Single-Molecule DNA Nanomanipulation: Detection of Promoter-Unwinding Events by RNA Polymerase

By A. Revyakin, J.-F. Allemand, V. Croquette, R. H. Ebright, and T. R. Strick

This article describes a nanomanipulation technique that makes it possible to mechanically and quantitatively stretch and supercoil a single linear DNA molecule. We show how this technique can be extended to the study of protein–DNA interactions that lead to DNA untwisting, particularly to the study of promoter unwinding by RNA polymerase during the initiation of transcription.

In our system, a linear 4-kb DNA molecule containing a single promoter is anchored at one end to a treated glass surface and at the other end to a small (1/22 m) magnetic bead (Fig. 1A). Using a magnetic manipulator, the bead is pulled on and rotated, resulting in supercoiling of the DNA (one negative supercoil per each clockwise rotation; one positive supercoil for each counterclockwise rotation). The three-dimensional position of the bead is determined in real time using videomicroscopy and software analysis, yielding the DNA end-to-end extension, l. Changes in l as a function of force, F, and supercoiling, n, are calibrated. The system is used to observe, in real time, protein–DNA interactions that affect supercoiling. For example, when RNA polymerase is introduced and allowed to bind to and unwind promoter DNA, the corresponding changes in DNA supercoiling and end-to-end extension can be observed by monitoring the bead position in real time.

The system is prepared as follows. One end of the 4-kb DNA fragment containing a single promoter is ligated to a 1-kb multiply biotin-labeled DNA fragment, and the other end is ligated to a 1-kb multiply digoxigenin-labeled DNA fragment. Reaction of the resulting DNA fragment with a streptavidin-coated magnetic bead results in attachment of the DNA fragment to the bead, through multiple linkages, to the biotin-labeled end. Deposition of the resulting bead-attached DNA fragment onto an

Fig. 1. Experimental system. (A) DNA is tethered between a magnetic bead and a glass surface as described in the text. Magnets located above the sample impose a vertical stretching force that can be changed by increasing or decreasing the distance between the magnets and the bead. Rotating the magnets causes the bead to rotate in a synchronous manner, supercoiling the tethered DNA, with each clockwise rotation introducing one negative supercoil and each counterclockwise rotation introducing one positive supercoil. The bead can be viewed under the microscope, and image analysis is used to determine the position of the bead above the surface and hence the extension of DNA, \( l \). In an appropriate range of supercoiling, each rotation of the bead results in the introduction or removal of one plectonemic supercoil (plectoneme or loop; three plectonemes are shown) and a corresponding change in DNA extension, \( \Delta l_{cal} \approx 60 \text{ nm} \) (because the contour length of a plectoneme is \( \approx 60 \text{ nm} \), but its contribution to vertical extension of the DNA is 0 nm). (B) Calibration curve showing DNA extension \( l \) vs rotation in RNA polymerase buffer (see text) at 34°C for \( F = 0.3 \text{ pN} \). Rotation \( n \) is the number of turns applied to the 4-kb (1.3 \( \mu \text{m} \)) DNA via the magnets and the bead. The degree of supercoiling, \( \sigma = n/Lk_0 \), is the number of rotations of the bead, \( n \), divided by the natural linking number, \( Lk_0 = N/h \), where \( N \) is the number of base pairs and \( h \) is the number of base pairs per helical turn of the DNA (\( h = 10.4 \)). As the DNA is progressively over- or underwound at this low force, its extension drops regularly as additional plectonemes are formed. The dataset is collected at constant force, \( F \), by rotating the magnets while their height above the sample is kept constant. In these conditions, each additional rotation (i.e., plectonemic supercoil) causes the extension to decrease by about 60 nm. As the force increases, this contraction rate drops according to \( F^{0.4} \), lowering ionic strength causes an increase in \( \Delta l_{cal} \). (C) Force–extension data obtained on a single 4-kb DNA molecule at \( \sigma = 0 \). The solid line is a fit to the worm-like chain model of DNA elasticity (see text), giving a persistence length \( \xi \approx 50 \text{ nm} \) and a crystallographic length of about 1.3 \( \mu \text{m} \).
antidigoxigenin-coated glass surface results in attachment to the surface, through multiple linkages, at the digoxigenin-labeled end. Multiple linkages between the DNA fragment and the bead and the DNA fragment and the surface torsionally constrain the DNA with respect to the bead and surface and thereby couple supercoiling of the DNA to rotation of the bead relative to the surface.

Application of a magnetic field gradient above the surface makes it possible to pull on the bead, lifting the bead from the surface and stretching the DNA. The gradient is generated by a pair of magnets separated by a small gap (Fig. 2). Translating the magnets toward or away from the glass surface causes the force to increase or decrease, respectively. The stretching force can be measured by analyzing the Brownian motion of the bead and the end-to-end extension of the DNA, and forces from 10 fN to 100 pN can be applied and measured using this technique.

Rotating the bead by rotating the magnetic field mechanically and quantitatively twists the DNA. Control over the angular displacement of the bead is achieved by the magnetic field mentioned earlier. The bead becomes magnetized in a fixed orientation, causing it to behave effectively as a compass needle. Rotating the magnetic field causes the bead to rotate in a completely synchronous fashion. Each clockwise turn of the magnets unwinds the DNA by one turn, and each counterclockwise turn overwinds the DNA by one turn. A user-selected number of turns can be applied to the DNA in a fully controlled, fully reversible fashion.

In an appropriate range of supercoiling, each rotation of the bead causes a large, \(~60\text{-nm}\) change in DNA end-to-end extension (see Fig. 1B), as it introduces or removes one plectonemic supercoil (which has a contour length of \(~60\text{ nm}\) but which contributes \(~0\text{ nm}\) to DNA end-to-end extension \(l\)). Systematically rotating the bead and monitoring the extension yields a calibration curve that relates extension to supercoiling (Fig. 1B). Thereafter, supercoiling can be determined simply by measuring extension and referring to the calibration curve. Continuous monitoring of extension gives real-time information on changes in DNA supercoiling, and thus makes possible real-time measurements of the topological effects of protein–DNA interactions.\(^3\)

For a constant linking number, as is the case with this system when the magnets are not rotating, any change in DNA twist, \(T_w\), must produce a corresponding change in DNA writhe, \(W_r\) (represented by plectonemic supercoils), according to the equation \(\Delta T_w = -\Delta W_r\). We reasoned that

Fig. 2. Experimental setup. A treated glass capillary tube (1 × 1 × 50 mm, Vitrocom) is mounted on an inverted microscope (Olympus) and connected at its ends to buffer reservoirs. DNA is tethered at one end to the “floor” of the capillary and at the other to a micrometer-sized magnetic bead (Dynal or New England Biolabs, not shown to scale). The microscope is equipped with a high-NA 60 or 100× oil immersion lens (Olympus). The capillary and the objective are temperature controlled to within 0.1°C using Peltier modules (Marlowe Electronics) and a temperature controller module (Wavelength Electronics, not shown). The fine focus of the objective is controlled by mounting it on a piezoelectric element (Physik Instrumente) operating in closed loop mode and driven with a PC-based interface. A pair of neodymium–iron–boron magnets (1 × 1 × 0.5 cm, Magnet Sales) is mounted north–south on motorized translation and rotation stages that are piloted using a PC-based controller (Physik Instrumente). A CCD camera (Jai CVM30) connected to the microscope relays images of the magnetic bead at video rates to the PC via a frame grabber (PCVision, Coreco Imaging). The computer uses prototype image processing routines (XVIN®) to extract from this video the 3D position of the bead (to within about 10 nm). The vertical (z) position of the bead above the surface is determined by analysis of bead diffraction rings, which increase regularly in size as the bead moves away from the focal plane of the objective. The lateral (x and y) position of the bead is determined using two-dimensional particle tracking algorithms. From the position of the magnetic bead above the surface, the DNA end-to-end extension, $l$, is measured. Measurement of the bead mean-squared fluctuations perpendicular to the direction of stretching, $< \delta x^2 >$, yields the stretching force, $F = k_B T l / < \delta x^2 >$, where $k_B$ is Boltzmann’s constant and $T$ is the absolute temperature.

C. Gosse and V. Croquette, Biophys J. 82, 3314 (2002).
promoter unwinding by RNA polymerase (or any other protein–DNA interaction that causes a local change in DNA twist) should be observable using this system. Similar to traditional topological assays on supercoiled DNA, the approach exploits the fact that a change in twist upon promoter unwinding will result in a corresponding change in writhe (plectonemic supercoils). In this system the change in writhe is detected as a change in DNA end-to-end extension (Fig. 4).

**Experimental Setup**

A sketch and description of the experimental setup are provided Fig. 2. For the study of interactions between RNA polymerase and supercoiled DNA, this configuration has several advantages.

First, the magnetic manipulator is a user-friendly and robust tool for nanomanipulation experiments. The main magnets, made of high-grade neodymium–iron–boron and spaced by ~1 mm, generate a magnetic field gradient that drops off exponentially over distances on the millimeter scale. As a result, the force imposed on the bead is not significantly affected by either changes in the DNA extension (no greater than 1 μm) or the surface position (due, for instance, to thermal drift).

Second, beads of different diameters can be employed to give the system different force and noise characteristics. With 1-μm-diameter magnetic beads, forces of about 1–2 pN and 1-s timescales—an important range in the study of protein–DNA interactions—can be accessed. Forces of up to about 100 pN can be reached using 4.5-μm-diameter beads, but for pN-scale forces, these beads do not achieve the temporal resolution of the smaller ones.

Third, reversible rotation of the bead, and corresponding supercoiling of the tethered DNA, is accomplished easily by rotating the magnets.

**Methods and Protocols**

For these DNA nanomanipulation experiments, a 4-kb linear DNA is labeled at one end (on both strands) with digoxigenin groups and at the other end (again on both strands) with biotin groups. Linear 4-kb DNA molecules are generated from a GC-rich DNA template by polymerase chain reaction (PCR). The template contains a unique promoter site of interest. One end of the 4-kb molecule is ligated to a DNA fragment labeled with biotin groups and the other end to a DNA fragment labeled with digoxigenin groups. The DNA is then attached to streptavidin-coated magnetic beads. This bead–DNA construct is then introduced into a glass capillary tube that has been functionalized with antidigoxigenin and to which the bead–DNA constructs bind at the remaining free DNA end.
The mechanical properties of the DNA are calibrated. This calibration is then used to measure RNA polymerase–DNA interactions via changes in overall DNA supercoiling.

Preparation of flow cell

In order to absorb antidigoxigenin to the glass surface, the surface must first be made hydrophobic. A simple procedure involving plasticization is presented, followed by an alternative procedure involving silanization. We then present procedures for functionalization and blocking of the resulting hydrophobic surface and assembly of the surface into a flow cell.

Preparing a Hydrophobic Surface by Plasticization

**Materials**

- Square glass capillary, $1 \times 1 \times 50$ mm (Vitrocom)
- Nitric acid (Sigma)
- Polystyrene, $M_w \approx 280,000$, 0.1% (w/v) in toluene (Sigma)
- Tygon tubing R3603, i.d. 0.0402 in., o.d. 0.1082 in. (Kimberly-Clarke)
- Argon

1. Capillary tubes are cleaned in a nitric acid bath for 4 h and then rinsed extensively with water and dried with a stream of clean argon.
2. One end of the capillary is dipped into the polystyrene solution so as to draw in about 15 μl.
3. The capillary is slowly tipped at one end and then the other so as to coat the entire inner surface with the polystyrene solution. This should be done no more than two or three times, as a thick polystyrene layer will be more unstable than a thin one.
4. Excess solution is wicked out of the capillary, which then is dried with a stream of clean argon.
5. The ends of the capillary are fitted with a 2-cm length of Tygon tubing.

Preparing a Hydrophobic Surface by Silanization

**Materials**

- Square glass capillary (as described earlier)
- Phenyltrimethoxysilane (United Chemical)
- Ethanol, anhydrous (Pharmco)
- Acetic acid, pure (glacial) (Fisher)

1. Capillary tubes are cleaned in nitric acid and dried as described earlier.
2. A 95% ethanol solution in $H_2O$ is adjusted to pH 5.0 with acetic acid.
3. Silane is added to the 95% ethanol solution to a final concentration of 2% and incubated with stirring for 5 min at room temperature.
4. Capillaries are incubated overnight at room temperature in the solution under gentle agitation.
5. Capillaries are rinsed briefly with ethanol and cured at 100°C for 30 min. The ends of the capillary are then fitted with Tygon tubing.

**Functionalization and Blocking of Hydrophobic Surface**

**Materials**
- Surface-modified capillary (see earlier description)
- Phosphate-buffered saline (PBS; Fluke)
- Polyclonal antidigoxigenin (Roche)
- Bovine serum albumin fraction V (BSA; Roche); stock is 50 mg/ml in H2O
- Polyglutamic acid, $M_w$ 1500–3000 (Sigma); stock is 10 mg/ml in PBS
- Standard buffer (SB; 10 mM potassium–phosphate buffer, pH 8.0, 0.1 mg/ml BSA, 0.1% Tween 20)
- NaN3 (Sigma)

1. One hundred microliters of PBS containing 0.1 mg/ml polyclonal antidigoxigenin is injected into the capillary, which is then placed in a humid chamber and left to incubate overnight at 37°C.
2. A blocking solution of SB containing 10 mg/ml BSA, 3.3 mg/ml of polyglutamic acid, and 3 mM sodium azide is injected into the capillary, which is then placed in a humid chamber and left to incubate for at least 2 days at 37°C. (The resulting blocked capillary can be stored at 4°C for up to 2 weeks.)

**Assembly of Capillary into Flow Cell**

**Materials**
- Blocked surface-modified capillary (see earlier description)
- Syringe pump (Fisher)
- Microscope system (see Fig. 2)
- Thermally regulated capillary holder (10 × 10-cm aluminum plate with 1.2 × 50-mm slot and attached Pelletier modules)
- PBS
- 3-μm-diameter tosylactivated polymer beads (Bangs Laboratories)

1. To use a surface for experiments, the capillary is rinsed gently twice with 1 ml PBS to remove the blocking solution, inserted into a thermally regulated capillary holder, and connected to buffer-exchange reservoirs at each end (500-μl plastic wells, one connected to a syringe pump and the
other to a waste reservoir). The holder is attached to the microscope stage, and the microscope is focused on the “floor” of the capillary.

2. One hundred microliters of a 1:1000 dilution of 3-μm tosylactivated polymer beads in PBS is injected into the capillary. The beads are left to settle and bind to the surface for 30 min, or until they cease to move, at room temperature. (The beads later serve as references to correct for surface drift.)

3. A gentle (0.1–0.3 ml/min; typically 20 min) flow of PBS is established to rinse out unbound reference beads. (Each field of view as observed through the CCD camera should contain three to five beads.)

4. One to 2 ml of buffer SB is flowed (0.2–0.3 ml/min, typically 10 min) through the capillary.

Preparation of DNA Constructs for Nanomanipulation

Preparation of DNA Fragment

As mentioned previously, the DNA fragment under study should be ~4 kb in length, corresponding to an end-to-end extension under stretching of up to ~1.3 μm. (For longer DNA fragments, real-time measurements of extension show higher noise relative to expected signal.) The DNA fragment should contain a centrally located promoter and should otherwise be G/C-rich (to avoid nonspecific melting of A/T-rich regions). For this construct, the rrnB P1 promoter was amplified from plasmid pTZ19rrnBP1 (S. Nechaev and K. Severinov, unpublished results) using the add-on PCR primers GAGAGAGGTACCGGTTGAATGTTGCGCGGTCAG and GAGAGAGGTACCGTTGTTCCGTGTCAGTGGTG. The resulting 94-bp DNA fragment (spanning positions −78 to +16, where +1 is the transcription start site) was then cloned into the unique KpnI site of the Thermus aquaticus rpoC gene (located at position 2889 downstream of the rpoC ORF), yielding plasmid pARTaqRpoC/rrnBP1. The same procedure can be used to prepare constructs for the study of other promoter sequences.

Materials

Add-on PCR primer “MluI”: gagagaacgctgacctctccacagcg (10 μM)
Add-on PCR primer “NolI”: gagagagcggcgcgactcgetctgcgtcagct (10 μM)
pARTaqRPOC/rrnBP1 plasmid, 1:200 dilution of DNA miniprep Dimethyl sulfoxide (DMSO; Sigma) dNTP mix, 2.5 mM each (Roche)

Thermostable DNA polymerase, high fidelity, 3 U/µl, and 10× PCR buffer (Roche)
*Mlu*I and *Not*I restriction enzymes (New England Biolabs)
Qiaquick gel extraction kit (Qiagen)
Spin column, S400HR (Amersham)
Thermal cycler
Lyophilizer

PCR solutions and temperature cycles are set up as follows.

<table>
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<tr>
<th>Ingredient</th>
<th>Volume (µl)</th>
<th>Step</th>
<th>Temperature (°)</th>
<th>Time (min)</th>
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<td>0.5</td>
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<td>2</td>
<td>68</td>
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<td>94</td>
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<tr>
<td>H₂O</td>
<td>34</td>
<td>7</td>
<td>68</td>
<td>7</td>
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1. Prepare five tubes of the aforementioned mixture (250 µl total).
2. Place in thermal cycler and incubate for 5 min at 95°.
3. To each tube add 0.5 µl of DNA polymerase and pipette up and down. (Tubes should be left in the thermal cycler at 95° during this step.)
4. Run the PCR program shown above.
5. Purify the 4-kb PCR product on 0.7% agarose gel containing 0.5 µg/ml ethidium bromide and extract the 4-kb product. Typical yields are on the order of 2 µg (~1 pmol).
6. Digest product with restriction enzymes *Mlu*I and *Not*I per instructions of the manufacturer for 1 h at 37°. Heat inactivate for 20 min at 65°.
7. Purify product using the spin column as per instructions of the manufacturer.
8. Adjust the concentration of the product to 50 nM.

*Preparation of 1-kb Labeled DNA Fragments*

Two 1-kb DNA fragments—one labeled with dUTP-biotin and the other labeled with dUTP-digoxigenin—are generated by PCR using the same template. One primer for the biotin–labeling PCR incorporates a *Not*I restriction site for subsequent cleavage and ligation with the 4-kb
DNA fragment. One primer for the digoxigenin-labeling PCR incorporates a *Mlu*I restriction site for subsequent cleavage and ligation with the 4-kb DNA fragment.

**Materials**

Add-on PCR primer "*Mlu*I", 10 μM
Add-on PCR primer "*Not*I", 10 μM
Primer "TaqRpoC-1" TCCTGGCGCAGGTAGATGAG, 10 μM
Primer "TaqRpoC-2" CTGATGCAAAAGCCCTCGGG, 10 μM
Plasmid pARTaqRpoC/rrnBP1, 1:200 dilution of miniprep DNA (see earlier description)

Thermostable
Thermostable DNA polymerase, high fidelity, 3U/μl, and 10× PCR buffer (Roche)
DMSO (Sigma)
dNTP mix, 2.5 mM each (Roche)
Biotin-16-dUTP, 1 mM (Roche)
Digoxigenin-11-dUTP, alkali stable, 1 mM (Roche)
Qiaquick PCR cleanup kit (Qiagen)
Spin column S400HR (Amersham)
Thermal cycler
Lyophilizer

PCR volumes and temperature cycles are as follows.

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<tr>
<th>Ingredient</th>
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<th>Dig labeling</th>
<th>Step</th>
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<th>Time (min)</th>
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<td>1</td>
<td>58</td>
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<td>dNTPs</td>
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<tr>
<td>H₂O</td>
<td>34</td>
<td>34</td>
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</tr>
</tbody>
</table>

1. Prepare five tubes of 50 μl of the aforementioned mixture (250 μl total per labeling reaction).
2. Place in thermal cycler and incubate for 5 min at 95°C.
3. To each tube, add 0.5 µl of DNA polymerase and pipette up and down. (Tubes should be left in the thermal cycler at 95°C during this step.)
4. Run the PCR program shown above.
5. Purify the 1-kb PCR products on 0.7% agarose gel containing 0.5 µg/ml ethidium bromide and extract the 1-kb product. Typical yields are ~2 µg (1 pmol).
6. Digest purified, biotin-labeled DNA with NotI for 1 h at 37°C as per manufacturer’s instructions. Heat inactivate enzyme for 20 min at 65°C. Digest purified, digoxigenin-labeled DNA with MluI for 1 h at 37°C as per manufacturer’s instructions. Heat inactivate enzyme for 20 min at 65°C.
7. Purify product using the spin column as per instructions of the manufacturer.
8. Adjust the concentration of the product to 200 nM.

**Ligation of 4-kb DNA Fragment to 1-kb-Labeled DNA Fragments**

**Materials**

- Purified DNA fragments (see earlier description)
- T4 DNA ligase, 10 U/µl and 10× reaction buffer (New England Biolabs)
- EDTA (Sigma)
- Tris–HCl, pH 8 (Sigma)

<table>
<thead>
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<th>Ingredient</th>
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</tr>
<tr>
<td>1 kb Dig-labeled fragment (200 nM)</td>
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</tr>
<tr>
<td>1 kb Biotin-labeled fragment (200 nM)</td>
<td>2.5</td>
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<tr>
<td>10× T4 DNA ligase buffer</td>
<td>2</td>
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<tr>
<td>H₂O</td>
<td>8</td>
</tr>
<tr>
<td>T4 DNA ligase (10 U/µl)</td>
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</tbody>
</table>

1. Mix items listed and incubate for 3 h at room temperature.
2. Inactivate ligase by the addition of EDTA to 10 mM followed by a 10-min incubation at 65°C.
3. Dispense into 5-µl aliquots and store at −20°C.
4. For binding to magnetic beads, the DNA construct is diluted to ~50 pM with 10 mM Tris, pH 8.0, 10 mM EDTA. Dilute solution can be stored at 4°C for 3–6 months before the proportion of DNA
molecules observed to be supercoiled under the microscope decays by about half.

Anchoring of DNA to Beads

**Materials**

SB (see above)
PBS (see above)
BSA (see above)
1-μm-diameter magnetic beads, 10 mg/ml (New England Biolabs or Dynal) dig- and bio-labelled 4-kb.
DNA fragment (see above)

1. Wash 10 μl of 1-μm magnetic beads in 200 μl PBS supplemented with 1 mg/ml BSA.
2. Resuspend beads in 10 μl PBS supplemented with 1 mg/ml BSA.
3. Deposit a 0.5-μl drop of DNA fragment (50 pM) at the bottom of a small microfuge tube.
4. Load a wide-bore pipette tip with 90 μl of SB.
5. Deposit the 10 μl of beads onto the drop of DNA.
6. Immediately dilute the reaction with the 90 μl SB. This should be done by gently depositing the SB onto the bead + DNA solution.
7. Resuspend the beads to homogeneity by tipping the tube upside down (without causing the liquid to drop) or spinning the tube between thumb and forefinger.

Anchoring of DNA to Flow Cell

**Materials**

Flow cell (see above)
DNA fragment tethered to beads (see above)
SB (see above)

1. Before injecting the bead-DNA mixture into the flow cell, the magnets should be moved at least 2 cm away from the flow cell.
2. Inject 15 μl of the bead-DNA mixture into one of the plastic reservoirs connected to the capillary tube.
3. Inject another 20-50 μl of SB into the reservoir to ensure that the majority of beads enter the capillary and are distributed evenly along the length of the capillary.
4. Allow magnetic beads to sediment and incubate for ~15 min at room temperature. Magnetic beads should be seen to move about on the surface and not appear immobile. If beads appear immobile, reblock surface for
1–2 h by incubating with BSA and polyglutamic acid as described. [Other possible ways of reducing nonspecific interactions include exposing the surface for 5–10 min to a 1 M solution of NaN₃ or a 10% solution of sodium dodecyl sulfate, the surface can also be exposed to a 10-mg/ml solution of polyglutamic acid in PBS.]

5. Establish a gentle flow (150–200 μl/min, typically 20 min) of SB to remove unbound beads from the surface. (Buffer is injected into the input reservoir using a motorized syringe pump.) The output reservoir is drained by gravity feed. Approximately every 2 min a rod with a small (2-mm diameter) magnet at the end is slowly passed just over the capillary to lift unbound beads off the surface and into the flow field.)

6. Turn off flow and move magnets as close as possible to the sample without coming into contact, causing DNA molecules to extend away from the surface. (Forces range from ~0.5 to 1 pN, depending on the bead.)

Rapid Selection of a Single Supercoilable DNA

At forces between 0.5 and 1 pN, a magnetic bead tethered by a single 4-kb DNA molecule hovers ~1 μm above the surface and displays rapid, constrained Brownian motion. Beads tethered by a single supercoilable molecule can be identified rapidly by observing the behavior of beads as the magnets are rotated. (Changes in the vertical position can be judged by eye by focusing the image slightly above the bead; the bead will appear to grow larger as it moves away from the focal plane toward the surface.)

The magnets first are rotated 15–20 turns counterclockwise (in the direction of positive supercoiling). For a bead associated with a single supercoilable DNA molecule, the DNA molecule will form ~15 plectonemic supercoils, and the bead will be observed to move toward the surface. After returning to the initial position, the magnets are then rotated 15–20 turns clockwise (in the direction of negative supercoiling). For a bead associated with a single supercoilable DNA molecule, the bead will not be observed to move toward the surface upon rotation 15–20 turns clockwise, because under such levels of negative supercoiling DNA denatures and extends. (If the bead does descend in this case, it is probably tethered to the surface by two DNA molecules.)

Calibration of Bead Image, Torsional Zero of DNA, and Force

*Calibration of Bead Image*

The bead is tracked in x, y, and z at 30 Hz to a resolution of ~10 nm using the software package XVIN, which contains image treatment routines. A reference bead attached to the surface is tracked simultaneously
to correct for surface drift. Parallel illumination of the sample permits observation of diffraction rings around the beads, thus allowing for measurement of the z position.

The x and y positions of a bead from one frame to the next are determined by correlating the bead profile with its mirror image obtained by symmetry about the position of the bead at the previous frame (see Fig. 3). To measure the z position of the bead, an initial calibration of its diffraction ring pattern as a function of distance from the objective must be performed. Then, when the objective position is fixed, changes in the ring pattern of the bead can be converted into vertical displacements of the bead relative to the focal plane.

To perform this calibration, the DNA is first extended by increasing the force to \( \sim 1 \) pN. (This reduces the bead’s Brownian motion.) The image is then focused just above the bead, and the objective is stepped away (i.e., focusing deeper into the sample) in 0.3-\( \mu m \), \( \sim 1 \)-s increments. At each step, the radial intensity profile of the diffraction rings is measured and added to a calibration file. The process is then repeated on one of the tosylactivated polymer “reference” beads immobilized on the surface during preparation of the flow cell. The magnetic bead of interest is then made to recoil to the surface (by rotating the magnets \( \sim 25 \) turns counterclockwise and moving

![Fig. 3. Determination of vertical (z) displacement of bead via calibration of bead diffraction rings. (A) Images of a 1-\( \mu m \) bead obtained at different focus positions. Diffraction rings formed around the bead grow as the image is focused farther above the bead. [The value above each image represents the position (in \( \mu m \)) of the piezoelectric objective driver at which the image was taken.] (B) Calibration file consisting of a “stack” of bead images, where for each focus position (y axis, 0.3-\( \mu m \) increments) the intensity profile of the bead (gray scale) has been averaged over 360° and plotted (x axis) as a function of distance from the bead center. This calibration of the ring pattern as a function of focus position is then used to convert fluctuations in apparent ring size into vertical (z) displacement.](image-url)
the magnets ~1.5 mm from the sample), and the magnetic bead of interest and the immobilized reference bead are tracked simultaneously by comparing their diffraction patterns to the respective calibration files. This provides a reference for the position of the magnetic bead of interest when the DNA end-to-end extension is zero. Furthermore, tracking of both the bead of interest and the reference bead during experiments makes it possible to measure the position of the magnetic bead while correcting for thermal drift of the surface.

*Determining the Rotational Zero of the DNA*

The DNA end-to-end extension is measured as a function of supercoiling at a low stretching force ($F < 0.3$ pN) in the standard buffer (see Fig. 1B). The extension of DNA is greatest when the molecule is torsionally relaxed ($\sigma = 0$). The magnet rotation is adjusted so as to satisfy this condition before proceeding to the force calibration. This position is the “rotational zero” of the molecule.

In the regime where DNA extension changes linearly with rotation, an extra rotation of the bead generates an additional plectonemic supercoil (loop) along the DNA and its extension decreases by about 80 nm per turn (for experiments performed in the standard buffer and at forces of about 0.1 pN) (Fig. 1B). Calibration data must be acquired whenever the environmental conditions (ionic strength, temperature, or force) are changed. (Increasing ionic strength reduces the slope of the extension vs supercoiling curves, as the radius of plectonemic loops decreases when DNA charge is screened out.\(^4\) Increasing temperature decreases the natural linking number of the DNA, as the helical pitch increases with temperature.\(^10\) In our hands, the rotational zero of the molecule moves to lower $n$ by ~0.01 degrees/\(^\circ\)/bp (T.R.S., unpublished results). In addition, increasing force reduces the per-turn contraction rate of DNA.\(^4,7\))

*Force Calibration*

The force acting on the magnetic bead is measured by determining DNA end-to-end extension, $l$, and also the mean-square Brownian fluctuations of the bead perpendicular to the direction of stretching, $< \delta x^2 >$. These parameters are related to the stretching force, $F$, as $F = k_B T l / < \delta x^2 >$, where $k_B$ is Boltzmann’s constant and $T$ is absolute temperature.

The applied force and resulting DNA extension (see Fig. 1C) are measured for different positions of the magnets above the sample. The DNA must be torsionally relaxed during these measurements, and points should

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be taken in the 0.05 to 1 pN range. Fitting force–extension data to the worm-like chain model of polymer elasticity\textsuperscript{11,12} gives a persistence length of about 53 nm and a crystallographic length of about 1.3 \( \mu \text{m} \). A numerical solution is provided\textsuperscript{12}:

\[ F = \frac{k_B T}{\xi} \left( \frac{1}{4(1 - l/l_0)^2} - \frac{1}{4} + \frac{1}{l_0} + \sum_{i=2}^{7} a_i \left( \frac{l}{l_0} \right)^i \right) \]  

(1)

where \( \xi \) is the persistence length (\( \xi = 53 \) nm for DNA), \( l_0 \) is the crystallographic length (\( l_0 = 1.3 \mu \text{m} \) for 4 kb DNA) and \( a_2 = -0.5164228 \), \( a_3 = -2.737418 \), \( a_4 = 16.07497 \), \( a_5 = -38.87607 \), \( a_6 = 39.49944 \), \( a_7 = -14.17718 \).

**Force Calibrations Based on Stretching Transitions in Supercoiled DNA**

The following procedure yields an estimate of the stretching force exerted by the magnetic field gradient:

1. Rotate magnets clockwise \( \sim 25 \) turns (in the direction of negative supercoiling) and move magnets \( \sim 1-2 \) mm away from sample, allowing bead to recoil to surface.

2. Increase the force progressively by moving magnets closer to the sample (in \( \sim 0.2 \)-mm increments) until an abrupt increase in DNA extension occurs. The force at which this occurs, \( F_{-c} \), is 0.3 pN in SB at room temperature.\textsuperscript{4} (The abrupt increase in extension that occurs for negatively supercoiled DNA at \( F_{-c} \) is due to the formation of denatured regions in the DNA\textsuperscript{13} and the concomitant disappearance of plectonemic supercoils with negative topology.)

A similar procedure also holds for positively supercoiled DNA:

1. Rotate magnets counterclockwise \( \sim 25 \) turns (in the direction of positive supercoiling) and move magnets \( \sim 1-2 \) mm away from sample, allowing bead to recoil to surface.

2. Increase the force progressively by moving magnets closer to the sample (in \( \sim 0.2 \)-mm increments) until an abrupt increase in DNA extension occurs. The force at which this occurs, \( F_{+c} \), is 3 pN in SB at room temperature.\textsuperscript{4} (The abrupt increase in extension that occurs for negatively supercoiled DNA at \( F_{+c} \) is due to the formation of hypertwisted domains in

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the DNA\textsuperscript{2} and the concomitant disappearance of plectonemic supercoils with positive topology.\)

These transitions at limiting forces $F_{c}^{-}$ and $F_{c}^{+}$ can serve to estimate the force exerted on the bead for a given distance between the magnets and the sample. Increasing the salt concentration delays the onset of these secondary structural transitions and increases the limiting forces; thus in SB supplemented with 150 mM NaCl $F_{c}^{-} = 1$ pN.\textsuperscript{4}

It is important to keep in mind that when a negatively supercoiled and structurally homogeneous DNA is required (i.e., with no alternative DNA structures, such as denaturation bubbles, and forks), it must not be stretched by forces greater than $F_{c}^{-}$.

Application to the Study of Promoter Unwinding

Single-molecule techniques make it possible to observe in real time the interactions between a single protein and a single DNA molecule.\textsuperscript{3,7,14,15} This provides unique information on the catalytic step size of the enzyme, rate-limiting steps in the cycle, and the influence of torsion and force on different steps of the enzymatic cycle. Unwinding of a promoter site by RNA polymerase should affect the overall topology of the DNA molecule bearing the promoter\textsuperscript{5}; this may be detected in real time in a single-molecule assay using supercoiled DNA.

Detecting Promoter Unwinding

\textit{Materials}

\textit{Escherichia coli} RNA polymerase holoenzyme (Epicentre)

RNA polymerase reaction buffer (25 mM HEPES–NaOH, pH 7.9, 100 mM NaCl, 10 mM MgCl\textsubscript{2}, 3 mM 2-mercaptoethanol, and 200 \mu g/ml BSA)

Flow cell and calibrated DNA (see earlier description)

1. Rinse flow cell with RNA polymerase reaction buffer (0.1–0.3 ml/min, 3 ml total).
2. Set temperature to 34°C and DNA stretching force to $F \sim 0.3$ pN.
3. Calibrate DNA extension as a function of supercoiling as described earlier. In these conditions, changing the DNA rotation by one turn causes a change in DNA extension $\Delta l_{\text{calc}} \sim 60$ nm.


4. Rotate magnets to supercoil DNA by the desired amount (typically the DNA is supercoiled by seven turns to give $|\sigma| = 0.018$); direction of rotation depends on the sign of supercoiling one wishes to study.
5. Begin monitoring DNA extension by tracking bead position.
6. Rinse flow cell with $3 \times 100 \mu l$ of reaction buffer containing the desired concentration of RNA polymerase holoenzyme (typically $\sim nM$). After these preliminary washes, add another 200 $\mu l$ of reaction buffer containing RNA polymerase holoenzyme at the same concentration to the flow cell.
7. Terminate flow and verify that the DNA extension returns to position prior to injection (to ensure that the flow due to injection has not affected the system).
8. Monitor DNA extension as a function of time to observe RNA polymerase/DNA interactions.

Spatial Analysis of Unwinding Signal

Because promoter unwinding disrupts approximately one turn of the double helix (14 bp) in experiments using negatively supercoiled DNA, a single promoter unwinding event should result in the loss of approximately one plectonemic supercoil and a $\Delta l_{obs} \sim 60$-nm increase in the distance between the bead and the surface (Fig. 4A). Reversal of promoter unwinding should be accompanied by reappearance of the plectonemic supercoil and a return of the bead to its initial position. Analogously, in experiments with positively supercoiled DNA, a single reversible promoter unwinding event should result in the transient gain of approximately one plectonemic supercoil and a $\sim 60$-nm decrease in end-to-end extension (Fig. 4B). The loss and appearance of the plectonemic supercoil amplify the signal, as a unit change in local DNA twist is converted into a large ($\sim 60$ nm) change in overall DNA extension. It is important to point out that, for the forces considered here, the contribution of the bubble to extension is essentially zero.

Temporal Analysis of Unwinding Signal

Temporal analysis of the unwinding signals may also be performed on such time traces (Fig. 4). One can measure the time interval $t_{closed}$ between consecutive promoter unwinding events (related to the rate of formation of

(i.e., the lifetime of the unwound complex). Statistical analysis of the distribution of both intervals and lifetimes of unwound complexes can provide information on the kinetics of the reaction pathway. The dependence of lifetime distributions on DNA supercoiling, protein concentration, temperature, nucleotides, effectors, and activators (such as CAP protein) should then allow for a better understanding of the mechanisms involved in promoter unwinding by bacterial RNA polymerase.

**Signal-to-Noise Analysis**

We now consider the temporal and spatial resolution afforded by this system.

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Thermal agitation of the magnetic bead generates noise of amplitude $\delta z$ in the real-time measurement of the DNA extension. This noise precludes measurements on the subsecond scale. However, this noise can be reduced to yield a robust signal-to-noise ratio (signal of $\sim 60$ nm and noise of $\sim 15$ nm give a signal-to-noise ratio of $\sim 4$) by averaging the real-time signal over a $\sim 1$-s timescale.

The rationale for the aforementioned conclusion is as follows: fluctuations (noise), $\delta z$, in bead $z$ position are related to the random thermal (Langevin) force, $F_L$, acting on the bead and the stiffness, $k_z$, of plectonemically supercoiled DNA: $\delta z = F_L/k_z$. The rms Langevin force is given by $F_L = \sqrt{4k_B T \cdot 6\pi \eta r \Delta f}$, where $\Delta f$ is the bandwidth in Hertz and $6\pi \eta r$ is the viscous drag coefficient of the bead. With the viscosity of water $\eta = 10^{-3}$ poise, the bead radius $r = 0.5 \mu m$, and $k_z \sim 7 \times 10^{-7}$ N/m (for negatively supercoiled 4-kb DNA at a force of $\sim 0.3$ pN), we obtain $F_L \sim 10 fN$. $\sqrt{\Delta f}$ and $\delta z \sim 15$ nm $\sqrt{\Delta f}$. Thus as the bandwidth increases (i.e., as temporal resolution is improved), there is less time over which to average out thermal fluctuations and the noise level increases (i.e., the accuracy of spatial measurements worsens). With a bandwidth of 1 Hz (achieved by signal averaging over 1 s), we have an acceptable compromise between temporal and spatial resolution with a signal-to-noise ratio of 4.

Detecting DNA Bending/Compaction

Formation of the RNA polymerase–promoter open complex is known to be accompanied by apparent compaction of DNA of 5–30 nm due to introduction of a net bend in the DNA and wrapping of the DNA on and around the surface of RNA polymerase (C. Rivetti, N. Naryshkin, and R. H. Ebright, unpublished results). Apparent compaction of DNA will result in different signals—and therefore will be detectable and quantifiable—in experiments with positively supercoiled DNA or negatively supercoiled DNA (Fig. 5A). Apparent DNA compaction will cause a net decrease in the end-to-end extension of the DNA regardless of the sign of the supercoiling. Thus, in experiments with negatively supercoiled DNA, promoter unwinding will cause DNA extension to increase, but associated DNA compaction will partly offset the increase in extension (Fig. 5A). (The two effects will subtract from one another, yielding a smaller signal than if no compaction occurs.) Conversely, in experiments with positively supercoiled DNA, promoter unwinding will cause DNA extension to decrease, and associated DNA compaction will cause DNA extension to decrease further (Fig. 5B). (The two effects will add.) Thus compaction will be observable as a difference in signal amplitude observed upon promoter
unwinding on positively vs negatively supercoiled DNA and will be quantifiable as one-half the difference in signal amplitude (see legend to Fig. 5). We note that DNA bending may produce a similar effect. In principle, it should therefore be possible to distinguish between protein–DNA interactions that result solely in a change of local DNA twist and those that result in full or in part from a local change in DNA compaction and/or bending.

Concluding Remarks

We described a nanomanipulation technique that is used to quantitatively supercoil a single DNA molecule. Once the response of the DNA to mechanical changes in supercoiling has been calibrated, the system can be used to observe in real-time interactions between a single enzyme molecule and its supercoiled DNA substrate. We discussed applying this method to the study of RNA polymerase-induced promoter unwinding, as well as bending/compaction of the DNA by RNA polymerase. With this assay it will be possible to quantitatively study the role of DNA sequence
and supercoiling, temperature, nucleotides, effectors, and activators in formation of the transcription bubble. This technique may be applicable to the real-time study of a variety of protein–DNA interactions that cause deformation of the DNA, such as the unwinding of origins of replication or the binding of transcription factors.

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Simple Fluorescence Assays Probing Conformational Changes of Escherichia coli RNA Polymerase During Transcription Initiation

By Ranjan Sen and Dipak Dasgupta

The promoter recognition by Escherichia coli RNA polymerase (RNAP) 70 holoenzyme is a multistep process involving complicated conformational changes in both DNA and RNA polymerases. From a detailed thermodynamic, kinetic, and biochemical characterization of this process, it has been established that RNAP first forms a closed complex (RPc) in which the promoter DNA is in a closed duplex state. Then RPc converts into an open complex (RPo) through multiple intermediate states (RPi). In open complex, the promoter is melted and ready to start transcription. This conversion from closed to open complex not only changes the state of DNA, but major conformational changes in RNAP also occur, which involve the “jaw closure” on the downstream part of the DNA. Each of these conformational states during the pathway of open complex formation could be potential targets of activator and repressor molecules. Therefore, it is important to probe each of these states structurally to understand the basic mechanism of regulation of gene expression (Fig. 1).