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Robustness of DNA looping across multiple cell divisions in individual bacteria

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DNA looping has emerged as a central paradigm of transcriptional regulation, as it is shared across many living systems. One core property of DNA looping–based regulation is its ability to greatly enhance repression or activation of genes with only a few copies of transcriptional regulators. However, this property based on a small number of proteins raises the question of the robustness of such a mechanism with respect to the large intracellular perturbations taking place during growth and division of the cell. Here we address the issue of sensitivity to variations of intracellular parameters of gene regulation by DNA looping. We use the *lac* system as a prototype to experimentally identify the key features of the robustness of DNA looping in growing *Escherichia coli* cells. Surprisingly, we observe time intervals of tight repression spanning across division events, which can sometimes exceed 10 generations. Remarkably, the distribution of such long time intervals exhibits memoryless statistics that is mostly insensitive to repressor concentration, cell division events, and the number of distinct loops accessible to the system. By contrast, gene regulation becomes highly sensitive to these perturbations when DNA looping is absent. Using stochastic simulations, we propose that the observed robustness to division emerges from the competition between fast, multiple rebinding events of repressors and slow initiation rate of the RNA polymerase. We argue that fast rebinding events are a direct consequence of DNA looping that ensures robust gene repression across a range of intracellular perturbations.

memory | noise | single-cell | microfluidics | robustness

Significance

It is well established that certain intracellular regulators can stabilize DNA loops to greatly enhance activation or repression of gene transcription. In vitro and in vivo ensemble measurements have determined that only a few copies of regulators are, in fact, needed to stably form DNA loops. In view of such a small number, we address the issue of sensitivity of gene regulation by DNA looping to variations of intracellular parameters in individual growing *Escherichia coli* bacteria. Surprisingly, we find that DNA looping from the *lac* system is robust to a range of perturbations, including divisions during which cells can maintain tight repression over many generations. We propose molecular hypotheses compatible with the observed robustness across a range of intracellular perturbations.

Some genetic regulatory systems in bacteria are known to use only a few repressors to maintain low levels of expression, such as *lac*, *ara*, and lysogenic regulations (1–3). The diversity of these examples underlines the importance for these systems to have selected certain molecular mechanisms for efficiently maintaining low expression levels together with low levels of repressors. The *lac* operon is arguably among the most studied genetic regulatory systems of this class and is known to utilize a higher-order structure of DNA, DNA looping, to efficiently repress the activity of the *lac* promoter using only a handful of copies of repressors (1, 4–6). While the strong repression mediated by DNA looping has clearly been established in vivo and in vitro, the fact that it relies on a small number of repressors to function makes this molecular mechanism potentially sensitive to intracellular perturbations. For example, a small number of repressors can fluctuate greatly at cell division, which may yield undesirable promoter leaks (7–9), and it is still an open problem to know whether DNA looping can maintain repression even across several divisions. Indeed, it is standard to assume that gene duplication and cell division may disrupt the looping structure and binding of the repressors to DNA (10), which would, consequently, limit the duration of repression intervals. Moreover, during cellular growth, DNA replicates and gene dosage increases as a function of time, which may dynamically alter the ratio of the number of DNA binding sites to that of repressors.

In light of these outstanding questions, we aimed at quantitatively characterizing how robust the repression of DNA looping is with respect to intracellular perturbations in individual growing bacteria. We combine several techniques to record and analyze the spontaneous leakiness of the *lac* system (Fig. 1). In our experiments, we monitor the spontaneous leakiness of the *lac* promoter, as a measure for the repression level of the promoter in the presence or absence of DNA looping. Using a microfluidic device, we record long time series associated with promoter leakiness in individual growing *Escherichia coli* cells across more than 40 generations. We use, as a starting point, the model by Vilar and Leibler (11) that proposed that the change of free energy associated with DNA looping formation is equivalent to the existence of a very large "local" repressor concentration, effectively hundred times larger than the "global," wild-type repressor concentration (12). One key prediction of this model is that repression by means of DNA looping is robust to fluctuations of repressor concentration,

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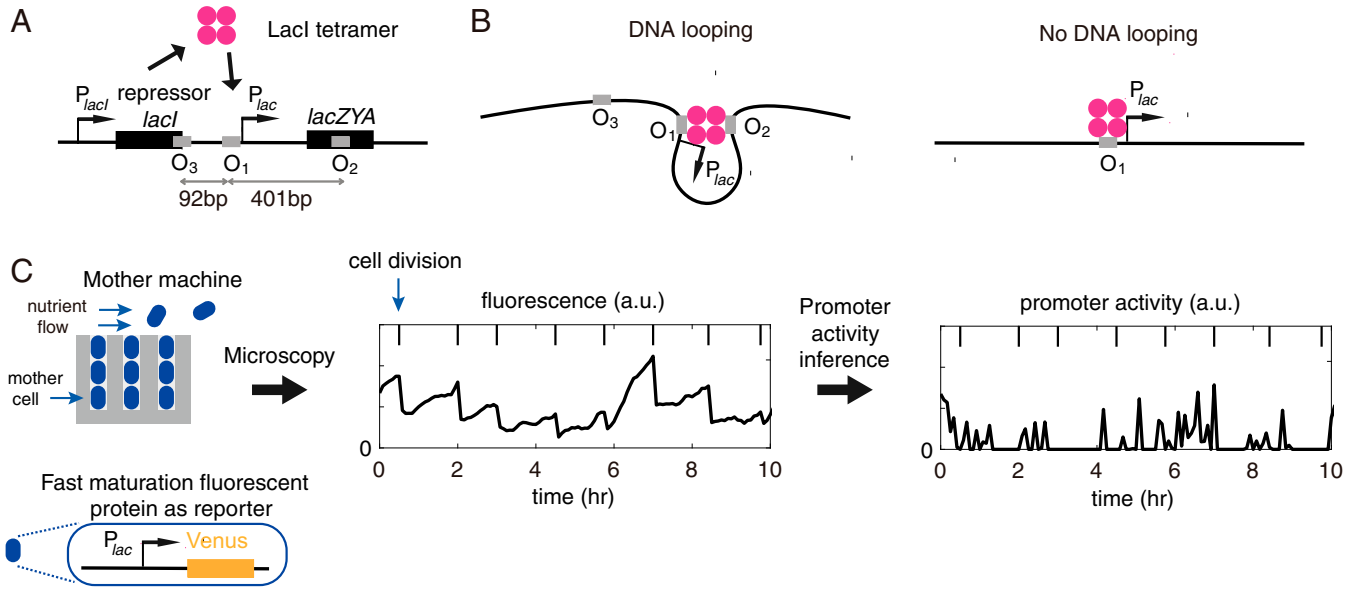

Fig. 1. Monitoring multigenerational leakiness of the endogenous *lac* promoter in single cells. (*A* and *B*) Wild-type *lac* operon maintains a low level of expression by the means of DNA looping. (*A*) Genetic organization of the *lac* system, where O_i denote the operators *i* that are DNA-specific sequences where the repressor LacI binds. LacI is fully functional as a tetramer (36), and can bind to any of three possible operators. (*B*) Cartoon that illustrates how several operators can mediate the formation of DNA looping (*Left*) while one operator alone cannot *(Right)*. (*C*) We developed an experimental platform to monitor promoter activity for low-expression systems across multiple cell divisions.

while repression in the absence of DNA looping is sensitive to fluctuations. Here, we experimentally investigate the consequences of this theoretical hypothesis in the broader context of cell division at the single-cell level.

Experimental Design

Due to the very low leaking rate, we monitor the promoter activity from single cells and across many division cycles using a microfluidics device, called the "mother machine" (13, 14). In this device, cells grow under chemostatic conditions. The mother cell is trapped at the bottom of a microfluidic channel, while daughter cells are washed away when they exit the channel. In our experiments, to estimate promoter activity, we use the production rate of a fluorescent reporter driven by a copy of the native *lac* promoter. To measure gene expressions with an improved temporal resolution and signal-to-noise ratio, we use a fast-maturating fluorescent protein [VenusNB, maturation half-time 4.1 ± 0.3 min (15)], together with an optimized ribosome binding site to maximize the yield of translation of the fluorescent reporter (16). Maximizing the yield of translation of fluorescent reporter per messenger RNA (mRNA) helps us to detect small transcriptional bursts that may not be directly detectable at the single-cell level. It was found earlier that a large fraction of cells do not contain even one copy of the fluorescent protein controlled by the *lac* operon (17); thus the fluorescent level of most of the cells is often nearing the level of autofluorescence. Under this condition of such a low expression level, we further developed a probabilistic algorithm that explicitly takes into account the fluctuations of autofluorescence background to robustly discriminate promoter activity from background noise (*SI Appendix*).

Cells were cultured overnight in M9 medium with 0.4% glycerol as a carbon source. The native *lac* operon is under the combinatorial control of both *lac* repressor and cAMP, Cyclic adenosine monophosphate, Receptor Protein (CRP) (18). It is well established that carbon sources such as glycerol or glucose are associated with relatively higher or lower cAMP levels (19), and that cAMP–CRP can either promote a high transcription initiation rate or mediate repression with DNA looping (18). However, it was shown that, in the absence of inducer (i.e., our conditions), both carbon sources yield similar low levels of promoter activity (18), and, therefore, the particular choice of carbon source is not expected to profoundly affect the spontaneous leakiness of the promoter (*SI Appendix*, Fig. S7).

Overnight, cells were loaded in the mother machine and cultured at exponential growth by steadily flushing them with fresh media (≥40 h; 30 °C; also see *SI Appendix*, Fig. S6 for control experiments at 35 °C). Phase contrast and fluorescence images (Zeiss Axiovert 200M microscopy) were captured for each field of view with a dwell time of 5 min (15). We used an open-source software, Molyso (20), to perform cell segmentation and lineage tracking, and further customized that software for proofreading (*SI Appendix*).

Statistics of Promoter Leakiness with and without DNA Looping

The regulatory regions of the *lac* operon consist of one main operator O_1, and of two auxiliary operators O_2 and O_3 (Fig. 1*A*). When a tetramer of LacI repressor binds two operators, for example, O_1–O_2 or O_1–O_3, it can form a stable DNA loop (Fig. 1 *B*, *Left*). Previous population measurements suggest that, with only ∼10 repressor tetramers (21), the *lac* operon can maintain a repression level ∼100 times stronger than in the absence of DNA looping when there is only the single operator, O_1, present (1, 22). To compare the statistics of spontaneous leaky events with and without DNA looping, we investigated two *E. coli* strains: one that carries all three operators, denoted as the "Loops" strain and another one that only carries the main operator O_1, denoted as the "No-loop" strain (Fig. 1*B* and *SI Appendix*, Table S1). Without DNA looping, the promoter exhibits frequent transcriptional bursts (Fig. 2 *A*, *Left*). By contrast, in the presence of DNA looping, the promoter leaks unfrequently, and transcriptional bursts are separated by very

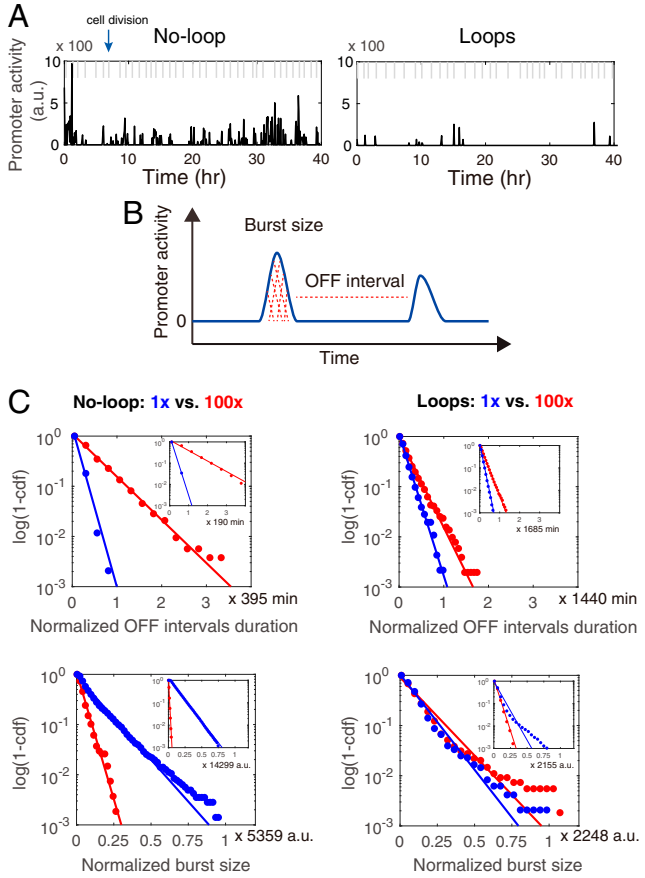

Fig. 2. Statistics of promoter activity with and without DNA looping in single cells. (*A*) Examples of promoter activity from single-cell traces of (*Left*) the No-loop and (*Right*) the Loops strain across multiple cell divisions. (*B*) We use two key physical quantities to characterize the dynamics of promoter activity: the duration of OFF intervals and the burst size of pulses. (*C*) Cumulative distributions ($P(X \geq x)$) of statistics with various repressor concentrations (denoted by color). Dots represent the statistics from experiments, lines give the fitting (linear fits in semilog space), and *Insets* give the statistics and the fitting from simulated time series in the absence of cell division using the Vilar and coworkers model. In each panel and each *Inset*, the statistics are normalized with the maximum value from the blue dots so that the blue curve ends around one. Number of OFF intervals included in the analyses is as follows: Loops (n = 462), 100×/Loops (n = 511), No-loop (n = 1,447), and 100×/No-loop (n = 529). Slopes of the fitting of OFF intervals before normalization are given as follows: Loops (-0.00446 min^{-1}), 100×/Loops (-0.00289 min^{-1}), No-loop (-0.01838 min^{-1}), and 100×/No-loop (-0.00497 min^{-1}).

long periods of time that can exceed, sometimes, 10 cell cycles (Fig. 2 *A*, *Right*).

We characterized the dynamics of the promoter activity using two quantities: the duration of OFF intervals and the transcriptional bursts size (Fig. 2*B*; number of lineages ≥50). Surprisingly, we find that the OFF intervals from the Loops strain follow an exponential distribution similar to the simpler No-loop strain (Fig. 2*C*). Such a memoryless statistical process was not expected for the Loops strain, because the OFF intervals were, on average, longer than several cell cycles, and complex statistics would have been more in line with the multiple-step processes that accompany cell division. On the other hand, the burst size of the Loops and No-loop strains have a linear region in the semilog space but followed by a long tail (Fig. 2*C*). The Loops strain, overall, exhibits significantly longer OFF intervals (OFF mean = 202 min, SE ± 10, or, on average, 2.8 cell cycles) and smaller burst size (213 [SE] ± 11 a.u.) compared to the No-loop strain (OFF = 47 ± [SE] 1 min or 0.6 cell cycles, burst size = 592 [SE] ± 18 a.u.).

Next, we test one key prediction of the Vilar and coworkers model (11, 12), that is, the promoter leakiness is insensitive to repressor concentration in the presence of DNA looping. Consequently, we investigate how sensitive repression mediated by DNA looping is to a high concentration of repressors versus using a strain that carries only the main operator O_1 that cannot form loops. To perform this experiment, we constructed two strains, 100×/Loops and 100×/No-loop, for which the concentration of repressors is ∼100 times larger than that in the Loops and No-loop strains (*SI Appendix*). Under those conditions, the OFF intervals still follow exponential distributions (Fig. 2*C*). Again, the burst size of 100×/Loops has a long tail, but not the 100×/No-loop strain. However, in the 100×/No-loop strain, promoter leakiness is very sensitive to the increase of the repressor concentration, with OFF intervals increasing to 178 [SE] ± 8 min (or 2.6 cell cycles) and burst size to 210 [SE] ± 9 a.u. By contrast, 100×/Loops is mostly insensitive to the increase in LacI repressor concentration and shows only slightly longer OFF intervals (292 [SE] ± 15 min or 4.5 cell cycles) than those of the Loops strain. As for the distributions of the burst size, they are similar (223 [SE] ± 13 a.u.), indicating that the typical burst size of the Loops strain has already been reduced to its lowest limit, which, we reasoned, may be associated with the synthesis of only one mRNA per pulse. The burst size of the 100×/No-loop strain also reached a similar limit in the presence of high repressor concentration. Our first observations are in agreement with the predictions of the Vilar and coworkers model. The observed insensitivity may directly stem from the saturation of the operator site in the presence of DNA looping with high "local" but low global concentration of the repressors. In principle, in the absence of DNA looping, this saturation may be achievable with high global concentration as well, but this condition might not be biologically favored, as it could become toxic to the cell.

However, that OFF intervals across many divisions in the Loops strains follow exponential distributions is unexpected, because it is the signature of a memoryless one-step process associated with a single rate. We hypothesize that this one-step process that controls the statistics of OFF intervals in the Loops strains is largely dominated by the unbinding of repressors from the operator O_1, regardless of whether the auxiliary operators O_2 and O_3 are bound or not.

To check this hypothesis, we genetically removed one additional operator (O_2 or O_3) from the Loops strain, and find that the OFF intervals of those strains that can form only one loop (either O_1–O_2 or O_1–O_3) follow exponential distributions as well (Fig. 3*A*). Furthermore, we observe that the O_1–O_2 one-loop strain exhibits qualitatively similar timescales of the OFF intervals (204 [SE] ± 9 min) as the Loops strain that has the possibility to form multiple alternative loops (Fig. 3*A*). This result indicates that only one loop, O_1–O_2, dominates the statistics of the OFF intervals in the Loops strain and that the possibility of forming different loops only mildly affects the statistics of OFF intervals.

To evaluate the impact of cell division on the OFF intervals, we performed stochastic simulations of gene expression using the Vilar and coworkers model (12) (*SI Appendix*). We attempted to model cell division by periodically forcing the simultaneous unbinding of the repressors from the operators; however, this approach failed to reproduce long and exponentially distributed intervals as well as the robustness to repressor concentration in the Loops strain (*SI Appendix*, Fig. S8). By contrast, stochastic simulations in the absence of cell division can reproduce the experimentally observed statistics (Fig. 2 *C*, *Insets*), indicating that

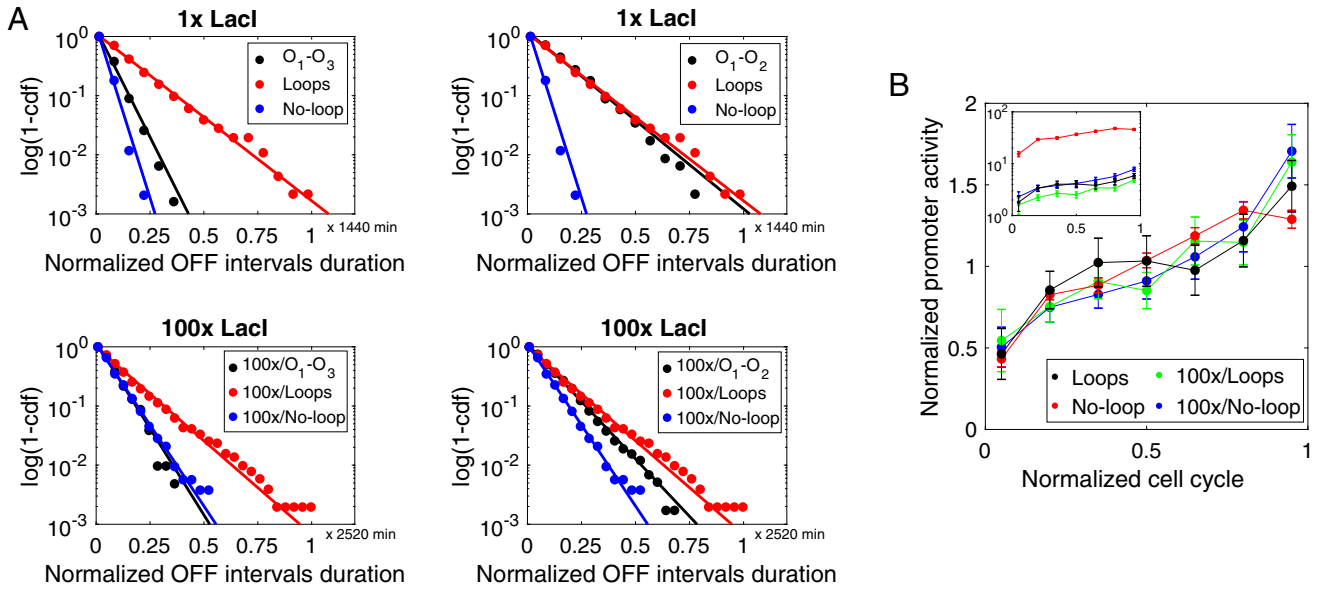

Fig. 3. Robustness of repression from distinct configurations of DNA loops and cell cycle dependence of promoter activity. (*A*) One-loop vs. No-loop or Loops strains configurations. OFF intervals for one-loop strains with either (O_1–O_2) or (O_1–O_3) configuration combined with 1× and 100× LacI background all exhibit exponential distributions. We compare cumulative distributions ($P(X \geq x)$) for four one-loop strains with those of the Loops and No-loop strains. In each panel, the OFF intervals were normalized with the maximum value of the red dots. Number of OFF intervals included in the analyses is as follows: O_1–O_3 one-loop (n = 1,239), O_1–O_2 one-loop (n = 463), 100×/O_1–O_3 one-loop (n = 207), and 100×/O_1–O_2 one-loop (n = 583). Slopes before normalization are as follows: O_1–O_3 one-loop (-0.01162 min^{-1}), O_1–O_2 one-loop (-0.00478 min^{-1}), 100×/O_1–O_3 one-loop (-0.00534 min^{-1}), and 100×/O_1–O_2 one-loop (-0.00355 min^{-1}). (*B*) Promoter activity as a function of time span between two cell division events. Time span between two successive division events is normalized by the total duration of the division time. Promoter activity is normalized by the mean of each strain. Mean promoter activity is computed using $N \geq 50$ cells. Error bars represent the SE. *Inset* gives unnormalized promoter activity for each strain.

DNA looping confers strong robustness to the perturbations associated with cell division.

A Theoretical Model without Cell Division.

Most observations in our experiments can be qualitatively understood with a three-state model extended from the Vilar and coworkers model (11, 12): 1) state B, where the repressor LacI is bound to O_1; 2) state E, where the operator O_1 is empty and freed from RNA polymerase; and 3) state TS, where O_1 is cleared from RNA polymerase and transcription starts (regardless of the states of O_2 or O_3). We further assume that only one transcript is produced in state TS and that the system returns to state E immediately. The transitions between states are described by

$$B \underset{k_b}{\overset{k_u}{\rightleftarrows}} E \xrightarrow{k_t} TS,$$

where k_b is the effective binding rate for the repressors to O_1, k_u gives the unbinding rate for a repressor from O_1 (0.10 min^{-1}), and k_t is the effective transcription rate (20 VenusNB $\cdot$ min^{-1}). Without DNA looping, repressors follow a simple ON–OFF dynamics; thus k_b scales linearly with the repressor concentration n_R ($n_R = 10$ molecules per cell in 1× LacI strain and $n_R = 1{,}000$ in 100× LacI). For a strain with DNA looping, when the system is in state E, one of its auxiliary operators is most likely bound to a repressor, given the free-energy difference between a bound and a free operator. For an E–B transition, either a repressor from the rest of the cell, denoted as "global," binds to O_1 or the repressor that has already bound to an auxiliary operator binds rapidly to O_1, denoted as "local." The free-energy difference of the looping formation, $e^{-\Delta G_l}$, is effectively equivalent to a very large "local" concentration n_L ($n_L = 0$ for No-loop, 1,080 for Loops) (11, 12). Considering both situations, we have $k_b = k_{on}(n_R + n_L)$, where k_{on} is the binding rate for a single repressor (0.28 per molecule per min).

An OFF interval consists of one or multiple rounds of E–B transitions before the system goes to state TS. The probability of l rounds to occur is $P_l = \alpha^{l-1}(1-\alpha)$, with $\alpha = k_b/(k_b + k_t)$ as the probability of entering state B from E. Considering that $k_u \ll k_b$, as implied by the physical parameters of the system, the timing at which l unbinding events happen is given by the composition of l exponential decays, which can be described by the Erlang distribution, $w_{t|l} = k_u e^{-k_u t}(k_u t)^{l-1}/(l-1)!$ resulting in a distribution of waiting times between transcriptional events (OFF intervals) $w_t = \sum_l w_{t|l} P_l = e^{-(1-\alpha)k_u t} k_u (1-\alpha)$. Thus, the average duration of the OFF interval is $\tau_{\mathrm{OFF}} = \int_0^\infty t w_t \, dt = \frac{1}{k_u}(1 + (k_b/k_t))$. Theoretical calculations suggest that the ratio of τ_{off} between the Loops strains with 1× and 100× repressor concentration is ∼2, but that the ratio between the No-loop strains with 1× and 100× is ∼14 (*SI Appendix*). Consequently, the model qualitatively predicts the great sensitivity of the No-loop strains and the robustness of the Loops strains to repressor concentration.

On the other hand, the burst size of a pulse is proportional to the number of E–TS transitions before the system goes to state B, equivalently, the number of transcripts. Starting from state E, the probability of entering state TS is $\beta = k_t/(k_b + k_t)$. The probability to produce r transcripts in a pulse is $P_r = \beta^{r-1}(1-\beta)$, a geometric distribution with an average number $\langle r \rangle = \sum_r r P_r = \frac{1}{1-\beta} = \frac{k_t}{k_{on}(n_R+n_L)} + 1$. When $n_R + n_L$ is large, $\langle r \rangle \to 1$. Theoretical calculations suggest the No-loop strain is expected to have more than one transcript per pulse (estimated as approximately eight); by contrast, the other three conditions with DNA looping or a high concentration of repressors are predicted to have only about one transcript per burst. These predictions are in line with our experimental

observations showing that the burst size cannot be reduced further in the Loops strain, even when we drastically increase the repressor concentration.

Correlation between Promoter Activity and Gene Dosage during Cell Growth

As described by the Cooper–Helmstetter relation (23), gene expression depends on global factors such as gene dosage (24). As the cell grows, DNA replicates in such a way that the average copy number of chromosomes is maintained after division, but, between two division events, the gene dosage increases. In *E. coli*, it has been reported that the promoter activity of a gene with a high expression level is correlated with the phase of cell cycle (25), and this has been quantitatively measured in ref. 26. Under full induction (i.e., the removal of the repressors), expression is constitutive, and the promoter activity of the *lac* operon exhibits a flat region at the early phase of the cell cycle, and gradually increases to about twice its initial level (26). In our experiments, we monitor how the spontaneous leakiness of the repressed promoter correlates with the cell cycle in the presence and absence of DNA loops. Although the absolute promoter activity greatly varies across our four strains, for example, the promoter activity of the No-loop strain is ∼10 times larger than that of the Loops strain, they all show positive correlations with the cell cycle (Fig. 3 *B*, *Inset*). Furthermore, after normalizing by the mean promoter activity, all the curves from the different strains collapse (Fig. 3*B*), indicating that the promoter activity in all strains has the same dependence on the cell cycle in the presence or the absence of DNA looping. We interpret the increase of promoter activity within the cell cycle as a consequence of gene dosage increase due to DNA replication during the cell cycle(see *SI Appendix*, Text and Fig. S5 for alternative analyses).

Discussion

The extension of Vilar and coworkers model and stochastic simulations without considering DNA replication and cell division is able to reproduce several key observations from our experiments, including memoryless distributions of the OFF intervals and extremely long OFF intervals with DNA looping, as well as its robustness to variations of repressor concentration. Given that bound repressors will unbind DNA during replication (10), and that a new copy of *lac* operon will be created, the structure of DNA looping may be affected, and the observation of multigenerational OFF intervals is unexpected, especially in the presence of very few repressors (∼10 tetramers; see ref. 21). Remarkably, neither the variations due to gene dosage within cell cycles nor perturbations associated with division events limit the long OFF intervals and alter the associated simple exponential distribution.

In the No-loop strain, the ab initio search time for LacI to bind free O_1 is >30 s (27, 28), but it only takes ∼3 s for the RNA polymerase to start transcription (29). Thus, the No-loop strain is expected to be sensitive to the removal of the bound repressors onto DNA during growth, as observed in our experiments. By contrast, the timescale for long repression intervals observed in the Loops strain can be reconciled with the short lifetime of the DNA loop measured in vitro (30). Using Chen et al. (30) measurements and refs. 31 and 32, we estimated that the in vivo loop lifetime was of the order of 10^3 s, and that of the open loop was ∼1 s. These values are similar to those used in our theoretical model that provides a simple hypothesis for the existence of OFF intervals longer than division cycles. When O_1 is unoccupied, we hypothesize that there is competition between RNA polymerase initiation and repressor rebinding events. Using the assumption of high "local" repressor concentration from the Vilar and coworkers model, we reason that the rebinding of the repressor onto the operator after either spontaneous unbinding or DNA replication is fast. For example, the typical timescale for the RNA polymerase initiation (∼3 s) is about an order of magnitude slower than the timescale to reform a loop (∼0.1 s, estimation from our model); consequently, transcription initiation is statistically allowed to happen only once every 30 spontaneous unbinding events. Along the same lines, we hypothesize that "local" repressor concentration is shared between the old and new DNA copies during or immediately after replication, which remains high relative to the DNA copy number that increases only by about a factor of 2. Additionally, the number of replication events that can potentially perturb repressor binding is small. Then, we shall expect the mean OFF intervals in the presence of DNA looping to be on the order of 30 times the loop lifetime (10^3 s) measured in vitro, that is, about seven cell cycles, in line with our experiments. While we assessed the statistical origin of the robustness of repression, it is still not clear how DNA looping can mechanistically conserve a high "local" concentration of repressors across divisions when DNA duplicates, and additional experiments, most likely at the single molecule level in live cells, may be able tackle this open question.

In summary, we report the robustness of DNA looping to intracellular perturbations across multiple cell cycles. While a small copy number of repressors is present in the cell, we find that repression with DNA looping is robust to variations of intracellular environment, such as repressor concentration, cell divisions, and detailed configurations of DNA loops. We speculate that similar robustness plays a crucial rule in other genetic regulatory systems beyond the *lac* operon.

Materials and Methods

All *E. coli* strains in this study were constructed using lambda red recombineering (33, 34), and the list of strains is available in *SI Appendix*, Table S1. For a typical experiment, cells were cultured in M9 media overnight, and then loaded into a mother machine microfluidics device for microscopy (Zeiss Axiovert 200M). Microscopy images were analyzed based on the software Molyso (20). Simulations of gene expression were performed following ref. 12. A full description of materials and methods can be found in *SI Appendix*.

Data, Materials, and Software Availability. Time series for the fluorescent signals have been deposited in Harvard Dataverse (35). The codes are available at https://github.com/changsysbio/ProbabilisticInferenceForPromoterActivity.

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