# Robustness of synthetic oscillators in growing and dividing cells

Joris Paijmans,[1] David K. Lubensky,[2] and Pieter Rein ten Wolde[1]
[1]*AMOLF, Science Park 104, 1098 XG Amsterdam, The Netherlands*
[2]*Department of Physics, University of Michigan, Ann Arbor, Michigan 48109-1040, USA*
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Synthetic biology sets out to implement new functions in cells, and to develop a deeper understanding of biological design principles. Elowitz and Leibler [Nature (London) **403**, 335 (2000)] showed that by rational design of the reaction network, and using existing biological components, they could create a network that exhibits periodic gene expression, dubbed the repressilator. More recently, Stricker *et al.* [Nature (London) **456**, 516 (2008)] presented another synthetic oscillator, called the dual-feedback oscillator, which is more stable. Detailed studies have been carried out to determine how the stability of these oscillators is affected by the intrinsic noise of the interactions between the components and the stochastic expression of their genes. However, as all biological oscillators reside in growing and dividing cells, an important question is how these oscillators are perturbed by the cell cycle. In previous work we showed that the periodic doubling of the gene copy numbers due to DNA replication can couple not only natural, circadian oscillators to the cell cycle [Paijmans *et al.*, Proc. Natl. Acad. Sci. (USA) **113**, 4063 (2016)], but also these synthetic oscillators. Here we expand this study. We find that the strength of the locking between oscillators depends not only on the positions of the genes on the chromosome, but also on the noise in the timing of gene replication: noise tends to weaken the coupling. Yet, even in the limit of high levels of noise in the replication times of the genes, both synthetic oscillators show clear signatures of locking to the cell cycle. This work enhances our understanding of the design of robust biological oscillators inside growing and diving cells.

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## I. INTRODUCTION

Synthetic biology strives to implement new functions in living cells, and to develop a deeper understanding of biological design principles, using a modular rational design of biochemical reaction networks [1–3]. As synthetic biology becomes more mature, the goal is to design robust, stable, and tunable networks [4–7] that are resilient to the effects of intrinsic noise and stochastic gene expression [8–12]. In oscillators, enhanced robustness has been achieved via the design of the reaction network at the single cell level [13–18], and by connecting multiple cells through quorum sensing [19–21]. These analyses, however, have generally ignored a potentially major source of perturbation to synthetic oscillators: The periodic gene replication and cell division that occur in any growing cell [22,23]. Cell division introduces noise due to the binomial partitioning of the proteins [24,25]. Moreover, we recently showed that circadian oscillators can lock to the cell cycle via the periodic discrete gene duplication events arising from DNA replication during the cell cycle [26]. Here we study in detail how two synthetic oscillators are affected by the cell cycle, and especially by these discrete replication events.

The mechanism by which cellular oscillators can couple to the cell cycle is generic and pertains to any biochemical oscillator in growing and dividing cells. Since the genes need to be replicated during the cell cycle, and because the transcription rate is often proportional to the gene copy number in a cell [10,27], the cell cycle can cause a periodic doubling in the transcription rate of the clock-related genes. While the mechanism of coupling is generic, it is best understood in the context of an oscillator consisting of one clock protein, which is a transcription factor that negatively autoregulates the expression of its own gene [26]. The periodic doubling of the gene copy number due to DNA replication leads to a periodic doubling of the gene density. This means that the synthesis rate of the clock protein depends on the phase of the clock with respect to that of the cell cycle: if the gene is expressed when its gene density is maximal, then the amplitude of the protein concentration will be maximal as well. This increases the amplitude of the oscillation, and since the subsequent decay of the protein concentration does not depend on the gene density, the rise in amplitude will increase the period of the oscillation. The period of the oscillation thus depends on the phase of the oscillator with respect to that of the cell cycle, and, as for any nonlinear oscillator, this allows the cell cycle to strongly influence the synthetic oscillator [28].

The two synthetic oscillators that we study are the repressilator, developed by Elowitz and Leibler [1], and the dual-feedback oscillator, developed by Stricker and co-workers [13]. Both oscillators have been reconstructed in *E. coli*. In our previous work, we showed by mathematical modeling that both oscillators can lock to the cell cycle [26]. Also, the authors of [29] found, independently, by combining modeling with experiments, that the dual-feedback oscillator can be entrained by the cell cycle. Here we study how the coupling strength depends on the noise in gene replication, and, following earlier work [26], on the positions of the genes on the DNA.

To systematically study the effects of the cell cycle on the repressilator and the dual-feedback oscillator, we use computational models that describe how these systems evolve in time using mean-field chemical rate equations. The repressilator consists of three different genes, each of which expresses a transcription factor that represses the next gene in the cycle [1]. This effectively creates autorepression of the genes with a delay, which causes oscillations in the concentration of the three proteins. The dual-feedback oscillator consists of two

 

genes, one coding for an activator and one for a repressor protein [13]. The activator enhances the expression of both genes, while the repressor represses the expression of both genes. Because repressor monomers have to form a tetramer before being active, while activators only have to form dimers, the expression of the genes is only repressed with a delay after being activated by the activators. This delay will cause oscillations in the protein concentrations.

We modify the original computational models of the synthetic oscillators to include the periodic doubling of the mRNA production rate with the cell cycle. We consider the scenario that the synthetic oscillators are incorporated into the chromosome, although we will also discuss the fact that in the experiments the oscillators are implemented on plasmids present at a high copy number [1,13]. Under typical slow growth conditions, *E. coli* has one chromosome at the beginning of the cell cycle, in which case the gene copy number goes from 1 to 2 over the course of the cell cycle. At high growth rates, corresponding to cell division times shorter than the replication time of the DNA (on the order of 40 min), the chromosome can have multiple replication forks, which means that the gene copy number can be larger. Here, we only consider the regime in which the cell division time is on the order of the DNA replication time or longer, such that the gene copy number rises from $N = 1$ at the beginning of the cell cycle to $2N = 2$ at the end. To quantify the sensitivity of the network to the cell cycle, we investigate the effect on the peak-to-peak time in the protein concentrations related to the oscillator for different periods of the cell cycle.

Unlike the Kai circadian clock, these two genetic oscillators comprise more than one operon that shows significant time variation in its expression. This introduces important time scales to the problem: If the genes pertaining to the oscillator are placed at a distance on the chromosome, there is a time delay between when they are replicated. The synthetic oscillators studied here have an intrinsic period that is on the order of hours [1,13], which is similar to the time scale of DNA replication, which takes at least 40 min. Consequently, the time delay can, depending on the reaction network, have a strong effect on the period of the oscillations.

Both synthetic oscillators can lock to the cell cycle for a wide range of cell division times, but, as we reported in our earlier work [26], the effect depends critically on the positioning of the genes on the chromosome: Where the repressilator shows almost no locking when the genes are placed adjacently, the dual-feedback oscillator experiences the strongest effect in this case, and locking decreases as the genes are placed further apart.

The pronounced effect of varying the delay between replication of different genes suggests that synthetic oscillators should also be sensitive to stochastic variation in replication times. Our major goal here is thus to understand how such variation contributes to noise in the period of cellular oscillators. The noise in the replication time is the result of two stochastic processes: The timing of initiation of DNA replication and the progression of DNA replication. Stochasticity in the initiation of replication has the same effect on all the genes on the chromosome; a fluctuation in the initiation time propagates to the replication times of all the genes, leaving the interval between the gene replication times unchanged. In contrast, stochasticity in replication progression introduces temporal fluctuations in the time between the replication of different genes.

Our simulation results show that, for physiological levels for the noise in the gene replication times, the effects of gene replications on the period of the oscillations are strongly attenuated. However, clear signatures of the cell cycle are observable, especially around the 1:1 locking region. We then address the question of which noise source has the strongest effect on attenuating the effects of the cell cycle: initiation or progression of DNA replication. To find out, we study the effects of the cell cycle in two different scenarios: either there is noise in the initiation of replication, such that the timing between replicating different genes is fixed, or the noise is limited to the progression of replication such that the initiation time is fixed and the timing between genes is stochastic. Our results reveal that noise in the initiation of DNA replication reduces the effect of locking much more than noise in DNA replication progression. This is because at biologically relevant noise levels, the standard deviation in the initiation of DNA replication is much larger than that in the progression of replication. Nevertheless, even with high levels of noise in the initiation of DNA replication, the effects of locking are still clearly present for cell division times around the oscillator's period. Our results thus predict that synthetic oscillators will be perturbed by the cell cycle in growing and dividing cells, when the oscillators are implemented on the chromosome.

Below, we provide an overview of the models for the repressilator, the dual-feedback oscillator, and the models for the cell cycle. First we give a description of a completely deterministic cell cycle, and then we introduce stochasticity in the model by changing the time at which DNA replication is initiated and the time it takes to replicate the DNA to stochastic variables. To determine how strongly the oscillators are coupled to the cell cycle, we study how the period of the oscillators scales with the cell division time.

## II. THEORY

To study the effect of the cell cycle on synthetic oscillators, we will use the ordinary differential equation models of the repressilator [1] and dual-feedback oscillator [13], as described in those papers. As we argue in more detail in [26], the key quantity connecting the cell cycle and the oscillator is the gene density, $G(t)$, i.e., the gene copy number per unit cell volume. Because the protein production rate is proportional to the gene copy number, discrete gene replication events cause sudden doubling of the production rate (at least in prokaryotes [27,30]). We include the effects of the discrete gene replication events by making the mRNA production rates due to transcription of each gene $i$ proportional to the gene density $G_i(t) = g_i(t)/V(t)$ [26]. Here $g_i$ is the gene copy number of gene $i$, which switches from 1 to 2 during the cell cycle, and $V(t)$ is the cell volume, which exponentially doubles in size during a cell division time $T_d$. When $g(t)$ and $V(t)$ are deterministic functions of time, $G(t)$ is a periodic function with a period of the cell-division time $T_d$. Note that when we set $G_i(t) = 1$, the models reduce to the original limit cycle oscillators as introduced in [1] and [13].

### A. Repressilator

The repressilator consists of three genes, which sequentially repress each other's expression. As schematically shown in Fig. 1(a), the first gene represses the expression of the second, which represses the third gene, which in turn represses the expression of the first again [1]. To take into account gene replication, the expression of mRNA is proportional to the gene density $G_i(t)$,

$$\frac{dm_i(t)}{dt} = -m_i(t) + \frac{G_i(t)}{\bar{G}_i}\frac{\alpha}{1+[p_{i-1}(t)]^n} + \alpha_0,$$
$$\frac{dp_i(t)}{dt} = -\beta p_i(t) + \gamma m_i(t). \quad (1)$$

Here, $m_i$ and $p_i$ are the concentrations of mRNA and proteins ($i \in \{1,2,3\}$), respectively, both rescaled with the constant of half-maximum repression $K_M$. The transcription rate is assumed to be proportional to the instantaneous gene density $G_i(t)$; importantly, the gene density can differ between the three genes when they are positioned differently on the chromosome; see Fig. 1, panels (b) and (c). We normalize the gene density by the time-averaged gene density, $\bar{G}_i = (1/T_d)\int_0^{T_d} G_i(t)dt$, which depends on the phase of the cell cycle at which the gene is duplicated.

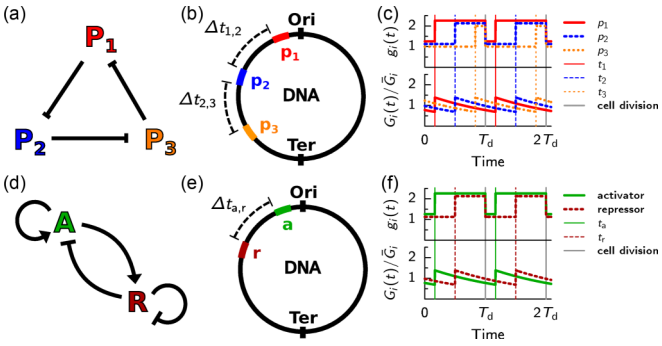


FIG. 1. Models for the synthetic oscillators and cell cycle. (a) Network architecture of the repressilator [1]: $P_1$ represses the production of $P_2$, $P_2$ represses $P_3$, and $P_3$ represses $P_1$ again. (b) Illustration of a circular chromosome, with the origin (Ori) and termination (Ter) of replication. When the three genes $p_1$, $p_2$, and $p_3$ are placed at a distance on the chromosome, there are temporal delays $\Delta t_{1,2}$ and $\Delta t_{2,3}$ between when the genes are replicated. (c) Gene copy numbers (top) and gene densities (bottom) of the genes $p_1$ (red, solid), $p_2$ (blue, dashed), and $p_3$ (orange, dotted), respectively. They are replicated at times $t_1$, $t_2$, and $t_3$, respectively, as indicated by the vertical lines. The thick gray vertical lines indicate cell divisions. For the gene copy number, lines are shifted vertically for clarity. (Bottom) Gene densities for each gene, normalized by their average. (d) Network architecture of the dual-feedback oscillator [13]: The activator ($A$) autoactivates its production and enhances the production of the repressor ($R$). The repressor autorepresses its production and suppresses the production of the activator. (e) Schematic of the circular chromosome. The genes for the activator ($a$) and repressor ($r$) are placed at different positions on the DNA, such that there is a temporal delay, $\Delta t_{a,r}$, between their respective replication times. (f) Gene copy numbers (top) and gene densities (bottom) of the genes $a$ (green, solid) and $r$ (red, dashed), respectively. Genes $a$ and $r$ are replicated at times $t_a$ and $t_r$, respectively, indicated by the vertical lines. The thick gray vertical lines indicate cell divisions. For the gene copy number, lines are shifted vertically for clarity.

TABLE I. Parameters corresponding to the cell-cycle models. The parameters of the original models of the repressilator and the dual-feedback oscillator are given in the captions of Figs. 3 and 5, respectively. SD stands for standard deviation.

| Parameter | Value | Definition and motivation |
|---|---|---|
| $\alpha_{\text{init}}$ | 0.2 | Fraction of $T_d$ when replication starts [31] |
| $T_{\text{rep}}$ | 40 min | Mean DNA replication time in *E. coli* |
| $\Delta t_{1,2}/T_{\text{rep}}$ | $0, \frac{1}{14}, \frac{1}{5}, \frac{1}{2}$ | Time between gene replications (repressilator) |
| $\Delta t_{a,r}/T_{\text{rep}}$ | $0, \frac{1}{8}, \frac{1}{2}, 1$ | Time between gene replications (dual-feedback) |
| $\sigma_{\text{rep}}$ | 0.35 $T_{\text{rep}}$ | SD in DNA replication progression [32] |
| $\sigma_{\text{init}}$ | 0.20 $T_d$ | SD in initiation of DNA replication [32] |

The mRNA expression has a basal rate $\alpha_0$ and an enhanced rate $\alpha$, which is repressed by protein $p_{i-1}$, where $i-1$ is mod 3, with a Hill coefficient $n$; here, following the original paper [1], time is rescaled in units of the mRNA lifetime, and protein concentrations are in units of the concentration necessary for half-maximal repression. In the second equation, $\beta$ is the protein decay rate over the mRNA decay rate and $\gamma$ is the translation efficiency, i.e., the average number of proteins produced per mRNA molecule. The parameters of the original model are given in the caption of Fig. 3, and the parameters corresponding to the cell cycle are given in Table I.

### B. Dual-feedback oscillator

The dual-feedback oscillator, schematically shown in Fig. 1(d), consists of two genes, one coding for an activator and one for a repressor [13]. The activator enhances the expression of both genes, while the repressor represses the expression of both genes. Since the genes have identical promoters, the temporal expression of the two proteins is similar. The model we employ is presented in the supporting information of [13], but to take into account the periodic variations in the gene density, we have modified the equations describing the transcription of mRNA of the activator and repressor,

$$P_{0,0}^{a/r} \xrightarrow{\frac{b_{a/r}}{\bar{G}_{a/r}}G_{a/r}(t)} P_{0,0}^{a/r} + m_{a/r},$$
$$P_{1,0}^{a/r} \xrightarrow{\frac{\alpha b_{a/r}}{\bar{G}_{a/r}}G_{a/r}(t)} P_{1,0}^{a/r} + m_{a/r}. \quad (2)$$

Here $P_{m,n}^{a/r}$ denotes the promoter of the ($a$)ctivator/($r$)epressor gene, with $m = 0,1$ activator protein and $n = 0,1$ repressor protein bound to it, respectively. The mRNA $m_{a/r}$ of the activator ($a$) and repressor ($r$) is transcribed with a rate $(\alpha)b_{a/r}G(t)$, which depends on the state of the promoter and on the gene density $G_{a/r}(t)$. See [33] for a complete set of differential equations and parameters of the model. The parameter values are given in the caption of Fig. 5. Genes can be placed at a distance from each other on the chromosome, as shown in Figs. 1(e) and 1(f), which introduces a delay between when they are replicated. The intrinsic period of this oscillator without the driving by the gene density is ~40 min, and we want to study the behavior of the oscillator in a wide window of cell-division times around this period. Because

in our model of the cell cycle $T_d$ always needs to be longer than the DNA replication time of 40 min, it is convenient to study the dual-feedback oscillator with an intrinsic period that is longer than the current 40 min. To obtain a longer clock period, we use the experimental observation in [13] that the clock period scales with temperature via the Arrhenius law. Toward that end, we scale all time-dependent rate constants, $k_i$, in the dual-feedback model to obtain rescaled rate constants, $k_i'$, using

$$k_i' = k_i \exp(-\Theta_{cc}[1/T - 1/T_{ref}]), \tag{3}$$

where $k_i$ is the rate constant at the reference temperature $T_{ref}$ of 310 K, and $\Theta_{cc} \approx 8300$ K is a constant. We will evaluate the model at a temperature of 303 K where the clock has an intrinsic period of about 73 min.

### C. Cell-cycle model

The time at which a gene is replicated depends on the timing of two major events, which divide the cell cycle into three distinct intervals: The time between the start of the cell cycle and initiation of DNA replication, the replication time of the chromosome, and, after this has finished, the time until cell division. As we argued in [26], cell division has a smaller effect on the oscillator as compared to gene replication, as both the cell volume and the gene copy number divide by 2 at cell division, leaving the important gene density unchanged. Therefore, in our model we assume there is no stochasticity in the division time $T_d$, which we keep fixed. Furthermore, we assume that the *E. coli* cells grow slowly, such that the division time is always longer than the DNA replication time. In this case, there are at most two origins of replication per cell, and we do not have to take into account the effects of multiple replication forks [34].

Because it is still poorly understood how the cell coordinates the replication and division cycles, in this work we employ a simple model for the cell cycle. Evidence emerged that initiation of chromosome replication is triggered at a fixed density of the origin of replication (Ori), $\hat{G}_{Ori}$, independent of a cell's division time [31,35]. Given that the density of the Ori depends on the cell volume $V(t)$, $G_{Ori} = 1/V(t)$, the time and precision of initiation of DNA replication is set by the evolution of the cell volume and the precision of the sensor for $G_{Ori}(t)$. Because we consider the slow growth regime where at the beginning of the cell cycle there is only one origin of replication, and because we assume that the initial volume is independent of the growth rate, it follows that the average time at which DNA replication is initiated is at a fixed fraction $\alpha_{init}$ of the division time $T_d$, $\Delta t_{init} = \alpha_{init} T_d$, with a standard deviation $\sigma_{init}$. We choose, based on data presented in [31], $\alpha_{init} = 0.2$. The time it takes to replicate the chromosome depends on the speed of the DNA polymerase, which in turn can depend on the cell's physiological state [32]. For simplicity, we assume that the mean time to replicate the whole chromosome is $T_{rep} = 40$ min with a standard deviation given by $\sigma_{rep}$, both independent of the cell's division time. In this work, we consider two models for the timing of gene replications: one in which both the initiation and the progression of DNA replication are deterministic, such that gene replications occur at the same phase in each cell cycle, and one in which we introduce noise in these two processes. The effects of noise in the initiation and progression of DNA replication on gene replication times is illustrated in Fig. 2.

#### 1. Deterministic model

The first model is completely deterministic. Indeed, when we assume the evolution of the cell volume, $V(t)$, to be deterministic and that DNA replication initiates exactly when $G_{Ori}(t) = \hat{G}_{Ori}$, then the evolution of $G_{Ori}(t)$ becomes fully deterministic. Clearly, since both the initiation and the progression of DNA replication are deterministic, the respective genes are copied at the same times in each cell cycle [see Figs. 2(a) and 2(b)]. Furthermore, in our model the first gene of the oscillator is next to the origin of replication, such that the time this gene is replicated is $t_1 = \Delta t_{init} = \alpha_{init} T_d$. Note that it is not important when exactly during the cell cycle the gene is replicated, as it only changes the gene density by a prefactor, which we compensate for by normalizing $G_i(t)$ by its mean $\bar{G}_i$. However, as we will see, the time between the replication of the different genes is important. Genes can be placed apart on the DNA, which introduces a time delay, $\Delta t_{i,j}$, between when the genes $i$ and $j$ are copied, respectively. The times during the cell cycle when the genes $p_1$, $p_2$, and $p_3$ are replicated for the repressilator, and the times when the activator and repressor genes are replicated for the dual-feedback oscillator, are

$$\begin{aligned} t_1 &= \Delta t_{init}, & t_a &= \Delta t_{init}, \\ t_2 &= t_1 + \Delta t_{1,2}, & t_r &= t_a + \Delta t_{a,r}, \\ t_3 &= t_2 + \Delta t_{2,3}. \end{aligned} \tag{4}$$

#### 2. Stochastic model: Noise in the initiation and progression of DNA replication

For the second model, we again assume that the evolution of the cell volume is deterministic, but we turn replication progression and replication initiation into stochastic processes. Due to stochasticity in the progression of DNA replication, the time interval between the gene replication events becomes stochastic, as illustrated in Fig. 2(c). We assume the time it takes to replicate the full chromosome follows a Gaussian distribution with a mean $T_{rep} = 40$ min and standard deviation $\sigma_{rep}$ that is proportional to the replication time $T_{rep}$. When the standard deviation in the DNA replication time is the result of many independent stochastic steps, the time between replicating genes $i$ and $j$, $\delta\tau_{i,j}$, which on average takes a time $\Delta t_{i,j}$, will therefore also be Gaussian-distributed with a standard deviation of $\sqrt{\Delta t_{i,j}/T_d}\,\sigma_{rep}$.

Stochasticity in the initiation of replication affects the replication times of all genes equally; indeed, the time between copying two different genes, $\Delta t_{i,j}$, is constant, as is shown in Fig. 2(d). This stochasticity in the timing of the initiation can come from the sensing limit of measuring $G^{Ori}(t)$, or because of stochasticity in the evolution of the cell volume (which, however, we assume progresses deterministically in this scenario). In our model, the time of initiation of DNA replication, $\delta\tau_{init}$, is a stochastic variable drawn from a Gaussian probability distribution with a mean $\alpha_{init} T_d$ with a standard deviation $\sigma_{init}$. Assuming the standard deviation in measuring $G_{Ori}(t)$, $\sigma_{G_{Ori}}$, is small, the standard deviation in the

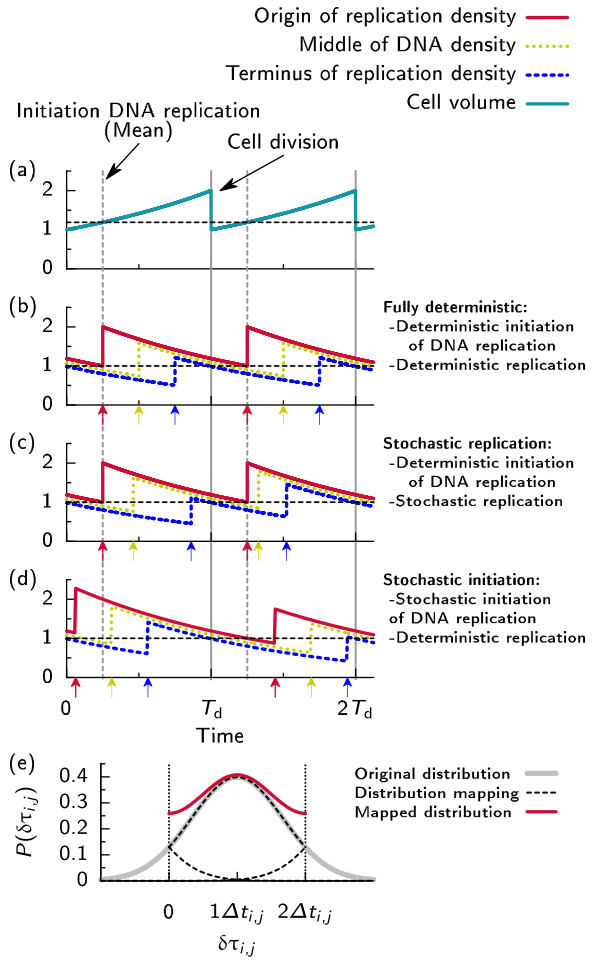


FIG. 2. Models to determine the gene replication times. (a) Time trace of cell volume, which is a deterministic function of time in all models, where cell division occurs with a period $T_d$, indicated by the vertical solid gray lines. The vertical dashed gray lines indicate the times at which DNA replication is initiated when the timing of the initiation of replication is deterministic. The horizontal dashed lines show the volume (a) or the concentration of the origin of replication (b)–(d), at which DNA replication, on average, initiates. (b)–(d) Time traces of the density of the origin of replication of the chromosome (red solid line), a gene precisely half-way between the origin and terminus of replication (green dotted line) and the terminus of replication (blue dashed line). Arrows below the $x$ axis indicate the replication times of these sites. Note that the gene densities show no discontinuity at cell division. All gene densities are normalized by the critical density for replication initiation. (b) Fully deterministic model. Initiation of replication and the replication of the two genes occur at fixed times each cell cycle. (c) When there is stochasticity in DNA replication progression, the timing between initiation of replication and the replication of genes further along the DNA becomes stochastic. (d) When the initiation of replication is stochastic, but the replication rate is constant, all replication events move in concert, and the time between initiation and replication of the genes is fixed. (e) Probability density of gene replication times $P(\delta\tau_{i,j})$. A tentative replication time $\delta\tau'_{i,j}$ drawn from a Gaussian distribution (gray thick line) could lie outside the domain $[0,2\Delta t_{i,j}]$ (dotted vertical lines). In this case, $\delta\tau'_{i,j}$ is mapped back onto this domain by mirroring the value across the nearest domain boundary (black dashed lines). After the mapping, the replication times follow a flatter distribution (red solid line).

initiation time is

$$\sigma_{\text{init}} = \left|\frac{d(\Delta t_{\text{init}})}{dG_{\text{Ori}}}\right|_{G_{\text{Ori}}=\hat{G}_{\text{Ori}}} \sigma_{G_{\text{Ori}}}. \tag{5}$$

DNA replication is initiated when $G_{\text{Ori}} = V_0^{-1}\exp[-\ln(2)/T_d\,\Delta t_{\text{init}}] = \hat{G}_{\text{Ori}}$, where $V_0$ is the cell volume after cell division. Solving this equation for the initiation time gives $\Delta t_{\text{init}} = -T_d \ln(\hat{G}_{\text{Ori}}\,V_0)/\ln(2)$. Then, from Eq. (5) it follows that the standard deviation in the initiation time is $\sigma_{\text{init}} \sim T_d\sigma_{G_{\text{Ori}}}$. Therefore, in our model, the standard deviation in the initiation time is proportional to $T_d$.

Assuming that the two stochastic processes are independent, the replication times of the genes for the repressilator and dual-feedback oscillator become, respectively,

$$\begin{aligned} t_1 &= \delta\tau_{\text{init}}, & t_a &= \delta\tau_{\text{init}}, \\ t_2 &= t_1 + \delta\tau_{1,2}, & t_r &= t_a + \delta\tau_{a,r}, \\ t_3 &= t_2 + \delta\tau_{2,3}. \end{aligned} \tag{6}$$

Because in our model the division time is fixed in each cell cycle, we have to constrain the values of the replication times to lie within the finite interval $[0,T_d]$. First we choose $\delta\tau_{\text{init}}$, and we constrain it to lie within $[0,(T_d - T_{\text{rep}})]$. Then we draw a value for $\delta\tau_{1,2}$ and constrain it to lie within the interval that is symmetric around its mean value $\Delta t_{1,2}$, $[0,2\Delta t_{1,2}]$. Similarly, we draw a value for $\delta\tau_{2,3}$ constrained to the interval $[0,2\,\Delta t_{2,3}]$. For the dual-feedback oscillator, the times $\delta\tau_{a,r}$ are constrained to the interval $[0,2\,\Delta t_{a,r}]$. When a tentative replication time $\delta\tau'$ lies outside this interval because, for instance, it has a negative value, we map it back on the interval by mirroring the value across the nearest boundary $\delta\tau' \to -\delta\tau'$. We apply a similar mapping when the tentative value lies to the right of the domain, as shown in Fig. 2(e).

Recent single-cell experiments revealed the coefficient of variation (CV) in the time of initiation of DNA replication, $\text{CV}_{\text{init}} = 0.7$, and in the time of replicating the DNA, $\text{CV}_{\text{rep}} = 0.16$, in slow-growing *E. coli* cells [32]. Given our models for stochasticity in replication times (including the fact that the initiation times are constrained to lie in the windows discussed above), we find that standard deviations of $\sigma_{\text{init}} = 0.2T_d$ and $\sigma_{\text{rep}} = 0.35T_{\text{rep}}$ give similar coefficients of variation. All parameters are listed in Table I.

## III. RESULTS

Here we study how the peak-to-peak times of the oscillations of the repressilator and the dual-feedback oscillator depend on the cell-division time. Furthermore, we illuminate the effects of the position of the genes on the DNA and the role of stochasticity in the replication times. Preliminary work on the effect of gene positioning was reported in the supporting information of [26].

### A. Repressilator

We first consider the scenario in which the three genes are close together on the chromosome, such that, to a good approximation, they are replicated at the same time, and the timing of DNA replication is fixed. In Fig. 3(a), we show the mean peak-to-peak time, $T_{\text{PtP}}$, in the concentration of $P_1$,

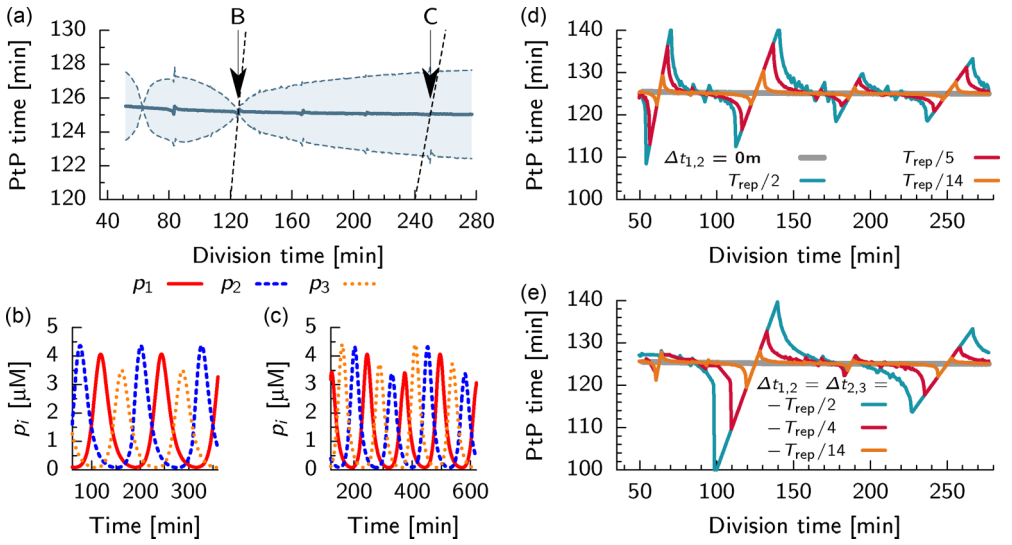


FIG. 3. The repressilator [1] can strongly lock to the cell cycle, and the strength of locking depends sensitively on how the genes are positioned on the DNA. (a) Average (solid line) and standard deviation (shaded region) of the peak-to-peak time $T_{\mathrm{PtP}}$ as a function of the division time, where the time between replicating genes is $\Delta t_{1,2} = \Delta t_{2,3} = 0$. The repressilator has an intrinsic period of $T_{\mathrm{int}} = 125$. The locking regions around $T_{\mathrm{int}}$ and $2T_{\mathrm{int}}$ are almost absent. (b) and (c) Representative time traces of the concentrations of the three repressilator proteins, $p_1(t)$ (red, solid), $p_2(t)$ (blue, dashed), and $p_3(t)$ (orange, dotted), for the cell-division times indicated by the arrows in panel (a). (b) When $T_d = T_{\mathrm{int}}$, the oscillations are very regular (almost no variance in the PtP-times), but each protein concentration has a different amplitude. (c) At $T_d = 2T_{\mathrm{int}}$, all three protein concentrations switch between a small and a large amplitude in successive oscillation cycles. Panels (d) ($\Delta t_{1,2} > 0$) and (e) ($\Delta t_{1,2} < 0$) show the effect of varying the timing of replication of the three genes, assuming $\Delta t_{2,3} = \Delta t_{1,2}$. We show results for four values of $\Delta t_{1,2}$, given in the legend, and expressed as a fraction of the mean DNA replication time $T_{\mathrm{rep}}$. In panels (d) and (e), from top to bottom, the value of $\Delta t_{1,2}$ decreases. For clarity, we only show the average peak-to-peak time as a function of $T_d$, not the standard deviation. Remarkably, for all $\Delta t_{1,2} \neq 0$, there is significant locking. Clearly, the timing of gene replication can markedly affect locking, which means that the spatial distribution of the genes over the chromosome can be of critical importance in the interaction between the clock and the cell cycle. Parameters used in all simulations of the repressilator (from [1]): $\alpha_0 = 2.16 \times 10^{-3}$, $\alpha = 2.16$, $\beta = 0.2$, $\gamma = 20$, and $n = 2$. (Figure adapted from [26].)

for different cell division times, $T_d$. Clearly, locking is not very strong: The locking regions—the range of cell division times where the mean peak-to-peak time of the repressilator is equal to a multiple of $T_d$—are very small. The only effect of locking is that in these very small windows the variance in the peak-to-peak time is strongly reduced. The reason why locking is weak is that while the genes are replicated at the same time, they are expressed at different times. This means that gene replication has a different effect on the expression level of each of the three genes. Hence, even when the cell-cycle period $T_d$ is approximately equal to the oscillator's intrinsic period $T_{\mathrm{int}}$, $T_d \approx T_{\mathrm{int}}$, where $T_{\mathrm{int}}$ is the oscillator's period when there would be no effects from the cell cycle, e.g., $G_i(t) = 1$, the oscillation of each protein concentration has a different amplitude, as shown in Fig. 3(b). This makes it harder for all three protein oscillations to get the same period as that of the cell cycle, and become locked to it. Interestingly, Fig. 3(c) shows that when the cell-cycle time is twice the intrinsic clock period, the pattern of alternating smaller and larger oscillation amplitudes can still be observed for each of the respective protein concentration profiles. This observation can be used to detect the effect of periodic gene replication experimentally.

We now consider a scenario in which the different genes are replicated at different times during the cell cycle, which corresponds to a situation in which the genes are located at different positions on the chromosome. We assume that the gene for protein $p_1$ is close to the origin of replication, such that it is copied at the moment DNA replication is initiated. We consider two scenarios for the order of the genes on the DNA. In the first scenario [panel (d)], genes are placed on the DNA in the order of their interaction in the biochemical reaction network, $p_1, p_2, p_3$ [see Fig. 1(a)]: The gene for $p_2$ is copied a time $\Delta t_{1,2}$ after $p_1$, and $p_3$ is copied a time $\Delta t_{2,3}$ after $p_2$. In the second scenario [panel (e)], genes are in order of maximal expression: $p_3, p_2, p_1$ [see Figs. 3(b) and 3(c)], which corresponds to negative values of $\Delta t_{1,2}$ and $\Delta t_{2,3}$. Throughout this work, we will use the condition $\Delta t_{1,2} = \Delta t_{2,3}$. Interestingly, while the locking regions are very small when the genes are replicated at the same time [$\Delta t_{1,2} = 0$, panel (a), gray lines in panels (d) and (e)], replicating them at different times introduces marked locking: both for $\Delta t_{1,2} > 0$ [panel (d)] and $\Delta t_{1,2} < 0$ [panel (e)] strong locking is observed. Even more strikingly, the 1:1 locking region is largest when genes are replicated in the order of maximal expression, and when the distance between them is the largest [panel (e)]. This can be understood by noting that when genes are replicated in the order of maximal expression, shifting the phase of the clock with respect to that of the cell cycle has then the strongest effect on the amplitude and hence the period of the clock oscillations, which underlies the phenomenon of locking, as explained in [26].

To see if locking persists in the presence of physiological levels of noise in gene replication times, we change the gene replication times $t_1$, $t_2$, and $t_3$ into stochastic variables

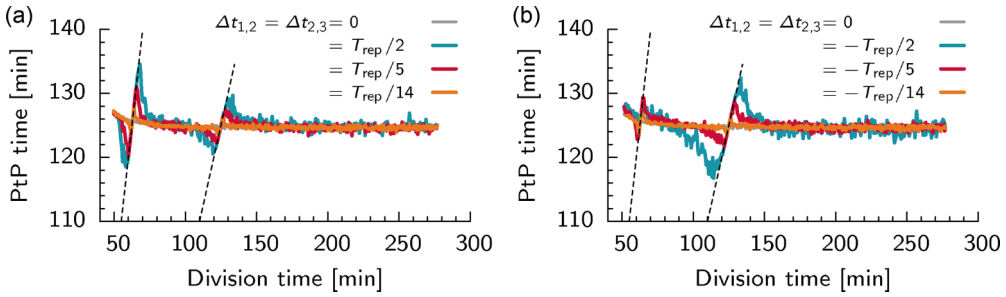


FIG. 4. In the repressilator, locking persists in the presence of physiological levels of noise in the gene replication times. In all panels the solid lines show the peak-to-peak time in the activator concentration for different periods of the cell division time $T_d$. Standard deviation in $T_{PtP}$ omitted for clarity, but it is similar in all panels. The amplitudes of the functions decrease as the time interval $\Delta t_{1,2}$ decreases, as given in the legend. We used the physiologically motivated values for the standard deviations in the timing of the initiation, $\sigma_{init} = 0.2T_d$, and the progression, $\sigma_{rep} = 0.35T_{rep}$, of DNA replication. The two panels show a different order of the genes $p_1$, $p_2$, and $p_3$ with $\Delta t_{1,2} > 0$ (a) and $\Delta t_{1,2} < 0$ (b). Clearly, at these noise levels, locking is strongly reduced compared to the deterministic case [see Figs. 3(d) and 3(e)], but it is still clearly observable around $T_d = T_{int}$.

via Eq. (6). Our results reveal that both when $\Delta t_{1,2} > 0$ [Fig. 4(a)] and when $\Delta t_{1,2} < 0$ [Fig. 4(b)], the coupling of the repressilator to the cell cycle is strongly attenuated. However, the effects of the cell cycle are still clearly observable around the 1:1 locking region and when $T_d = 0.5T_{int}$. For division times longer than the intrinsic period of the oscillator, all signatures of locking have disappeared.

### B. Dual-feedback oscillator

Figure 5(a) shows strong locking of the dual-feedback oscillator to the cell cycle. We assume here that the genes are located next to each other on the chromosome, so that their time-varying gene densities are the same. Clearly, the widths of the locking regions are very large; they are even larger than those observed for our simple negative feedback oscillator studied in [26]. In Fig. 5(b) we show a time trace of the irregular oscillations around a cell-division time of $T_d = 98$ min. Figure 5(c) shows that the amplitude of the oscillations alternates between a high and a low value when the cell-division time $T_d$ is about twice the intrinsic clock period of $T_{int} = 74$ min, due to periodic gene replication every other clock period. We thus conclude that also the dual-feedback oscillator can strongly lock to the cell cycle and that this effect should be observable experimentally.

Figures 5(d) and 5(e) show the result of varying the moment of gene replication for the two genes. Again, in this model, the first gene of the oscillator is placed next to the origin of replication such that it is replicated at initiation of DNA replication, and the second gene is replicated with a mean delay $\Delta t_{a,r}$ later. For positive $\Delta t_{a,r}$, the activator is replicated before the repressor, and vice versa for negative $\Delta t_{a,r}$. We vary the time delay between the replication of the two genes, as $\Delta t_{a,r} = 0$, $T_{rep}/8$, $T_{rep}/2$, and $T_{rep}$ [panel (d)] and minus these values [panel (e)], where $T_{rep}$ is the mean replication time of the DNA. It is seen that in both scenarios the strength of locking decreases upon increasing the distance between the genes on the DNA: The strongest entrainment is observed when the genes are replicated at the same time during the cell cycle (gray lines), in stark contrast to the behavior of the repressilator. While in the repressilator the locking increases with the distance between the genes, the dual-feedback oscillator shows the opposite behavior. Interestingly, though, in the dual-feedback oscillator locking still persists when the genes are placed at a maximum distance from each other.

To see if locking persists in the presence of noise in the timing of gene replications, we changed the time of replication of both genes, $t_a$ and $t_r$, into stochastic variables via Eq. (6). The noise strongly attenuates the effects of the cell cycle, both for positive [Fig. 6(a)] and negative [Fig. 6(b)] $\Delta t_{a,r}$, as compared with the deterministic result of Fig. 5. However, the peak-to-peak times of the dual-feedback oscillator are still perturbed around the 1:1 locking region, especially in the case $\Delta t_{a,r} > 0$.

### C. What attenuates the effects of the cell cycle more: Stochasticity in the initiation or progression of DNA replication?

Comparing Fig. 3 with Fig. 4 for the repressilator and Fig. 5 with Fig. 6 for the dual-feedback oscillator, it is clear that noise in gene replication times has a significant effect on the coupling between the cell cycle and these synthetic oscillators. In our model, noise in the replication times is the result of noise in the initiation and in the progression of DNA replication. We want to know which of these two sources of stochasticity is key for reducing the coupling between the cell cycle and the oscillator.

To find out whether the initiation or the progression of DNA replication is more important for attenuating the effects of gene replications, we studied two models for the noise in the replication times. In the first model, there is only noise in the progression of replication, such that the time intervals between replicating different genes, $\delta\tau_{1,2}$ and $\delta\tau_{a,r}$, are stochastic variables but the time of initiation of DNA replication is deterministic, $\Delta t_{init} = \alpha_{init} T_d$ [see Fig. 2(c)]. In the second, the initiation of DNA replication, $\delta\tau_{inti}$, is stochastic but the progression of replication is deterministic such that $\Delta t_{1,2}$ and $\Delta t_{a,r}$ are fixed each cell cycle [see Fig. 2(d)]. We will use the same values for the standard deviations $\sigma_{init}$ and $\sigma_{rep}$ of the two noise sources as before.

In Fig. 7 we show the effects of the cell cycle on the period of the repressilator when there is only noise in the progression of replication [panels (a) and (b)], or when there is only noise in the initiation of DNA replication [panels (c) and (d)]. Clearly,

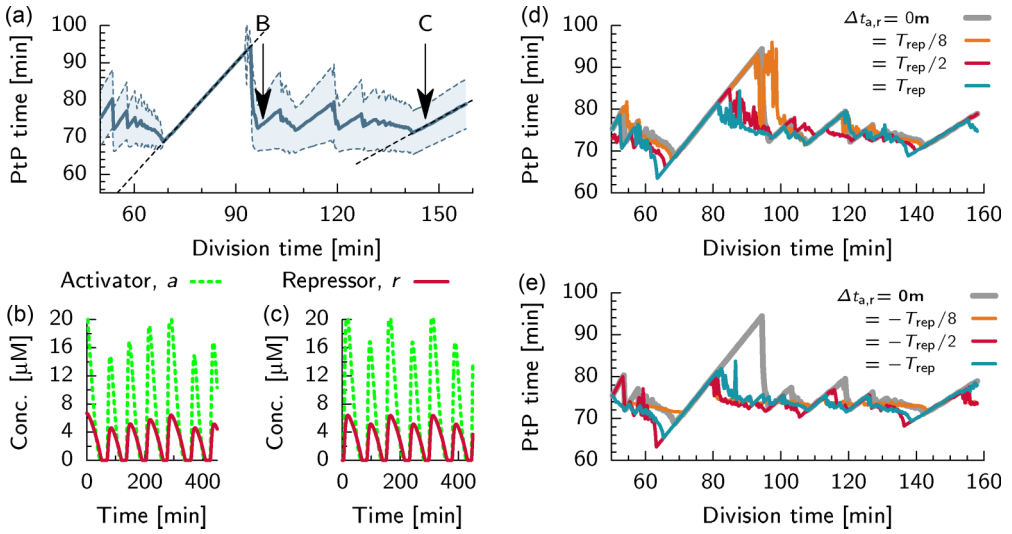


FIG. 5. The dual-feedback oscillator [13] can strongly lock to the cell cycle, and the strength of locking depends on the temporal order in which the genes are replicated during the cell cycle. The intrinsic period of the oscillator $T_{\rm int} = 73$ min. (a) Average (solid line) and standard deviation (shaded region) of the peak-to-peak time $T_{\rm PtP}$ as a function of the division time $T_d$ when both genes are replicated simultaneously, $\Delta t_{a,r} = 0$. There is a wide region of cell division times (around $T_d = T_{\rm int}$) where the oscillator has a $T_{\rm PtP}$ equal to the cell cycle (left dashed line). (b) and (c) Representative time traces for the division times indicated by the arrows in panel (a). Shown are the activator and repressor concentrations $a(t)$ (green, dashed) and $r(t)$ (red, solid), respectively. At a cell-division time of $T_d = 98$ min (b), just outside the region where the oscillator is locked to the cell cycle, the time traces show very irregular behavior resulting in a large variance in the PtP times. At $T_d = 2T_{\rm int}$ (c), the oscillations switch between a small and a large amplitude in successive oscillation cycles, a signature of the periodic gene replications. (d) and (e) The effect of the order of gene replication during the cell cycle. For clarity, only the average peak-to-peak time as a function of $T_d$ is shown, not the standard deviation. Values of $\Delta t_{\rm a,r}$ are given in the legend, and they are written as a fraction of the DNA replication time $T_{\rm rep}$. The amplitudes of the functions increase as the time interval $\Delta t_{a,r}$ decreases. (d) Positive $\Delta t_{a,r}$; the repressor gene is replicated after the activator gene. (e) Negative $\Delta t_{a,r}$; the repressor gene is replicated before the activator gene. Remarkably, contrary to the behavior of the repressilator, locking decreases with increasing time delay between replicating genes $\Delta t_{a,r}$. This illustrates that the influence of the cell cycle on the clock depends in a nontrivial way on the architecture of the clock and on the nature of the driving signal. Parameters used in all simulations of the dual-feedback oscillator (from [13]): $b_a = b_r = 0.36$ min$^{-1}$, $\alpha = 20$, $k_{-a} = k_{-r} = 1.8$ min$^{-1}$, $t_a = t_r = 90$ min$^{-1}$, $d_a = d_r = 0.54$ min$^{-1}$, $k_{fa} = k_{fr} = 0.9$ min$^{-1}$, $k_{da} = k_{dr} = k_t = 0.018$ min$^{-1}$ mol$^{-1}$, $k_{-da} = k_{-dr} = k_{-t} = 0.000\,18$ min$^{-1}$, $k_l = 0.36$ min$^{-1}$, $k_{ul} = 0.18$ min$^{-1}$, $\gamma = 1080$ mol/min, $c_e = 0.1$ mol, $\gamma = 2.5$, $\epsilon = 0.2$, $k_a = 0.059$ min$^{-1}$, and $k_r = 0.018$ min$^{-1}$. For the full set of equations describing the model, see [33]. The rate constants $k_a$ and $k_r$ set the rate at which activators and repressors bind to the promoter, respectively. In the original experiment, these rates can be tuned by the concentration of the inducers arabinose and IPTG. For the value of the rate constants given, we used [IPTG] = 2 nM and [ara] = 0.7%. (Figure adapted from [26].)

when there is only noise from replication progression, both for positive [panel (a)] and negative [panel (b)] $\delta\tau_{1,2}$, the width of the locking regions is almost the same as compared to the deterministic case [see Figs. 3(d) and 3(e)]. The effects of the cell cycle are not significantly attenuated by the noise in the DNA replication progression. However, when the noise is due

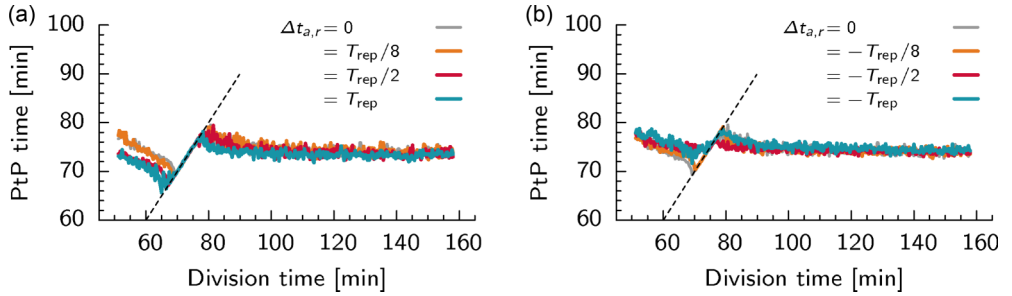


FIG. 6. In the dual-feedback oscillator, locking persists in the presence of physiological levels of noise in the gene replication times. In all panels, solid lines show the peak-to-peak time in the activator concentration for different periods of the cell division time $T_d$. Standard deviation in $T_{\rm PtP}$ omitted for clarity, but it is similar in all panels. The amplitudes of the functions are similar for different time intervals $\Delta t_{a,r}$. Legends are defined in Fig. 5. We used the physiologically motivated values for the standard deviations in the timing of the initiation, $\sigma_{\rm init} = 0.2T_d$, and the progression, $\sigma_{\rm rep} = 0.35T_{\rm rep}$, of DNA replication. The two panels show a different order of the activator and repressor gene with $\Delta t_{a,r} > 0$ (a) and $\Delta t_{a,r} < 0$ (b). As observed for the repressilator, locking is strongly reduced compared to the deterministic case [see Figs. 5(d) and 5(e)], but it is still clearly observable around $T_d = T_{\rm int}$.

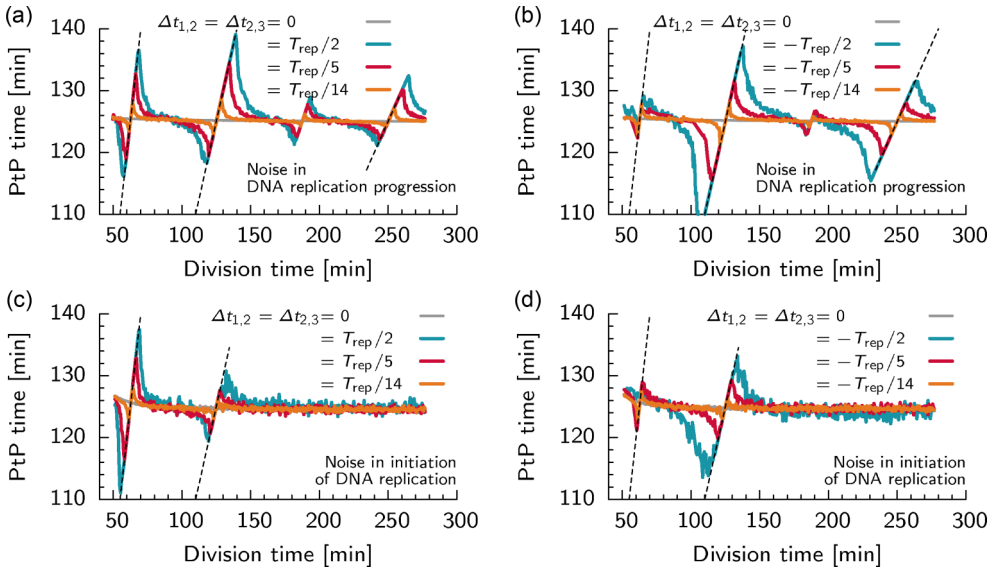


FIG. 7. In the repressilator, stochasticity in the initiation of DNA replication plays the dominant role in attenuating the effects of gene replications. The top row, panels (a) and (b), shows results with only noise in the progression of DNA replication, $\sigma_{rep} = 0.35T_{rep}$, and the bottom row, panels (c) and (d), corresponds to the situation in which there is only noise in replication initiation, $\sigma_{init} = 0.2T_d$ (see Fig. 2). In both panels, solid lines show the peak-to-peak time $T_{PtP}$ in the oscillations of $P_1$ as a function of the cell division time $T_d$. Standard deviation in $T_{PtP}$ omitted for clarity, but it is similar in all panels. Legends are defined in Fig. 3. The amplitudes of the functions decrease as the time interval $\Delta t_{1,2}$ decreases. (a),(b) When there is noise in the time intervals between the gene replication events, but the initiation of DNA replication is fixed, locking seems little affected compared to the deterministic case [see Figs. 3(d) and 3(e)]. (c),(d) When there is noise in the initiation of DNA replication, but the time between replications is fixed, the effects of the cell cycle almost disappear for division times $T_d > T_{int}$, in both ways of ordering the genes. However, strong locking persists at the 1:1 locking region and for $T_d < T_{int}$. Comparing with panels (a) and (b), noise in the initiation of DNA replication seems to be more effective in protecting the clock against the cell cycle.

to the initiation of replication [panels (c) and (d) for positive and negative $\delta\tau_{1,2}$, respectively], all signatures of coupling disappear for $T_d > T_{int}$, and the width of the 1:1 locking region is strongly reduced compared to the case of a deterministic cell cycle. We conclude that the decrease in locking to the cell cycle is due predominantly to the stochasticity in the initiation time of DNA replication.

For the dual-feedback oscillator we obtain similar results. In Fig. 8 we show the effects of the cell cycle on the period of the dual-feedback oscillator when there is only noise in the progression of replication [panels (a) and (b)], or when there is only noise in the initiation of DNA replication [panels (c) and (d)]. When there is only noise due to the progression of DNA replication, both for positive [panel (a)] and negative [panel (b)] $\delta\tau_{a,r}$, strong signatures of locking persist, especially around $T_d = T_{int}$ and $T_d = 2T_{int}$. Again, stochasticity in DNA replication progression does not attenuate the coupling to the cell cycle much. When the source of noise is due to stochasticity in the initiation of DNA replication, almost all effects of the cell cycle on the peak-to-peak time of the dual-feedback oscillator have disappeared; only when $T_d = T_{int}$ can locking still be observed. Clearly, also for the dual-feedback oscillator the initiation of DNA replication has the biggest effect on the coupling between the cell cycle and the oscillator.

We observe that, both for the repressilator and the dual-feedback oscillator, the initiation of DNA replication is dominant in attenuating the effects of the cell cycle. Why is this the case? An oscillator couples to the cell cycle by maintaining a specific phase relation between the phase of the oscillator and that of the gene density, as explained in [26]. When the standard deviation in the replication times is of the same order as the intrinsic period of the oscillator, it becomes impossible to maintain this phase relation, and the oscillator cannot couple to the cell cycle. Because in our model the standard deviation in the initiation of replication is proportional to $T_d$, while the standard deviation in replication *progression* is constant, initiation of DNA replication will be the dominant source of noise when $T_d > T_{int}$. Indeed, for $T_d > T_{int}$, the stochasticity in the initiation of DNA replication will be so large that the clock no longer couples to the cell cycle (see Figs. 4 and 6). For $T_d \leqslant T_{int}$, the stochasticity in the initiation of DNA replication is much smaller. Moreover, the noise in DNA replication progression is so small that the coupling of the clock to the cell cycle is not much weakened by it [see Figs. 7(a) and 7(b) and Figs. 8(a) and 8(b)]. This explains why for $T_d \leqslant T_{int}$, noise in DNA replication does not appreciably attenuate the locking of the clock to the cell cycle.

## IV. DISCUSSION

Discrete gene replication events, present in all cells, can have marked effects on the period of circadian clocks [26]. We wanted to know how gene replications affect the robustness of two renowned synthetic oscillators built in *E. coli*: the repressilator by Elowitz *et al.* [1] and the dual feedback oscillator by Stricker *et al.* [13]. Using computational modeling, we show how the peak-to-peak time of the oscillators depends on the cell division time, the position of the genes on the DNA, and the noise in the gene replication times.

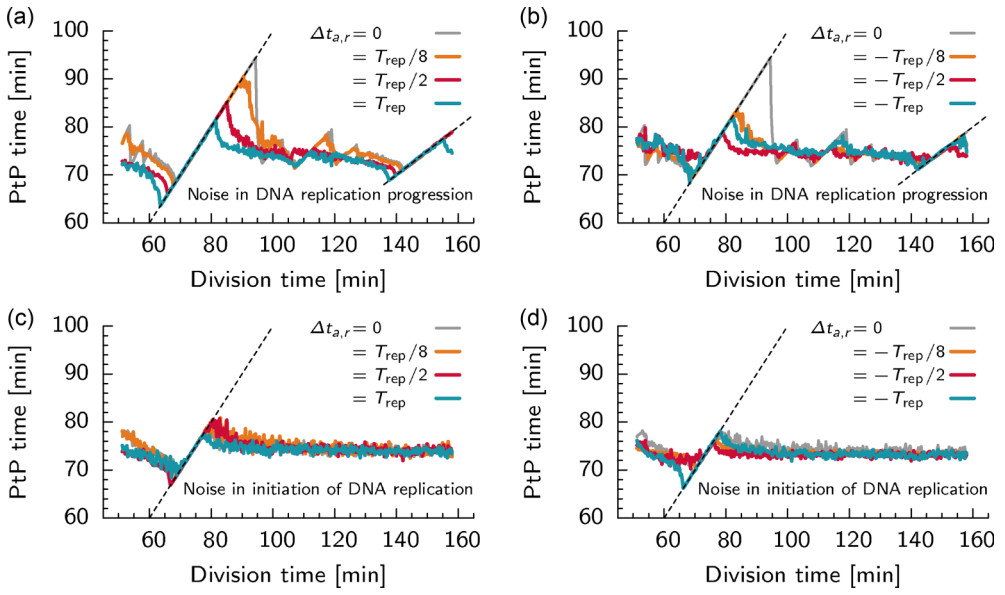


FIG. 8. In the dual-feedback oscillator, stochasticity in the initiation of DNA replication plays the dominant role in attenuating the effects of gene replications. In both panels, solid lines show the peak-to-peak time in the activator concentration for different periods of the cell division time $T_d$. Standard deviation in $T_{\mathrm{PtP}}$ omitted for clarity, but it is similar in all panels. Legends are defined in Fig. 5. The amplitudes of the functions decrease as the time interval $\Delta t_{1,2}$ increases. We compare a scenario with only noise in DNA replication progression, with a standard deviation $\sigma_{\mathrm{rep}} = 0.35T_{\mathrm{rep}}$, panels (a) and (b), to a scenario with only noise in the initiation of DNA replication, with a standard deviation $\sigma_{\mathrm{init}} = 0.2T_d$, panels (c) and (d). (a),(b) As observed for the repressilator, with noise in replication progression but not in replication initiation, locking is little affected, compared to the deterministic case [see Figs. 5(d) and 5(e)]. (c),(d) In the opposite scenario, with noise in the initiation of DNA replication but not in the progression of replication, most signatures of locking disappear both when the activator or repressor gene is replicated first. Only around $T_d = T_{\mathrm{int}}$ does locking persist. Clearly, comparing with panels (a) and (b), noise in the initiation of DNA replication has a stronger attenuating effect on locking.

We find that both synthetic oscillators can lock strongly to the cell cycle, where the oscillator's peak-to-peak time is equal to a multiple of the cell division time, over a wide range of division times. Remarkably, the effect depends strongly on how the genes of the oscillator are located on the chromosome. The distance between the genes introduces a temporal delay between the moments at which the different genes of the oscillators are replicated, which affects the period of the oscillations. Increasing the distance between genes has an opposite effect on the two oscillators: Whereas the repressilator exhibits almost no locking when the genes are positioned close together yet strong coupling over a wide range of $T_d$ when the temporal delay is increased, the dual-feedback oscillator shows the strongest coupling to the cell cycle at negligible temporal delay between gene replications. For both models, the signature of the gene replication events should be clearly visible in the amplitude of the time traces of the protein concentrations.

It is well known that the timing of key events during the cell cycle, such as the start of DNA replication, the duration of chromosome replication, and cell division exhibit high levels of stochasticity [36,37], which will propagate to the replication times of the oscillator's genes. To investigate how strong noise in the timing of gene replication affects the oscillator's coupling to the cell cycle, we introduced two noise sources in our model of the cell cycle: one when DNA replication is initiated and one in the time it takes to replicate the chromosome. Using physiologically relevant values for the standard deviations in the timing, we found that noise in gene replication times strongly attenuates the effects of the cell cycle. However, observable signatures of locking remain for division times equal to and shorter than the oscillator's intrinsic period. For these cells, the standard deviation in gene replication times becomes smaller than the oscillator's intrinsic period, making it possible for the clock to lock to a certain phase of the gene density, which sets the peak-to-peak time. We then asked which of these two sources is more important in attenuating the coupling between the cell cycle and the oscillator. Toward that end, we made two models for stochasticity in the replication times: one with only noise in the replication progression, and the other with only noise in the time of replication initiation. We found that noise in the initiation of DNA replication has a stronger effect than that in the progression of DNA replication. The reason is that, at physiologically motivated values, the standard deviation in the time of replication initiation is much larger than the standard deviation in the time of replicating the chromosome. We thus conclude that the initiation of DNA replication is mainly responsible for attenuating the effects of the gene replications on the repressilator and dual-feedback oscillator.

Throughout this work, we assume the genes reside on the bacterial chromosome. Importantly, however, the synthetic oscillators were originally constructed on plasmids, which are often present in large copy numbers ranging from 10 to 100. Moreover, experiments indicate that these plasmids are copied at random times during the major part of the cell cycle [38]. Based on our observation that multiple chromosome copies that are replicated asynchronously strongly reduce the strength of locking [26], we expect that, at these high plasmid copy

numbers, the synthetic oscillators exhibit no clear signatures of locking. Indeed, the original study on the dual-feedback oscillator does not report any effects from the cell cycle, even when the growth rate is comparable to the oscillator's intrinsic period where locking is expected to occur [13]. Signatures of locking were observed for the dual-feedback oscillator in the experiments of [29], but only in the bidirectional system, in which gene replication not only affects the oscillator, but conversely the oscillator also regulates the time when plasmids are replicated during the cell cycle. In the unidirectional system, in which the discrete gene replication events (still) affect the oscillator but the oscillator does not control gene replication, no signatures of locking were observed. This is in line with our predictions, since in these experiments the genes reside on high copy-number plasmids [29], such that the coupling strength is negligible. However, in the mathematical model for the cell cycle and dual-feedback oscillator introduced in [29], the authors assume all plasmids are replicated simultaneously, such that their model of the cell cycle becomes similar to our model with initial gene copy number $N = 1$. It is therefore remarkable that they find no signatures of locking in their models with unidirectional coupling, since we would predict strong locking. However, this discrepancy could be resolved by recognizing that in the model of [29], the cell division time deviates strongly from the intrinsic period of the dual-feedback oscillator. Therefore, the division time of the cell cycle lies outside the 1:1 locking window, which is probably why locking is not observed in their model. Our analysis predicts that locking can be observed in the case of unidirectional coupling from the cell cycle to the oscillator, when the genes are put on the chromosome, and the cell division time and the oscillator's intrinsic period are similar. Conversely, in order to prevent locking, it seems beneficial to construct the oscillator on high copy-number plasmids.

Given our result that stochasticity in replication times attenuates the effects of the cell cycle on the oscillator, it seems natural to expect that also intrinsic noise, caused by the stochastic nature of chemical reactions and the limited number of proteins, can help to reduce locking. However, in our earlier work [26], we made the observation that intrinsic noise can, in fact, widen the 1:1 locking region. Apparently, where stochasticity in the replication times attenuates the effects of gene replications, intrinsic noise in the reaction network can enhance the effects. The effects of intrinsic noise and the interplay with locking to the cell cycle are thus expected to be intricate, demanding a much more detailed study. We leave this for future work.

The genes of biological oscillators such as circadian clocks do reside on the chromosome, and the periods of these oscillators are often unaffected by the cell cycle [39]. One approach to understand how these natural clocks are so resilient to perturbations from the cell cycle is to construct synthetic oscillators in growing and dividing cells. The dual-feedback oscillator studied in this work, based on a coupled positive and negative feedback architecture regulating gene expression, has been predicted to produce robust oscillations [14,17]: The amplitude and period do not depend critically on specific parameter values, and oscillations persist in a wide range of temperatures and growth media [13,21]. However, these models do not take the effect of gene replications into account, and in the experiments the genes reside on high copy-number plasmids, potentially abolishing any effect of the cell cycle. Our results suggest that the relatively simple design of the dual-feedback oscillator implemented on the chromosome might not be very robust in growing and dividing cells, since its period scales with that of the cell cycle. Clearly, to test the predictions of our analysis, it would be of interest to implement this oscillator on the chromosome, which is now increasingly being done in synthetic biology [7]. Comparing the unstable synthetic oscillators with their evolved stable counterparts found in, e.g., *S. elongatus* and *N. crassa* could elucidate why the latter feature a remarkably more complex reaction network, including, for example, post-translational modification of the proteins [26,40].

## V. METHODS

Both the repressilator and the dual-feedback oscillator models are described using ordinary differential equations, and they are propagated using MATHEMATICA 8 (Wolfram Research). For each value of $T_d$, we generated a single time trace of about 200 oscillations for the repressilator and 100 oscillations for the dual-feedback oscillator. To allow the oscillations to settle down to a steady state, we discarded the first 10 oscillations in the system.

To simulate the (stochastic) gene replication events, for each gene $n$ in the model we generated a list of replication times, $\tau_i^n$, using Eqs. (4)–(6). The gene copy number for this gene, $g^n(t)$, equals 1 when $t < \tau_i^n$, and 2 when $t > \tau_i^n$, modulo $T_d$. The discrete gene replication events enter the models via the gene density, $G^n(t) = g^n(t)/V(t)$, where $V(t) = \exp[\ln(2)/T_d \bmod(t, T_d)]$ is the cell volume [26].

To find the peak-to-peak times, $T_{\mathrm{PtP}}$, in the ODE simulations (including those with noise in the gene replication times), we used the built-in methods of MATHEMATICA to return all local extrema in the concentration of $p_1$ (repressilator) or the activator (dual-feedback oscillator). These extrema correspond to the time points $t_i$ where the concentration is higher, in the case of a maximum, or lower, in the case of a minimum, than its two immediate neighbors. As is standard for numerical solution of differential equations, the spacing $t_i - t_{i-1}$ between successive time points was determined adaptively by the algorithm to meet imposed precision bounds, but it never exceeded 0.2 h. We then checked if a given local minimum is the lowest point within an interval of $\pm 3/4$ the oscillator's intrinsic period, $T_{\mathrm{int}}$, centered on the minimum; if so, we defined this point as the global minimum of a single oscillation cycle. If there existed a local extremum with a lower value, we repeated this procedure around the lower point until we found a point that was the lowest within a time interval of $\pm 3/4 T_{\mathrm{int}}$. The same procedure is used to find the local maxima of the oscillations. The peak-to-peak time is then calculated by subtracting the times of two consecutive minima; we verified that subtracting the times of two consecutive *maxima* yielded essentially the same results.

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