Analysis of Genetic Toggle Switch Systems Encoded on Plasmids

Adiel Loinger and Ofer Biham
Racah Institute of Physics, The Hebrew University, Jerusalem 91904, Israel
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Genetic switch systems with mutual repression of two transcription factors, encoded on plasmids, are studied using stochastic methods. The plasmid copy number is found to strongly affect the behavior of these systems. More specifically, the average time between spontaneous switching events quickly increases with the number of plasmids. It was shown before that for a single copy encoded on the chromosome, the exclusive switch is more stable than the general switch. Here we show that when the switch is encoded on a sufficiently large number of plasmids, the situation is reversed and the general switch is more stable than the exclusive switch. These predictions can be tested experimentally using methods of synthetic biology.

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Regulation processes in cells are performed by networks of interacting genes, which control each other's expression [1,2]. In recent years these networks have been studied extensively in different organisms. It was proposed that the networks exhibit a modular structure [3–5]. In particular, they include modules or motifs which may perform specific functions and appear in different parts of the network. Common examples of such motifs are the autoregulator [6] and the feed forward loop [4].

In addition to the genetic circuits found in natural organisms, in recent years it has became possible to construct synthetic circuits of a desired architecture [7,8]. These circuits are constructed from available components, namely, genes and promoters. They do not require the manipulation of the structure of proteins and other regulatory elements at the molecular level. These genes and promoters are often inserted into plasmids rather than on the chromosome. This is advantageous because plasmids are easier to manipulate and give rise to a stronger signal. Important examples of synthetic circuits, which were constructed on plasmids in *Escherichia coli*, include the genetic toggle switch [9] which exhibits bistability and the repressilator [10] which exhibits oscillations.

Measurements of gene expression in single cells have shown significant variability in populations of genetically identical cells [11–14]. These results focused the attention on the role of fluctuations and noise in gene expression processes [15–19]. The stochastic analysis is required because some of the proteins and their binding sites appear in low copy numbers. The copy number of the plasmids on which the circuit is encoded can be used as a control parameter for the noise level in the circuit. This is due to the fact that one copy of the circuit is encoded on each plasmid. Thus, increasing the number of plasmids enhances the synthesis rate of proteins and increases the copy numbers of free and bound proteins. Therefore, the plasmids copy number is an important parameter, which should be taken into account in theoretical and computational studies of such circuits. In previous studies, much attention was given to the control of the plasmids copy number itself [20,21]. Recently, it was shown that the plasmids copy number affects the behavior of genetic circuits encoded on them [22–24]. In a recent paper [25] we performed a comprehensive analysis of the repressilator. In particular we have studied the effect of the plasmids copy number of the behavior. We have found that as the number of plasmids is increased the fluctuations are reduced and the stochastic simulation results coincide with those obtained from the rate equations.

In this Letter we analyze the effect of the plasmids copy number on the genetic toggle switch system. The toggle switch consists of two genes, a and b, which negatively regulate each other by transcriptional regulation (Fig. 1). This system was constructed on plasmids using methods of synthetic biology [9]. It was found that under suitable conditions this system exhibits bistability, namely, two stable steady states. In each of these states, one of the proteins (A or B) appears in a high copy number, while the other protein is suppressed. Induced transitions between the two states were demonstrated.

In subsequent theoretical work [26–29], the conditions for bistability were elucidated. Taking into account the effect of noise and fluctuations, spontaneous transitions were found to take place between the two states. The stability of the switch can be characterized by the switch-

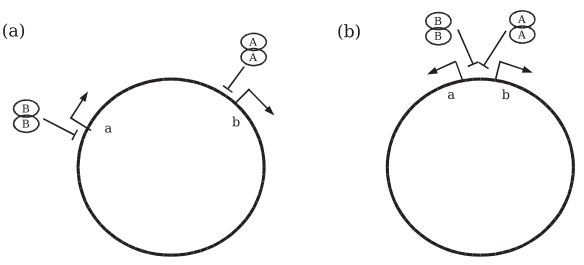

FIG. 1. Schematic illustrations of the general switch (a) and the exclusive switch (b), encoded on a plasmid. The transcriptional regulation is performed by dimers.

ing time τ, namely, the average time between spontaneous transitions. Two variants, the general switch and the exclusive switch, were studied and compared in Refs. [27]. The difference between these two variants is that in the exclusive switch there is an overlap between the two promoter sites, so they cannot be occupied at the same time. The studies in Refs. [27] focused on the case in which the switch system is encoded on the chromosome, namely, each gene and its promoter site appears in a single copy. In addition, cooperative binding was assumed, where the transcriptional regulations are performed by dimers composed of two identical proteins rather than a single one. It was found that both variants exhibit bistability for a broad range of parameters. The exclusive switch was found to be more stable than the general switch. This can be understood as follows. For a spontaneous transition to take place two events must happen. First, a few proteins of the minority type must be synthesized and form dimers. This may happen due to fluctuations in the number of bound repressors of the dominant protein. Second, one of the minority dimers must bind to the promoter site of the dominant gene and suppress the synthesis of the majority protein. The second event is more likely to happen in the case of the general switch since the promoter site is vacant. In the exclusive switch the minority repressor cannot bind as long as the dominant repressor is bound.

Here we examine the effect of the number of plasmids, n, on the stability of the switch. Following Warren and ten Wolde [27] we focus on two variants of the switch: the general switch and the exclusive switch (Fig. 1). The transcriptional regulation is performed by dimers, A_2 and B_2, where A_2 negatively regulates the expression of b and B_2 negatively regulates the expression of a. For simplicity we consider the symmetric case, in which the parameters of the a and b genes and their products are identical. We also ignore the mRNA level and take the processes of transcription and translation as a single step of protein synthesis, with a maximal rate g (s^{-1}) per plasmid. The proteins degrade at a rate μ and the dimers degrade at a rate $\tilde{\mu}$ (s^{-1}). The association rate of two A (B) proteins that form a dimer is γ_0 and their dissociation rate is γ_1 (s^{-1}). The binding rate of a dimer to the specific promoter site on the DNA is α_0 and the unbinding rate is α_1. In the case of the general switch, these processes can be illustrated by the following set of rate equations:

$$\begin{aligned}\frac{d[A]}{dt} &= g(n-[r_B]) - \mu[A] - \gamma_0[A]^2 + \gamma_1[A_2],\\ \frac{d[A_2]}{dt} &= \gamma_0[A]^2 - \gamma_1[A_2] - \tilde{\mu}[A_2]\\ &\quad - \alpha_0[A_2](n-[r_A]) + \alpha_1[r_A]\\ \frac{d[r_A]}{dt} &= \alpha_0[A_2](n-[r_A]) - \alpha_1[r_A],\end{aligned} \tag{1}$$

where $[A]$ is the copy number of A proteins in the cell, $[A_2]$ is the copy number of free A_2 dimers, and $[r_A]$ is the copy number of A_2 dimers bound to the b promoter. These equations are complemented by three symmetric equations for $[B]$, $[B_2]$, and $[r_B]$. Since the transcription in each gene takes place only in the absence of a bound repressor and the number of such genes is $(n-[r_B])$, the synthesis rate of A proteins is $g(n-[r_B])$. The equations for the exclusive switch are nearly identical, except that the $\alpha_0[A_2]\times(n-[r_A])$ term is replaced by $\alpha_0[A_2](n-[r_A]-[r_B])$.

The rate equations presented above involve a mean-field approximation and do not account for the discrete nature of the protein and their binding sites and for the fluctuations in the copy numbers. To account for these effects one needs to perform stochastic analysis, using numerical integration of the master equation [15–17,30,31] or Monte Carlo simulations [15,32,33].

The results presented below were obtained using Monte Carlo simulations of these processes, which enable one to follow the time evolution of a specific realization of the system in a single cell. It also enables one to perform ensemble averages and obtain all the relevant statistical information. The switching times can also be computed using the "forward flux sampling" method [34], which is more efficient computationally, and yields similar results. The parameters that were used in the simulations are $g = 0.05$, $\mu = 0.005$, $\gamma_0 = 0.2$, $\gamma_1 = 0.01$, $\tilde{\mu} = 0.0025$, $\alpha_0 = 0.2$, and $\alpha_1 = 0.01$ (s^{-1}). These are typical parameters for bacteria such as *E. coli* [2]. The results and conclusions presented here are not sensitive to the specific choice of these parameters.

In Fig. 2 we present the switching time τ (namely, the average time between spontaneous switching events) vs the number of plasmids, n, for the general switch (○) and for the exclusive switch (×). In both circuits, adding more plasmids increases the switching time, making the switch more stable. This is consistent with the general picture, according to which increasing the number of plasmids

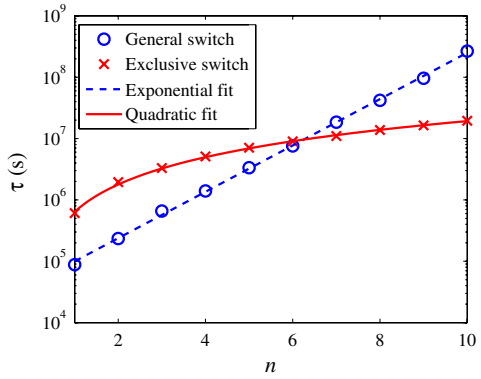

FIG. 2 (color online). The switching time τ for the general switch (○) and for the exclusive switch (×) vs the number of plasmids, n. In both cases τ is a monotonically increasing function of n. For the exclusive switch $\tau(n)$ can be approximated by a quadratic fit (solid line), while for the general switch it can be approximated by an exponential fit (dashed line). For a small number of plasmids the exclusive switch is more stable, up to a crossover point beyond which the general switch is more stable.

reduces the effects of fluctuations, approaching the deterministic limit in which $\tau \to \infty$. In the exclusive switch, τ increases quadratically with n. In the general switch, τ increases exponentially with n. For a small number of plasmids the exclusive switch is more stable than the general switch, in agreement with Refs. [27]. However, since τ increases more rapidly as a function of n for the general switch, there is a crossover point such that for a larger number of plasmids the general switch is more stable. It should be noted that the quadratic dependence of $\tau(n)$ is nonuniversal. In other cases a different polynomial dependence can be obtained. For instance, the exclusive switch without cooperative binding exhibits linear dependence.

We will now try to interpret and explain these results. As the number of plasmids increases, the minority dimers need to bind to a larger number of promoter sites in order to flip the state of the switch. As a result, τ increases as a function of n. To explain why τ increases more rapidly for the general switch, consider a case in which the A proteins are dominant and the expression of gene b is suppressed. When a B_2 dimer is formed and binds to the a promoter, the production of A proteins from this particular plasmid is suppressed. In the exclusive switch there is an additional effect: by binding to the promoter, the production of B proteins is enabled because the bound B_2 dimer prevents the binding of an A_2 dimer to this plasmid. Therefore, once a B_2 dimer is bound, it opens the way to a fast production of B proteins, making it easier for a transition to take place.

The difference in the switching process between the general switch and the exclusive switch is demonstrated in Fig. 3, where we show an example of the dynamic behavior of the bound repressors during a single switching event. In the general switch, B_2 dimers gradually attach while A_2 dimers remain bound [Fig. 3(a)]. During this process, the expression of the b genes encoded on all plasmids is suppressed. Thus, under these conditions the formation of B proteins and dimers and their binding to

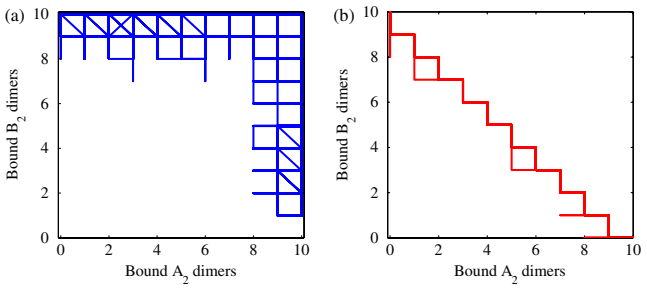

FIG. 3 (color online). Realization of a representative transition path corresponding to one transition from the B dominated to the A dominated state for the general switch (a) and for the exclusive switch (b), encoded on 10 plasmids. The path is shown in the plane representing the numbers of bound A_2 and B_2 dimers. In the general switch the number of bound A_2 dimers builds up, followed by a decrease in the number of bound B_2 dimers. In the exclusive switch A_2 dimers replace bound B_2 dimers one by one.

different plasmids are independent, low probability processes. As a result, the switching time increases exponentially with the number of plasmids.

In the exclusive switch, the switching from the A dominated to the B dominated state occurs when A_2 dimers detach from the promoter site and are replaced by B_2 dimers [Fig. 3(b)]. In this case, the binding of a B_2 dimer to a plasmid facilitates the synthesis of additional B proteins. These B proteins form B_2 dimers, enhancing their binding probability to additional plasmids. Thus, the binding processes of successive B_2 dimers are not independent. As a result, the switching time does not increase as fast as a function of n as in the general switch.

To summarize the situation: in the exclusive switch it is more difficult for the minority dimer to bind. However, once it is bound to one of the plasmids, its effect on the state of the switch is much stronger. Therefore, the advantage of the exclusive binding which makes the switch more stable when it is encoded on a single plasmid (or on the chromosome), becomes a disadvantage when the number of plasmids is large.

It should be noted that changing the number of plasmids is fundamentally different than changing the parameters of the circuit encoded on a single plasmid. Naively, one may expect that increasing the number of plasmids from 1 to n will have a similar effect as increasing the production rate, g, and the binding and unbinding rates α_0 and α_1 by a factor of n. This scaling actually works for simple networks such as the autorepressor, but not for the toggle switch. In Fig. 4 we present Monte Carlo simulation results for the number of free A_2 (blue or black) and B_2 (red or gray) dimers vs time for the general switch. In Fig. 4(a) we present results for a single plasmid, where its parameters are scaled according to $g \to ng$, $\alpha_0 \to n\alpha_0$, and $\alpha_1 \to n\alpha_1$, with $n = 5$. In Fig. 4(b) we show the results for $n = 5$ plasmids. In this case the switch is much more stable and the average switching time is longer by 2 orders of magnitude. These results show that adding plasmids is fundamentally different than changing the parameters of the circuit encoded on a single plasmid.

We have also studied the effect of fluctuations in the copy number of the plasmids themselves. We find that this additional source of noise causes a certain reduction in the switching time. This is, however, a quantitative effect, while the qualitative results and conclusions regarding the behavior of the general and exclusive switch systems remain valid.

In summary, noise and fluctuations play an important role in the dynamics of genetic networks. This noise is not only due to the low copy numbers of mRNAs and proteins, but also due to the low copy numbers of the binding sites involved in transcriptional regulation. For genes encoded on the chromosome, there is typically only one promoter site for each gene. However, for genes encoded on plasmids the number of promoter sites increases with the number of plasmids, providing control over the fluctuation

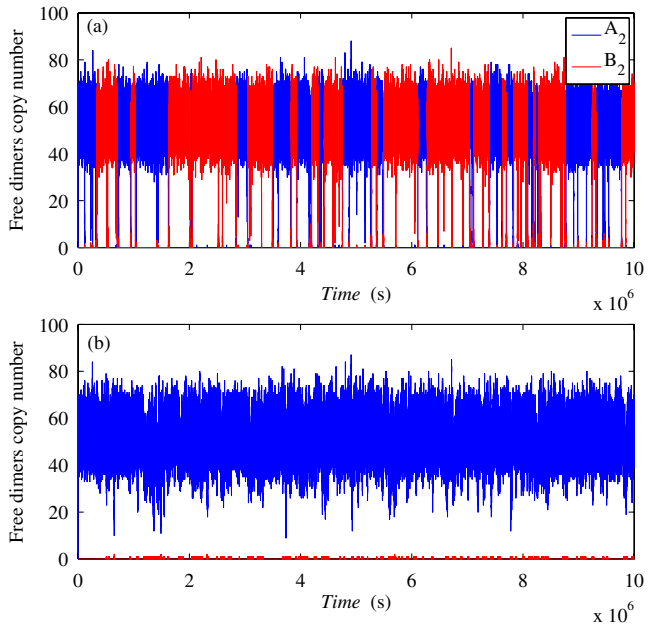

FIG. 4 (color online). The number of free A_2 (blue or black) and B_2 (red or gray) dimers vs time in the general switch: (a) for a single plasmid, with parameters scaled according to $g \rightarrow ng$, $\alpha_0 \rightarrow n\alpha_0$, and $\alpha_1 \rightarrow n\alpha_1$, with $n = 5$; (b) for a circuit encoded on 5 plasmids. In the second case the switching time is longer by 2 orders of magnitude, clearly showing that increasing the number of plasmids is markedly different from rescaling of the parameters by the same factor.

level. Thus, plasmids may serve as an excellent laboratory for the study of noise in genetic regulatory networks.

The plasmids copy number has a strong effect on the behavior of genetic circuits, particularly those that involve feedback such as the genetic switch. We have shown that increasing the number of plasmids dramatically enhances the stability of the switch. We have also shown that varying the number of plasmids is fundamentally different from changing the rate constants of cellular processes. The state of a switch encoded on a single plasmid is controlled by a single bound transcription factor. As a result, a single event driven by fluctuations may lead to a spontaneous transition. For a switch encoded on multiple plasmids, the transition is a more complex process, which requires a series of events resulting in flipping the states of the promoters on all the plasmids.

Methods of synthetic biology enable one to test experimentally the predictions presented above on the effect of the plasmid copy number on the dynamical properties of genetic circuits. These issues should be taken into account in the design of artificial networks that perform desired functions in the cell.

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