

# The antiparallel loops in *gal* DNA

Giuseppe Lia<sup>1</sup>, Szabolcs Semsey<sup>2</sup>, Dale E. A. Lewis<sup>3</sup>, Sankar Adhya<sup>3</sup>,  
David Bensimon<sup>4</sup>, David Dunlap<sup>5</sup> and Laura Finzi<sup>6,\*</sup>

<sup>1</sup>Department of Chemistry, Harvard University, Cambridge, MA, USA, <sup>2</sup>Department of Genetics, Eötvös Lóránd University, H-1117 Budapest, Hungary, <sup>3</sup>Laboratory of Molecular Biology, NIH, Bethesda, MD, USA, <sup>4</sup>Laboratoire de Physique Statistique, Ecole Normale Supérieure, Paris, France, <sup>5</sup>Department of Cell Biology and <sup>6</sup>Department of Physics, Emory University, Atlanta, GA, USA

Received March 8, 2008; Revised June 2, 2008; Accepted June 4, 2008

## ABSTRACT

**Interactions between proteins bound to distant sites along a DNA molecule require bending and twisting deformations in the intervening DNA. In certain systems, the sterically allowed protein–DNA and protein–protein interactions are hypothesized to produce loops with distinct geometries that may also be thermodynamically and biologically distinct. For example, theoretical models of Gal repressor/HU-mediated DNA-looping suggest that the antiparallel DNA loops, A1 and A2, are thermodynamically quite different. They are also biologically different, since in experiments using DNA molecules engineered to form only one of the two loops, the A2 loop failed to repress *in vitro* transcription. Surprisingly, single molecule measurements show that both loop trajectories form and that they appear to be quite similar energetically and kinetically.**

## INTRODUCTION

The activity of promoters is often regulated by the interaction between proteins that are simultaneously bound to distant DNA segments to form a loop. Such complexes may be called enhanceosomes (1–3) or repressosomes (1,4–6), depending on their effect on transcription. The loop of DNA might, in principle, follow either a parallel or an antiparallel trajectory (7), and the particular trajectory can be influenced by requirements of the protein–DNA and protein–protein interactions, flexibility of protein–protein interfaces, binding of architectural proteins and length of the intervening DNA. In some systems, the scheme of protein–DNA and protein–protein interactions would allow more than one parallel or antiparallel geometry (7–9).

The Gal repressosome is a ternary nucleoprotein complex that represses transcription of the *gal* operon in *Escherichia coli*. Assembly of the Gal repressosome

requires direct interaction of GalR dimers bound to two operator sites ( $O_E$  and  $O_I$ ) separated by 113 bp. This long-range interaction is mediated by the transcriptional cofactor HU and negative DNA supercoiling (10). GalR dimers form a V-shaped, stacked tetramer in the repressosome (6,11,12). Binding of the symmetric GalR dimers to the operators could lead to four different DNA trajectories with respect to the DNA sequence, two of which are parallel (P1 and P2), while two are antiparallel (A1 and A2) (4,7). The relative stacking arrangements of the two operator-bound GalR dimers are different within each trajectory, and elastic energy calculations suggest that the A1 antiparallel GalR/HU–DNA loop (Figure 2a) is more stable than either of the parallel loops or the A2 antiparallel loop (4,8). The major difference between the two antiparallel trajectories results from the stacking of the operator-bound dimers. As a consequence of the 60° angle between the two dimers in the GalR tetramer, the DNA was calculated to be under-twisted (by slightly different amounts) in both antiparallel trajectories (8).

The HU protein stabilizes the GalR-mediated DNA loop by bending the DNA near the apex of the loop (1,10). Both single-molecule manipulations using magnetic tweezers (10) and AFM visualization of DNA loops (13) predicted an antiparallel DNA trajectory in the repressosome, suggesting that HU binding does not assist formation of repressosomes containing a parallel DNA loop. These measurements, however, were unable to distinguish between the two alternative antiparallel configurations. Recently instead, only one (A1) of the two alternative antiparallel loops was found to repress *gal* transcription (4). Two explanations were proposed for this observation: (i) the A2 loop is thermodynamically disfavored such that it either does not form or forms with such thermodynamics and/or kinetics that it fails to repress transcription, or (ii) the A2 and A1 loops are geometrically/topologically different. Here, we test these hypotheses using magnetic tweezers to detect and characterize loop formation in DNA molecules in which engineered operator sequences and Gal repressor proteins formed either the A1 or A2 antiparallel loop.

\*To whom correspondence should be addressed. Tel: +1 404 727 4930; Fax: +1 404 727 0873; Email: lfinzi@emory.edu

## MATERIALS AND METHODS

### Preparation of DNA

Linear DNA fragments of plasmid pSA580, ~3.6 kb in length, were used as tethers. The original plasmid had been modified to contain properly arranged hybrid operator sites for oriented binding of the GalR<sup>A16T</sup>/GalR<sup>V15T,T322R</sup> heterodimers (6). The arrangement of the hybrid operator sites determined the mutual orientation of the active tetramerization interfaces of the operator-bound GalR heterodimers, allowing only either A1- or A2-type loop formation. The resulting plasmids were linearized by digestion with KpnI and SacI. Two 'tails' were synthesized with biotin or digoxigenin-labeled nucleotides by PCR of the multiple cloning site of a pBS plasmid comprising the restriction sites for KpnI and SacI. After restriction of these labeled tails, they were ligated to the complementary ends of the linear fragment of interest.

### Stretching and twisting single DNA molecules

All looping experiments were performed in the presence of 25 nM GalR and 50 nM HU. One end of a single molecule of DNA was attached to the glass surface of a microscope flow-chamber (previously coated with antidigoxigenin) and a paramagnetic bead 2.4 μm in diameter (DYNAL MyOne beads coated with streptavidin) was attached to the other end. A pair of permanent magnets, above the microscope stage were used to gently attract the tethered, magnetic bead and effectively stretch the DNA with molecular-scale forces (14). Furthermore, rotation of the magnets causes synchronous rotation of the bead to enable twisting of the DNA tether, which does not swivel at either the glass or bead surfaces due to multiple attachments. The extension,  $l = \langle z \rangle$ , of the molecule of the DNA was monitored with an error of ~10 nm with 1 s averaging using 3D, video-rate tracking of the bead (15). The horizontal motion of the bead  $\langle \Delta x^2 \rangle$  allowed the determination of the tension in the molecule via the equipartition theorem:  $F = k_B T / \langle \Delta x^2 \rangle$  with 10% accuracy. Mechanical drift in the data was eliminated using differential tracking of a second bead stuck on the surface.

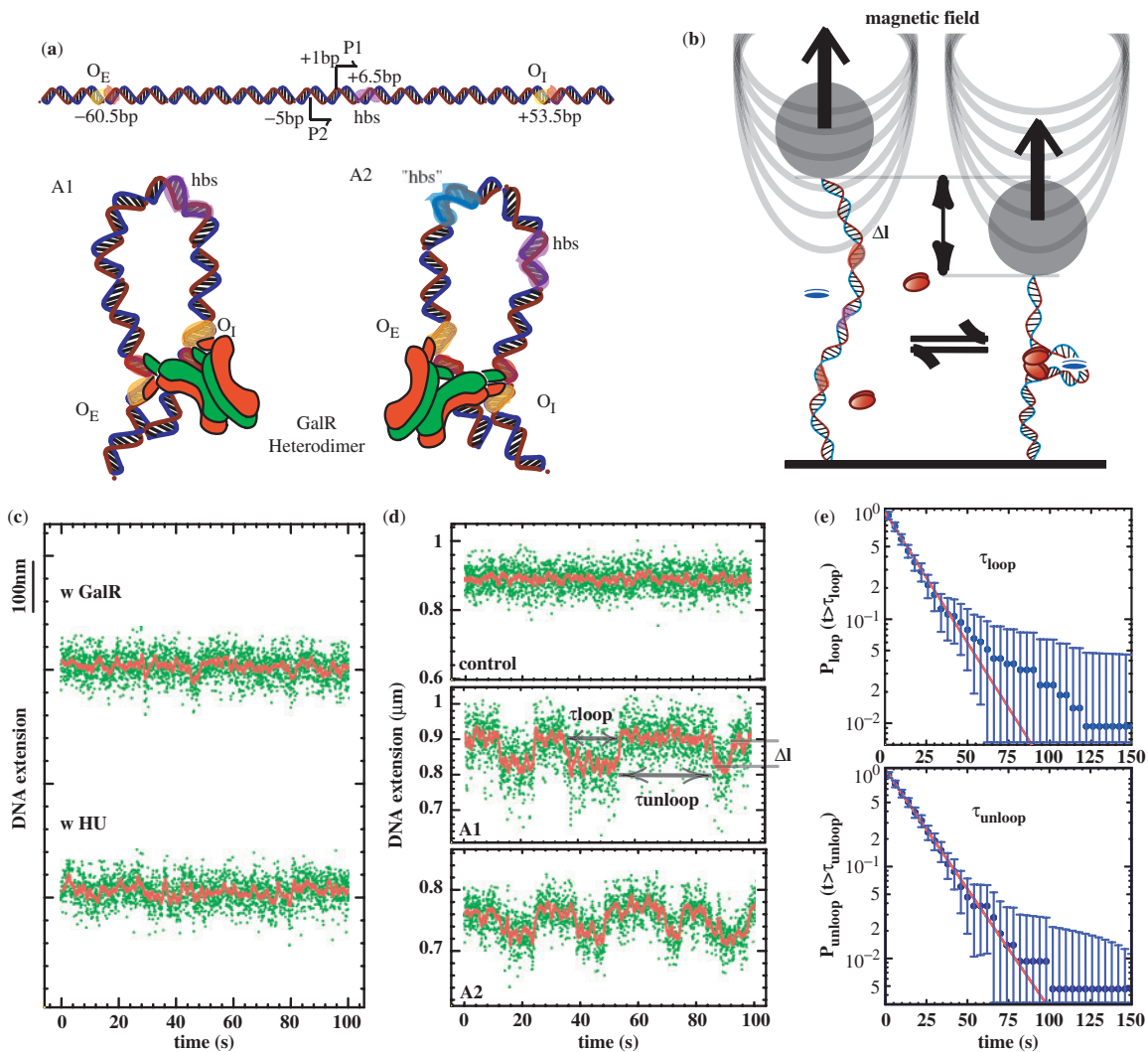
### Loop detection in length versus time data

Data were analyzed as described previously (10). In brief: traces with transitions between longer (unlooped) and shorter (looped) lengths were best fitted to the raw data  $l(t)$  (filtered using a 1 s window) using a sliding Heaviside (step) function:  $l_{\text{step}}(t) = s\theta(t-t_1) + l_1$  defined over a time window of size  $T_{\text{av}}$ . In other words, for every data point,  $t$ , of the data set, the parameters of the step function,  $s$ ,  $t_1$  and  $l_1$ , were fitted such as to minimize the error  $(l(t) - l_{\text{step}}(t))^2$  in the time window  $t_0 < t < t_0 + T_{\text{av}}$ , where only one transition is expected. Finally, the parameters, that consistently scored best ( $\chi^2$ -test), were selected as steps. The time intervals between successive looped and unlooped steps were included in histograms of  $\tau_{\text{unlooped}}$  (or  $\tau_{\text{looped}}$ ) corresponding to the time spent in the longer (or shorter) state.

## RESULTS

In order to characterize differences and similarities between the two antiparallel loops, A1 and A2 illustrated in Figure 1a, we used previously engineered A1 and A2 DNA molecules (4; see also Materials and methods section). These sequences contain hybrid GalR operators formed of half-sites, which determine the orientation of binding by a GalR hybrid (GalR<sup>A16T</sup>/GalR<sup>V15T,T322R</sup> mutant; GalR heterodimer, for brevity). Since this heterodimer also contains only one active surface for tetramerization, the operator-bound GalR heterodimers can only form either A1- or A2-type loops (Figure 1a). This oriented heterodimer loop formation strategy was a modification of the principle of Zhou *et al.* (16–18). Measurements were then performed using magnetic tweezers to stretch and twist a single DNA molecule between a paramagnetic microsphere and the glass surface of a microscope flow-chamber. In this pendulum-like system, fluctuations of the  $x$  or  $y$  positions of the microsphere allowed determination of the tension in the DNA (19). In addition, time-resolved records of the position of the microsphere along the tension axis revealed a telegraphic-like signal with alternating looped (short) and unlooped (long) configurations and their lifetimes (Figure 1d). The GalR or HU protein alone did not induce loop formation (Figure 1c).

We monitored looping mediated by GalR heterodimer or wt GalR and HU proteins in DNA molecules maintained at a constant negative supercoiling of 3% ( $\sigma = -0.03$ ) (10) and constant tension. We repeated these assays in the range of forces between 0.7 and 1.2 pN. Loop formation was undetectable at lower forces due to low signal-to-noise ratios and was prevented by higher tension. Wt GalR can interact with both A1 and A2 DNA with no orientation specificity; in this case, loops with either trajectory can in principle form. The distribution of the dwell times in the looped or the unlooped state was fit with an exponential decay function to determine the average lifetimes,  $\tau_{\text{loop}}$  and  $\tau_{\text{unloop}}$ , at a particular force (Figure 1d). In all cases, the dependence was exponential, and the lifetimes obtained from measurements carried out on A1 and A2 DNA in the presence of HU and heterodimer or wild-type GalR are reported in Table 1. For each protein/DNA combination, increased tension diminished the loop lifetime and increased the unloop lifetime (Table 1 and Figure 2). This was true both for heterodimeric or wild-type Gal repressor, and lifetimes were similar for loops formed in A1 DNA molecules by heterodimeric or wild-type protein (Figure 2). However, we found that the average lifetime of the unlooped configuration in the presence of heterodimeric Gal repressor was shorter for A2 DNA with respect to that measured for A1 DNA molecules (Figure 2a). In addition, the lifetime of loops formed by heterodimeric Gal repressor in A2 and A1 DNA were commensurate at 0.7 pN of tension, but A2 loops endure half as long as A1 loops with 0.9 or higher tension. Extrapolation from the data in Figure 2b indicates that at tensions below 0.7 pN the A2 loops may last longer than the A1 loops. Unfortunately, the small loop was undetectable at tensions lower than 0.7 pN due to the lower signal-to-noise ratio.



**Figure 1.** Loop formation by the GalR and HU proteins on supercoiled DNA. (a, top) Graphic representation of the gal regulatory region. Top, the two promoters, *P1* and *P2*, are flanked by the gal operators, *OE* and *OI*. Using the transcriptional start site of the *P1* promoter as a reference for numbering, the HU-Binding Site (*hbs*) is located downstream of the promoters, at position +6.5 (42) in A1. For the A1 and A2 constructs, functional GalR tetramerization interfaces are marked in green; the inactivated ones are marked red. Arrowheads indicate directions of transcription. (a, bottom) GalR heterodimer-mediated A1 and A2 DNA loops. The major difference between the two trajectories results from the interaction of the operator-bound dimers. As a consequence of the 60° angle between the two dimers in the GalR tetramer, the DNA is thought to be unwound with respect to relaxed DNA in both antiparallel conformations (8). The site for HU binding in the A1 construct is colored pink. The putative site for HU binding “*hbs*” in the A2 construct is colored blue (see Discussion section). (b) Scheme of the experimental set-up. A single DNA molecule containing the GalR and HU-binding sites is anchored at one end to the glass surface and at the other end to a paramagnetic bead. In response to small magnets placed above the sample, the bead can be used to stretch and twist the DNA. Loop formation by GalR (red ovals) and HU (blue oval) reduces the extension by an amount,  $\Delta l$ . (c) Control experiments performed in the presence of only GalR or only HU. (d, top) Typical signal from an A1 DNA molecule in the absence of proteins. This is indistinguishable from that of an A2 DNA molecule. (d, center and bottom) Typical telegraph-like signal observed for A1 or A2 DNA molecules, respectively, at 0.9 pN in the presence of proteins. The green dots are raw data and the red line is the averaged signal (1 s). In all experiments, molecules were unwound by 3% ( $\sigma = -0.03$ ). From the trace, it is possible to measure the transition time ( $\tau_{loop}$  and  $\tau_{unloop}$ ) between the looped and unlooped state, as well the loop size. (e) Cumulative probability distribution of  $\tau_{loop}$  and  $\tau_{unloop}$  for all the transitions observed at 0.9 pN in the A1 DNA (error bars are statistical errors). The distributions are fitted by a single exponential giving a mean lifetime:  $\langle \tau_{loop} \rangle = 17.3 \pm 1.3$  s and  $\langle \tau_{unloop} \rangle = 16.7 \pm 0.9$  s.

Thermodynamic theory can be used to relate the average lifetime of each DNA configuration to the free-energy change involved in the looping reaction at given forces,  $\Delta G_{I,F}$ , according to:

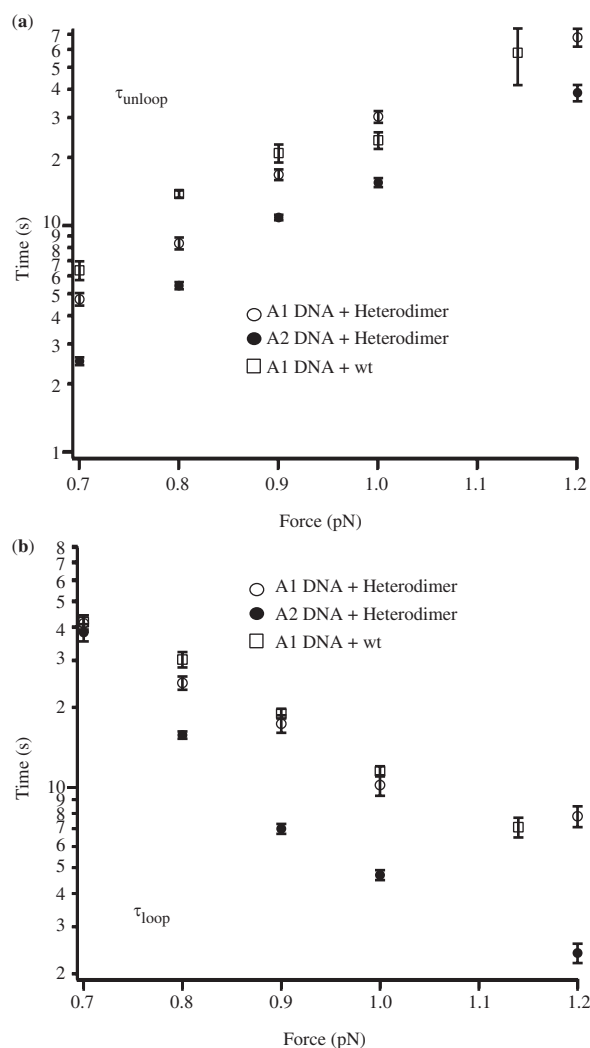
$$\frac{\tau_u(F)}{\tau_l(F)} = e^{\Delta G_{I,F}/k_B T},$$

where  $\tau_l(F)$  and  $\tau_u(F)$  are the average lifetimes for the looped and unlooped configurations at a particular force,  $\Delta G_{I,F}$  is the free-energy difference between the looped and unlooped states (column 6 in Table 1),  $k_B$  is Boltzman’s constant and  $T$  is the temperature. A plot of  $\Delta G_{I,F}$  as a function of force is shown in Figure 3a. The related probability of loop formation  $P_{loop} = t_l/(t_l + t_u)$ ,

**Table 1.** Kinetic and thermodynamic values (mean  $\pm$  SD) measured and calculated respectively from magnetic tweezers assays

Force (pN)	Event number	$\tau_{\text{loop}}$ (S)	$\tau_{\text{unloop}}$ (S)	$\tau_{\text{unloop}}/\tau_{\text{loop}}$	$\Delta G_{I,F}$ ( $k_B T$ )	$\Delta l$ (nm)
<b>A1 DNA heterodimer</b>						
0.7	100	41.1 $\pm$ 2.9	4.7 $\pm$ 0.3	0.1 $\pm$ 0.01	-2.3 $\pm$ 0.1	79.9 $\pm$ 2.5
0.8	152	24.6 $\pm$ 1.4	8.3 $\pm$ 0.5	0.3 $\pm$ 0.03	-1.2 $\pm$ 0.1	78.9 $\pm$ 2.3
0.9	215	17.3 $\pm$ 1.3	16.7 $\pm$ 0.9	1.0 $\pm$ 0.1	0 $\pm$ 0.1	75.0 $\pm$ 1.8
1.0	196	10.2 $\pm$ 0.9	30.1 $\pm$ 1.8	3.0 $\pm$ 0.3	1.1 $\pm$ 0.1	57.9 $\pm$ 1.9
1.2	136	7.8 $\pm$ 0.7	67.5 $\pm$ 6.1	8.6 $\pm$ 1.1	2.2 $\pm$ 0.1	60.1 $\pm$ 2.9
<b>A2 DNA heterodimer</b>						
0.7	165	38.0 $\pm$ 2.8	2.5 $\pm$ 0.1	0.1 $\pm$ 0.01	-2.3 $\pm$ 0.1	72.4 $\pm$ 1.3
0.8	152	15.7 $\pm$ 0.5	5.4 $\pm$ 0.2	0.3 $\pm$ 0.02	-1.2 $\pm$ 0.1	69.3 $\pm$ 1.5
0.9	103	7.0 $\pm$ 0.3	10.8 $\pm$ 0.3	1.5 $\pm$ 0.1	0.4 $\pm$ 0.1	66.6 $\pm$ 1.9
1.0	280	4.7 $\pm$ 0.2	15.4 $\pm$ 0.7	3.3 $\pm$ 0.2	1.2 $\pm$ 0.1	66.1 $\pm$ 1.8
1.2	100	2.4 $\pm$ 0.2	38.4 $\pm$ 3.2	16.0 $\pm$ 1.9	2.8 $\pm$ 0.1	56.2 $\pm$ 2.0
<b>A1 DNA wt</b>						
0.7	183	41.5 $\pm$ 2.5	6.3 $\pm$ 0.6	0.2 $\pm$ 0.02	-1.6 $\pm$ 0.1	81.5 $\pm$ 1.4
0.8	277	30.1 $\pm$ 2.0	13.7 $\pm$ 0.5	0.5 $\pm$ 0.04	-0.7 $\pm$ 0.1	82.0 $\pm$ 1.4
0.9	208	18.9 $\pm$ 0.8	20.8 $\pm$ 1.9	1.1 $\pm$ 0.1	0.1 $\pm$ 0.1	57.4 $\pm$ 0.7
1.0	251	11.5 $\pm$ 0.5	23.7 $\pm$ 2.0	2.1 $\pm$ 0.2	0.7 $\pm$ 0.2	53.8 $\pm$ 1.2
1.1	135	7.1 $\pm$ 0.6	57.7 $\pm$ 16.2	8.1 $\pm$ 2.4	2.1 $\pm$ 0.3	51.0 $\pm$ 1.5

$\tau_{\text{loop}}$  and  $\tau_{\text{unloop}}$  are the average lifetimes for the looped and unlooped configurations, respectively, calculated from the dwell time distributions in each case. From the data, it is possible to directly extract the free energy for the looping reaction at a given force,  $\Delta G_{I,F}$ , using the following equation:  $\Delta G_{I,F} = k_B T \ln(\tau_{\text{unloop}}/\tau_{\text{loop}})$ .  $\Delta l$  is the average change in the DNA length associated with looping.

**Figure 2.** Dependence of the mean lifetime on the externally applied force for the (a) unlooped and (b) looped configuration.

which was calculated from the aggregate time spent in the looped state as a fraction of the total observation, was practically indistinguishable across the range of forces employed as shown in Figure 3b.

The DNA shortening due to loop formation ( $\Delta l$ , in Figure 1c) was observed to change with the applied force (Figure 4). This force dependence can be explained as follows. At lower forces, the additional DNA unwinding introduced by loop formation generates extra compensatory plectonemes outside the loop (20); as a consequence  $\Delta l$  is large (14,19,21). At high forces, such plectonemes do not form and instead negatively supercoiled DNA denatures locally. This local, torque-induced melting absorbs any change in twist (22), due to loop formation, via a change in the amount of denaturation, thereby the overall change in extension,  $\Delta l$ , is closer to the effective loop size.

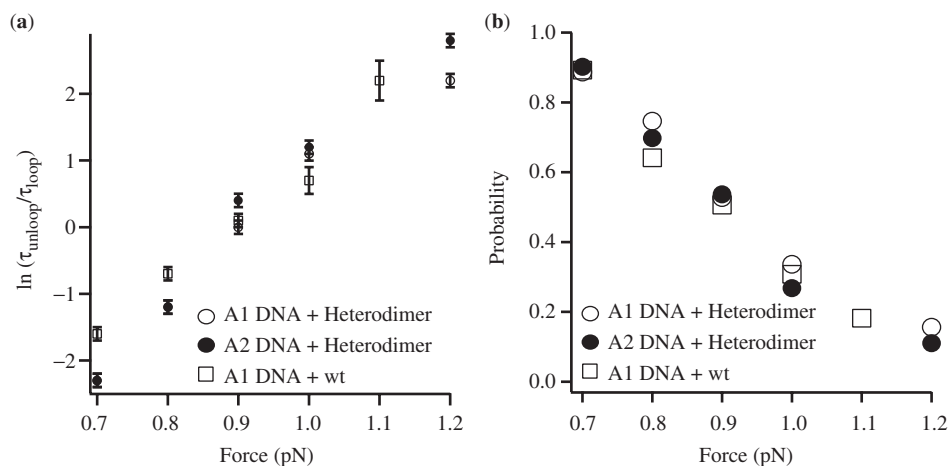
## DISCUSSION

### DNA loop formation

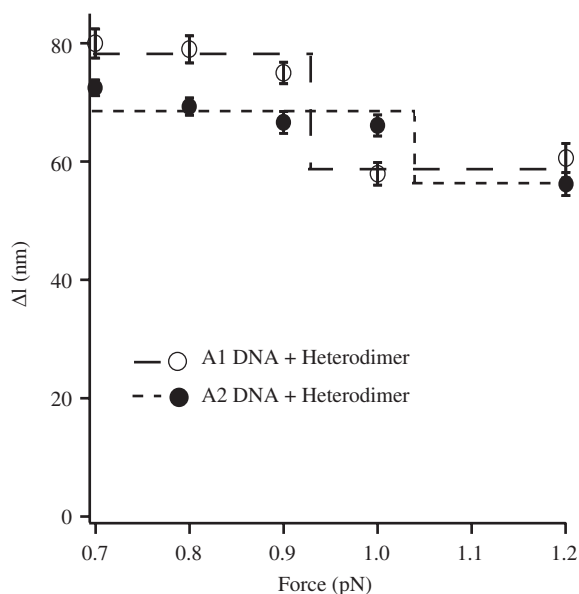
Interactions between proteins bound to well-separated sites on a DNA molecule require bending and twisting deformations in the intervening DNA. Double-stranded DNA is a semi-flexible polymer, with a persistence length of  $\sim 50$  nm ( $\sim 150$  bp) (23–25). DNA segments shorter than the persistence length do not easily bend. However, many DNA ‘transactions’ require formation of short DNA loops (150 bp or less). The feasibility of loop formation over distances shorter than its persistence length depends on the intrinsic shape and flexibility of the DNA sequence, the phasing of the binding sites being juxtaposed, supercoiling of the DNA, in concert with the effect of architectural proteins.

In many systems, protein–DNA and protein–protein interactions can produce loops with different geometries. However, not all these geometries are energetically equivalent and loops with a specific DNA trajectory may be





**Figure 3.** (a) Dependence of the free energy for loop formation,  $\Delta G_{L,F}$ , on the stretching force. (b) Probability of loop formation as a function of force. The probability was calculated as the ratio between the aggregate time spent in the looped configuration and the total observation time.



**Figure 4.** Force dependence of the change in DNA length ( $\Delta l$ ) associated with heterodimer/HU-mediated loop formation of A1 and A2. The dashed/dotted lines show the amplitude of the total change in  $\Delta l$  observed in each case over the force range investigated.

preferred (1,8,26–28). For example, in the case of the Gal repressosome, which contains a 113-bp long DNA loop mediated by two Gal repressor dimers and the HU protein, there are four possible DNA trajectories. Two of these trajectories have antiparallel DNA at the entry/exit points (A1 and A2), while two other trajectories have parallel DNA (P1 and P2) (6,8). The DNA exiting an antiparallel loop must curve under tension and therefore formation of this kind of loop would be expected to cause a larger change in the overall end-to-end distance of the molecule (larger  $\Delta l$ ) than formation of a parallel loop where the exiting DNA is straight. However, parallel loops are generally more strained than antiparallel ones (9). Stereochemical models of GalR/HU-DNA loops

confirm this and also predict that the A1 loop is much more stable than the A2 loop. In addition, they also predicted that, as a consequence of the  $60^\circ$  angle between the two dimers in the GalR tetramer, the DNA is similarly under-twisted in both conformations (8). These structural predictions might be relevant to *in vitro* transcription assays, in which the heterodimer and the HU protein repressed transcription from DNA engineered to form A1 but not A2 configurations. Note, however, that these twist calculations were performed on relaxed DNA.

Instead, the magnetic tweezing assays reported here indicate that the A1 and A2 loops formed with similar energies in DNA, which was unwound by an amount similar to that found in the plasmids used in *in vitro* transcription assays and *in vivo* (Table 1 and Figure 3a). The two loops had nearly equivalent probabilities of formation,  $P_{\text{loop}}$  (Figure 3b), which in all cases studied was about 50% at  $F \sim 0.9$  pN (force at which  $\tau_{\text{loop}} \sim \tau_{\text{unloop}}$ ). As expected, the change in free energy for loop formation,  $\Delta G_{L,F}$ , rose with increasing force (i.e. tension destabilizes the loop in the DNA). Therefore, there is no thermodynamic reason to expect a functional difference in transcriptional repression between A1 and A2 loops. In fact, the only significant difference is that the A2 loop forms and breaks down more frequently than the A1 loop having shorter loop and unloop lifetimes, at least for tensions above 0.7 pN (Figure 2). With the current understanding of repression, it is difficult to relate this observation to RNA polymerase activity.

Of course, *in vitro* transcription assays utilize supercoiled plasmids that are not under so much tension. However, it would not be rigorous to extrapolate the experimental lifetime data to zero force given the necessarily small range of forces investigated, the distance from the zero point, and the logarithmic scale of the y-axis of Figure 2. In addition, loop lifetimes in the absence of force may not be relevant. Evidence is accumulating in the literature (29) that DNA is under tension *in vivo* and several motor enzymes, such as RNA polymerase have been reported to exert large forces on the topologically constrained DNA (30). Furthermore, DNA molecules negatively supercoiled by 6% have a built-in entropic tension of about 0.5 pN (31).

### Alternative DNA trajectories in transcriptional regulation

Thermodynamic stability plays a key role in the function of DNA loops that regulate transcription. Formation of a transient DNA loop may accompany transcriptional activation when a distant, DNA-bound transcription factor directly interacts with RNA polymerase to close the DNA loop. For transient loops, different DNA trajectories might support similar levels of transcriptional activation (32). Instead, DNA loops that repress transcription are generally quite stable. Repressors involved in DNA loop closure can inhibit RNA polymerase action directly (33,34) [e.g. sterically hindering RNA polymerase binding (35–37) or contacting the promoter-bound RNA polymerase to inhibit transcription initiation (38)] or indirectly by reducing the effective torsional flexibility of DNA (39,40) as in the case of loop formation. Loop formation by repressors may be enhanced by accessory DNA-binding proteins that bend the DNA at a critical segment or contribute stabilizing protein–protein interactions. Especially for the transcriptionally repressive cases, thermodynamic characterization of the macromolecular complex permits quantitative prediction of the probability of DNA loop formation (40,41), which might therefore also predict transcription efficiency.

However, we found that *gal* DNA loops with A1 and A2 trajectories form with similar energies and probabilities in the range of forces investigated, when DNA is untwisted to the level found *in vivo* and in *in vitro* transcriptional assays. Therefore, the failure of the A2 loop to repress transcription cannot be explained on the basis of thermodynamics alone. Previously it has been proposed that, failure of the A2 trajectory to repress *in vitro* transcription of naturally supercoiled DNA may result from destabilization of the DNA loop by RNA polymerase (6). Calculations performed on relaxed DNA show that, despite the fact that the A1 and A2 loops have similar overall structure, the direction of local DNA bending is different; the DNA surface that is inside the A1 loop apex is turned halfway outside in the A2 loop. One consequence is that the HU-binding site, which is experimentally observed at position +6.5 in the A1 loop, shifts to –14.5, a structurally equivalent position with respect to the loop apex in the A2 loop [Figure 1a and Figure 6 in (4)]. A structural-instead of a sequence-dependent binding site is consistent with the very high nonspecific binding affinity of HU for DNA. Thus, these calculations suggest that in the A2 trajectory, RNA polymerase may easily transition from closed to open complex, facilitating transcription (40), or evict HU from the –13.5/–14.5 site, which overlaps the –10 promoter element. Our data support this idea. In our experimental conditions, DNA may be already unwound by an amount sufficient to abrogate the energetic difference between the A1 and A2 loop and yet maintain the structural difference between A1 and A2 loops found in (4).

Furthermore, we speculate that, given the similarity between the lifetime and  $\Delta l$  data relative to the interaction between heterodimer and wt GalR and A1, the A1 trajectory is preferred in the wild-type case. This is also to be expected given the similar transcriptional repression by A1 and wt loops but not by A2 loops.

In summary, our data show unequivocally that thermodynamic probabilities of Gal repressor/HU-induced alternate DNA loops failed to quantitatively predict their physiological effect. Therefore, in order to predict transcription modulations due to different DNA loop trajectories, one must carefully consider not only looping probabilities but also how DNA supercoiling affects the double helix topology and how this may impact the interactions of proteins associated with a given trajectory. Single molecule experiments such as those described here are very useful for the characterization of this effect. Furthermore, they emphasize how local tension in the DNA may alter the formation of repressive loops. A better understanding of this and the effect of supercoiling on macromolecular complex formation is emerging from such work.

### ACKNOWLEDGEMENTS

We thank Dr Kim Sneppen at the Niels Bohr Institute for helpful discussion. We also thank our colleagues in our laboratories for their assistance. This work was supported by Human Frontier Science Programme Organization (RGP0050/2002-C to L.F. and S.A.); Intramural Research Program of the National Institutes of Health, National Cancer Institute, Center for Cancer Research (1Z01BC010017 to S.A.); János Bolyai fellowship of the Hungarian Academy of Sciences (to S.S.). Funding to pay the Open Access publication charges for this article was provided by LF's Emory startup.

*Conflict of interest statement.* None declared.

### REFERENCES

- Courey, A.J. and Jia, S.T. (2001) Transcriptional repression: the long and the short of it. *Genes Dev.*, **15**, 2786–2796.
- Kim, T.K., Kim, T.H. and Maniatis, T. (1998) Efficient recruitment of TFIIIB and CBP-RNA polymerase II holoenzyme by an interferon-beta enhanceosome *in vitro*. *Proc. Natl Acad. Sci. USA*, **95**, 12191–12196.
- Maniatis, T., Falvo, J.V., Kim, T.H., Kim, T.K., Lin, C.H., Parekh, B.S. and Wathel, M.G. (1998) Structure and function of the interferon-beta enhanceosome. *Cold Spring Harb Symp Quant Biol.*, **63**, 609–620.
- Semsey, S., Tolstorukov, M.Y., Virnik, K., Zhurkin, V.B. and Adhya, S. (2004) DNA trajectory in the Gal1 repressosome. *Genes Dev.*, **18**, 1898–1907.
- Fisher, A.L. and Caudy, M. (1998) Groucho proteins: transcriptional corepressors for specific subsets of DNA-binding transcription factors in vertebrates and invertebrates. *Genes Dev.*, **12**, 1931–1940.
- Geanakopoulos, M., Vasmatazis, G., Lewis, D.E.A., Roy, S., Lee, B. and Adhya, S. (1999) GalR mutants defective in repressosome formation. *Genes Dev.*, **13**, 1251–1262.
- Semsey, S., Virnik, K. and Adhya, S. (2005) A gamut of loops: meandering DNA. *Trends Biochem. Sci.*, **30**, 334–341.
- Geanakopoulos, M., Vasmatazis, G., Zhurkin, V.B. and Adhya, S. (2001) Gal repressosome contains an antiparallel DNA loop. *Nat. Struct. Biol.*, **8**, 432–436.
- Mehta, R.A. and Kahn, J.D. (1999) Designed hyperstable lac repressor center dot DNA loop topologies suggest alternative loop geometries. *J. Mol. Biol.*, **294**, 67–77.
- Lia, G., Bensimon, D., Croquette, V., Allemand, J.F., Dunlap, D., Lewis, D.E.A., Adhya, S.C. and Finzi, L. (2003) Supercoiling and denaturation in Gal repressor/heat unstable nucleoid protein

- (HU)-mediated DNA looping. *Proc. Natl Acad. Sci. USA*, **100**, 11373–11377.
11. Geanakopoulos, M. and Adhya, S. (2002) Genetic analysis of GalR tetramerization in DNA looping during repressosome assembly. *J. Biol. Chem.*, **277**, 33148–33152.
  12. Semsey, S., Geanakopoulos, M., Lewis, D.E.A. and Adhya, S. (2002) Operator-bound GalR dimers close DNA loops by direct interaction: tetramerization and inducer binding. *EMBO J.*, **21**, 4349–4356.
  13. Virnik, K., Lyubchenko, Y.L., Karymov, M.A., Dahlgren, P., Tolstorukov, M.Y., Semsey, S., Zhurkin, V.B. and Adhya, S. (2003) 'Antiparallel' DNA loop in gal repressosome visualized by atomic force microscopy. *J. Mol. Biol.*, **334**, 53–63.
  14. Strick, T.R., Allemand, J.F., Bensimon, D. and Croquette, V. (2000) Stress-induced structural transitions in DNA and proteins. *Annu. Rev. Biophys. Biomol. Struct.*, **29**, 523–543.
  15. Strick, T.R., Charvin, G., Dekker, N.H., Allemand, J.-F., Bensimon, D. and Croquette, V. (2002) Tracking enzymatic steps of DNA topoisomerases using single-molecule micromanipulation. *C. R. Physique*, **3**, 595–618.
  16. Meibom, K.L., Kallipolitis, B.H., Ebright, R.H. and Valentin-Hansen, P. (2000) Identification of the subunit of cAMP receptor protein (CRP) that functionally interacts with CytR in CRP-CytR-mediated transcriptional repression. *J. Biol. Chem.*, **275**, 11951–11956.
  17. Zhou, Y.H., Busby, S. and Ebright, R.H. (1993) Identification of the functional subunit of a dimeric transcription activator protein by use of oriented heterodimers. *Cell*, **73**, 375–379.
  18. Zhou, Y.H., Pendergrast, P.S., Bell, A., Williams, R., Busby, S. and Ebright, R.H. (1994) The functional subunit of a dimeric transcription activator protein depends on promoter architecture. *EMBO J.*, **13**, 4549–4557.
  19. Strick, T.R., Allemand, J.F., Bensimon, D., Bensimon, A. and Croquette, V. (1996) The elasticity of a single supercoiled DNA molecule. *Science*, **271**, 1835–1837.
  20. Lia, G., Praly, E., Ferreira, H., Stockdale, C., Tse-Dinh, Y.C., Dunlap, D., Croquette, V., Bensimon, D. and Owen-Hughes, T. (2006) Direct observation of DNA distortion by the RSC complex. *Mol. Cell*, **21**, 417–425.
  21. Strick, T.R., Allemand, J.F., Bensimon, D. and Croquette, V. (1998) Behavior of supercoiled DNA. *Biophys. J.*, **74**, 2016–2028.
  22. Strick, T.R., Croquette, V. and Bensimon, D. (1998) Homologous pairing in stretched supercoiled DNA. *Proc. Natl Acad. Sci. USA*, **95**, 10579–10583.
  23. Hagerman, P.J. (1981) Investigation of the flexibility of DNA using transient electric birefringence. *Biopolymers*, **20**, 1503–1535.
  24. Shore, D., Langowski, J. and Baldwin, R.L. (1981) DNA flexibility studied by covalent closure of short fragments into circles. *Proc. Natl Acad. Sci. USA Biol. Sci.*, **78**, 4833–4837.
  25. Smith, S.B., Finzi, L. and Bustamante, C. (1992) Direct mechanical measurements of the elasticity of single DNA-molecules by using magnetic beads. *Science*, **258**, 1122–1126.
  26. Kim, S. and Landy, A. (1992) Lambda-Int protein bridges between higher-order complexes at 2 distant chromosomal loci. *Attr. Science*, **256**, 198–203.
  27. Lavoie, B.D., Shaw, G.S., Millner, A. and Chaconas, G. (1996) Anatomy of a flexer-DNA complex inside a higher-order transposition intermediate. *Cell*, **85**, 761–771.
  28. Nunesduby, S.E., Smithmungo, L.I. and Landy, A. (1995) Single base-pair precision and structural rigidity in a small Ihf-induced DNA loop. *J. Mol. Biol.*, **253**, 228–242.
  29. Blumberg, S., Pennington, M.W. and Meiners, J.C. (2006) Do femto Newton forces affect genetic function? A review. *J. Biol. Phys.*, **32**, 73–95.
  30. Wang, M.D., Schnitzer, M.J., Yin, H., Landick, R., Gelles, J. and Block, S.M. (1998) Force and velocity measured for single molecules of RNA polymerase. *Science*, **282**, 902–907.
  31. Charvin, G., Allemand, J.F., Strick, T.R., Bensimon, D. and Croquette, V. (2004) Twisting DNA: single molecule studies. *Contemp. Phys.*, **45**, 383–403.
  32. Lilja, A.E., Jenssen, J.R. and Kahn, J.D. (2004) Geometric and dynamic requirements for DNA looping, wrapping and unwrapping in the activation of *E. coli* glnAp2 transcription by NtrC. *J. Mol. Biol.*, **342**, 467–478.
  33. Friedman, A.M., Fischmann, T.O. and Steitz, T.A. (1995) Crystal structure of lac repressor core tetramer and its implications for DNA looping. *Science*, **268**, 1721–1727.
  34. Dodd, I.B., Perkins, A.J., Tsemitsidis, D. and Egan, J.B. (2001) Octamerization of lambda CI repressor is needed for effective repression of P(RM) and efficient switching from lysogeny. *Genes Dev.*, **15**, 3013–3022.
  35. Jacob, F. and Monod, J. (1961) Genetic regulatory mechanisms in synthesis of proteins. *J. Mol. Biol.*, **3**, 318–326.
  36. Johnson, A., Meyer, B.J. and Ptashne, M. (1978) Mechanism of action of Cro-protein of bacteriophage-lambda. *Proc. Natl Acad. Sci. USA*, **75**, 1783–1787.
  37. Schlax, P.J., Capp, M.W. and Record, M.T. (1995) Inhibition of transcription initiation by lac repressor. *J. Mol. Biol.*, **245**, 331–350.
  38. Roy, S., Semsey, S., Liu, M.F., Gussin, G.N. and Adhya, S. (2004) GalR represses galP1 by inhibiting the rate-determining open complex formation through RNA polymerase contact: a GalR negative control mutant. *J. Mol. Biol.*, **344**, 609–618.
  39. Choy, H.E. and Adhya, S. (1992) Control of Gal transcription through DNA looping - inhibition of the initial transcribing complex. *Proc. Natl Acad. Sci. USA*, **89**, 11264–11268.
  40. Choy, H.E., Park, S.W., Parrack, P. and Adhya, S. (1995) Transcription regulation by inflexibility of promoter DNA in a looped complex. *Proc. Natl Acad. Sci. USA*, **92**, 7327–7331.
  41. Vilar, J.M.G. and Saiz, L. (2005) DNA looping in gene regulation: from the assembly of macromolecular complexes to the control of transcriptional noise. *Curr. Opin. Genet. Dev.*, **15**, 136–144.
  42. Aki, T. and Adhya, S. (1997) Repressor induced site-specific binding of HU for transcriptional regulation. *EMBO J.*, **16**, 3666–3674.