Quantitation of the DNA tethering effect in long-range DNA looping in vivo and in vitro using the Lac and lambda repressors

David G. Priest, University of Adelaide
Lun Cui, University of Adelaide
Sandip Kumar, Emory University
David Dunlap, Emory University
Ian B. Dodd, University of Adelaide
Keith E. Shearwin, University of Adelaide

Journal Title: Proceedings of the National Academy of Sciences
Volume: Volume 111, Number 1
Publisher: National Academy of Sciences | 2014-01-07, Pages 349-354
Type of Work: Article | Final Publisher PDF
Publisher DOI: 10.1073/pnas.1317817111
Permanent URL: https://pid.emory.edu/ark:/25593/s5rtq

Final published version: http://dx.doi.org/10.1073/pnas.1317817111

Accessed November 7, 2019 5:14 AM EST
Quantitation of the DNA tethering effect in long-range DNA looping in vivo and in vitro using the Lac and λ repressors

David G. Priest\textsuperscript{a}, Lun Cui\textsuperscript{a}, Sandip Kumar\textsuperscript{b}, David D. Dunlap\textsuperscript{b}, Ian B. Dodd\textsuperscript{a,1}, and Keith E. Shearwin\textsuperscript{a}

\textsuperscript{a}Discipline of Biochemistry, School of Molecular and Biomedical Science, University of Adelaide, Adelaide, SA 5005, Australia; and \textsuperscript{b}Department of Cell Biology, Emory University, Atlanta, GA 30322

Edited by Mark Ptashne, Memorial Sloan Kettering Cancer Center, New York, NY, and approved November 18, 2013 (received for review September 20, 2013)

Efficient and specific interactions between proteins bound to the same DNA molecule can be dependent on the length of the DNA tether that connects them. Measurement of the strength of this DNA tethering effect has been largely confined to short separations between sites, and it is not clear how it contributes to long-range DNA looping interactions, such as occur over separations of tens to hundreds of kilobase pairs in vivo. Here, gene regulation experiments using the Lac and λ CI repressors, combined with mathematical modeling, were used to quantitate DNA tethering inside \textit{Escherichia coli} cells over the 250- to 10,000-bp range. Although Lac and CI loop DNA in distinct ways, measurements of the tethering effect were very similar for both proteins. Tethering strength decreased with increasing separation, but even at 5- to 10-kb distances, was able to increase contact probability 10- to 20-fold and drive efficient looping. Tethering in vitro with the Lac repressor was measured for the same 600- to 3,200-bp DNAs using tethered particle motion, a single molecule technique, and was 5- to 45-fold weaker than in vivo over this range. Thus, the enhancement of looping seen previously in vivo at separations below 500 bp extends to large separations, underlining the need to understand how in vivo factors aid DNA looping. Our analysis also suggests how efficient and specific looping could be achieved over very long DNA separations, such as what occurs between enhancers and promoters in eukaryotic cells.

Interactions between proteins bound to separate sites on the same DNA molecule are critical in gene regulation and other DNA processes (1–4). The DNA separation between functionally interacting sites ranges from a few base pairs to hundreds of kilobase pairs, as in the case of some eukaryotic enhancers and their promoters. At short separations, it is clear that the DNA acts as a tether that keeps one site in the vicinity of the other so that the proteins at one site can find the other site in 3D space more efficiently than if they were free in solution (Fig. 1). Tethering is also a way to provide specificity because it aids interaction with linked sites but not unlinked sites. However, as the separation between the sites increases, this tethering effect becomes weaker, and it is not understood how the DNA linkage between widely separated sites, for example, between enhancers and promoters, provides the efficiency and specificity required for proper regulation.

The effect of DNA tethering can be quantified by the factor $j_{\text{LOOP}}$ (M), the effective molar concentration of one site on the DNA relative to the other, or as the free energy of the DNA looping reaction $\Delta G_{\text{LOOP}}$ (Fig. 1) (5–9). These parameters are interconvertible: $\Delta G_{\text{LOOP}} = -RTj_{\text{LOOP}}$ (kcal/mol); the reference $j$ is 1 M. The formation of a naked DNA loop is in itself an energetically unfavorable reaction ($\Delta G_{\text{LOOP}}$ is positive) under physiological conditions due to the enthalpic cost of DNA bending and twisting (particularly important for short DNA segments) and the entropic cost of restricted configurational freedom of the DNA (the major limitation for long DNA loops). Thus, protein-mediated DNA looping reactions are driven by thermodynamic linkage to favorable protein–protein and protein–DNA interactions (Fig. 1). Nevertheless, the DNA tether can help assemble DNA-protein complexes because the effective concentration of the DNA-bound protein at the distant site, $j_{\text{LOOP}}$, can be greater than the concentration of available free protein (Fig. 1). Despite its critical role in DNA looping interactions, there are few measurements of $j_{\text{LOOP}}$ in vivo, and these are mostly restricted to short site separations.

Many in vivo techniques used to detect DNA looping interactions can quantitate relative contact efficiencies but do not permit measurement of $j_{\text{LOOP}}$. Chromosome conformation capture (3C) and related methods have revealed a complex network of in vivo interactions, many over megabase pair DNA distances, between DNA sites in the genomes of organisms ranging from bacteria to humans (4, 10, 11). Assays using DNA recombinases have shown, as expected from DNA polymer theory, that specific contact efficiencies decrease with increasing DNA separation between the sites over the 1- to 100-kb range (12, 13). None of these methods reveal the fraction of the time that the sites are in contact.

$j_{\text{LOOP}}$ for naked DNA in vitro is quite low at long separations. DNA cyclization measurements show that $j_{\text{LOOP}}$ decreases with increasing tether length beyond ~500 bp, falling to ~10 nM at 4,000 bp (5, 14). Measurements of $j_{\text{LOOP}}$ for protein-induced looping give a similar picture. Most studies have focused on linear tethers <500 bp, finding variable $j_{\text{LOOP}}$ values generally below 100 nM (9). Lower $j_{\text{LOOP}}$ values are seen at longer separations: ~18 nM for Cre DNA recombination at 3,000 bp (15), whereas LacI DNA binding cooperativity was undetectable at

Significance

Proteins bound to DNA often interact with proteins bound elsewhere on the same DNA to regulate gene expression. The intervening DNA tethers the proteins near each other, making their interaction efficient and specific, but the importance of this tethering effect is poorly understood at large DNA separations. We quantitated tethering inside bacterial cells, using two different proteins at separations up to 10,000 bp, to show that tethering is strong enough to drive efficient interactions over these distances. The same interactions were ~10-fold weaker outside cells, implying that cellular factors enhance tethering. However, tethering was lost at a DNA separation of 500,000 bp inside bacteria, indicating special mechanisms inside eukaryotic cells to provide efficient and specific interactions over such distances.


The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

1To whom correspondence should be addressed. E-mail: ian.dodd@adelaide.edu.au.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1317817111/-/DCSupplemental.
(O1, O2, and O3) at O9, and three operators (Oid, a high affinity operator; O1; and a mutant operator, O') at O9, with a 300-bp spacing (center to center) between O9 and O2. Expression of lacZ was measured in the absence of Lac repressor (L0) or at two different Lac concentrations (L1 and L2), expressed from PlacUV5lacI or PlacUV5OidlacI constructs integrated at a separate chromosomal site (Fig. 24). The results showed, as expected, that the presence of O1 or Oid at O9 increased repression substantially, depending on the operator combination and the LacI concentration (Fig. 2B).

By fitting the data to a statistical-mechanical model of LacI repression (Fig. 2C), we were able to estimate a number of in vivo parameters (Fig. 2D). The model is similar to Han et al. (9), with the addition of RNA polymerase (RNAP) binding to PlacUV5 (24). The model assumes that LacI tetramers supplied from the WT lacI promoter has been estimated at 18 nM (25) (11 tetramers in a 1-fL volume) and was used for L1. The model does not permit determination of absolute binding constants, and KI was arbitrarily fixed to 1 nM. The relative dissociation constants obtained for the four operators are similar to those found by Garcia and Phillips (25) (0.22, 1, 4.5, and 335 for Kid, K1, K2, and K3, respectively). Western blotting and PlacI* and PlacO activities (Fig. S4) supported the estimate of the ratio of the high and low Lac repressor concentrations of L/L1 = 10.9. Other reporter measurements (SI Materials and Methods) supported the estimate of 18.1 background LacZ units from the PlacUV5lacZ reporter (Fig. 2D).

The fitting gives an estimate of jloop/L1 that is independent of the fixed values chosen for KI or L1. Assuming L1 = 18 nM gives jloop = 1.400 nM (for this 300-bp spacing), similar to estimates of ~1 μM for short lac operator spacings in vivo (6, 8, 19).

The upstream operator aided repression most strongly at the shortest spacing (242 bp). Although its effect weakened with increasing spacer length, there remained a significant effect of the upstream operator at 5,600 bp at the L1 concentration (Fig. 3A). The operator placed 500 kb away had no effect on repression.

The reporter data were fitted using the model and the parameters from Fig. 2D but allowing jloop/L1 to vary for each operator spacing (SI Materials and Methods). These jloop/L1 values (Fig. 3B and Fig. S5) show that the effective concentration of the operators relative to each other changes from 180-fold the L1 concentration of LacI in solution at 242 bp to 1.8-fold L1 at 5,600 bp. Thus, even at a 5,600-bp separation, a Lac repressor at the distal lac operator is seen by the promoter almost two times more frequently than all of the other Lac repressors in the cell combined. In contrast, the data for the 500-kb spacing was fitted with a jloop/L1 value of 0.99 (Fig. 3C); a Lac repressor bound 500 kb away contacts the proximal operator more frequently than any of the other ~11 Lac repressors in the cell.

The jloop/L1 values allow calculation of the fraction of looping for Oid and O2 at each separation and [lacI] (Fig. 3C). LacI looping is sensitive to concentration; at low concentrations, neither operator is occupied, whereas at high concentrations, looping is obstructed by the formation of doubly bound species (species 6; Fig. 2C). Maximal looping decreases with increasing spacing, but even at separations of 5,600 bp, Oid-O2 are looped at least 30% of the time over a 100-fold range of [lacI] (0.2–20 nM; Fig. 3C).

**Effect of Separation on Long-Range Looping by λ CI in Vivo.**

We used λ CI to obtain an independent measurement of the relationship between jloop and DNA separation in vivo. DNA looping by λ CI can be detected by repression of the λ PrM
promoter (7, 23, 27). At low concentrations, CI dimers form tetrmers on O1.O2.OL2 and OR1.OR2., activating PRM, and these tetrmers can interact to form an octamer-bridged DNA loop (Fig. 4d; free energy ΔGoct). The loop allows a dimer bound at OL to help a dimer bind to the weak OR, forming a trans-tetramer and repressing PRM (Fig. 4d; dimer-dimer interaction free energy ΔGtet). Thus, repression at moderate CI concentrations requires the distal OL site. With a statistical-mechanical model for CI regulation (7, 23), the free energy for the DNA looping reaction in vivo, ΔGvivo, can be extracted from a comparison of PRM activity + OL at high CI concentrations.

The looping reaction comprises the unfavorable DNA looping energy ΔGloop and a favorable protein–protein interaction ΔGPTN between DNA-bound CI tetrmers: ΔGoct = ΔGloop + ΔGPTN. Estimating ΔGPTN from a measure of the free energy of CI octamerization in vitro (~9.1 kcal/mol) (28, 29), it is possible to derive ΔGloop from ΔGoct (7) (SI Materials and Methods).

We made a series of OR.PRMP.lacZ reporters with OR at a range of distances from 150 to 10,000 bp upstream of OR (Fig. 4d, Figs. S1 and S2, and SI Materials and Methods). A chromosomally integrated CI gene (or vector only) provided 3.3 WT lysogenic units (WLUs) of CI (or no CI) (23). OL-dependent repression of PRM was strongest for the 250-bp spacing and became weaker with increasing distance (Fig. 4B and Fig. S6). However, even at 10,000 bp, the presence of OL aided repression of PRM. In contrast, OL 500 kb away on the chromosome gave no enhancement of PRM repression (Fig. S7).

The reporter data were fitted by allowing a difference in ΔGoct for each spacing but holding all other parameters fixed to the values obtained in our previous detailed analysis of OR-OR looping at 2,300-bp spacing (23) (SI Materials and Methods and Fig. S6). However, we could not achieve good fits to the data for the shorter spacers unless we decreased ΔGoct to ~3.4 kcal/mol, below our previous estimate of ~2.4 kcal/mol. The 2,300-bp data (23) are still reasonably well fitted using this revised value for ΔGoct and this value is in better agreement with the estimate of ΔGoct = ~3.2 kcal/mol obtained by single cell imaging of looping at 2,300 bp by CI in vivo (20).

The resulting ΔGloop estimates (Fig. 4C) have large errors, primarily reflecting uncertainty in the value for ΔGoct, which has the effect of shifting the whole ΔGloop vs. separation curve up or down. The lower ΔGoct value causes our ΔGloop estimates to be some 10-fold lower than before (7, 23) (Fig. 4C). However, our new 2,300-bp ΔGoct estimate agrees well with that derived from single cell imaging (20) (Fig. 4C). The λ CI values are also remarkably similar to those obtained for LacI with L1 = 18 nM (Fig. 4C). We also used our Lac model to analyze existing LacI looping data for 60- to 1,500-bp separations (21) and found a good match with the ΔGloop values from our LacI and CI data (Fig. S8).

These ΔGloop values produce efficient looping in the CI system, with OL-OR looped over 50% of the time at the 10,000-bp separation at 3.3 WLUs CI (Fig. 4D). Unlike the Lac system, the looped fraction increases with increasing CI concentrations (Fig. 4D) because the CI multimer in the looped complex, the octamer, does not form readily in solution, and loop-blocking species are only likely to form at considerably higher CI concentration (29).

Long-Range Looping by lac Repressor in Vitro. Previous studies indicate that LacI looping with short DNA tethers (~500 bp) is less efficient in vitro than in vivo. To test whether this difference holds for longer DNA tethers, we examined LacI looping in single molecules by TPM (9, 30–32), where looping of a DNA molecule attaching a bead to a surface can be detected as a restriction in the Brownian motion of the bead (Fig. 5A). The fragments were the same as the 600-, 900-, 1,200-, and 3,200-bp spacing in vivo constructs, but with O1 at O2 (Fig. 5A, Fig. S5, and SI Materials and Methods).

Looped and unlooped states were followed over time over a range of LacI concentrations. Excursion values from the motion records of all selected beads in one experimental condition were assembled in a histogram from which average looping probabilities were determined (Fig. 5B). As expected, the probability of looping goes through a maximum with respect to [LacI] (Fig. 5C). At LacI concentrations at which Oid is fully occupied, the decrease in looping with increasing [LacI] allows estimation of ΔGloop independently of the affinity of O1 (Fig. 5D), because looping or unlooping is a simple binding competition between bound or free LacI (9).

The 600-bp ΔGloop value obtained by TPM is 5.7-fold lower than the LacI and CI in vivo estimates (Fig. 5E), consistent with previous studies. Interestingly, this difference increases at longer separations: 11-fold at 1,200 bp and 45-fold at 3,200 bp (Fig. 4D). The TPM measurement of ΔGoct = 1.7 kcal/mol for CI looping at 2,300 bp (17) gives a ΔGloop value that is also approximately sevenfold lower than the in vivo CI and LacI estimates (Fig. 4D).

Discussion

DNA Can Foster Long-Range Interactions Even at Large Site Separations. We used two well-characterized bacterial systems where transcription is regulated by DNA looping to obtain measurements of ΔGloop in vivo for DNA tether lengths beyond 5 kb. The results for LacI...
... and λ CI were in remarkable agreement, especially considering the differences in looping mechanism, with formation of a LacI loop primarily a DNA–protein interaction (Figs. 1C and 2C) and formation of the CI loop dominated by a protein–protein interaction (Figs. 1B and 4A). The different modeling approaches and the distinct additional parameters needed to extract \( J_{\text{loop}} \) (\( L_1 \) for LacI and \( \Delta G_{\text{PTM}} \) for CI) make the estimates quite independent of each other. In addition, as discussed, our estimates for shorter spacings agree well with previous in vivo measurements (6, 19, 20).

At separations <300 bp, \( J_{\text{loop}} \) was >1 μM and decayed with separation roughly as a power law with exponent \( -1.2 \) (Fig. S10). In the 5- to 10-kb separation range, \( J_{\text{loop}} \) was still \( \sim \)20–40 nM, substantially higher than the 1.7 nM concentration of a single molecule within a cell volume of 1 fl (1 μm³). Thus, as long as the concentration of a protein in the cell is reasonably low, even a distant operator can strongly increase its effective local concentration. As a result, even at these distances, the fraction of time that the DNA sites spend looped can be considerable. For Oid-O2 at 5,600-bp separation, the fraction looped could be almost 40% (Fig. 3C). For λ OL-OR at 10-kb separation, the fraction looped can be at least 50% at CI concentrations >3 WLU (Fig. 4D).

This reasonably strong tethering effect at 5–10 kb disappears at a separation of 500 kb, where the distal operator has no detectable effect on regulation by LacI or CI. Thus, even at large separations, distance matters: a DNA site 10 kb away is much closer than a site 500 kb away. A question remains whether there is a gradual decline in \( J_{\text{loop}} \) over the 10- to 500-kb range or whether tethering is lost at separations well short of 500 kb. Simple extrapolation of the in vivo data in Fig. 5E (Fig. S10) suggests that substantial tethering could exist at separations of ∼100 kb. Previous measurements of relative contact efficiencies using sites for recombinases in bacteria have revealed a steady drop in relative contact efficiency over the 10- to 90-kb range (13). It may be possible to use our λ CI reporter system to quantitate \( J_{\text{loop}} \) at 100-kb separations, because \( J_{\text{loop}} \) values as low as 3 nM (\( \Delta G_{\text{act}} \) of ∼3 kcal/mol) should be measurable (Fig. 4A).

In Vivo Factors Increase \( J_{\text{loop}} \) at Long Separations. The in vitro \( J_{\text{loop}} \) values obtained for LacI looping by TPM were ∼5- to 45-fold lower and appeared to decay faster with separation than the in vivo values (Fig. 5E), fitting a power law with an exponent of ∼1.5 (Fig. S10).

Our TPM estimates are roughly comparable with published in vitro values. Our \( J_{\text{loop}} \) values of 75 nM for the 600-bp LacI loop is higher than the ∼10- and ∼30-nM values obtained for LacI by TPM for ∼100- and ∼300-bp loops, respectively, by Han et al. (9), but this is consistent with the expected increase in \( J_{\text{loop}} \) over these separations due to relief of enthalpy costs (5). The TPM results of Johnson et al. (26) indicate even poorer looping with short spacers, with \( J_{\text{loop}} \) values of 0.3–4 nM for different ∼100-bp LacI loops. Our 900-bp estimate of \( J_{\text{loop}} \) = 54 nM is similar to the \( J_{\text{loop}} \) = 37 nM obtained for an 870-bp loop formed by Cre recombinase (15). At the 3,200-bp spacing, our in vitro \( J_{\text{loop}} \) fell to >5 nM, which is comparable to 18 nM at 3,044 bp (14) and ∼10 nM for DNA cyclization at 4,000 bp (14). TPM analysis of CI looping at 2,300 bp gave \( J_{\text{loop}} \) = 24 nM (17), somewhat higher than our TPM values (Fig. 5E); however, this may be an overestimate because a low value of \( \Delta G_{\text{act}} \) was used.

Thus, we are confident that the in vivo–in vitro difference is real and applies over two orders of magnitude of the separation between sites (100–10,000 bp).

In vivo factors that increase flexibility or compaction of DNA such as DNA supercoiling and nonspecific DNA-binding proteins that bend or bridge DNA, such as the nucleoid protein HU, are thought to enhance short-range DNA looping (8, 33–36). It is not clear whether these factors also act at distances over which DNA bending and twisting are not limiting. However, increased DNA flexibility due to random binding of bend-inducing proteins such as nucleoid proteins or histones (35) should make the DNA more likely to wind back on itself, keeping it more compact and making it less likely that sites far apart on the DNA will be far apart in space. Unconstrained DNA supercoiling is implicated in aiding long-range looping in vivo (13). We note that our in vivo data can be reasonably well fitted by assuming that the DNA is a flexible polymer with an apparent persistence length of 27 nm (66 bp; Fig. S10). Relative FLP recombination rates over separations of 70–15,000 bp in human cells indicated a similar apparent persistence length of 27 nm (12).

Our data do not identify which factors are responsible for improved long-range looping in vivo. However, our work provides a quantitative target for the in vivo/in vitro difference—an ∼10-fold effect—and provides an experimental system to measure the effects of candidate factors by addition of factors in vitro or removal of factors in vivo.

Creating Efficient Long-Range Looping. Given a fixed \( J_{\text{loop}} \) between any two DNA sites, how can DNA looping be maximized? The LacI and λ CI proteins represent extremes of a biochemical continuum (Fig. 1). In the case of LacI, the protein–protein interaction of the operator can strongly increase its effective local concentration. As the concentration of a protein in the cell is reasonably low, even a distant operator can strongly increase its effective local concentration. As a result, even at these distances, the fraction of time that the DNA sites spend looped can be considerable. For Oid-O2 at 5,600-bp separation, the fraction looped could be almost 40% (Fig. 3C). For λ OL-OR at 10-kb separation, the fraction looped can be at least 50% at CI concentrations >3 WLU (Fig. 4D).

This reasonably strong tethering effect at 5–10 kb disappears at a separation of 500 kb, where the distal operator has no detectable effect on regulation by LacI or CI. Thus, even at large separations, distance matters: a DNA site 10 kb away is much closer than a site 500 kb away. A question remains whether there is a gradual decline in \( J_{\text{loop}} \) over the 10- to 500-kb range or whether tethering is lost at separations well short of 500 kb. Simple extrapolation of the in vivo data in Fig. 5E (Fig. S10) suggests that substantial tethering could exist at separations of ∼100 kb. Previous measurements of relative contact efficiencies using sites for recombinases in bacteria have revealed a steady drop in relative contact efficiency over the 10- to 90-kb range (13). It may be possible to use our λ CI reporter system to quantitate \( J_{\text{loop}} \) at 100-kb separations, because \( J_{\text{loop}} \) values as low as 3 nM (\( \Delta G_{\text{act}} \) of ∼3 kcal/mol) should be measurable (Fig. 4A).

In Vivo Factors Increase \( J_{\text{loop}} \) at Long Separations. The in vitro \( J_{\text{loop}} \) values obtained for LacI looping by TPM were ∼5- to 45-fold lower and appeared to decay faster with separation than the in vivo values (Fig. 5E), fitting a power law with an exponent of ∼1.5 (Fig. S10).

Our TPM estimates are roughly comparable with published in vitro values. Our \( J_{\text{loop}} \) values of 75 nM for the 600-bp LacI loop is higher than the ∼10- and ∼30-nM values obtained for LacI by TPM for ∼100- and ∼300-bp loops, respectively, by Han et al. (9), but this is consistent with the expected increase in \( J_{\text{loop}} \) over these separations due to relief of enthalpy costs (5). The TPM results of Johnson et al. (26) indicate even poorer looping with short spacers, with \( J_{\text{loop}} \) values of 0.3–4 nM for different ∼100-bp LacI loops. Our 900-bp estimate of \( J_{\text{loop}} \) = 54 nM is similar to the \( J_{\text{loop}} \) = 37 nM obtained for an 870-bp loop formed by Cre recombinase (15). At the 3,200-bp spacing, our in vitro \( J_{\text{loop}} \) fell to >5 nM, which is comparable to 18 nM at 3,044 bp (14) and ∼10 nM for DNA cyclization at 4,000 bp (14). TPM analysis of CI looping at 2,300 bp gave \( J_{\text{loop}} \) = 24 nM (17), somewhat higher than our TPM values (Fig. 5E); however, this may be an overestimate because a low value of \( \Delta G_{\text{act}} \) was used.

Thus, we are confident that the in vivo–in vitro difference is real and applies over two orders of magnitude of the separation between sites (100–10,000 bp).

In vivo factors that increase flexibility or compaction of DNA such as DNA supercoiling and nonspecific DNA-binding proteins that bend or bridge DNA, such as the nucleoid protein HU, are thought to enhance short-range DNA looping (8, 33–36). It is not clear whether these factors also act at distances over which DNA bending and twisting are not limiting. However, increased DNA flexibility due to random binding of bend-inducing proteins such as nucleoid proteins or histones (35) should make the DNA more likely to wind back on itself, keeping it more compact and making it less likely that sites far apart on the DNA will be far apart in space. Unconstrained DNA supercoiling is implicated in aiding long-range looping in vivo (13). We note that our in vivo data can be reasonably well fitted by assuming that the DNA is a flexible polymer with an apparent persistence length of 27 nm (66 bp; Fig. S10). Relative FLP recombination rates over separations of 70–15,000 bp in human cells indicated a similar apparent persistence length of 27 nm (12).

Our data do not identify which factors are responsible for improved long-range looping in vivo. However, our work provides a quantitative target for the in vivo/in vitro difference—an ∼10-fold effect—and provides an experimental system to measure the effects of candidate factors by addition of factors in vitro or removal of factors in vivo.

Creating Efficient Long-Range Looping. Given a fixed \( J_{\text{loop}} \) between any two DNA sites, how can DNA looping be maximized? The LacI and λ CI proteins represent extremes of a biochemical continuum (Fig. 1). In the case of LacI, the protein–protein interaction...
interactions that connect the two DNA-binding ends of the complex are strong, and the DNA-looping multimer, the tetramer, forms at exceedingly low concentrations (37). For CI, the assembly of dimers into tetramers, and tetramers into the loop-forming octamer is relatively weak so that the DNA looping complex does not form readily in solution (29).

Efficient LacI looping requires the protein concentration to be substantially below $j_{\text{LOOP}}$ and to lie close to the $K_D$ of both DNA sites (9, 26). At very long separations, where $j_{\text{LOOP}}$ is small, efficient looping by LacI is thus likely to be limited by difficulties in achieving reliable low protein concentrations inside cells due to gene expression noise.

Fig. 4. Effect of DNA separation on $\lambda$ CI looping in vivo. (A) Approach for measuring $j_{\text{LOOP}}$ with $\lambda$ CI. Structure of the OL-spacer-OR.PRM.lacZ reporters. CI activates PRM at low concentrations and represses it at high concentrations. Repression is dependent on the presence of OL and the free energy of loop formation between OL and OR, $\Delta G_{\text{oct}}$. (B) Decreased repression of PRM with increasing OL-OR separation. OR.PRM.lacZ reporters ± OL placed various distances upstream were assayed at 3.3 WT lysogenic units (WLU) of CI and in the absence of CI (SI Materials and Methods and Fig. S7). Also shown are previous $j_{\text{LOOP}}$ estimates for in vivo CI looping (square, ref. 7; triangle, ref. 23; red diamond, ref. 20); as well as the $j_{\text{LOOP}}$ values for LacI (Fig. 3C), assuming $L_1 = 18$ nM (blue circles). (D) Predicted fraction of looping for OL and OR at different separations up to 3.3 WLU, using $j_{\text{LOOP}}$ estimates from C.

Fig. 5. Measurement of $j_{\text{LOOP}}$ by TPM. (A) The TPM setup. (B) Examples of excursion vs. time traces for single beads, showing transitions between looped and unlooped states (left) and mean excursion probability distributions (right) for tethers with lac operators separated by 600–3,200 bp at 10 nM LacI. Different Lac loop conformations (two peaks) were distinguishable for the 900-bp loop. (C) The probability of looping vs. LacI concentration for the 900-bp separation. (D) $j_{\text{LOOP}}$ for each DNA was determined from the average fraction of time the DNA is looped ($p_{\text{looped}}$) at each [LacI], using the [LacI] dependence of loop blockage at [LacI] 0.5–20 nM (Oid was filled at 0.1 nM), according to the equation $p_{\text{unlooped}}/p_{\text{looped}} = 2K_1/j_{\text{LOOP}} + 2[LacI]j_{\text{LOOP}}$ (9) ($K_1$ was fixed at 1 nM). The slope of each $p_{\text{unlooped}}/p_{\text{looped}}$ vs. [LacI] plot equals $2j_{\text{LOOP}}$. (E) Comparison of the TPM $j_{\text{LOOP}}$ values with the in vivo values (LacI, blue; CI, brown). The red square shows a previous TPM estimate for $\lambda$ CI (17).
For CI, efficient looping requires the free energy of the protein–protein interaction, $\Delta G_{\text{PTN}}$, to compensate for the unfavorable $\Delta G_{\text{LOOP}}$. The interaction of two CI tetramers is estimated to provide $-9.1$ kcal/mol to drive the looping reaction (28, 29). However, this octamerization reaction alone would fail to give more than $50\%$ looping for DNA separations above $-1.000$ bp, where $\Delta G_{\text{LOOP}} > +9.1$ kcal/mol ($G_{\text{loop}} < 384$ nM). One way to improve CI looping would be to strengthen $\Delta G_{\text{PTN}}$ by increasing the strength of the interactions between protein subunits. However, such changes would tend to increase the formation of larger complexes in solution, which would block looping.

The efficiency of looping by $\lambda$ CI is increased by increasing the number of interacting proteins at each site. The additional bridge formed by the interaction of a CI dimer at each site, to form a trans-tetramer, effectively means that $\Delta G_{\text{PTN}}$ (octamerization) and $\Delta G_{\text{tet}}$ (tetramerization) combine to counterbalance $\Delta G_{\text{LOOP}}$. As suggested by Dröge and Müller-Hill (38), this approach of using a DNA scaffold to assemble at each site a large complex of proteins that individually interact weakly but in combination provide a strong protein–protein interaction seems optimal for creating strong looping at very long separations, such as in eukaryotic genomes. A similar method could also be used to make LacI-style looping more efficient. Additional factors at each site could permit a DNA loop to be bridged by two Lac tetramers.

However, strategies that use multiple interacting sites for the same protein to strengthen looping will eventually be limited by the formation of short DNA loops within each set of binding sites. A way around this limitation could be to combine different looping proteins. This kind of approach for making looping efficient has the advantage that specificity of looping could be provided by using a relatively small number of different looping proteins in different combinatorial patterns. Such a strategy may be necessary to provide both efficiency and specificity for the very long-range DNA interactions in eukaryotic genomes.

**Materials and Methods**

**Reporter and Expression Constructs.** The lacZ reporters and LacI and CI expression constructs were chromosomally integrated using the OSIP system and its precursors (23, 39) into MG1655 rph + lacI2347YA (SI Materials and Methods). Cells were grown at 37 °C in minimal medium (LacI looping strains) or in rich medium (CI looping strains) and assayed by a modified LacZ microtiter plate method (7) (SI Materials and Methods).

**Mathematical Modeling.** Data fitting to extract $\Delta G_{\text{loop}}$ values from the $\lambda$ CI reporter data was as previously described (23) with some modifications. Details of this and the LacI modeling are given in SI Materials and Methods.

**TPM.** TPM experiments were conducted as previously described (30–32) with some modifications (SI Materials and Methods). Distortion correction was improved by subtracting the motion of one to five stuck beads in the same field of view. Stringent selection of motion records based on symmetry and unlooped excursion amplitude was used. The mean square excursion of the bead was used to determine tether length by reference to a calibration curve with tethers of known length.

**ACKNOWLEDGMENTS.** We thank Laura Finzi, Iain Murchland, Julian Pietsch, and other members of the Shearwin, Dunlap, and Finzi laboratories, as well as the Children’s Hospital for discussion. We acknowledge Suleyman Ocuncuoglu and Weng Lee Lim in the Emory Physics Department for enhancements to bead tracking software and Kathleen Matthews for the Lac repressor. Support was from Human Frontiers Scientific Program Grant RGP0051, a University of Adelaide PhD scholarship to G.D.P. (G.D.P.), the China Scholarship Council (L.C.), Australian Research Council Grants DP110100824 and DP11010470, the WH Elliott Fellowship in Biochemistry and National and Medical Research Council (Australia) Grant APP1025549 (to I.B.D.), National Institutes of Health Grant R01GM084707A (to D.D.D.), and the Center for Pediatric Nanomedicine in the Department of Biomedical Engineering, Georgia Institute of Technology and Children’s Healthcare of Atlanta (D.D.D.).