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

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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 repressors, combined with mathematical modeling, were used to quantitate DNA tethering inside *Escherichia coli* cells over the 250- to 10,000-bp range. Although Lac and λ 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 $\lambda_{\text{LOOP}}$ (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: $\Delta G_{\text{LOOP}} = \kappa T \ln \lambda_{\text{LOOP}}$ (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).

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.

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Repressor in Vivo.

reporter (Fig. 2 CI can be detected by repression of the 1,400 nM (for this 300-bp spacing), similar to estimates CI repressors (6 PlacI Oid in competition with LacI (24). The cellular concentration j λ www.pnas.org/cgi/doi/10.1073/pnas.1317817111 (O at L2 values allow calculation of the fraction of ∼ operator is seen by the promoter almost two times PRM reporters with operator for DNA loops formed by LacI or SI values of the order of 1 DNA interactions are strong and loop formation is driven by protein–DNA interactions, as for the λ CI repressor.

4,000 bp (16). The only long-range tethered particle motion (TPM) study found jLOOP of ~24 nM for CI looping at 2,300 bp (17).

Most existing estimates of jLOOP in vivo have been obtained by analysis of DNA loop-length dependent repression of transcription by the Lac and λ CI repressors (6–8, 18–20). In such noncatalytic systems, the degree of repression reflects the fraction of the time that the repressor is bound at the promoter, allowing the thermodynamic stability of the looping interaction to be estimated. Analyses of data for LacI looping at short DNA separations (21, 22) have given jLOOP values of the order of 1 μM (6, 8, 19), which is much higher than would be expected based on in vitro estimates and modeling. However, at longer separations, in vivo looping by Lac repressor seems to be weak, with little effect seen at a spacing of 1,500 bp (21) and no effect at 4,300 bp (22), leading these authors to conclude that long-range looping by LacI is inefficient. In contrast, we estimated jLOOP of ~850 nM for λ CI DNA loops of 3.8 and 2.3 kb (7, 23), and single-molecule imaging in live Escherichia coli cells of CI-mediated interactions over a 2.3-kb separation gave results indicating a jLOOP of ~240 nM (20).

In theory, jLOOP for DNA loops formed by LacI or λ CI should be the same at large separations, where protein conformation effects are minimal. To resolve this apparent discrepancy and to extend the estimates of jLOOP for long DNA separations in vivo, we performed systematic repression-based jLOOP assays over the 300-bp to 10-kb range using the LacI and λ CI repressors in E. coli. To test whether the in vivo enhancement of looping seen at short separations holds at long separations, we measured jLOOP in vitro with TPM using the Lac repressor for 600- to 3,200-bp separations with the same DNA constructs. The results for the two different repressors were similar and show that long-range DNA looping in vivo is considerably more efficient than for naked DNA in vitro, indicating long distance suppressing factors inside cells. Strategies for maximizing looping efficiency are discussed.

Results

System to Estimate jLOOP Using Lac Repression in Vivo. We made chromosomally integrated lacZ reporters driven by the catabolite activator protein (CAP)-independent PlacUV5 promoter controlled by a proximal lac operator (OPl) at the normal O1 position (centered at +11) and an upstream distal lac operator (OD2; Fig. 2A). To characterize the system, we used three natural operators (O1, O2, and O3) at OPl, and three operators (Oid, a high affinity operator; O1; and a mutant operator, O’) at OD2, with a 300-bp spacing (center to center) between O2 and OPl. Expression of lacZ was measured in the absence of Lac repressor (L0) or at two different LacI concentrations (L1 and L2), expressed from PlacI–lacI or PlacP′–lacI constructs integrated at a separate chromosomal site (Fig. 2B). The results showed, as expected, that the presence of O1 or Oid at OD2 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 in competition with LacI (24). The cellular concentration of 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 PlacP′ and PlacP′ activities (Fig. S4) supported the estimate of the ratio of the high and low Lac repressor concentrations of L2/L1 ≈ 10.9. Other reporter measurements (SI Materials and Methods) supported the estimate of 18.1 background LacZ units from the PlacUV5-lacZ 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).

Effect of DNA Spacing on Lac Looping in Vivo. To measure jLOOP over longer distances, we used Oid–O2 reporters with operator spacings ranging from 242 to 5,600 bp. The sequence of spacer DNA can affect the efficacy of short Lac repressor systems, so we made two series of spacers: series 1 (300–5,600 bp) and series 2 (242–3,200 bp). We also inserted Oid (or O’) in the chromosome 500 kb away from O2 (SI Materials and Methods).

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 j LOOP/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 ranges 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 twice as much as 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 no 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 Repressor 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 tetromers on O1L.O1L2 or OR1.OR2, activating PRM, and these tetromers can interact to form an octamer-bridged DNA loop (Fig. 4A; free energy ΔGoctet). The loop allows a dimer bound at O2L to help a dimer bind to the weak OR3, forming a trans- tetramer and repressing PRM (Fig. 4A; 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, 22), the free energy for the DNA looping reaction in vivo, ΔGoct, 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 tetramers: ΔGoctet = 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 jloop from ΔGoctet (7) (SI Materials and Methods).

We made a series of OR.PRM.lacZ reporters with OL (or an OR2 sequence) at distances from 150 to 10,000 bp upstream of OR (Fig. 4B, Figs. S1 and S2, and SI Materials and Methods). A chromosomally integrated cl 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 different ΔGoctet for each spacing but holding all other parameters fixed to the values obtained in our previous detailed analysis of OL–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 ΔGoctet 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 ΔGoctet and this value is in better agreement with the estimate of ΔGoctet = ~3.2 kcal/mol obtained by single cell imaging of looping at 2,300 bp by CI in vivo (20).

The resulting jloop estimates (Fig. 4C) have large errors, primarily reflecting uncertainty in the value for ΔGoctet, which has the effect of shifting the whole jloop vs. separation curve up or down. The lower ΔGoctet value causes our jloop estimates to be some 10-fold lower than before (7, 23) (Fig. 4C). However, our new 2,300-bp ΔGoctet 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 Lac looping data for 60- to 1,500-bp separations (21) and found a good match with the jloop values from our LacI and CI data (Fig. S8).

These jloop 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 OL at OP (Fig. S4, Fig. S9, 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 jloop 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 jloop 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 ΔGoctet = 1.7 kcal/mol for CI looping at 2,300 bp (17) gives a jloop 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 jloop 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 the model predictions of the effect of DNA separation ($J_{\text{LOOP}}$) and [LacI] on the efficiency of looping. Average $J_{\text{LOOP}}$ values from series 1 and 2 were used for 300, 600, 900, and 1,800 bp.

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 µm$^3$). 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 $\geq 3$ 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 $\sim 100$ 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 quantify $J_{\text{LOOP}}$ at 100-kb separations, because $J_{\text{LOOP}}$ values as low as 3 nM ($\Delta G_{\text{act}}$ of $\sim 3$ 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 $\sim$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 $J_{\text{LOOP}}$ = 75 nM for the 600-bp LacI loop is higher than the $\sim 10$- and $\sim 30$-nM values obtained for LacI by TPM for $\sim$100- and $\sim$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 $\sim 6$ nM, which is comparable to 18 nM at 3,044 bp for Cre (15) and $\sim 10$ 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 23 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 $\sim 10$-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
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.

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For CI, efficient looping requires the free energy of the protein–protein interaction, ΔGPTN, to compensate for the unfavorable ΔGLOOP. 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 ΔGLOOP > +9.1 kcal/mol (ΔG < 384 nM). One way to improve CI looping would be to strengthen ΔGPTN 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 λ 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 ΔGPTN (octamerization) and ΔGtet (tetramerization) combine to counterbalance ΔGLOOP. 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 linker proteins 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 eukaryogenic genomes.

Materials and Methods

Reporter and Expression Constructs. The lacZ reporters and Lac and CI expression constructs were chromosomally integrated using the OSI system and its precursors (23, 39) into MG1655 rph ΔlacIΔZYA (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 ΔGloop values from the λ CI reporter data was as previously described (23) with some modifications. Details of this and the LacZ 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). Drift 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.

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