Published online 29 February 2008 *Nucleic Acids Research, 2008, Vol. 36, No. 8* **2505–2513**
doi:10.1093/nar/gkn071

Single-molecule manipulation reveals supercoiling-dependent modulation of *lac* repressor-mediated DNA looping

Davide Normanno[1,*], Francesco Vanzi[1,2] and Francesco Saverio Pavone[1,3]

[1]LENS, European Laboratory for Non-linear Spectroscopy, Università degli Studi di Firenze, Via N. Carrara 1, I-50019 Sesto Fiorentino (FI), [2]Dipartimento di Biologia Animale e Genetica 'Leo Pardi', Università degli Studi di Firenze, Via Romana 17, I-50125 Firenze (FI) and [3]Dipartimento di Fisica, Università degli Studi di Firenze, Via G. Sansone 1, I-50019 Sesto Fiorentino (FI), Italy

Received November 6, 2007; Revised January 7, 2008; Accepted February 5, 2008

ABSTRACT

Gene expression regulation is a fundamental biological process which deploys specific sets of genomic information depending on physiological or environmental conditions. Several transcription factors (including *lac* repressor, LacI) are present in the cell at very low copy number and increase their local concentration by binding to multiple sites on DNA and looping the intervening sequence. In this work, we employ single-molecule manipulation to experimentally address the role of DNA supercoiling in the dynamics and stability of LacI-mediated DNA looping. We performed measurements over a range of degrees of supercoiling between −0.026 and +0.026, in the absence of axial stretching forces. A supercoiling-dependent modulation of the lifetimes of both the looped and unlooped states was observed. Our experiments also provide evidence for multiple structural conformations of the LacI–DNA complex, depending on torsional constraints. The supercoiling-dependent modulation demonstrated here adds an important element to the model of the *lac* operon. In fact, the complex network of proteins acting on the DNA in a living cell constantly modifies its topological and mechanical properties: our observations demonstrate the possibility of establishing a signaling pathway from factors affecting DNA supercoiling to transcription factors responsible for the regulation of specific sets of genes.

INTRODUCTION

The metabolism of lactose in *E. coli* is subject to one of the most studied gene regulation processes. The seminal work by Jacob and Monod first demonstrated the lactose-dependent mechanism of repression of the genes of the *lac* operon (1). In the operon, the genes coding for proteins involved in lactose permeation and metabolism are clustered under the control of one promoter. In the absence of lactose, the repressor protein (LacI) binds to target sequences (the operators), preventing RNA polymerase from transcribing the operon genes. When available as the main source of energy, lactose is converted into allolactose, which inhibits LacI binding to DNA and switches gene transcription on. The *lac* operon has become a paradigmatic biological feedback machinery, representing, to date, a crucial system for understanding the mechanisms of gene regulation and the response of living organisms to changes in the environment. In the past four decades, a wealth of information has been provided by genetics, biochemistry and structural biology, leading to a detailed picture of the genetic elements of the *lac* operon, the atomic structure of the LacI protein, and the kinetics and thermodynamics of the LacI–DNA interaction (for a recent review see (2)). One fundamental aspect, emerging from several studies, is that the LacI tetramer can bind simultaneously to two operators on the DNA, forming a loop in the intervening sequence (3). The property of forming DNA loops is shared by other regulatory proteins and restriction enzymes (for a review on DNA looping see (4)). Like most other transcription factors, LacI is present in the cell at a very low copy number (about 10 molecules per cell); therefore, the ability of binding to multiple DNA

*To whom correspondence should be addressed. Tel: +34 669 231 321; Fax: +34 93 402 0183; Email: dnormanno@ibec.pcb.ub.es
Present address:
Davide Normanno, IBEC - Institut de Bioenginyeria de Catalunya, Parc Científic de Barcelona, Baldiri Reixac, 15-21 E-08028 Barcelona, Spain

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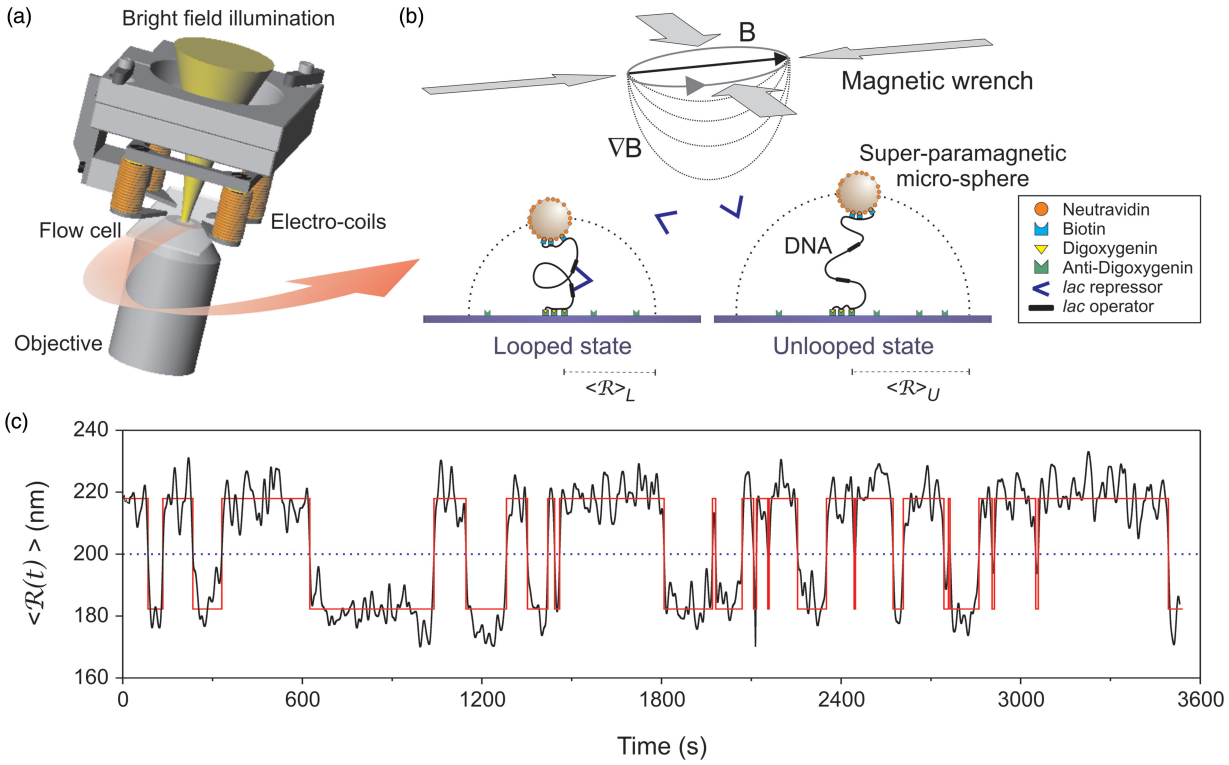

Figure 1. The single-molecule assay. (**a**) Rendering of the magnetic wrench. (**b**) Sketch (not to scale) of the LacI-DNA interaction in TPM. DNA molecules with multiple binding sites and containing two *lac* primary operators (305 bp apart) are employed in the TPM assay (see also 'Materials and Methods' section). (**c**) Experimental recording of the LacI-DNA interactions in the absence of DNA manipulation with 100 pM LacI in solution. The red line shows the two-state trace resulting from half-amplitude threshold analysis. The horizontal dotted line represents the threshold value used.

sites and forming loops is essential for increasing its local concentration at the target sequences and improving its repression efficiency (5–7). Looping of DNA, however, also leads to the possibility that the mechanical properties of DNA may play an essential role in modulating the binding and dissociation of regulatory proteins, such as LacI.

In the last few years an increasing number of theoretical studies have investigated the role of DNA mechanics on DNA–protein interactions (8–10). The importance of protein and DNA flexibility was investigated on LacI, demonstrating the effects of these physical parameters on the kinetics of looping and unlooping *in vitro* (11).

The effects of DNA topology and supercoiling on the binding of structural and regulatory proteins represent a novel concept in genetics and molecular biology, which may lead to an improved understanding of the complexity of gene expression regulatory processes. In normal conditions, *E. coli* DNA has a superhelical density of about −0.025. This value, however, was shown to change in response to environmental conditions (12–15). Thus, DNA supercoiling can play an important role in gene expression regulation (16), provided that the DNA-binding proteins involved in the regulatory processes are sensitive to supercoiling in the physiological range of this physical parameter.

In this work, we employed single-molecule micromanipulation techniques to experimentally investigate the modulation of LacI-induced DNA looping and unlooping kinetics in response to DNA supercoiling. A magnetic wrench was employed to perform experiments in the entropic regime of the DNA and to manipulate the degree of supercoiling of single DNA molecules containing two *lac* operators spaced 305 bp from each other. We demonstrate quantitatively the effects of supercoiling on the LacI–DNA interaction kinetics, as well as on the structural conformation of the LacI–DNA complex.

The experimental assay employed in this work consists in a combination of the Tethered Particle Motion (TPM) technique and magnetic manipulation, as shown in Figure 1a,b. TPM has been previously applied to the measurement of LacI–DNA interaction kinetics (11,17), as well as to other systems (among which are RNA polymerase (18), ribosomes (19), restriction enzymes (20), TATA-box binding protein (21), site-specific recombination (22)). In TPM, a DNA molecule is specifically attached to a glass cover slip at one end and to a micro-sphere at the other end. Measurements of the range of diffusion of the tethered micro-sphere (measured by the observable $\langle R(t) \rangle$, defined as the radius of the diffusion cloud (11)) report on the effective length of the DNA tether, allowing real-time detection of the DNA looping by LacI. Figure 1c shows a typical recording of LacI activity as detectable in a TPM experiment: the system switches between a looped state (corresponding to one LacI molecule simultaneously bound to both operators on the DNA) and an unlooped state, in which one or both

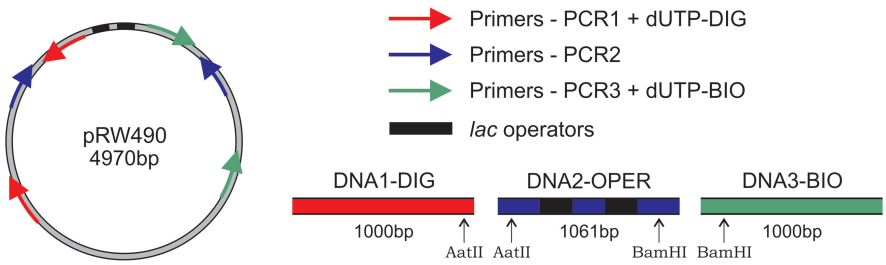

Figure 2. Engineered DNA molecules. The figure describes the construction of the DNA molecules used in the micro-manipulation experiments. Three different PCRs (about 1000 bp each) were performed, using the pRW490 plasmid (24) as template. The primers used in the different PCRs are color coded as shown on the right. Multiple tags are incorporated in PCR1 (digoxigenin) and PCR3 (biotin). On the right the three PCR products are shown with the restrictions sites used for restriction and ligation.

operators on the DNA are free, or occupied by different LacI molecules. The experimental novelty of the system presented in this work consists in employing a custom-made magnetic wrench (23) that allows the manipulation of DNA molecules under very low stretching forces (below 100 fN). The fundamental outcome of this technique is that it enables measurements in the entropic regime of DNA, allowing an experimental characterization of the effects exclusively due to DNA supercoiling and topology, decoupled from axial strain.

MATERIALS AND METHODS

DNA preparation

In order to manipulate DNA supercoiling, the DNA molecule needs to be torsionally constrained via multiple linkages at the attachments with the micro-sphere and with the glass surface. The DNA molecules used in the micro-manipulation experiments were obtained by ligation of three different segments, of approximately 1000 bp each, as shown in Figure 2. Each segment was amplified independently by PCR, using the pRW490 plasmid (24) as template. The two lateral segments of the final construct present multiple binding sites (digoxygenin tags on one segment, biotin tags on the other) in order to allow full torsional constraint. Multiple digoxygenin or biotin tags were introduced by adding, to the standard PCR reagents (Qiagen, Germany), 0.4 mM digoxygenin-11-dUTP (Roche, IN) or biotin-16-dUTP (Roche, IN). The central segment is 1061 bp long and contains two *lac* primary operators, spaced 305 bp from each other. Restriction enzymes (AatII and BamHI, New England Biolabs Inc., MA) were used, as shown in Figure 2, to produce segments with the appropriate sticky ends for ligation. Each segment was purified by agarose gel electrophoresis and gel extraction. Before ligation (performed with T4 DNA Ligase, New England Biolabs Inc., MA), the DNA was heated to 65°C for 20 min. The final construct (3061 bp long) was purified by extraction from electrophoretic gel. Multiple linkages (both on the coverslip-DNA and the microsphere-DNA interfaces) are formed when the geometry of the tags on the DNA and the disposition of the interaction partners on the bead or coverslip surface allow. Heterogeneities in the DNA–surface interaction geometries may lead to variability in the length of the effective tether (defined as the segment between the two innermost surface-immobilized tags). These heterogeneities would lead to a maximum range of $\langle R \rangle$ values between 200 and 350 nm, for the construct shown in Figure 2. We measured more than 100 tethers and found a Gaussian distribution of $\langle R \rangle$ with a mean value of 220 nm and a standard deviation of 30 nm. This corresponds to an average tether length of 1200 bp.

Tether assembly

Tethers were prepared weekly within a ~20 µl volume flow chamber assembled as described previously (11). A solution containing 200 µg/ml anti-Digoxigenin (Roche, IN) in phosphate-buffered saline buffer [2.7 mM KCl, 13.7 mM NaCl, 5.4 mM Na_2HPO_4, 1.8 mM KH_2PO_4 (pH 7.0)] was introduced into the flow chamber and incubated for 30 min to ensure optimal surface coating. The excess antibody was then removed by washing the chamber with ~1 ml of LBB buffer [10 mM Tris-HCl (pH 7.4), 200 mM KCl, 0.1 mM EDTA, 5% (v/v) dimethyl sulfoxide (DMSO), 0.2 mM DTT, 0.1 mg/ml α-casein]. Then, ~100 µl of LBB buffer containing DNA at a concentration of ~20 ng/ml were introduced into the flow chamber and incubated for 1 h. Unbound DNA was removed by washing with ~1 ml of LBB^- buffer (LBB lacking DTT and DMSO). Carboxylated super-paramagnetic micro-spheres (Merck Eurolab/Estapor, France) were prepared weekly by coating with Neutravidin (Pierce, IL), as described in (25), diluted in LBB^- buffer, introduced into the flow chamber and incubated for 1 h. Unbound micro-spheres were finally removed by washing the flow cell with ~1 ml of LBB buffer. The whole procedure was performed at room temperature in a water-saturated container.

Video microscopy and TPM data analysis

The sample was mounted on an inverted microscope (Eclipse TE300, Nikon, Japan). Bright-field time-lapse images of a region of interest containing only the tether under investigation were acquired at video rate (25 Hz, with 1/125 s as exposure time) with a CCD (Hamamatsu, Japan) and digitized with an A/D converter (National Instruments, TX). The software for instrumentation control, data acquisition and real-time processing was written in LabVIEW (v6.0, National Instruments,

TX). The position of the micro-sphere in the sample plane (x–y plane) was monitored in real time during the experiment using a centroid algorithm (19). Each video frame was de-interlaced, since the interlaced image clearly showed the shift in micro-sphere position occurring during the 20 ms elapsing between the acquisition of odd and even lines. Centroid calculation was performed separately, after background subtraction, on the odd and even lines of the image leading to an improved precision in the determination of centroid position. The range of mobility $\langle R(t)\rangle$ was obtained by filtering the 50 Hz x–y de-interlaced centroid data ($R(t)$) with a Gaussian filter with dead time $T_d = 6.75$ s (11,20). Tethers were chosen for recording on the basis of their range of mobility. Dwell-times were measured using a half-amplitude threshold method (26), eliminating all transitions that would give rise to events shorter than T_d (17). When the experimental recording showed a pronounced 'third-state' behavior (see Figure 5), the threshold was placed halfway between L_2 (the looped state with larger $\langle R(t)\rangle$, see below for more details) and U. The measured lifetimes (τ_L and τ_U) were corrected for missed events due to limited time resolution in the filtered data (equations (79) and (80) in (26)); errors were estimated by partial derivative analysis of such equations considering a Poissonian error on the observed lifetimes. Details regarding the choice of window (27) and the independence of the results obtained from the data analysis methods are discussed in Supporting Information S2.

Magnetic manipulation experiments

A custom-made magnetic wrench (23), constituted by four electro-coils (see Figure 1a), was employed to produce a uniform, remotely controllable magnetic field. In the sample plane, the magnetic field intensity applied during experiments was always 48 G. The measurements of looped and unlooped lifetimes at different degrees of supercoiling always began by checking the molecule against a set of selection criteria as follows. As a first check, the tether length and the diffusion cloud of the micro-sphere were probed in order to check the DNA length and to insure that the micro-sphere was tethered through a single DNA molecule (28). Then, the magnetic wrench was switched on and the reduction in tether length was controlled to be less than 10%. Finally, rotations were imposed to the bead to test if the DNA was torsionally constrained (i.e. devoid of single strand nicks). After these tests, LacI was added (always at a concentration of 100 pM in LBB) and the tethered micro-sphere diffusion was monitored in real-time and recorded, as in (11). The first recording (at least 1 h long) on each tether was always performed in the absence of any constraint (magnetic wrench off). These recordings and those performed with wrench on but no supercoiling, were used to measure the dwell-times and assess effects solely due to the magnetic field. As it is shown in Figure 4a, the interaction lifetimes are consistent, within experimental errors, demonstrating no measurable effect on the LacI–DNA looping kinetics due to stretching forces, when the force is below 100 fN (the stretching force exerted by the magnetic wrench during the manipulation experiments was directly measured according to the method introduced by Strick *et al.* (29), see Supporting Information S1 for details). Subsequently, the desired degree of supercoiling σ was imposed on the DNA, by rotating the magnetic field, and it was held by keeping the magnetic wrench switched on with the magnetic field oriented along a fixed axis. Once the torsional constraint was imposed on the system, a 1 h long recording was carried out, before rotating again the magnetic field. Each tether measurement, covering the full range of σ (between -0.026 and 0.026), would require at least 8 h of consecutive recording. In some cases, unspecific sticking of the micro-sphere to the surface would interrupt the measurement (in any case, the solution containing the protein was exchanged every 3–4 h).

RESULTS AND DISCUSSION

Modulation of LacI–DNA interaction lifetimes

The response of the DNA to externally imposed supercoiling in the combined TPM-magnetic manipulation experiment is reported in Figure 3. Figure 3a shows a recording of $\langle R(t)\rangle$ during a variety of manipulations (see figure legend for details). The data show the full reversibility of the topological variations induced in the DNA molecule with the magnetic wrench. Figure 3b shows $\langle R\rangle$ versus σ. In the range of σ explored in the measurements ($-0.026 < \sigma < 0.026$), DNA forms plectonemes (one plectoneme is formed or destroyed for each turn added or removed to the DNA molecule) and its extension decreases linearly for increasing $|\sigma|$ (as shown by the linear fits, red curves in Figure 3b).

The capability of manipulating DNA supercoiling demonstrated in Figure 3 was applied to study the influence of supercoiling on LacI–DNA looping interactions. Measurements of the looped and unlooped lifetimes were performed by dwell-time analysis as described in the Materials and Methods section; the results of the kinetic measurements are shown in Figure 4. Both the looped and unlooped lifetimes (τ_L and τ_U, reporting, respectively, on the kinetics of loop breakdown and formation) display a supercoiling-dependent modulation. Figure 4a shows that, in the absence of supercoiling (zero turns), τ_U (black symbols in Figure 4a) presents a maximum. This is in agreement with the minimum in the repression efficiency for a loop of 305 bp, expected according to the *in vivo* results by Bellomy *et al.* (30). On the other hand, adding or removing half a turn between the operators causes two minima in τ_U. This periodicity of one turn suggests that the observed modulation of τ_U depends on the relative orientation of the two operators with respect to the LacI–DNA binding domains. By changing the degree of supercoiling of the DNA, we induce different relative phases between the operators in the DNA molecule. This resulted in an overall modulation of a factor 2 in τ_U. On the other hand, the looped state lifetime τ_L (red symbols in Figure 4a) exhibits an overall modulation of a factor 3, in the range of σ explored. The looped complex stores bending and torsional mechanical energy, especially for loops with lengths smaller than or comparable to the

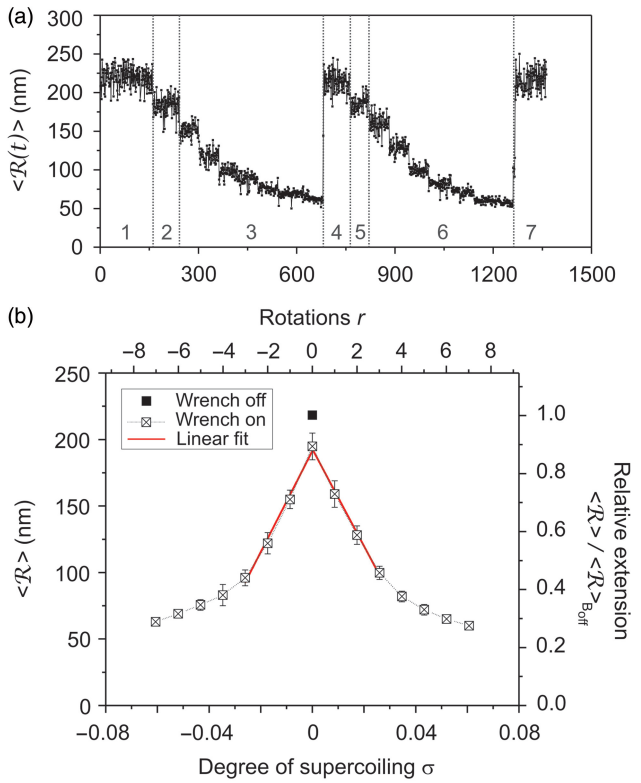

Figure 3. DNA under torsional constraints. (**a**) Raw data of a typical measurement of the tether length $\langle R(t)\rangle$ at different degrees of supercoiling σ. The temporal series of the manipulation is: (1) wrench off, (2) wrench on (with B = 48Gauss), (3) under-winding rotations, in steps of one turn, (4) wrench off, (5) wrench on, (6) over-winding rotations, in steps of one turn, (7) wrench off. (**b**) Tether length $\langle R\rangle$ vs. σ. $\langle R\rangle$ has been obtained by averaging the trace in (a) over all the data acquired for each σ value. $\langle R\rangle$ decreases linearly for values of $|\sigma|$ up to ~0.026, consistently with the formation of one plectoneme per rotation imposed, as expected in the DNA entropic regime (29). The angular coefficient of the linear fits to the data (red curves) are (-32 ± 1) nm/turn for positive values of σ and (33 ± 2) nm/turn for negative values of σ. The reduction due to the stretching force can be estimated from the data by considering the filled square (indicating the value $\langle R\rangle_{\mathrm{Boff}}$ in the absence of magnetic field) and the value corresponding to σ = 0, which is the tether length when the magnetic wrench is on (B = 48 Gauss). In this case $\langle R\rangle_{\sigma=0}/\langle R\rangle_{\mathrm{Boff}} = 0.92$, thus the magnetic field induces a reduction of $\langle R\rangle$ of the order of 10%. The upper scale in the graph reports the number of rotations r added or removed to the DNA molecule; the relationship between r and σ is: $\sigma = r \cdot h/L_C$, where h = 10.4 bp is the DNA helical repeat *in vitro*, and L_C = 1200 bp is the contour length of the DNA (see 'Materials and Methods' section). The right scale of the graph shows the relative extension of the tether length, calculated as $\langle R\rangle/\langle R\rangle_{\mathrm{Boff}}$.

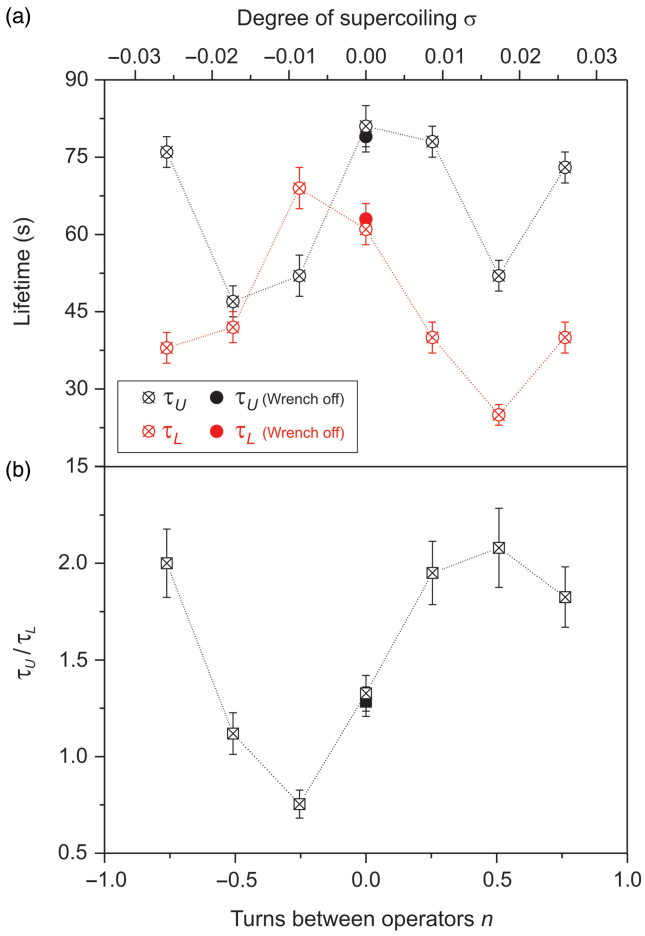

Figure 4. LacI-DNA interaction kinetics in the presence of supercoiling. (**a**) The figure reports the lifetimes of looped state (τ_L, red circles) and unlooped state (τ_U, black circles) at different degrees of supercoiling σ (top horizontal scale), converted in the bottom scale in terms of turns between operators n (conversion supposes supercoiling uniformly distributed across the DNA and n = σ 305 bp/10.4 bp, where 10.4 bp is the DNA helical repeat *in vitro*). The looped and unlooped lifetime values in the complete absence of manipulation (magnetic wrench off) are represented by filled circles and, within experimental errors, are consistent with the values obtained in the presence of the force exerted during the manipulation. (**b**) τ_U/τ_L ratio calculated from the data in (a).

DNA persistence lengths (about 150 bp for bending and 300 bp for twisting). Previous TPM experiments demonstrated that, in the absence of applied torque, the measured rate of loop breakdown corresponds to twice the rate of dissociation of LacI from a single operator (11,17). This observation suggests that loop bending strain does not significantly affect the rate of loop breakdown for a 305 bp loop in a DNA molecule not subject to external torque. When external torque is applied, supercoiling does affect the rate of loop breakdown as demonstrated by the data in Figure 4a. If DNA supercoiling acted on the system solely by modulating the energy barrier of the looping/unlooping reaction, one would expect τ_L and τ_U to display modulations with the same shape. However, τ_L does not display the symmetry observed for the unlooped lifetime with respect to positive and negative supercoiling. The asymmetry in the τ_L data indicates that supercoiling also modulates the partition of the looped state between different conformations, presumably characterized by different energies. Multiple looped conformations have indeed been observed in our TPM experiments as discussed below.

The full biochemical scheme underlying TPM measurements of LacI-induced DNA looping was previously discussed in detail (11). Briefly, the unlooped state (as observed in TPM) corresponds to a manifold of possible biochemical states and the rate of the looping reaction itself is governed by the LacI association rate constant for DNA operator (k_a) and by an effective concentration

of the free operator commonly referred to as J_M (31). Since supercoiling affects neither the equilibrium between different unlooped biochemical states (in fact, DNA mechanics has no role in those states) nor the value of k_a, the modulation observed in our measurements for τ_U reflects directly a modulation of J_M.

The overall influence of DNA supercoiling on the thermodynamic equilibrium between the unlooped and looped state can be robustly measured by the ratio of the measured lifetimes. Figure 4b shows the τ_U/τ_L ratio as a function of DNA supercoiling, demonstrating a clear sinusoidal modulation. This ratio is insensitive to the methods chosen for data analysis (see Supporting Information S2) and demonstrates that, in the experimental conditions tested *in vitro*, a variation of σ between -0.015 and $+0.015$ is associated with a smooth modulation of the looping propensity of the system by a factor of 3. A quantitative measurement of the equilibrium constant of the intramolecular looping reaction (K^*, see (24)) as a function of σ is shown and discussed in the Supporting Information S2.

Previous biochemical studies on LacI have demonstrated that supercoiling has an effect on the dissociation of repressor–operator complexes (32,33) and on the optimal interoperator distance for looping (34). Performing measurements only at negative supercoiling and with different pairs of primary and auxiliary operators of the *lac* operon (O_1O_3 or O_1O_2, instead of O_1O_1 as in our work), Eismann and Müller-Hill (33) found an oscillation of the LacI–DNA complex's half-life which is similar to our results in the same range of σ values. With respect to gel shift assays, our method has the advantage of allowing direct manipulation of the DNA molecule and, also, distinguishing looped and unlooped lifetimes, rather than measuring the resulting equilibrium dynamics (reported by the complex half-life, on a time scale of minutes).

The influence of DNA mechanics (particularly bending energy and torque) on the dynamics of looping by regulatory proteins introduces a fundamental concept in transcriptional regulation; allowing the DNA molecule to mediate modulation effects beyond its more traditionally accepted role of a passive substrate for binding and dissociation of transcription factors and RNA polymerase. That repression indeed depends on geometrical parameters (such as the distance between operators) has been shown for the *lac* operon *in vivo* (30,35). The results reported in Figure 4a provide the first experimental demonstration of a modulation of the kinetics of *lac* repressor looping and unlooping of DNA in a supercoiling-dependent fashion. Figure 4b summarizes the thermodynamic sensitivity of LacI–DNA looping to DNA supercoiling, thus demonstrating the possibility of this system to respond to changes in the DNA superhelical density. Furthermore, this modulation might be amplified *in vivo* by accessory proteins capable of varying the DNA bending and twisting persistence lengths. The effects observed in our measurements can be directly attributed to DNA supercoiling, since denaturation bubbles (that would affect the DNA bending flexibility) do not form in our regime of forces ($F < 0.1$ pN, (36)). These considerations indicate that the bending flexibility of the DNA is not the only player in the looping/unlooping process. In fact, looping/unlooping kinetics and complex stability are significantly affected also by DNA supercoiling. Recently, analogous conclusions have been proposed on theoretical grounds by Purohit and Nelson (9).

A direct observation of the importance of supercoiling in the formation of a protein–DNA complex at the single molecule level was first obtained on the Gal repressor (GalR) system, for which it was shown that GalR produces DNA looping only at negative supercoiling and in the presence of the accessory protein HU (37). However, this behavior might be related to the stretching force ($\sim$1 pN) applied to the DNA during those experiments. In this work we report the first micromanipulation experiments on DNA–protein interactions in the entropic regime of DNA. Theoretical studies have demonstrated the possibility of profound effects of DNA tension on the dynamics of the looping process (10,38); our experimental approach minimizes the tension applied, offering the opportunity of a direct measurement of the effects due exclusively to torque and DNA supercoiling.

In vivo experiments (35) have shown that repression efficiency is modulated on a length scale corresponding to the helical repeat of DNA. This modulation is determined by the variation of the relative phase between the *lac* operators in the DNA molecule (similarly to what was observed for the cyclization of short DNA segments (39,40)). In fact, the operators on the DNA must be appropriately oriented with respect to the DNA-binding sites on the LacI tetramer in order to form the repression loop. When the operators' orientation is varied by changing the spacing between the operators (i.e. the length of the intervening DNA segment), an alternation of maxima and minima in the repression efficiency can be observed (30,35,41). In the cell, the distances between operators are defined by the structure of the operon (42,43); however, the repression modulation due to operator phasing can be introduced by DNA supercoiling, as demonstrated by our results *in vitro*. Thus, in live cells, DNA mechanics (for example stresses and/or torsional constraints) and the action of accessory proteins (44,45) are likely essential players in mechanisms of modulation of gene expression regulation.

Modulation of loop geometry

The crystal structure of the LacI tetramer bound to short operator DNA fragments displays a V-shaped configuration (46). Nevertheless, alternative LacI–DNA looped complex configurations have been proposed (47,48), inferred from experimental observations (49–52) or suggested by numerical results (53–55). Structurally, it is plausible that the tetramerization domain represents a flexible hinge, suitable for drastic rotations of the two dimers relative to each other. It is, thus, possible that, in response to a specific interoperator geometry/sequence (50) or to an external strain (55), the LacI molecule may adopt a conformation different than the classical V-shape. Our experiments indeed confirmed this possibility. In fact, a fraction of the tethers observed during our experiments displayed three, rather than two, distinct levels of

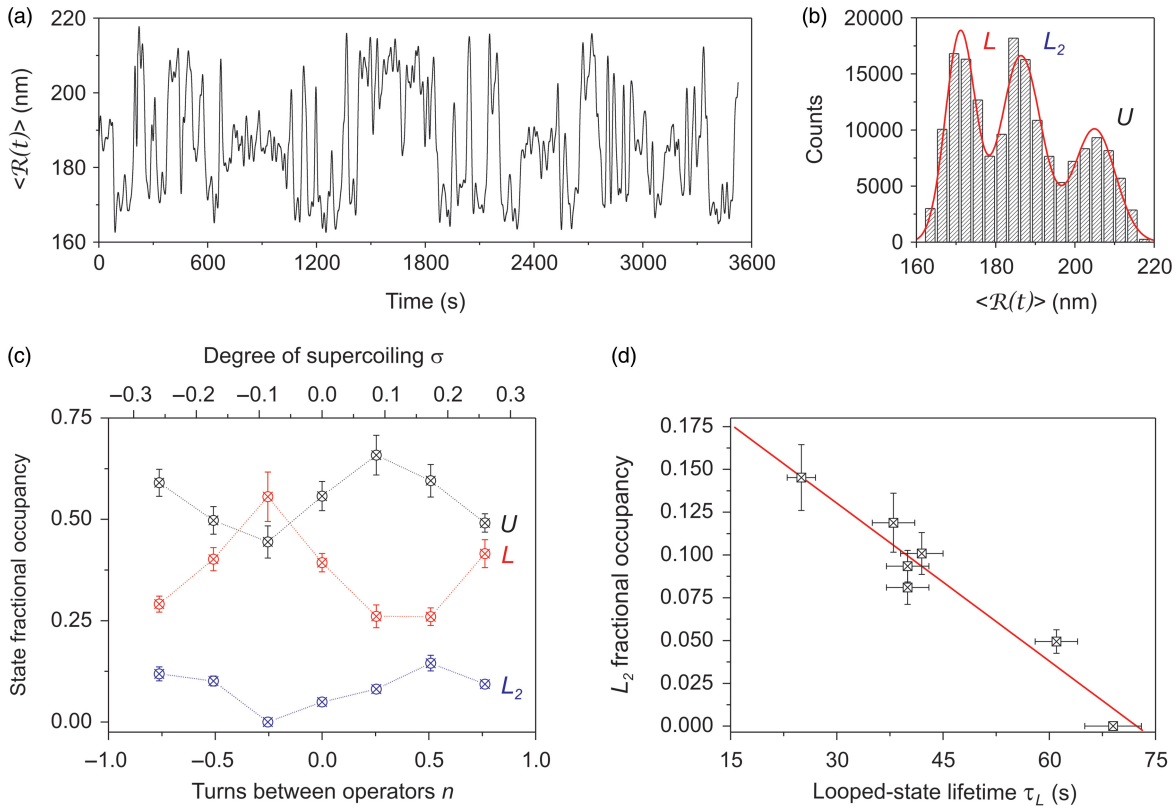

Figure 5. 'Third-state' in the LacI-DNA interaction. (**a**) Example of TPM experimental recording showing the 'three-state' behavior. The experimental recording was acquired with the magnetic wrench off. (**b**) Distribution of the $\langle R(t) \rangle$ signal reported in (a). The red line represents the best fit of three Gaussians to the data. (**c**) The state fractional occupancy is reported as a function of the DNA supercoiling for the unlooped state (U, black symbols), the looped state (L, red symbols), and the second looped state or 'third state' (L_2, blue symbols). The curves represent the (normalized) area under each peak for the whole data set (see Table I in Supplementary Data). (**d**) As indicated by the linear fit (red line), the fractional occupancy of the 'third-state' L_2 and the looped state lifetime τ_L result anti-correlated (slope = $(-3.1 \pm 0.3) \times 10^{-3} s^{-1}$, the correlation coefficient c has a value of -0.95, corresponding to a not-correlation probability $P_\gamma(|c| > 0.95) = 0.006$).

micro-sphere mobility (see Supplementary Table I for statistics). An example of experimental recording showing the three-state behavior is reported in Figure 5a,b. A possible explanation for the three-state behavior is the presence of an alternative configuration of the loop (L_2). Considering the limited spatial resolution of TPM measurements (~5 nm), such an alternative conformation should be characterized by a drastic structural rearrangement of the looped complex. If LacI was to assume a fully extended structure (i.e. with the two dimers forming an angle of 180° between each other) the distance between the DNA-binding sites would increase by about 10 nm with respect to that in the V-shaped loop. Our TPM data show a separation of about 10–15 nm between the two looped states (see Figure 5b), thus supporting the existence of two distinct looped conformations presumably corresponding to the LacI closed V-shaped (L) and linear extended (L_2) conformations (48,49).

The 'three-state' behavior has been observed in our experiments only when the torsionally constrained DNA (with multiple binding sites) was employed to form the tethers. Tethers with the same inter-operator distance, but with single binding sites, do not display the 'three-state' behavior (11,17). This observation indicates that topological constraints on the DNA (due to multiple linkages) enhance in some cases the probability of occurrence of L_2. The DNA construct shown in Figure 2 establishes extensive interactions with the surface of two massive objects (i.e. the micro-sphere and the glass cover slip). Multiple tags are randomly distributed in the DNA lateral segments and the tether assembly is governed by diffusion. Thus, the initial orientation of the micro-sphere magnetic dipole in the x–y plane has a tether-to-tether variability that spans the range between -0.5 and $+0.5$ turns, as demonstrated in Supplementary Figure S3d. In terms of inter-operator phasing, this corresponds to ± one-eighth of a turn. As shown in Figure 5c, the occupancy of the L_2 state critically depends on the supercoiling, reaching zero on the side of negative supercoiling. Thus, the tether-to-tether variability described above could easily explain the observation of the state L_2 only in a fraction of the tethers. On the other hand, the results in Figure 3 show a well detectable dependence on supercoiling, demonstrating that tether-to-tether variability is small compared to the amplitude of the observed phenomenon.

The TPM technique was already applied to the study of a 'three-state' behavior in the LacI–DNA interaction as a function of inter-operator distances: comparing constructs with operators at 153 bp and 158 bp distances (and with single binding sites at the ends) it was observed that a third

state in the micro-sphere mobility appeared only in the latter case (56). Therefore, the loop conformations are drastically affected by a change of half a turn in the relative phase between the operators. As discussed above, the manipulation imposed on the DNA molecules in our experiments can be assimilated to a change in the relative phase between the operators. A modulation in the partition of LacI–DNA loops between the different conformations is shown in Figure 5c, as a function of the degree of supercoiling. This modulation displays a negative correlation with the looped state lifetime τ_L (see Figure 5d). This suggests that the two loop conformations are characterized by significantly different lifetimes and when the loop conformation with longer lifetime (presumably the V-shaped loop) is energetically preferred (leading to a reduction of population of the third state, L_2), a larger τ_L is measured. On the contrary, when strains affect the V-shape loop stability, the L_2 loop is more likely to be formed, the 'three-state' behavior appears, and a lower value of τ_L is measured.

In conclusion, our single molecule work demonstrated a well-defined modulation of the looping and unlooping dynamics due to DNA supercoiling both in the negative and positive range. The thermodynamic equilibrium of the intramolecular looping reaction is similarly modulated by supercoiling, as demonstrated in Figure 4b. These results show that DNA supercoiling can indeed represent a powerful message relaying global effects of environmental changes onto specific responses of regulatory systems, provided that those systems are sensitive to supercoiling. Our work indicates that the *lac* operon could, in fact, be sensitive to small changes in the superhelical density of DNA *in vivo*. Finally, the structural geometry of the looped LacI–DNA complex itself is significantly affected by the mechanical strain present in the DNA molecule and can exhibit at least two distinct conformations.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

We thank Dr Marco Capitanio (LENS) for useful discussions and reading of the manuscript and Prof. Kathleen S. Matthews (Rice University) for stimulating discussion. D.N. acknowledges Prof. Maria Garcia-Parajo (IBEC) for support during the final part of manuscript preparation. This work was supported by the European Union Contracts RII3-CT-2003-506350, MTKD-CT 2004-509761. Funding to pay the Open Access publication charge was provided by MTKD-CT 2004-509761.

Conflict of interest statement. None declared.

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