Introduction

Looping between two DNA sites, mediated by transcription factors, is a ubiquitous mechanism in prokaryotic transcription regulation [1]. DNA looping brings two distal DNA sites into close proximity, enhancing interactions between transcription factors bound at separate sites or bringing transcription factors close to RNA polymerase at the promoter. Knowing when and how DNA loops in vivo is important to understand the role of DNA looping in gene regulation and cell decision-making; some studies found molecular details of gene regulation have little influence on gene expression levels in different operator mutants, we show quantitatively that DNA looping activates transcription and enhances repression. Further, we estimated the upper bound of the rate of conformational change from the unlooped to the looped state, and discuss how chromosome compaction may impact looping kinetics. Our results provide insights into transcription-factor-mediated DNA looping in a variety of operator and CI mutant backgrounds in vivo, and our methodology can be applied to a broad range of questions regarding chromosome conformations in prokaryotes and higher organisms.

Abstract

DNA looping mediated by transcription factors plays critical roles in prokaryotic gene regulation. The “genetic switch” of bacteriophage λ determines whether a prophage stays incorporated in the E. coli chromosome or enters the lytic cycle of phage propagation and cell lysis. Past studies have shown that long-range DNA interactions between the operator sequences O6 and O4 (separated by 2.3 kb), mediated by the λ repressor CI (accession number P03034), play key roles in regulating the λ switch. In vitro, it was demonstrated that DNA segments harboring the operator sequences formed loops in the presence of CI, but CI-mediated DNA looping has not been directly visualized in vivo, hindering a deep understanding of the corresponding dynamics in realistic cellular environments. We report a high-resolution, single-molecule imaging method to probe CI-mediated DNA looping in live E. coli cells. We labeled two DNA loci with differently colored fluorescent fusion proteins and tracked their separations in real time with ~40 nm accuracy, enabling the first direct analysis of transcription-factor-mediated DNA looping in live cells. Combining looping measurements with measurements of CI expression levels in different operator mutants, we show quantitatively that DNA looping activates transcription and enhances repression. Further, we estimated the upper bound of the rate of conformational change from the unlooped to the looped state, and discuss how chromosome compaction may impact looping kinetics. Our results provide insights into transcription-factor-mediated DNA looping in a variety of operator and CI mutant backgrounds in vivo, and our methodology can be applied to a broad range of questions regarding chromosome conformations in prokaryotes and higher organisms.

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Abbreviations: 3C, chromosome conformation capture; CDF, cumulative distribution function; PDF, probability density function; RBS, ribosome binding site; smFISH, single-molecule fluorescence in situ hybridization; TPM, tethered partial motion; WLU, Wild-type λ units.

* E-mail: xiao@jhmi.edu

¤ Current address: Integrative Systems Biology Unit, Okinawa Institute of Science and Technology, Onna-son, Okinawa, Japan

‡ These authors contributed equally to this work.

Transcription-Factor-Mediated DNA Looping Probed by High-Resolution, Single-Molecule Imaging in Live E. coli Cells

Zach Hensel¤, Xiaoli Weng*, Arvin Cesar Lagda*, Jie Xiao‡

Department of Biophysics and Biophysical Chemistry, Johns Hopkins University School of Medicine, Baltimore, Maryland, United States of America
Author Summary

One mechanism cells use to regulate gene expression is DNA looping, whereby two distant DNA sites are brought together by regulatory proteins. The looping then either enhances interactions between other regulatory proteins bound at the separate sites or brings those regulatory proteins close to RNA polymerase at the promoter. Recent work in bacteriophage λ has suggested that DNA looping mediated by a transcription factor called λ repressor CI plays a critical role in regulating the expression of λ genes and consequently in determining the fate of the host E. coli bacterial cells. CI-mediated DNA looping has been directly demonstrated in vitro, but it has only been indirectly inferred in vivo. For the current study we developed a method to visualize CI-mediated DNA looping in individual live E. coli cells. We labeled two DNA sites—one each side of the proposed loop—with differently colored fluorescent fusion proteins, allowing us to measure their separation with an accuracy of a few tens of nanometers. Using this method, we directly analyzed CI-mediated DNA looping, providing insight into how transcription factor-mediated DNA looping influences gene regulation in live E. coli cells. Our methodology can be applied to a broad range of questions regarding chromosome conformation in prokaryotes and higher organisms.

using fluorescent proteins fused to DNA-binding proteins bound to tandem arrays of hundreds of binding sites has been employed to visualize homologous chromosome pairing in yeast induced by double-strand breaks [17]; however, an array of several kilobases of binding sites makes this method unsuitable for studying DNA loops of only a few kilobases. In addition, the long array of tightly bound protein molecules may be detrimental to cells [18].

We developed a two-color, high-resolution imaging method to directly measure the end-to-end separation of two DNA sites 2.3 kb apart in live E. coli cells (Figure 1a). This method is based on the ability to precisely determine the location of a specific DNA site in vivo [19]. By expressing a fluorescent protein in fusion with a DNA-binding protein in a cell with only three tandem binding sites (spanning less than 100 bp), the resulting fluorescent spot is diffraction-limited, and the location of the binding site can be determined with sub-diffraction-limited precision by fitting its fluorescence profile to a two-dimensional Gaussian function [20]. By labeling two ends of a DNA segment with two unique sets of binding sequences and co-expressing corresponding fluorescent DNA-binding fusion proteins of different colors, the distance between the two DNA sites can be determined with a precision of a few tens of nanometers. An in vitro experiment employing the same principle measured intramolecular distances using organic dyes [21], but this approach has not been demonstrated in vivo with comparable resolution using fluorescent proteins.

We used our method to probe the mechanics and dynamics of DNA looping mediated by the bacteriophage λ repressor CI [22] in live E. coli cells and investigate its regulation of transcription from the CI promoter PRM. The λ repressor CI is an essential transcription factor in determining the fate of an E. coli cell infected by the bacteriophage λ. When CI is expressed, it represses lytic promoters to commit to an extraordinarily stable lysogenic state that persists for millions of generations [23–25]. However, upon induction by UV irradiation or other specific events, CI degradation can trigger an irreversible switch from lysogenic to lytic gene expression within one cell generation time [26].

The robustness of the λ regulatory circuit has been extensively studied. Among many important features of the system such as promoter-operator arrangement [27,28], CI autoregulation [3,29,30], and cooperative binding [31–34], DNA looping between the homologous rightward and leftward operators OR and OL, separated by 2.3 kb, was shown to play significant, fate-determining roles in the λ lifecycle [13,35]. Cooperative binding of CI dimers at the subsites OR1 and OL2 of OR represses the lytic promoter PR (reviewed in [36]) and simultaneously activates CI’s own promoter, PRM, by accelerating transcription initiation [37–39]. At higher CI concentrations, an additional CI dimer binds to OR3 and represses PRM [40].

As illustrated in Figure 1a, an octameric CI complex (with or without an additional CI tetramer) can mediate DNA looping by bridging OR and OL. These higher-order complexes result from interactions between CI dimers bound to subsites at OR2 and OL2, and were first identified in vitro by ultracentrifugation [41] and later visualized by EM [12] and AFM [42]. Looping dynamics were investigated in vitro using tethered particle motion (TPM) [43–46].

To gain quantitative insight into the relationship between CI-mediated DNA looping and transcription regulation, thermodynamic models and numerical simulations were developed [33,35,44,47–52]. Key parameters in these studies were the free energies of octameric and tetrameric CI interactions that mediate DNA looping [35]. These free energies specify the DNA looping probability at a given condition (temperature, CI concentration, etc.) and hence the extent to which distal DNA sites affect each other. To date, DNA-looping probabilities and free energies were either estimated indirectly in in vivo studies by measuring PRM and PR activities in various operator mutants with a priori assumptions of DNA looping states [35,49,51] or measured using purified components in vitro, where conditions differ from those in a cellular environment [42–46]. Consequently, these studies yielded varying estimates for the free energies of DNA looping and the degree to which DNA looping influences PRM activity. Hence, the roles of CI-mediated DNA looping in transcription regulation are still in debate [13,35,49,51,53].

In this study, we tracked the apparent separation between the OR and OL sites on a λ DNA segment (termed OR–OL DNA below) in real time in live E. coli cells, from which we obtained the first direct estimates of in vivo looping frequencies and kinetics for both wild-type DNA and for DNA carrying mutations in ORβ and OLβ. We also measured corresponding CI expression levels in these strains by counting the number of CI transcripts in individual cells. Applying these independent, in vivo measurements to a thermodynamic model, we were able to obtain looping free energies and quantify the influence of DNA looping on PRM expression. Furthermore, we discuss how the compaction of the E. coli chromosome may impact DNA looping kinetics. The methodology established in this work can be extended to a broad range of questions regarding chromosomal DNA conformation and/or gene activities in prokaryotes and higher organisms.

Results

High-Resolution Imaging of Two DNA Sites

We inserted the construct shown in Figure 1a into the E. coli chromosome. It contains three tandem tetO sites (tetO5) [54] and three tandem lacO sites (lacO7) [55] flanking the wild-type λ lysozyme sequence from OR to OL (including the PR, PRM and P2 promoters and the cl, reaC and reaB genes). In this construct, called λWT, CI is expressed from PRM and regulates its own expression. The
lacO-binding and tetO-binding proteins LacI and TetR (accession number P04483) were fused with red and yellow fluorescent proteins to generate LacI-mCherry and TetR-EYFP, and were expressed from an inducible plasmid (Figure 1b).

With the combination of strong induction, weak ribosome binding sites, and carefully controlled growth, we achieved sufficiently low LacI-mCherry and TetR-EYFP expression levels to detect distinct, diffraction-limited mCherry and EYFP spots in single cells. We then fit the fluorescence intensity profile of each individual spot with a two-dimensional Gaussian function to estimate its centroid position. The average localization precisions for individual spots of LacI-mCherry and TetR-EYFP were 17 and 14 nm, respectively (Figure S1a). Subsequently, we transformed EYFP coordinates into mCherry coordinates using fiducial data to calculate the vector between the mCherry and EYFP spots arising from LacI-mCherry and TetR-EYFP protein molecules bound to the same $O_R$–$O_L$ DNA segment. We called this vector $\mathbf{r}_{\text{lac/tet}}$ (Figure 1c). The magnitude of the vector, $\mathbf{r}_{\text{lac/tet}}$, is the two-dimensional projection of the distance between lacO$^3$ and tetO$^3$ onto the image plane; on average, it is proportional to the end-to-end distance between lacO$^3$ and tetO$^3$ in three dimensions. The total error for an $\mathbf{r}_{\text{lac/tet}}$ measurement, including fitting errors in determining centroid of individual spots (Figure S1a), registration errors in aligning EYFP and mCherry two-color images ($\sim$10 nm based upon experiments using fluorescent beads), and contributions from local fluorescent background, was on average $\sim$40 nm (see below). With very low TetR-EYFP and LacI-mCherry expression, it was inevitable that not all lacO$^3$ and tetO$^3$ sites were bound by fusion protein molecules. Furthermore, not all fusion protein molecules were fluorescent due to stochastic chromophore maturation. Figure 2a contains typical data showing that a subset of cells was successfully labeled at both sites. We analyzed all cells having distinct fluorescent spots in both emission channels to calculate $\mathbf{r}_{\text{lac/tet}}$. We expected $\mathbf{r}_{\text{lac/tet}}$ to decrease when DNA between lacO$^3$ and tetO$^3$ looped.

**Distinguishing Between Looped and Unlooped States**

To determine whether our two-color imaging method was sufficient to distinguish between looped and unlooped DNA in the crowded intracellular environment, we constructed two control strains (Table 1). In the positive control $\lambda R$, the centers of lacO$^3$ and tetO$^3$ sites are separated by 66 bp (Figure 1d). The outmost lacO$^{sym}$ and tetO$^+$ sites are separated by less than 40 nm (Figure S2a). The close proximity of lacO$^3$ and tetO$^3$ mimicked permanently looped DNA. In the negative control $\lambda \Delta O_L$, we inserted the $\lambda$ sequence from $O_R$ up to but not including $O_L$ between lacO$^3$ and tetO$^3$ (Figure 1c). The resulting $\lambda \Delta O_L$ DNA has comparable length as the wild-type $\lambda$ DNA, but CI-mediated DNA looping between $O_R$ and $O_L$ is abolished.

We first examined $\lambda null$ and $\lambda \Delta O_L$ in two-color fluorescence images to determine whether we could discriminate between looped and unlooped DNA by eye. We obtained at least sixty 20-frame movies (100 ms exposures; 2 s total) for each strain in each of three independent experiments. Typical fluorescence images are shown in Figure 2a and b. Crosstalk between the two emission channels was negligible, as bright mCherry and EYFP spots only appeared in the corresponding channel but not the other. Figure 2c and d show 1 s of typical data for individual $\lambda null$ and $\lambda \Delta O_L$ spots. Representative movies for the two strains and others discussed below are included as Movies S1, S2, S3, S4, S5, S6. As expected for a permanently looped configuration, the positive control $\lambda null$ exhibited overlapping EYFP and mCherry spots
Arrows highlight molecules that exclusively appeared in mCherry (magenta, top) and EYFP (green, middle) channels, indicating a lack of significant crosstalk between the two channels. Squares show a spot that appeared in both channels. In the overlay image (bottom), fluorescence images were bandpass filtered and background was subtracted. Only cells having both mCherry and EYFP fluorescence were used in analysis. Scale bar, 2 μm. The color image and scale bar are repeated in (b–e). (b) Fluorescent images of λnull. Scale bar, 1 μm. (c–e) Timelapse images of spots acquired every 100 ms; (c) and (d) are spots in white squares in (a) and (b), respectively, and (e) shows an additional spot, whose apparent separation can be easily detected by eye. Top and middle rows show mCherry and EYFP channels, respectively, and bottom rows show two-color overlays on brightfield images. (f–h) Trajectory vectors from fitting fluorescence data for spots in (c–e). Coordinates are in nm. Vertices indicate the f vector and subsequent time points are connected by lines that are colored to indicate elapsed time.

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**Figure 2. High-resolution imaging of lacO and tetO sites separated by 66 bp (λnull) or 2.3 kb (λΔO).** (a) Fluorescent images of λnull. Arrows highlight molecules that exclusively appeared in mCherry (magenta, top) and EYFP (green, middle) channels, indicating a lack of significant crosstalk between the two channels. Squares show a spot that appeared in both channels. In the overlay image (bottom), fluorescence images were bandpass filtered and background was subtracted. Only cells having both mCherry and EYFP fluorescence were used in analysis. Scale bar, 2 μm. The color image and scale bar are repeated in (b–e). (b) Fluorescent images of λΔO. Scale bar, 1 μm. (c–e) Timelapse images of spots acquired every 100 ms; (c) and (d) are spots in white squares in (a) and (b), respectively, and (e) shows an additional λΔO spot, whose apparent separation can be easily detected by eye. Top and middle rows show mCherry and EYFP channels, respectively, and bottom rows show two-color overlays on brightfield images. (f–h) Trajectory vectors from fitting fluorescence data for spots in (c–e). Coordinates are in nm. Vertices indicate the f vector and subsequent time points are connected by lines that are colored to indicate elapsed time.

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**Table 1.** Descriptions of strains and plasmids used in this study.

| Strain Name | Genotype |
|-------------|----------|
| λnull       | MG1655 λlacI:λacO2tetO' |
| λWT         | λnull lacO2tetO':(O2–O2) |
| λΔO         | λWT ΔO1 |
| λO3         | λWT O3r1 |
| λO3null     | λWT O3r1 |
| λΔO3P$_{\text{tet}}$Cl$_{-}$ | λΔO3 P$_{\text{tet}}$ Cl$_{-}$ |
| λΔO3P$_{\text{tet}}$Cl$_{-}$/P$_{\text{tet}}$ | λΔO3 P$_{\text{tet}}$ Cl$_{-}$/P$_{\text{tet}}$ (pACL18) |
| λCFC147D    | λWT d(G147D) |
| λCFC147D/C0/C1trans | λCFC147D (pACL17) |
| pZH102R33Y29 | pLau53 [82] pBad-lac-mCherry-(tetR-EYFP) |
| pZH102R33TD | pLau53 pBad-lac-mCherry-EYFP |
| pACL18      | pACYC184-cPm |
| pACL17      | pACYC184-cPm |

The 2.3-kb, wild-type phage λ sequence from O2 to O1, was incorporated into the E. coli chromosome in λWT. Strains λO3, λO3 , and λWT(CFC) contain the r1 [30], OL3–4 [35], and CFC(CFC) mutations, respectively. Curly brackets indicate fusion products expressed from a single ribosome binding site. These are shorthand strain names; names used in our lab are listed in Table S4.

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**DNA Looping Probed in Live E. coli Cells**
mean separations, \( \langle r^{\text{lac/tet}} \rangle \), were 47 (\( N = 1,153 \)) and 71 nm (\( N = 979 \)) for \( \lambda \text{null} \) and \( \lambda \Delta O_2 \) respectively (results and measurement errors summarized in Table 2). Peaks in \( P(r^{\text{lac/tet}}) \) plots centered at \( \sim 40 \) nm, reflecting our experimental precision in determining \( r^{\text{lac/tet}} \); that is, \( O_k - O_2 \) molecules with \( r^{\text{lac/tet}} \) below \( 40 \) nm could not be distinguished from each other. Hence, it was more meaningful to compare distributions of \( r^{\text{lac/tet}} \) at large values where \( r^{\text{lac/tet}} \) distributions differed most prominently. The cumulative probability of \( r^{\text{lac/tet}} \) being \( 75 \) nm or more was \( \sim 40\% \) for \( \lambda \Delta O_2 \) and only \( \sim 15\% \) for \( \lambda \text{null} \) (Figure 3b).

Furthermore, two-dimensional distributions of \( r^{\text{lac/tet}} \) vectors (Figure S4) were clearly wider for \( \lambda \Delta O_2 \) than for \( \lambda \text{null} \). Thus, by
examining $P^{lac/tet}$ distributions, we could distinguish between the looped and unlooped control strains, suggesting that this approach could be used to probe CI-mediated DNA looping.

**Compact Conformation of Unlooped DNA $\lambda\Delta O_2$ Does Not Depend on Transcription or Nonspecific CI Binding**

We measured the mean end-to-end separation $\langle r^{lac/tet} \rangle$ for $\lambda\Delta O_2$, at 71-nm, much shorter than the ~200-nm distance expected for B-form DNA with a typical 30-nm in vitro persistence length [57]. While such a result is expected given the many factors known to compact prokaryotic chromosomes [58], it is possible that nonspecifically bound CI on the $\lambda\Delta O_2$ DNA and/or $P_{RM}$ transcription activity could influence the $r^{lac/tet}$ distribution, as indicated by a series of recent studies in vitro and in higher eukaryotic systems [46,59,60].

To examine these possibilities, we first compared the $r^{lac/tet}$ distribution of the $\lambda\Delta O_2$ strain to that of a control strain $\lambda\Delta O_2 P_{RM} cI^+/cI^{00}$ (Table 1, Figure S5a and b). In this control strain, promoter $P_{RM}$ was mutated to abolish transcription and the cI start codon was eliminated, but CI binding to OR was unaffected (Figure S5c, d, and e). In addition, we expressed CI from a plasmid at ~9 times its level in $\lambda$W (Table S8). We found that the $r^{lac/tet}$ distributions of the $\lambda\Delta O_2$ and $\lambda\Delta O_2 P_{RM} cI^+/cI^{00}$ strains were indistinguishable (Figure S5a and b), demonstrating that the compact $\lambda\Delta O_2$ distribution does not depend on $P_{RM}$ transcription. Furthermore, $r^{lac/tet}$ distributions for the same $\lambda\Delta O_2 P_{RM} cI^+/cI^{00}$ strain with or without the CI-expressing plasmid were indistinguishable (Figure S5c, d, and e). In addition, we expressed CI from a plasmid known to compact prokaryotic chromosomes [58], it is possible that $\lambda\Delta O_2$, with or without the CI-expressing plasmid were indistinguishable (Figure S5c, d, and e) distributing the $r^{lac/tet}$ distributions for the same $\lambda\Delta O_2 P_{RM} cI^+/cI^{00}$ strain with or without the CI-expressing plasmid were indistinguishable (Figure S5c, d, and e). In addition, we expressed CI from a plasmid known to compact prokaryotic chromosomes [58], it is possible that $\lambda\Delta O_2$, with or without the CI-expressing plasmid were indistinguishable (Figure S5c, d, and e).

**In Vivo Observations of DNA Looping**

We next investigated DNA looping in the context of wild-type and mutant $O_R$ $O_L$ DNAs. In $\lambda$W, the wild-type $\lambda$ sequence from $O_R$ through $O_L$ was inserted between lacO7 and tetO7. CI could bind all $O_R$ and $O_L$ sites to mediate looping with both octameric and tetrameric CI complexes (Figure 1a). In $\lambda$O93 and $\lambda$O93, mutations in $O_R$ and $O_L$ essentially eliminated CI binding to these operators at lysogenic CI concentrations (Table 1) [35,61].

We measured $r^{lac/tet}$ for these three strains and found that $r^{lac/tet}$ distributions differed significantly from those of the positive and negative controls $\lambda$null and $\lambda\Delta O_2$, $\langle r^{lac/tet} \rangle = \langle r^{lac/tet} \rangle$, except $p=0.004$ for $\lambda$W and $\lambda$null, with $P^{(r^{lac/tet})}$ and $C^{(r^{lac/tet})}$ being intermediate to those of the controls (Figure 3c and d). Mean $r^{lac/tet}$ values for the three strains also fell in between those of $\lambda$null and $\lambda\Delta O_2$ (Table 2). The wild-type strain had lower $\langle r^{lac/tet} \rangle$ than $\lambda$O93 and $\lambda$O93, and its distribution differed from those of the mutant strains with moderate to high significance $p=0.001$ and $0.048$ for $\lambda$O93 and $\lambda$O93, respectively); $r^{lac/tet}$ distributions for $\lambda$O93 and $\lambda$O93 were indistinguishable from each other ($p=0.493$). The trend of $\lambda$null $\lambda$W $\lambda$O93 $\lambda$O93 $\lambda\Delta O_2$ for $\langle r^{lac/tet} \rangle$ was reproduced in three independent experiments (Figure S1b). Assuming that a DNA molecule in the $\lambda$W, $\lambda$O93, and $\lambda$O93 strains is in either a looped or unlooped state, the intermediate $\langle r^{lac/tet} \rangle$ values of the three strains suggested that the fraction of looped DNA molecules (herein termed looping frequency) could be estimated by comparing $r^{lac/tet}$ distributions of these strains to those of the looped and unlooped controls $\lambda$null and $\lambda\Delta O_2$.

To further investigate whether the observed DNA looping in the $\lambda$W, $\lambda$O93, and $\lambda$O93 strains could be abolished by eliminating CI cooperative binding rather than by deleting $O_L$, we constructed a control strain $\lambda CI^{G147D}$ (Table 1). This strain differs from $\lambda$W by a CI mutation G147D known to be defective in pairwise cooperative interaction [62,63]. Structural evidence suggests that cooperative binding interfaces are shared for pairwise binding to adjacent operator sites and the formation of CI tetramers or octamers via DNA loops [64]. We found that the $r^{lac/tet}$ distribution of the $\lambda CI^{G147D}$ strain was indistinguishable from that of $\lambda\Delta O_2$ (Figure S5f and g, Table S7). We note that this G147D mutant also diminishes $P_{RM}$ transcription because of its weakened ability to form a CI tetramer at the $O_R$ and $O_L$ sites; hence its expression level is lower than that with wild-type CI (Table S8). Therefore, we constructed another control strain $\lambda CI^{G147D}/\lambda CI^{G147D}$, in which the CI-G147D mutant protein was expressed constitutively at ~11 times the CI expression level in $\lambda$W from a plasmid transformed into the $\lambda CI^{G147D}$ strain (Table S8). We found that $r^{lac/tet}$ distribution of this strain was indistinguishable from that of the $\lambda\Delta O_2$ and the $\lambda CI^{G147D}$ strains, demonstrating that DNA looping could be abolished by eliminating CI cooperative binding.

**Estimating DNA Looping Frequency from $C^{(r^{lac/tet})}$**

To quantitatively examine how operator mutations influence DNA looping, we estimated looping frequencies for $\lambda$W, $\lambda$O93, and $\lambda$O93 by assuming a simple model. In this model, DNA molecule can only exist in one of two states, looped or unlooped, with $r^{lac/tet}$ distributions for each state resembling those of the looped and unlooped controls, $\lambda$null and $\lambda\Delta O_2$, respectively. Therefore, the distribution $P^{(r^{lac/tet})}$ or $C^{(r^{lac/tet})}$ for one of the three strains is the linear combination of that of $\lambda$null and $\lambda\Delta O_2$, with their distributions weighted by the looping frequency, $f$:

$$C^{(r^{lac/tet})} = f C^{(r^{lac/tet})}_{\lambda\Delta O_2} + (1-f) C^{(r^{lac/tet})}_{\lambda\Delta O_2}. \text{Using this model, we found that the looping frequency was 79%}$$

| Strain | $P_{RM}$ Measurements | Mean $r^{lac/tet}$ (nm) | Median $r^{lac/tet}$ (nm) | Looping Frequency | CI Expression Level (WLU) |
|--------|------------------------|-------------------------|--------------------------|-------------------|--------------------------|
| $\lambda$null | 1,153 | 47±1 | 41±1 | N/A | N/A |
| $\lambda\Delta O_2$ | 979 | 71±1 | 63±2 | N/A | 1.38±0.05 |
| $\lambda$W | 962 | 52±1 | 45±1 | 79±6% | 1.00±0.05 |
| $\lambda\Delta O_2$ | 784 | 59±1 | 50±2 | 53±7% | 2.50±0.07 |
| $\lambda\Delta O_2$ | 781 | 56±1 | 48±1 | 60±7% | 2.51±0.07 |

Errors are all 1 s.e.m. as estimated from 1,000 bootstrapped samples.

Table 2. Summary of measurements and sample statistics in this study.
for λWT, and reduced to 53% for λOβ3− and 60% for λOγ3− (results with errors summarized in Table 2). The results were indistinguishable within error regardless of whether cumulative or probability density distributions were used, or whether data points from all frames or only the first frame of each molecule’s movie were used (Table S1). The looping frequencies for λOβ3− and λOγ3− were indistinguishable from each other within error, suggesting a similar role of Oβ3 and Oγ3 in loop formation. Reduced looping frequencies of λOβ3− and λOγ3− compared to λWT suggest that while a CI octamer at Oβ12 and Oγ12 is sufficient to loop DNA, the resulting loop can be further stabilized by an additional CI tetramer only if both Oβ3 and Oγ3 are intact. To our knowledge, these measurements provide the first quantitative in vivo estimates of DNA looping frequencies that are independent of gene regulation models.

Estimating DNA Looping Kinetics

In the above analyses, we only utilized $r_{\text{lac/tet}}$, the magnitude of the $r_{\text{lac/tet}}$ vector, and discarded information about the direction of $r_{\text{lac/tet}}$ and its evolution in time. Looping frequencies estimated from $r_{\text{lac/tet}}$ distributions are analogous to equilibrium constants and lack kinetic information. While many DNA molecules only exhibited fluorescent spots in both EYFP and mCherry channels for one or two consecutive frames due to photobleaching, some molecules had fluorescent spots lasting for several consecutive frames in both channels (Figure 2c–h; also see $r_{\text{lac/tet}}$ plots from molecules with many frames in Figure S3). By analyzing how $r_{\text{lac/tet}}$ evolves in time, we can obtain additional information about DNA looping kinetics.

We calculated the autocorrelation of $r_{\text{lac/tet}}$ (the average dot product of two $r_{\text{lac/tet}}$ vectors separated by a time lag) up to 0.5 s for each strain using all movies in which fluorescent spots in both channels lasted two or more frames (Figure 4a). The autocorrelation curves of all strains showed an initial drop of ~2,500 nm2 at the first time lag, corresponding to uncorrelated errors in determining $r_{\text{lac/tet}}$. After the initial drops, all autocorrelation curves showed positive correlation values that were approximately constant at time lags up to 0.5 s.

The observation of near constant autocorrelation values after the first time lag for all the strains indicated that the conformation of each DNA molecule, characterized by both the magnitude and orientation of $r_{\text{lac/tet}}$, persisted for at least 0.5 s. This provides a lower limit for the amount of time it takes for two DNA sites in the relaxed, unlooped state to move relative to each other and potentially form a DNA loop, and thus an upper limit of ~2 s−1 for the rate of DNA looping. The plateau values are related to the averaged mean end-to-end separations—λΔO2 has the highest autocorrelation plateau and λWT, λOβ3−, and λOγ3− have intermediate values because they contain a mixture of looped and unlooped DNA molecules.

Single-Molecule Measurements of CI Expression Levels

Next, we measured average CI expression levels, $\langle CI \rangle$, in all strains in order to understand to what different extent DNA looping influences $P_{\text{Rm}}$ regulation. We used single-molecule fluorescence in situ hybridization (smFISH, [2,63,66]), in which multiple fluorescently labeled oligonucleotides probe targeted nonoverlapping regions of $cI$ mRNA, to count the number of $P_{\text{Rm}}$ transcripts per cell for one or more transcripts. (c) Distribution of $P_{\text{Rm}}$ transcripts per cell determined by smFISH. The average expression level in wild-type λ units (WLU) is defined as the mean number of transcripts per cell in a given strain divided by the mean transcript number in λWT cells.

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Figure 4. DNA looping kinetics and CI expression levels. (a) Vector dot-product autocorrelation for $r_{\text{lac/tet}}$ time trajectories for each strain. Plots show the average dot product of two $r_{\text{lac/tet}}$ vectors separated by a given time lag. Error bars show 1 s.e.m. calculated by bootstrapping. (b) Typical smFISH images for λnull, which has no $P_{\text{Rm}}$ transcripts, and for all other strains. Top, brightfield images showing a group of fixed cells for each strain. Bottom, maximum-intensity projections of fluorescence image stacks. Spots indicate one or more transcripts. (c) Distribution of $P_{\text{Rm}}$ transcripts per cell determined by smFISH. The average expression level in wild-type λ units (WLU) is defined as the mean number of transcripts per cell in a given strain divided by the mean transcript number in λWT cells.
magnitude below the levels of all other strains; false positives arise when nonspecifically bound probes occasionally co-localize to create a fluorescent spot above the detection threshold. Typical smFISH images of the five strains are shown in Figure 4b. We quantified the number of transcripts in each individual cell by dividing the total intensity of fluorescent spots in each cell by the average intensity of a single-transcript spot (Figure 4c). We then determined $\langle |C| \rangle$ in wild-type λ units (WLU) by dividing the average number of transcripts in cells of a given strain by the average number of transcripts in λWT cells. We found that deleting $O_{3L}$ increased $\langle |C| \rangle$ to $\approx 1.4$ WLU (Table 2), indicating that the DNA loop formed between $O_{3L}$ and $O_{4R}$ in λWT enhances $P_{RM}$ repression. Mutating either $O_{3L}$ or $O_{3R}$ further increased $\langle |C| \rangle$ to $\approx 2.5$ WLU. These observations are consistent with previous observations that although $O_{3L}$ is 2.3 kb away from the $P_{RM}$ promoter, it has as important a role as $O_{3R}$ in repressing $P_{RM}$ at lysogenic CI concentrations [13]. This suggests that $P_{RM}$ was not strongly repressed by CI binding to $O_{3L}$ in the absence of a tetrameric interaction with an additional dimer at $O_{3R}$. Finally, elevated $\langle |C| \rangle$ in $\lambda O_{3S}$ relative to $\lambda O_{3}^*$ indicated that DNA looping could also activate $P_{RM}$, which was likely mediated by the binding of a CI octamer at $O_{4R}$ and $O_{4R}$, and was consistent with recent in vivo [49,51] and in vitro [53] experiments.

### Evaluating Looping Free Energies and Transcription Activation Using a Thermodynamic Model

We have shown that reduced looping frequencies in $\lambda O_{3S}$ and $\lambda O_{3}^*$ compared to that in λWT corresponded to increased expression levels of CI in the two strains, and that unlooped $\lambda O_{3}$ has a higher expression level than the λWT strain. To establish a quantitative framework that explains all observed relationships between looping and CI expression levels, we refined a thermodynamic model, with which we estimated looping free energies and transcriptionation rates for DNA-looping free energies and parameters such as specific and nonspecific DNA binding of CI were fixed at the values used by Dodd et al. [35]. The wild-type CI concentration was fixed to $150$ molecules/cell based upon our previous experiment in which CI molecules were counted at the single-molecule level in a similar strain at similar growth conditions [3]. The CI degradation rate was fixed to a half-life equal to the observed 2-h doubling time in our experiments.

The four free parameters were adjusted to best fit our experimental measurements of looping frequencies and CI expression levels. Modeled looping frequencies and CI expression rates at different CI concentrations are shown in Figure 5a and b. The best fit estimated $\Delta G_{\text{oct}}$ and $\Delta G_{\text{tet}}$ at 0.3 and $-3.2$ kcal/mol, respectively, and the CI expression rates at 1.9 nM/s and 4.5 nM/s for unlooped $k_{\text{unlooped}}$ and looped $k_{\text{looped}}$ DNA when CI binds $O_{4R}$. These results suggest that the DNA looping mediated by only a CI octamer is not strongly favored, while looping mediated by both an octamer and tetramer is the dominant configuration if all six binding sites are bound by CI dimers. Note that a small, positive $\Delta G_{\text{oct}}$ is consistent with measured looping frequencies greater than 50% for $\Delta L O_{3S}$ and $\Delta L O_{3}^*$, as one unlooped configuration could lead to multiple looped configurations (Table S2). The higher CI expression rate from the looped configuration suggests that, in the absence of $O_{4R}$ binding, bringing the distal $O_{3L}$ and $O_{4R}$ sites together to form a DNA loop activates $P_{RM}$ to 2.4 times the unlooped level.

To test how sensitive the fitting results were to two fixed parameters that are poorly defined in previous work, we varied CI expression levels and nonspecific DNA binding affinity. We found that across the examined ranges, octameric looping energies, $\Delta G_{\text{oct}}$, were consistently near 0 and tetrameric looping energies, $\Delta G_{\text{tet}}$, were strongly favorable between $-2.8$ to $-4.6$ kcal/mol (Table S3). Similarly, CI expression rates $k_{\text{unlooped}}$ and $k_{\text{looped}}$ remained close to the original fit values, giving activation ratios between 1.7 and 2.5 (Table S3). We also verified that our fit parameters were unique—as shown in Figure 5c and d, the values of fit parameters corresponded to a well-defined minimum in the sum of squared residuals in the four-dimensional (two free energies and two expression rates) parameter space (Figure 5c and d). Hence we conclude that the four fit parameters resulted from the model were robust and well defined.
Discussion

In this work, we directly measure the end-to-end separation between two DNA sites separated by only 2.3kb on the E. coli chromosome with high spatial resolution and report the first estimates of CI-mediated DNA looping frequencies in live E. coli cells. We improved a thermodynamic model to estimate the free energies of DNA looping as well as the degree to which DNA looping enhances transcription regulation. Combining independent, single-molecule measurements of looping frequencies and CI expression levels increased confidence in this model. Our results provide insight into transcription-factor-mediated DNA looping in vivo, and the new method reported here also has the potential to address questions beyond DNA looping, including understanding of chromosome structure and dynamics in vivo. In the following, we compare our results with previous work, and discuss unique information provided by our new method.

Differences with In Vitro Looping Measurements

Our estimated looping frequencies of 79% for λWT and greater than 50% for λO3− and λO23− are larger than those observed in vitro by TPM and AFM, where looping frequencies at lyogenic CI concentrations were approximately 60% with wild-type operators and 10%–40% in the absence of OR3 and OR3 [42,44,46]. As looping frequency is directly linked to looping free energy, comparison of AG values showed the same trend: AGtet values estimated in these in vitro experiments were similar to our estimate of 2.3.2 kcal/mol, while in vitro AGoct values were 1–2 kcal/mol higher than ours [44,46].

Significantly different AGoct values likely resulted from differences between naked DNA in an in vitro environment and the compact, protein-decorated E. coli chromosome in the crowded cellular environment. Factors such as supercoiling and nonspecific, “histone-like” DNA-binding proteins could compact DNA and lead to more frequent encounters between OR and OL. Our observation that the unlooped λΔO2 DNA was extremely compact...
estimated that CI-mediated DNA looping activates P of these binding sites. Furthermore, results are directly comparable as all strains used in results are consistent with previous observations [13, 49, 51, 53].

One important assumption we employed in calculating looping frequencies is that that looped and unlooped λWT, λO23−, and λO23− DNA molecules had similar distributions to those of the looped control λW and unlooped control λA01, respectively. It is possible that the unlooped states in the λWT, λO23−, and λO23− strains were more compact than that in λA01 if after a DNA loop breaks O2–Oz DNA does not always completely relax before it reforms again. In such a case, looping frequencies estimated using the linear-combination model would be upper limits on the true looping frequencies. Nevertheless, as we show above, our looping frequency estimates broadly agree with expectations from previous studies. Since this simple model only requires one free parameter and gives reasonable results, it is unnecessary to invoke more complicated models.

Effects of DNA Looping on Transcription Regulation

By comparing looping frequencies and corresponding CI expression levels in λWT, λA01, λO23−, and λO23−, we showed that loop stabilization by the CI tetramer between O23 and O23 is important for efficient Pram repression, and that looping mediated by a CI octamer at O21 and O22 is important for Pram activation. We note that while it is possible that the presence of tetO2 and lacO3 binding sites flanking Oz–Oz DNA may influence CI binding and/or transcription, this influence is negligible. It is because CI expression levels in these strains measured using smFISH are comparable to that of a wild-type λ lysogen (Table S8), and our results are consistent with previous observations [13, 49, 51, 53]. Furthermore, results are directly comparable as all strains used in this study are identical with respect to the presence and positioning of these binding sites.

Combining these results in our thermodynamic model, we estimated that CI-mediated DNA looping activates Pram to 2.4 times its level when the DNA does not loop. This compares well to earlier estimates of 2–4 fold [49], and 1.6-fold for a high-expression Pram mutant [53]. Another study did not find looping activates transcription, modeling CI-concentration-dependent Pr and Pram activities without invoking activation via looping (by assuming klooped = kunlooped) [35]. A later study indicated that this discrepancy may have resulted from different constructs used in the earlier study [49].

The molecular basis for DNA loop-enhanced Pram activation is unclear. One possibility is that a CI dimer bound to O22 interacts with RNA polymerase to a greater extent if it is part of a higher-order CI octamer [33]. Alternatively, a recent work showed that a DNA UP element proximal to O2 [49, 69] enhances CI expression from Pram in looped DNA by contacting the α-C-terminal domain of RNA polymerase [51]. The activation mechanism could be clarified in future experiments measuring both looping frequency and Pram activity while varying operator and UP element sequences and introducing CI mutations affecting operator binding, oligomerization, and RNA polymerase interaction.

Kinetics of DNA Looping

We estimated the time scale a DNA molecule stays in a particular state by calculating the autocorrelation function of the \( r_{\text{bac/tet}} \) vector (Figure 4a). The \( r_{\text{bac/tet}} \) vector was strongly correlated for at least 0.5 s, suggesting that a particular DNA conformational state, either compact or extended, persisted for at least 0.5 s. This implies an upper limit of 2 s\(^{-1}\) for the rate of loop formation from the extended state. This upper bound of transition rate is in the range of what was observed in a previous TPM experiment, in which looped and unlooped states lasted for tens of seconds [44], and argues against a significantly faster rate used in a recent computer simulation (~60 s\(^{-1}\)) [50]. We note that although it is possible that transient CI unbinding does not necessarily lead to immediate and complete DNA conformational relaxation at our measurement time scale, the autocorrelation analysis puts an upper limit for the true transition rate between the looped and unlooped states. The same concern also applies to in vivo 3C and in vitro TPM experiments.

Slow transitions between looped and unlooped states imply that low or high expression states resulting from a particular DNA conformation could be long-lived, potentially committing a cell to a particular fate. Supporting this is a recent study that suggested that a single unlooping event could trigger induction of the lac operon [5]. We were unable to obtain time trajectories long enough to clearly identify looped/unlooped transitions for single DNA molecules. Development of brighter, faster maturing, and more photostable fluorescent proteins or in vivo labeling with synthetic fluorophores [70, 71] will help in increasing the number of measurements made on one DNA molecule, possibly enabling accurate measurement of DNA looping kinetics in vivo.

The Short End-to-End Separation of λA01 Reflects the High Compactness of Chromosomal DNA

We observed very small end-to-end separation for the unlooped control \( \langle r_{\text{bac/tet}} \rangle = 71 \text{ nm} \). This distance was shorter than expected from modeling the unlooped DNA as a noninteracting worm-chain with an in vitro persistence length of 50 nm [72], but consistent with the recently observed extreme bendability of short DNA molecules [73]. A noninteracting chain with an equivalent \( \langle r_{\text{bac/tet}} \rangle \) to that of λA01 would have a persistence length of only 3 nm, which is physically infeasible. Our measurements of indistinguishable conformational distributions in the absence of Pram transcription and the presence of CI overexpression suggest that neither transcription nor nonspecifically bound CI played a major DNA-compacting role in our experiments. Furthermore, C. crescentus chromosomal DNA segments of ~5 kb were found to be similarly compact and consistent with Brownian dynamics simulations of supercoiled DNA [74].

We attribute the small end-to-end separation observed for λA01 to the high compaction of the E. coli chromosome in the crowded cellular environment. While the exact molecular mechanisms responsible for compaction remain unclear, previous studies found that in vitro binding of the histone-like HU proteins [75] (accession numbers P0A0CF0, P0A0CF4) and in vivo mammalian chromatin packing [76] reduced the apparent persistence length of DNA. Hence, it is possible that nucleoid-associated proteins such as HU may bring distal DNA sites together by protein–protein interactions and/or affect local DNA conformations by introducing bends and relieving torsional strain [77]. Another important factor could be negative supercoiling, which has been shown to compact the chromosomal DNA globally [78]. However, the exact effect of negative supercoiling on a 2.3-kb DNA segment is difficult to predict, because negative supercoiling could also introduce...
extended, pletonomic structures that promote large separations between DNA sites on relatively short length scales [78].

Potential Applications

Our two-color, high-resolution method can be applied to examine how chromosomal location, DNA length, genetic background, and growth conditions affect the distance between any two DNA sites on the E. coli chromosome. Furthermore, the spatial organization of the E. coli chromosome can be determined by systematically measuring \( \lambda \) distributions between DNA sites throughout the chromosome. This method is similar to how chromosome conformation capture was used to generate a 3D model of the C. crescentus chromosome [79], but with significantly improved spatial resolution and without potential artifacts from fixation.

Materials and Methods

Strain and Plasmid Construction

A plasmid, pS2391, containing lacO\(^{3}\) and tetO\(^{3}\) (the tetO\(^{3}\) sequence [54] was used for each repeat in tetO\(^{3}\) sites) was synthesized by Genewiz, Inc. Segments of \( \lambda \) DNA (\( O_{g} \) through \( O_{2} \) for \( \lambda WT \), \( O_{g} \) up to but not including \( O_{2} \) for \( \lambda AO_{2} \)) from the wild-type lysogen JL5392 (a gift from John Little, University of Arizona) were amplified by PCR. This DNA was sequenced and inserted between lacO\(^{3}\) and tetO\(^{3}\) using the In-Fusion PCR cloning system (Clontech). A kanamycin-resistance cassette flanked by BamHI sites was amplified by PCR and inserted after lacO\(^{3}\). For strains with mutated operators, mutations r1 [80], \( O_{3} \)–\( 4 \) [13], and \( O_{2}^{1147d} \) [62] were introduced to the \( \lambda WT \) template via QuickChange (Agilent). A plasmid carrying the \( P_{\text{Bab}^{2}} \) promoter \( \lambda AO_{2}^{3} \) was constructed by overlapping PCR mutagenesis using complementary primers carrying the desired mutations, flanked by a forward primer that sits at the EcoRI site on the upstream end of the operon and a reverse primer at the ClaI site in the \( \text{recA} \) gene downstream of \( \text{cI} \). The 1.13 kb PCR product was introduced to the \( \lambda AO_{2} \) plasmid by restriction ligation.

This procedure resulted in seven plasmids that were used as templates in subsequent chromosome insertion: pZH105 (\( \lambda WT \)), pZHO16 (\( \lambda AO_{3} \)), pZH107 (\( \lambda WT \)), pZHI07r1 (\( \lambda AO_{3} \)), pZH107r3–4 (\( \lambda AO_{3} \)), pACL006 (\( \lambda WT^{1147d} \)), and pACL007 (\( \lambda AO_{2}^{3} \)). Note that we use shorthand names such as \( \lambda null \) here for clarity; corresponding names used in our laboratory are \( \lambda WTG147D \), and pACL007 (\( \lambda AO_{2}^{3} \)).

To express the CI protein in trans from a plasmid, we constructed the plasmid pACL18, which has the wild-type \( \text{cI} \) ORF driven by a constitutive promoter, \( P_{\text{Bab}^{2}} \), inserted at the EcoRI site. Two sets of at least nine strains were transformed with pACL18, and CI protein expression was monitored using anti-CI antibodies. The expression of CI from the plasmid pACL18 was significantly higher than that from the chromosomal ortholog, indicating that the plasmid \( \lambdaWT \) template is transcribed more efficiently.

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We compared growth rates for the parent strain MG1655 to the experimental strain \( \lambda null \) to determine whether inserting the lacO\(^{3}\) and tetO\(^{3}\) construct into the chromosome and/or inducing expression from the reporter plasmid introduced a significant growth defect. Under induction growth conditions (~27°C, M9 media with 0.4% glycerol and 1 × MEM amino acids) starting at OD\(_{600}\)=0.1 and observing 8 h of growth, we measured doubling times of 2.7 h for MG1655 and 3.4 and 3.3 h for \( \lambda null \) harboring the reporter plasmid (in the absence and presence of 0.3% L-arabinose, respectively), indicating that there is no large growth defect associated with the insertion of the tandem operator sites into the chromosome and/or the expression of TetR-EYFP and LacI-mCherry fluorescent fusion proteins (Figure S6c).

Imaging Conditions

In each experiment, samples of all strains were placed on separate gel pads in the same growth chamber. Two sets of at least 30 movies were acquired for each strain, with the second set acquired in the reverse order to minimize any bias possibly introduced by observing some strains in a particular order. All images were acquired within less than one cell doubling time.

Cells were put on a gel pad made of 3% low-melting-temperature SeaPlaque agarose (Lonza) in M9 with glucose and imaged on an Olympus IX-81 inverted microscope with a 100× oil immersion objective (Olympus, PlanApo 100× NA 1.45) and additional 1.6× amplification. Images were split into red and yellow channels using an Optosplitt II adaptor (Andor) and captured with an Ixon DU-895 (Andor) EM-CCD with a 13-μm pixel width using MetaMorph software (Molecular Devices). Laser illumination was provided at 514 nm by an argon ion laser (Coherent I-308), which also pumped a rhodamine dye laser (Coherent 599) tuned to ~570 nm. A quarter-wave plate further benefits from a large Stokes shift, fast chromophore maturation rate, and high brightness relative to other monomeric RFPs [84], was inserted in place of ECFP. We also created a tandem LacI-mCherry-EYFP reporter, which was used as a fiducial marker, by inserting the linker sequence from the tandem-dimer fluorescent protein tdTomato [84] in between mCherry and EYFP.

To accurately localize a fluorescent spot arising from only a few fluorescent protein molecules above the background of unbound molecules within a cell, we reduced the reporter expression level by weakening the ribosome binding sites (RBSs). Weakened RBS sequences were designed using an online RBS calculator [85]. For example, the RBS for TetR-EYFP was the consensus AGGAGG Shine-Delgarno sequence in the parent plasmid pLau53. Our reporter plasmid had an ACCAGG Shine-Delgarno sequence, with a predicted ~300-fold decrease in the TetR-EYFP translation rate. All sequences including chromosome insertions were verified by sequencing (Genewiz Inc). Reporter plasmids are described in Table 1.

Growth Condition

For all experiments reported in this study, cells were grown and imaged at room temperature (~25°C) in M9 minimal media supplemented with MEM amino acids (Sigma). Cells were grown overnight with 0.4% glucose and 50 μg/ml carbenicillin to an optical density (OD\(_{600}\)) of 0.4. After centrifugation at room temperature, cells were resuspended at OD\(_{600}\)=0.2 with 0.4% glycerol plus 0.2% L-arabinose and grown for 2 h (~1 cell cycle) to induce LacI-mCherry and TetR-EYFP expression. Cells were then resuspended at OD\(_{600}\)=0.2 with 0.4% glucose and grown for another 2 h before immediate observation to allow time for fluorescent protein chromophores to mature.

We compared growth rates for the parent strain MG1655 to the experimental strain \( \lambda null \) to determine whether inserting the lacO\(^{3}\) and tetO\(^{3}\) construct into the chromosome and/or inducing expression from the reporter plasmid introduced a significant growth defect. Under induction growth conditions (~27°C, M9 media with 0.4% glycerol and 1 × MEM amino acids) starting at OD\(_{600}\)=0.1 and observing 8 h of growth, we measured doubling times of 2.7 h for MG1655 and 3.4 and 3.3 h for \( \lambda null \) harboring the reporter plasmid (in the absence and presence of 0.3% L-arabinose, respectively), indicating that there is no large growth defect associated with the insertion of the tandem operator sites into the chromosome and/or the expression of TetR-EYFP and LacI-mCherry fluorescent fusion proteins (Figure S6c).
(Thorlabs) was used to circularly polarize excitation light. Emitted light was split by a long-pass filter, and the red and yellow images were filtered using HQ630/60 and ET540/30 bandpass filters (Chroma).

**Measuring and Analyzing $r$**

Images were inspected manually using a custom MATLAB script to identify spots that appeared in both EYFP and mCherry images. Images from all strains were displayed in random order without knowing the strain identity to avoid bias in spot selection. Pixel intensities within 3 pixels of the initial spot location were fitted with a symmetric, two-dimensional Gaussian distribution to estimate spot coordinates. The variance of the fit distribution was constrained to be less than 2 pixels. Spot-fitting error was estimated by scrambling residuals from a fit to the fluorescence data in 10 random permutations, adding them to the data, and fitting the resulting images; the reported error for a spot is the standard deviation of the distances between these fits and the initial fit to the raw data. Fitting error distributions are shown in Figure S1a.

The LacI-mCherry-EYFP tandem dimer (Figure S2b) in which the two fluorescent proteins were directly fused together was used to acquire fiducial control points to transform between the mCherry and EYFP coordinate systems. A projective transform was calculated from the control points using the cp2tform function in MATLAB. We found that relatively simple, global transformations were sufficient to transform coordinates of fluorescent beads (Tetraspeck, Invitrogen) with $\sim$10-nm registration error in our microscope setup, and did not see any further improvement with a locally weighted transformation used in in vitro two-color experiments [21]. This transformation was also used to generate the overlay images in Figure 2, Figure 3, and all supplemental movies. Fluorescent beads were not used as fiducial markers because the beads’ emission spectra were different from those of the fluorescent proteins. Analysis was restricted to molecules in which mCherry and transformed EYFP coordinates were separated by less than 200 nm. Separations beyond this threshold were rare ($\sim$1% of data, see two-dimensional distributions in Figure S4) and did not correlate with strain identity in any reasonable way. They possibly arose from data in which cells contained two labeled copies of O$_{R}$-O$_{L}$ DNA.

After transformation into a uniform coordinate system, $P(\text{lac/tet})$ was calculated from the mCherry and EYFP coordinates and multiplied by an 81-nm pixel size (resulting from 160x magnification on a CCD with a 13-μm pixel width). Probability and cumulative distributions $P(\text{lac/tet})$ and $C(\text{lac/tet})$ were calculated for 10-nm bins using the kernel smoothing probability density estimation function in MATLAB, restricting the density to positive values and employing a uniform kernel width small enough to follow empirical cumulative density distributions without any systematic errors. Significant differences between $P(\text{lac/tet})$ distributions were determined using a two-sample Kolmogorov–Smirnov test; two-tailed Student’s t tests of sample means returned smaller, more significant p values. Errors in $\langle P(\text{lac/tet}) \rangle$ and $\langle |C| \rangle$ were determined by calculating the means of 1,000 bootstrapped samples; the reported error is the standard deviation of the calculated means. Looping frequencies were estimated by least squares fitting of 1,000 bootstrapped distributions (control distributions were also randomized on each iteration) and their error was calculated similarly.

**Single-Molecule Fluorescence in Situ Hybridization (smFISH)**

Concentration measurements by smFISH followed a previously described protocol [66]. Transcripts from $P_{R}$ were labeled with a mixture of 42 oligonucleotides labeled with CAL Fluor Red 610 (Biosearch Technologies), 31 of which hybridized to $\text{d1}$ (11 targeted sequences not found in *E. coli* and did not cause a problematic level of false positives). Table S5 lists all 42 oligonucleotides. Labeled cells were imaged with 561-nm excitation at six imaging planes separated by 200 nm z-depth with negligible photobleaching. For each frame, fluorescent spots were automatically detected and fit to a Gaussian using a custom MATLAB routine. Nearly all molecules appeared in multiple image slices; the slice with the largest fit amplitude was kept. The integrated fluorescence of spots was observed to be quantized with one or a few molecules localized within one diffraction-limited spot. The intensity of one transcript was estimated from the distribution of spot intensities, and the number of molecules contributing to each spot was estimated from this quantization. The number of transcripts in each cell was estimated from the sum of the number of molecules in each spot within that cell. Alternatively, the number of molecules in one cell is proportional to its integrated fluorescence; this measurement provided the same average expression levels within error. The experiment was repeated to ensure that differences in labeling efficiency between samples were not responsible for differences in the number of detected molecules; combined data from both experiments were used for analysis.

**Simulation of $P(\text{lac/tet})$**

To generate simulated $P(\text{lac/tet})$ distributions, we first generated 10,000 random radial distances for a chain with a contour length $L$ and persistence length $P$ from a worm-like, noninteracting chain model using a Gaussian distribution with Daniels’ approximation, which is accurate in the regime $L/P = \kappa << 1$ [72]:

$$P(r) \propto r^2 e^{-\frac{3r^2}{4}} \left(1 - \frac{5\kappa}{4} + 2r^2 - \frac{33\kappa r^4}{80\kappa} \right).$$

Each simulated $r$ was projected onto the plane at a random angle to give a distance $r'$. Simulated spots were placed at $(x,y)$ coordinates $(0,0)$ and $(r',0)$. The MATLAB function mvnrnd was then used to simulate normally distributed measurement error with a standard deviation of 22 nm to the coordinates of each simulated spot. This procedure was sufficient to simulate the Annull distribution (Figure S2c) using a fixed end-to-end distance of 22-nm approximate distance between the centers of the lacO$^R$ and lacO$^L$ sites; Figure S2a). Note here that the simulation is simplified in that it assumes that each spot has the same 22-nm localization error. In reality, localization error varies between different spots (Figure S1a) and there are other sources of measurement error. These differences may explain the slight deviation of the simulated distribution from the experimental distribution. The same procedure was used to estimate the $\langle P(\text{lac/tet}) \rangle$ expected for 2.3-kb, B-form DNA with a 50-μm persistence ($\sim$200 nm) as well as the apparently persistence length (3 nm) implied by the 71-nm $\langle P(\text{lac/tet}) \rangle$ observed for λA$O_l$.

**Thermodynamic Modeling**

Additional descriptions of thermodynamic states are listed in Table S3. Parameter values were determined by first scoring a wide range of parameter values and then searching closer and more finely grained parameter ranges to manually minimize the sum of the squares of the differences between experimental and modeled values for looping frequency and CI expression level. We then refined this fit by least-squares minimization using MATLAB. This was done using a minimized model that only accounted for states likely to be populated near or above lyogenic
CI concentrations (e.g., disregarding states in which \( O_B^1 \) and \( O_B^2 \) are unbound by CI). Using the same parameters and accounting for all 176 possible states (122 unique states accounting for degeneracy) did not significantly change the fit results. Fitting with this much more complex model gave octameric and tetrameric looping free energies of 0.6 and \(-3.3 \pm \sigma \text{ kcal/mol and unlooped and looped expression rates of 2.1 and 5.3 nM/min. When determining parameters, rates were expressed in terms of changes in concentration per unit time; we followed earlier work in assuming that in a typical \( E. coli \) cell, a single molecule is at a concentration of \( \sim 1.47 \text{nM} \).)

We do not report any estimate of fitting error; instead, we present only the parameters most consistent with our data and assumptions. Figure 5c and d shows that fit parameters were well-determined at a given combination of wild-type CI concentration and nonspecific binding parameters. As noted in the main text, varying these two parameters changed the absolute best-fit parameters, but did not dramatically change our conclusions. Furthermore, fixed parameters of previous studies were determined in a number of separate experiments employing different methods at temperatures other than 25°C; a rigorous estimate of modeling error would require knowing the error in the measurements of fixed parameters in our experimental conditions.

The basal CI expression rate, \( k_{\text{basal}} \), was arbitrarily fixed at \( k_{\text{basal}}/5 \); this did not have any significant impact on determining other parameters, as our measurements were all at or above lysogenic [CI], where \( O_B^2 \) is almost always bound by a CI dimer. Additionally, the fraction of free CI dimers was fixed at its value for 150 CI molecules per cell at a given concentration of nonspecific binding sites and nonspecific binding affinity. Fixing the concentration of free CI dimers is a reasonable approximation if (1) nearly all CI molecules are in dimers and (2) the number of free nonspecific binding sites is not significantly changed by nonspecifically bound CI dimers.

Image Representation in Figures

Figure 2a–e, Figure 4b, and Movies S1, S2, S3, S4, S5, S6 were prepared using NIH ImageJ [86]. Raw fluorescence image intensities were scaled linearly from the lowest to highest values in region shown. For EYFP/mCherry overlay images, brightfield images were inverted and converted to 8-bit RGB. Fluorescence images were bandpass filtered and background subtracted before being used to generate magenta (mCherry) and green (EYFP) 8-bit images that were added to the brightfield image. The EYFP images were first transformed in MATLAB using the imtransform function and the same fiducial data that were used to transform EYFP spot locations into mCherry coordinates. For smFISH images (Figure 4b), the value of each pixel is the maximum value of that pixel in six images collected at different z-axis positions. Intensities for all images were scaled linearly from the minimum to the maximum of all pictures (117–4,840 counts in 16-bit images).

Supporting Information

Figure S1 Spot fitting and experimental error analysis. (a) Distribution of fitting errors for EYFP (green), mCherry (red) localizations, and \( r_{\text{lac/tet}} \) (black). Errors were estimated using a bootstrapping procedure by fitting raw data to a Gaussian distribution. The residuals from this fit were then randomly rearranged and added back to the data in 10 different permutations. The reported error is the standard deviation of the distance between these 10 locations and the initial fit location. Error in \( r_{\text{lac/tet}} \) was determined similarly; from the 10 bootstrapped EYFP and mCherry fits, 100 distances were obtained and the error was estimated as the standard deviation of the difference between these distances and the distance determined from fitting the raw data. (b) A compilation of all data from three separate experiments was used for all analysis in the main text. Here, \( \langle \Delta \text{O}_{\text{EYFP}} / \Delta \text{O}_{\text{mCherry}} \rangle \) is shown for the individual experiments. Error was estimated as the standard deviation of the means of 1,000 bootstrapped distributions. Except for one sample (\( \Delta \text{O}_{\text{EYFP}} \), day 3), the estimated mean separations for all days followed the trend \( \Delta \text{O}_{\text{WT}} < \Delta \text{O}_{\text{EYFP}} \approx \Delta \text{O}_{\text{LacI}} < \Delta \text{O}_{\text{OLPRM}} \).

Figure S2 Estimate of positive control dimensions and apparent end-to-end distance distribution. (a) The maximum distance between TetR-EYFP and mCherry-LacI chromophores was approximately assuming straight DNA. All distances are in nm. Here, bound fusion proteins are shown on the same face of a DNA molecule, but this needs not be the case. Dimers of DNA-binding proteins were based on Protein Data Bank (PDB) entries for TetR (1QPI [87]) and LacI (1FEA [88]). Both fluorescent proteins are shown using the entry for GFP (1GFL [89]). Protein structures images generated using VMD [90]. (b) In an alternative positive control that was used to collect fiducial data for image registration, the plasmid pZHI02R35TD encodes the tandem-dimer reporter LacI-mCherry-EYFP. (c) The \( \Delta \text{O}_{\text{LacI}} \) PDF for the null control (black line; 1 s.e.m. shown in red as in Figure 3a) is shown with the distribution of 10,000 numerically simulated end-to-end distances for two sites separated by 22 nm, randomly projected onto the 2D plane, and subjected to 22-nm localization error for both ends (dashed black line). PDFs were calculated using methods described in main text. See Materials and Methods for simulation details. (TIF)

Figure S3 Plots showing trajectories of \( r_{\text{lac/tet}} \) vectors for all data from all strains for every molecule that was fit in both the EYFP and mCherry images for at least 8 consecutive frames (800 ms). Green and magenta lines are single-color trajectories for TetR-EYFP and LacI-mCherry spots, respectively; the corresponding \( r_{\text{lac/tet}} \) trajectory with time color-coded from blue to red is plotted on top at the same length scale. Coordinates are in nm. (PDF)

Figure S4 Two-dimensional distributions of the \( x \) and \( y \) components of \( r_{\text{lac/tet}} \) vectors. (a) A cartoon describes the calculation of the \( x \) and \( y \) components of the \( r_{\text{lac/tet}} \) vector. In the projected image, the \( r_{\text{lac/tet}} \) vector has two components determined by the arbitrary orientation of the detector. (b–f) Heat maps of the distribution of the \( x \) and \( y \) components of \( r_{\text{lac/tet}} \) vectors of each strain. Plots were generated by binning the data for all \( r_{\text{lac/tet}} \) into 5 nm \( \times \) 5 nm bins. The resulting 2-dimensional distribution was then filtered with a Gaussian kernel (with a width similar to spot-localization precision) to approximate the smoothed distributions. Each image is colored by the probability of the \( r_{\text{lac/tet}} \) vector falling within a given bin according to the scale bar in (b). (TIF)

Figure S5 Experiments showing the effects of transcription, nonspecific CI binding and higher-ordered CI oligomer on DNA looping. (a) End-to-end distance (\( \Delta \text{O}_{\text{P/R55I}} \) ) distributions (PDF) for \( \Delta \text{O}_{\text{null}} \) (red), \( \lambda \Delta \text{O}_{\text{P/R55I}} \) (blue), \( \lambda \Delta \text{O}_{\text{P/R55I}} \) (purple), and \( \lambda \Delta \text{O}_{\text{P/R55I}} \) (green). The PDF is estimated for 10-nm bins. (b) Cumulative density of \( \Delta \text{O}_{\text{P/R55I}} \) (CDF) for \( \Delta \text{O}_{\text{null}} \) (red), \( \lambda \Delta \text{O}_{\text{P/R55I}} \) (blue), \( \lambda \Delta \text{O}_{\text{P/R55I}} \) (purple), and \( \lambda \Delta \text{O}_{\text{P/R55I}} \) (green).
The CDF is estimated for 10-nm bins. (c) DNA sequence for the P_{gam} \text{ of } \lambda^k mutant in comparison to the wild-type sequence. Mutated nucleotides are shown in red. (d) Gel shift assay monitoring the binding of wild-type CI protein. Lane 1–4, CI at concentrations of 0, 150, 300, and 600 nM binding to a 150-bp DNA fragment (20 nM) amplified from the plasmid pZH107 carrying the wild-type P_{gam} DNA sequence. Lane 5–8, CI at concentrations of 150, 0, 300, and 600 nM (note loading order) binding to a 150-bp DNA fragment (20 nM) amplified from the plasmid pACU007 carrying the P_{gam} \text{ of } \lambda^k sequence. Lane 9: empty. Lane 10–13, CI at concentrations of 0, 150, 300, and 600 nM binding to a 140-bp DNA fragment (20 nM) amplified from the E. coli his promoter region, which CI does not bind specifically. Reaction mixtures were incubated in a buffer (10 nM Tris pH 8.0, 50 mM KCl, 1 mM MgCl$_2$, 10% glycerol, 100 µg/ml BSA, 1 mM DTT) at room temperature for 10 min. Samples were electrophoresed in Bio-Rad 4–20% Gradient TBE gels (Bio-Rad, Hercules, CA) in a cold room and then stained with Ethidium Bromide for 30 min. (e) Fraction of bound DNA (intensity of low-bandpass filtered using the program ImageJ [91]. The overlay images are scaled to be twice as large as the single-color images. Scale bars correspond 4 µm in the small, single-color images and 2 µm in the overlay image. Ten consecutive image frames are shown in real time (10 frames per second); the movie is looped 5 times. 

**Figure S6** Growth rate comparisons. (a, b) Strains used in thermodynamic modeling were diluted from exponential growth to low optical densities in M9 minimal media supplemented with 0.4% glucose and carbonate as described in the main text. OD600 was measured over 10 h of growth for two replicate experiments. Strains are λΔO$_2^k$ (WT, red), λO$_2^k$ (green), and λΔO$_2^k$ (purple). Doubling times calculated using the Microsoft Excel LOGEST function range from 1.7 to 2.5 h. Two independent replicates are shown. (c) Growth rates for the parent E. coli strain MG1655 (blue) were compared to those of the control strain λnull in which the lac operon is replaced with a construct incorporating the lacO$^a$ and tetO$^a$ binding site arrays and which harbors the plasmid pZH102R33Y29 which expresses both TetR-EYFP and LacI-mCherry fluorescent fusion proteins upon arabinose induction. Strains were grown in M9 minimal media supplemented with 0.4% glycerol and λnull was grown in both the absence (red) and presence (green) of 0.3% L-arabinose. Doubling times were 2.7 h for MG1655 and 3.4 and 3.3 h for λnull in the absence and presence of L-arabinose, respectively.

**Movie S1** Fluorescence movie montage for strain λnull corresponding to the data in Figure 2c. Single-color images for TetR-EYFP (top left) and LacI-mCherry (top right) data have intensities scaled linearly from the lowest to the highest pixel values in the first image in each time series. Before creating the overlay images (bottom), single-color images were background subtracted and bandpass filtered using the program ImageJ [91]. The overlay images are scaled to be twice as large as the single-color images. Scale bars correspond 4 µm in the small, single-color images and 2 µm in the overlay image. Ten consecutive image frames are shown in real time (10 frames per second); the movie is looped 5 times. 

**Movie S2** Fluorescence movie montage for strain λΔO$_2^k$ corresponding to the data in Figure 2d. Single-color images for TetR-EYFP (top left) and LacI-mCherry (top right) data have intensities scaled linearly from the lowest to the highest pixel values in the first image in each time series. Before creating the overlay images (bottom), single-color images were background subtracted and bandpass filtered using the program ImageJ [91]. The overlay images are scaled to be twice as large as the single-color images. Scale bars correspond 4 µm in the small, single-color images and 2 µm in the overlay image. Ten consecutive image frames are shown in real time (10 frames per second); the movie is looped 5 times.

**Table S1** Looping frequencies were estimated from alternate data sets using either all data or only the data from the first
frames (for molecules appearing in more than one sequential frame) and fitting either probability (PDF) or cumulative (CDF) distributions. The first row results for each strain were reported in the main text.

(DOCX)

Table S2 States used in thermodynamic modeling. We used free-energy parameters that were described by Dodd et al. [35]. States that will not be populated near lysogenic CI concentrations (e.g., those without O1 or O2 bound) are ignored; the reference state (ΔG=0) has CI dimers bound to O1 and O2. A state with O2 free of CI is included to show activation in Figure 5a and b, but does not significantly change fit parameters; because O2 and Og2 binding is highly cooperative, we do not model states with only one or the other operator bound. The degeneracy term indicates how many microstates exist with identical CI dimer binding patterns and free energies. A particular macrostate may have several microstates that differ in terms of parallel or antiparallel looping configurations or in the identity of binding sites participating in cooperative interactions (either through looping or through adjacent dimers). Here, we also list whether a state is looped (1 for looped; 2 for unlooped) as well as its transcription rate, adjacent dimers). Here, we also list whether a state is looped (1 for looped; 2 for unlooped) as well as its transcription rate, r

(DOCX)

Table S3 Thermodynamic model fitting using alternative choices for wild-type CI concentration (expressed here in molecules/cell; in the model, 1 molecule per cell is equivalent to 1.47 nM and the fraction of CI molecules that are in the form of free dimers. The approximation of a constant free-dimer fraction is reasonable if specifically bound CI dimers (up to 6 dimers composed of 12 monomers) do not make up a large fraction of total CI and if CI concentration is sufficiently high that almost all CI molecules are in dimeric complexes. The free-dimer fractions used here were calculated assuming the absence of specific binding sites using the parameters for nonspecific binding site affinity and free energies. A particular macrostate may have several microstates that differ in terms of parallel or antiparallel looping configurations or in the identity of binding sites participating in cooperative interactions (either through looping or through adjacent dimers). Here, we also list whether a state is looped (1 for looped; 2 for unlooped) as well as its transcription rate, k (0; 1 for kbasal; 2 for kunlooped; 3 for klooped). The free energy of state 2 is called ΔG2 below.

(DOCX)

Table S4 Names of new strains used in this study (as used internally in our lab) and shorthand names used in the main text.

(DOCX)

Table S5 Sequences of oligonucleotide probes for single-molecule fluorescense in situ hybridization (smFISH) experiment. Asterisks indicate probes that do not hybridize specifically with any E. coli sequence. All other probes hybridize nonoverlapping sequence in the cI coding region of the mRNA transcript from the P_E promoter.

(DOCX)

Table S6 Measurement statistics for experiment comparing ΔG_N distributions for looped and unlooped control strains to ΔG_N distributions for strains lacking O2 and having weakened P_E promoters. CI expression levels measured by smFISH for wild-type phage lambda lysogen JL5392 and additional strains. For strains with replicate experiments (N, number of independent experiments), errors indicate standard deviation. The expression levels were normalized to wild-type units (WTUs) using the WT strain.

(DOCX)

Table S7 Measurement statistics for experiment comparing ΔG_N distributions for looped and unlooped control strains to ΔG_N distributions for strains in which CI harbors the G147D mutation with and without the overexpression of CI(G147D) from a plasmid. Errors for the ΔG_N measurements are all 1 s.e.m. as estimated from 1,000 bootstrapped samples. Note that ΔG_N distributions display small, day-to-day variability between experiments (see Figure S1, this table, Table 2, Table S7), but the trends stays the same for a given set of experiments.

(DOCX)

Table S8 CI expression levels measured by smFISH for wild-type phage lambda lysogen JL5392 and additional strains. For strains with replicate experiments (N, number of independent experiments), errors indicate standard deviation. The expression levels were normalized to wild-type units (WTUs) using the WT strain.

(DOCX)

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Author Contributions

The author(s) have made the following declarations about their contributions: Conceived and designed the experiments: ZH JX ACL XW. Performed the experiments: ZH ACL XW. Analyzed the data: ZH ACL XW. Contributed reagents/materials/analysis tools: ZH ACL XW. Wrote the paper: ZH JX ACL XW.

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