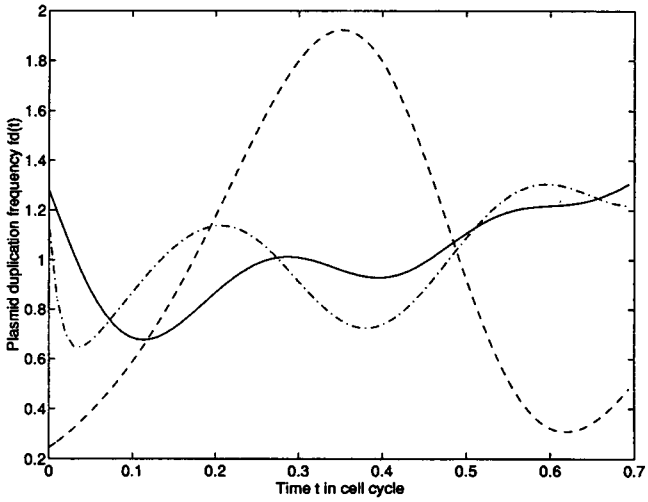


FIGURE 2 Duplication frequency $f_D(t)$, as in Fig. 1, in the rom$^-$ case with $n = 250$ and $k_{II}/k_H = 7000$ for different, average copy numbers. Copy number 1.5 (– – –), 3.0 (– · – · –), and 4.0 (——).

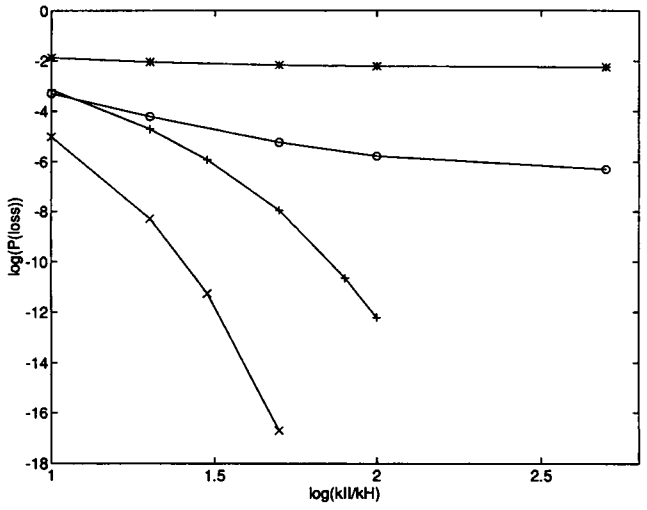


FIGURE 3 Logarithm of the probability P(loss), defined in Eq. 33, of generating a daughter cell lacking plasmid upon cell division, plotted as a function of the logarithm of the ratio k_{II}/k_H in Eq. 5. The average number of plasmids per cell is four. Plots are made for rom$^-$ and $n = 1$ in Scheme 6 (*), for rom$^+$ and $n = 1$ (○), for rom$^-$ and $n = 250$ (+) and for rom$^+$ and $n = 250$ (×).

by the fact that Rom reduces plasmid losses for any value of the number of steps (n) in the mechanism (Scheme 6) by which RNA I inhibits RNA II.

DISCUSSION

The present work explores a number of consequences of "multiple-step inhibition" in replication control of plasmids and compares these with more conventional mechanisms. The theory treats ColE1 plasmids in particular, but its results may have more general implications. It predicts the outcome of a number of experiments involving new ColE1 constructs and may be significant for the understanding of plasmid evolution in a population genetic perspective.

The most spectacular consequences of multiple-step inhibition arise for ColE1 plasmids that have an active partitioning mechanism (Gerdes et al., 1985). We have therefore focused attention on the unusual condition that ColE1 is par$^+$, in the hope that such constructs will provide experimental evidence for a mechanism that we think may occur in many other contexts (e.g., Brantl and Wagner, 1994) and may be of quite general interest.

It is shown how inhibition of RNA II by RNA I may enable single copies of ColE1 to "measure" the cell volume $v(t)$ with extreme precision, allowing duplication at well-defined times in the cell cycle (Fig. 1). This requires that inhibition of R_{II} by R_I occurs with uniform probability in a narrrowly distributed time window (Brenner and Tomizawa, 1991), and that the probability that R_{II} will avoid inhibition by R_I is small (large ratio between k_{II} and k_H). Then, the duplication frequency is, in the single-copy, rom$^-$ case proportional to $e^{\text{cons tan t}\cdot v(t)/v(0)}$, where the "constant" in the exponent is large. Accordingly, an increase in the cell volume $v(t)$ by a factor of 2 can be transformed, in principle, to an arbitrarily large increase in plasmid duplication frequency. There exists an upper limit to the size of the "constant" in the exponent, and therefore to how sharply the duplication probability can respond to changes in $v(t)$. This is set by an upper bound to the initiation frequency of transcription of the gene for R_{II}. Further precision in duplication time can be obtained by Rom, whose action makes the exponent above depend on the square of $v(t)$. When the inhibition window is dominated by a single step and has a broad time distribution (Brendel and Perelson, 1993), the duplication rate depends parabolically on $v(t)/v(0)$, prohibiting a sharply defined duplication time irrespective of the magnitude of k_{II}/k_H.

The existence of a sharply defined inhibition window can be tested by plasmids with two changes from wild-type ColE1. The first modification is an increase in the rate k_{II} of initiation of R_{II} by a stronger promoter, and the second is an increase in the rate of synthesis of R_I, so that the average copy number per cell drops from about 20 to between 1 and 2. Meselson-Stahl experiments have been applied to the R1 plasmid system (Nordström, 1983), and similar methods could be used to find out how the time between succesive plasmid duplications (the "eclipse time") depends on the ratio k_{II}/k_H. If the eclipse time becomes long in relation to the generation time, when k_{II}/k_H increases, this would favor the B and T model. Another experimental possibility is to directly measure the distribution of duplication times in the cell cycle (Cooper and Helmstetter, 1968). The proposed narrowing of the distribution of duplication times by the action of Rom can be studied in similar experiments.

Also in the multiple copy number case, which is the normal phenotype for ColE1 plasmids, the sharply defined inhibition window has interesting and experimentally testable properties. One is that plasmid losses at cell division, due to the occurrence of single copies in cells just about to divide, are drastically reduced when k_{II}/k_H increases (Fig. 3).

Our analysis also suggests that an important function of the Rom protein is that its action reduces plasmid losses upon cell division: Fig. 3 reveals that both for single- ($n = 1$ in Scheme 6) and multiple- ($n = 250$) step inhibition, plasmid losses are orders of magnitude lower in rom$^+$ than in rom$^-$ cases.

The "precision" of the suggested ColE1 mechanism depends on dissipation of free energy: the more R_{II} is inhibited and degraded per plasmid replication, the more sharply the copy number control responds to small differences in plasmid and R_I concentrations. The mechanism is similar to multiple-step (Freter and Savageau, 1980; Ehrenberg and Blomberg, 1980) proofreading (Hopfield, 1974; Ninio, 1975) schemes. For these, a small difference in enzyme binding energy for cognate and noncognate substrates is transformed, through repeated selection, to a very large difference between cognate and noncognate product formation flows. Furthermore, proofreading mechanisms become increasingly accurate with a larger dissipation of free energy (Ehrenberg and Blomberg, 1980; Hopfield, 1974; Ninio,

1975). In fact, schemes for repeated proofreading (Freter and Savageau, 1980; Ehrenberg and Blomberg, 1980) and schemes for repeated inhibition (Scheme 6 above) are mathematically equivalent. They both amplify a small difference in an input parameter (binding constant and plasmid concentration) to a large difference in an output parameter (accuracy and probability of plasmid duplication, respectively) through the accumulated effect of choices at repeated branch points.

Repeated inhibition may be quite ubiquitous in plasmid copy number control: the copy number of the pIP501 plasmid is post-transcriptionally regulated by the interaction between an antisense RNA, RNA III, and mRNA, RNA II, for a replication-limiting protein RepR. RNA III induces transcriptional termination of RNA II (attenuation) (Brantl et al., 1993) by acting in a time window set by transcription of about 160 bases between the RNA III target sequence on RNA II and the RNA II terminator (Brantl and Wagner, 1994). Regular transcription, without pausing, would give hypersensitive responses in RepR synthesis to RNA III variations.

Theory also suggests that R1 plasmids with copy numbers between 1 and 2 can duplicate at a well-defined time in the cell cycle (Ehrenberg and Sverredal, 1995). This would be accomplished by the *cis* action of a large number of replication proteins, RepA, whose synthesis is inhibited by a small RNA molecule (CopA), which is antisense to the leader, CopT, of RepA mRNA (CopT) (see Nordström and Wagner, 1995, for a review of copy number control in R1).

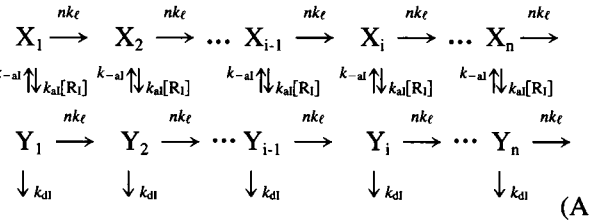
One population-genetic aspect of cell-plasmid systems concerns the optimal average copy number per cell. Such a number can, in principle, be calculated by identifying the maximal growth rate of the host cell population for different numbers of plasmids per cell (cf. Ehrenberg and Kurland, 1984). A cost-benefit analysis would have to take into account the burden of doubling the plasmid DNA pool during the cell cycle and the turnover of R_{II} as well as the large turnover rates of both Rom and R_I, that are necessary to keep their concentrations nearly proportional to the plasmid concentration (Keasling and Palsson, 1989a). A smaller plasmid pool is advantageous in that less DNA has to be synthesized. However, here the demands on the copy number control system will be stricter, because a higher dissipation of free energy is required to reduce plasmid losses. One expected outcome of such a calculation is that for large plasmids the optimum is associated with a lower copy number than it is for small plasmids. It is therefore conceivable that present-day ColE1, which is characterized by multiple copies and slow turnover of RNA II (k_{II}/k_H small) (Brendel and Perelson, 1993), has an evolutionary precedent with larger size, smaller copy numbers, and with a much more precise replication control (k_{II}/k_H large). To be consistent we must also assume that this precedent used a mechanism for active partitioning of plasmids among the daughter cells, i.e., that there were par^+ variants.

When the ColE1 copy number increases above 2, coordination between cell and plasmid cycles is lost (Fig. 2). This is because when one plasmid duplicates, the concentration of R_I increases and this delays the next duplication, until further cell volume expansion has sufficiently reduced the concentration of R_I . Therefore there is a strong tendency for duplications to stay apart in time, in contrast to the synchronized initiations of replication at multiple origins of chromosomal DNA (Skarstad et al., 1986).

APPENDIX 1: MULTIPLE STEP INHIBITION OF R_{II} BY R_I

The present section is devoted to a discussion of the conditions under which Scheme 6 of the main text is valid.

X_i is the state where an RNA polymerase (RNAP) is just transcribing base i of the gene coding for the inhibition window of the initiator RNA, R_{II}, and where there is no contact between the antisense RNA, R_I, and R_{II} upstream of the inhibition window. Y_i is also a transcription state i, but here R_I is unstably bound to R_{II} before duplex formation. This is illustrated in Scheme A1.1:



(A1.1)

RNAP enters the inhibition window in state X_1, assuming that the upstream binding site for R_I is unoccupied at this time point. From X_1 the state Y_1 is reached by association of R_I to R_{II}, which occurs with the rate constant $k_{aI} \cdot [R_I]$. Alternatively, the state X_2 is reached from X_1 by transcription of one base, occurring with rate constant nk_ℓ. From Y_1 the system may return to X_1 by dissociation of R_I from R_{II} with rate constant k_{-aI}. Alternatively, the state Y_2 is reached by transcription (rate constant nk_ℓ). Finally, duplex formation (and inhibition) may occur with the rate constant k_{dI}. The system then moves, according to these same rules, through its different states X_i and Y_i, until it either leaves to the right from state X_n or Y_n by transcription or leaves the diagram by duplex formation and inhibition along one of the vertical branches from states Y_i. Q_0, first used in Eq. 5 in the main text, is the probability that the system A1.1 moves from its starting point in state X_1 all the way to X_n or Y_n and leaves to the right by transcription without duplex formation at any point in the diagram. Q_0 can be found by setting up algebraic equations for the average times, x_i and y_i, that the system spends in its states X_i and Y_i, respectively. Defining $z_i = x_i + y_i$, one obtains Q_0 from the expression

$$Q_0 = z_n \cdot nk_\ell. \tag{A1.2}$$

Define $P_i(t)$ and $Q_i(t)$ as the probability that the system is in state X_i and Y_i, respectively, at time t, given that it started in state X_1 at time 0. The following differential equations are valid

$$\begin{aligned}
\frac{dP_1}{dt} &= -P_1 \cdot (nk_\ell + k_{aI}[R_I]) + Q_1 \cdot k_{-aI} \\
\frac{dQ_1}{dt} &= P_1 \cdot k_{aI}[R_I] - Q_1 \cdot (nk_\ell + k_{-aI} + k_{dI}) \\
\frac{dP_{i+1}}{dt} &= -P_{i+1} \cdot (nk_\ell + k_{aI}[R_I]) + Q_{i+1} \cdot k_{-aI} + P_i \cdot nk_\ell \\
\frac{dQ_{i+1}}{dt} &= P_{i+1} \cdot k_{aI}[R_I] - Q_{i+1} \cdot (nk_\ell + k_{-aI} + k_{dI}) + Q_i \cdot nk_\ell \\
& \qquad i = 1, 2, \cdots n-1.
\end{aligned} \tag{A1.3}$$

The initial conditions are that $P_i(0) = 1$ and that all other probabilities are zero at time 0. Integrating these equations from zero time to infinity, using the definitions

$$x_i = \int_0^\infty P_i(t)\,dt, \qquad y_i = \int_0^\infty Q_i(t)\,dt \tag{A1.4}$$

and that $P_i(\infty) = Q_i(\infty) = 0$, leads to the equations

$$\begin{aligned} -1 &= -x_1 \cdot (nk_\ell + k_{aI}[R_I]) + y_1 \cdot k_{-aI} \\ 0 &= x_1 \cdot k_{aI}[R_I] - y_1 \cdot (nk_\ell + k_{-aI} + k_{dI}) \\ 0 &= -x_{i+1} \cdot (nk_\ell + k_{aI}[R_I]) + y_{i+1} \cdot k_{-aI} + x_i \cdot nk_\ell \\ 0 &= x_{i+1} \cdot k_{aI}[R_I] - y_{i+1} \cdot (nk_\ell + k_{-aI} + k_{dI}) + y_i \cdot nk_\ell \\ & \qquad i = 1, 2, \cdots n-1. \end{aligned} \tag{A1.5}$$

To obtain the algebraic equations in A1.5 we have neglected time variations in the concentration $[R_I]$, because the time the system spends in the inhibition window (~5 s) is much shorter than the generation time (>1 000s) of the host cell.

With the definitions

$$z_i = x_i + y_i, \qquad \alpha_i = \frac{y_i}{z_i}, \tag{A1.6}$$

z_n takes the form

$$z_n = \frac{1}{nk_\ell} \cdot \prod_{i=1}^{n} \frac{1}{1 + k_{dI} \cdot \alpha_i / nk_\ell}, \tag{A1.7}$$

with

$$\begin{aligned} \alpha_1 &= \frac{k_{aI} \cdot [R_I]}{nk_\ell + k_{-aI} + k_{dI} + k_{aI} \cdot [R_I]}, \\ \alpha_{i+1} &= \frac{k_{aI} \cdot [R_I] + nk_\ell \cdot \alpha_i}{nk_\ell + k_{-aI} + k_{dI} \cdot (1 - \alpha_i) + k_{aI} \cdot [R_I]} \\ & \qquad i = 1, 2, \cdots n-1. \end{aligned} \tag{A1.8}$$

Q_0 can now be computed from Eqs. A1.2 and A1.7–8 for any values of the rate constants. The α values in Eq. A1.8 will in general vary with the index i, but they will tend to converge to a constant α. This can be found by setting $\alpha_{i+1} = \alpha_i = \alpha$ in the lower equation in A1.8 and by solving the second-degree equation for α. We will assume, as in the main text, that the mechanism is far from saturated with inhibitor. This means that the α values are much smaller than 1, so that quadratic terms can be neglected, which leads to an equation of first degree in α. The solution is

$$\alpha = \frac{k_{aI} \cdot [R_I]}{k_{-aI} + k_{dI} + k_{aI} \cdot [R_I]}. \tag{A1.9}$$

When, in addition, $k_{-aI} + k_{dI}$ is larger than nk_ℓ, α_1 is close to the limiting α value in Eq. A1.9, and the convergence from α_1 to α is rapid. It is then a good approximation to set the α_i values constant and equal to the α in Eq. A1.9 for all values of i. In this case Q_0 takes the simple form

$$Q_0 = \left(\frac{1}{1 + k_{dI} \cdot \alpha / nk_\ell}\right)^n \approx \exp\left(-\frac{k_{dI} \cdot \alpha}{k_\ell}\right). \tag{A1.10}$$

If $k_{-aI} + k_{dI}$ is smaller, instead of larger, than nk_ℓ, then the mechanism retains its properties over a broad range of conditions, provided that it operates far below maximum inhibition at saturating concentrations of RNA I and Rom. Expression A1.10 may be generalized to take into account how the α_i values converge toward α. For a large number, n, of steps the generalization is (M. Pavlov, personal communication)

$$Q_0 \approx \exp\left[-\frac{k_{dI} \cdot \alpha}{k_\ell}\left(1 - \frac{1 - e^{-\gamma}}{\gamma}\right)\right]. \tag{A1.11}$$

The parameter γ measures how fast α_i converges toward α in relation to the length of the inhibition time window. γ is given by

$$\gamma = \frac{k_{-aI} + k_{dI} + k_{aI}[R_I]}{k_\ell}. \tag{A1.12}$$

The notion of "hypersensitivity" introduced in the main text requires that the probability Q_0 of no inhibition is quite small. This means that the dynamic range of Q_0, which we define as the reciprocal of the minimum of Q_0, obtained at saturating concentration of RNA I, has to be a large number. The dynamic range, DR, is determined by the average time, $\tau_\ell = 1/k_\ell$, that transcription is in the inhibition window, by the number of steps, n, in the window as well as by the rate, k_{dI}, of duplex formation:

$$DR = \left(1 + \frac{k_{dI} \cdot \tau_I}{n}\right)^n. \tag{A1.13}$$

This expression follows immediately from Eq. A1.7 with the saturation condition $\alpha_i = 1$. For a given large value of the product the dynamic range is dramatically increased by multiple-step inhibtion. The conditions that make Scheme 6 in the main text a good approximation and lead to "hypersensitivity" can be summarized as the following two inequalities:

$$1 \ll \frac{1}{Q_0} \ll DR. \tag{A1.14}$$

When $k_{aI} \cdot [R_I]$ is neglected in the denominator of Eq. A1.9, which corresponds to nonsaturating conditions, α is

$$\alpha = \frac{k_{aI} \cdot [R_I]}{k_{-aI} + k_{dI}}. \tag{A1.15}$$

In this case inhibition is completely specified by a second-order rate constant k_{12}, as in Eq. 1 of the main text, and Scheme 6 is valid. Under these conditions the rate constant k_{12} is related to microscopic parameters according to

$$k_{12} = \frac{k_{aI}}{1 + k_{-aI}/k_{dI}}, \tag{A1.14}$$

which is Eq. 11 of the main text.

A similar type of argument leads to Eq. 12 in the main text for the rom^+ case.

APPENDIX 2: RELATIONS BETWEEN PLASMID AND INHIBITOR CONCENTRATIONS

According to Eq. 4 of the main text the concentrations of both R_I and Rom are approximately proportional to the plasmid concentration y. In both cases the proportionality arises from a similar consideration, and we will therefore treat a general case with an inhibitor, I, that is synthesized with rate k_S per plasmid and is degraded with the first-order rate k_D. Thus k_S is k_I for R_I and k_R for Rom. k_D is ϵ_I for R_I and ϵ_R for Rom. The differential equation that governs the concentration, [I], of the inhibitor I is now (Ehrenberg and Sverredal, 1995)

$$\frac{d[I(t)]}{dt} = k_S \cdot y - (k_D + k_H) \cdot [I(t)].$$

In realistic cases the growth rate k_H of the host system is much smaller than the degradation rate k_D of the inhibitor, and hence k_H can be neglected in relation to k_D (i.e., Brendel and Perelson, 1993). Indeed, if this were not the case the plasmid control system would fail. The differential equation then becomes

$$\frac{d[I(t)]}{dt} = k_S \cdot y - k_D \cdot [I(t)]. \qquad (A2.1)$$

Its general solution is given by (Ehrenberg and Sverredal, 1995)

$$[I(t)] = [I(0)] \cdot e^{-k_D t} + k_S \cdot e^{-k_D t} \cdot \int_0^t e^{k_D u} \cdot y(u)\, du. \qquad (A2.2)$$

This solution has a characteristic relaxation rate, k_D, which determines how well $[I(t)]$ follows $y(t)$.

For very large values of k_D the contributions to the integral in Eq. A2.2 are dominated by u values close to t, because the exponential factor increases very rapidly as u approaches the upper integration limit t. If y does not vary too rapidly with time, it is then possible to set $y(u) \approx y(t)$ in the integrand and move y to the outside of the integral sign. Integration of the remaining exponential term shows that in this case $[I(t)]$ is indeed proportional to $y(t)$. Accordingly, if one goes to the limit of large (infinite) values of k_S and k_D, the inhibitor concentration becomes exactly proportional to y for $t > 0$:

$$\lim es[I(t)] = \frac{k_S}{k_D} \cdot y(t) \qquad k_S, k_D \to \infty. \qquad (A2.3)$$

In this limit the expressions in Eq. 4 of the main text are exact, so that this is one example illustrating how increased "precision" is associated with enhanced dissipation of free energy.

One obvious problem of copy number control is rapid adjustment of the inhibitor concentration after a plasmid duplication event, where y jumps from an initial ($y_{initial}$) to a final (y_{final}) value. Neglecting slow variations in y due to cell growth, Eq. A2.2 shows that for this special case the response in inhibitor concentration is

$$[I(t)] = \frac{k_S}{k_D} \cdot (y_{initial} \cdot e^{-k_D t} + y_{final} \cdot (1 - e^{-k_D t})). \qquad (A2.4)$$

According to Eq. A2.4 the inhibitor concentration reaches its new, "correct" value in a time equal to the inverse of the degradation rate constant k_D.

I thank Kurt Nordström for bringing attention to the difference between the B and T and B and P models and Michail Pavlov for carrying out numerical integrations as well as for deriving Eq. A1.11, and both of them for helpful suggestions concerning the manuscript. I also wish to thank Donald Crothers for his comments on Rom action.

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