Biophysical Journal, Volume 99

Supporting Material

Bending the rules of transcriptional repression: Tightly looped DNA directly represses T7 RNA polymerase

Troy Lionberger and Edgar Meyhöfer

Supporting Material

Minicircle Template Sequences

Templates were designed to include the following features (color-coded according to sequences below):

1.) EcoRV site: provides blunt ends for first sequence ("A") used as substrate for minicircle cyclization procedure (PvuII site used for "B" sequence).
2.) EcoRI site: cohesive end generation for insertion of second species ("A") into pRSET plasmid (BamHI site for "B" sequence).
3.) Nt.BbvCI site: site recognized by a nicking enzyme capable of nicking the non-template strand.
4.) T7 Promoter sequence: the strong (class III) Φ10 bacteriophage T7 promoter sequence.
5.) **Elongation Complex Formation Sequence**: sequence capable of being transcribed in the absence of UTP, stalling T7RNAP ECs at +20 site until UTP is present.
6.) MB Recognition Sequence: sequence from which transcribed RNA will hybridize to 2′-o-methyl-RNA molecular beacons.

Minicircle size (bp)		
	100	…ATCGGATCCTAATACGACTCACTATA**GGGAG**ACCACAACCAC **CACC**TGAATTCCAGCTGTAAGAAACCATTTCGATACCCCCGATGT CATCCTCAGCGAT…
	106	…ATCGGATCCTAATACGACTCACTATA**GGGAG**ACCACAACCAC **CACC**TctgGAATTCCAGCTGTAAGAAACCAtgcTTTCGATACCCCCG ATGTCATCCTCAGCGAT…
	108	…ATCGGATCCTAATACGACTCACTATA**GGGAG**ACCACAACCAC **CACC**TctgaGAATTCCAGCTGTAAGAAACCAtgcaTTTCGATACCCCC GATGTCATCCTCAGCGAT…
	Note: lower case letters are sequences inserted to change the size of the minicircle template.	

Table S1 Minicircle Template Sequences.

Minicircle Template Synthesis

Sufficient quantities of minicircle templates were created by modifying the ligation-assisted minicircle accumulation technique previously described (23). Briefly, two double-stranded DNA sequences (each the length of the desired minicircle template) were designed such that the first half of one sequence is complementary to the second half of the other. Consequently, upon melting into single-stranded DNA, the DNA can reanneal as either double-stranded DNA or a doubly-nicked minicircle. After ligation by a thermophilic DNA ligase, however, the closed minicircle is incapable of melting, and hence accumulates in solution. Both sequences were amplified by PCR and restricted with a blunt-end restriction enzyme. *Taq* DNA ligase (New England Biolabs, Inc., Beverly, MA) was then added to 12-15µg of each product, then thermocycled for 15 cycles as follows: melt at 95°C for 20 seconds, plunged to 4°C and held for 1 minute, ligate at 65°C for 20 minutes. After digesting any remaining linear DNA using exonucleases I and III (New England Biolabs), the

monomeric circular DNA was estimated by denaturing PAGE to comprise ~99% of the exonuclease-resistant product (see Fig. S1) and was used without further purification.

Substrates for the ligation-assisted minicircle accumulation technique were prepared by inserting the 100 bp, 106 bp, or 108 bp minicircle sequences (above) into a pRSET plasmid (Invitrogen). Two sequences were designed for each minicircle species, such that the first half of one sequence ("A") is complementary to the second half of the other ("B"). E. coli cells (DH5-α) were transformed with plasmids containing the correct inserts (either A or B) and grown in 1L of Luria-Bertani broth. Plasmids were extracted using a PureLink HiPure Plasmid Maxiprep Kit (Invitrogen, Carlsbad, CA). The A and B sequences were extracted from plasmid backbone by restriction with either EcoRV (A species) or PvuII (B species), and purified using a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA).

Relating the Bal-31 Sensitivity Data to the Residual Twist within DNA Minicircles

In order to qualitatively determine the relative differences between the torsional stress sustained within each minicircle species, we use Bal-31 nuclease (New England Biolabs) to identify DNA minicircles that are untwisted (and hence, susceptible to local melting and subsequent recognition as single-stranded DNA by Bal-31). These data (see Fig. 3 *C*) suggest that the 100 bp minicircle (which is quickly digested by Bal-31) is highly untwisted, the 106 bp minicircle (resistant to Bal-31) is overtwisted, and the 108 bp minicircle (slowly digested) is modestly untwisted. The observed sensitivity of the minicircle constructs to Bal-31 also suggests that the helical pitch of the minicircles differs from conventionally accepted values for B-DNA, typically within the range of roughly 10.4-10.5 bp/turn. It should be noted that these values represent the average value of helical pitch measured over long DNA sequences. However, it is known that intrinsic twist imparted to the double helix of DNA is strongly sequence-dependent, ranging from 28-40°/bp (1). It is expected that sequence effects to the helical pitch will be averaged over long lengths of DNA and exaggerated as DNA becomes shorter. Therefore, particularly short DNA sequences (such as DNA minicircles) may exhibit different helical pitches.

The helical pitch of DNA is also sensitive to ionic strength and temperature, making it difficult to quantitatively define the exact magnitude of the residual DNA twist within each minicircle if these conditions are changed. To confirm our conclusion from the Bal-31 digestions that the magnitude of overtwist in the 106 bp minicircle is larger than magnitude of untwist in the 108 bp minicircle, we cyclized a 104 bp minicircle. We observe that the 104 bp minicircle is distributed between two topoisomers (one overtwisted, the other untwisted, see Fig. S2), while all other minicircles cyclize to single topoisomer. The observed cyclization behavior suggests that the linear 104 bp DNA templates must be a semi-integral number of helical turns. While precise quantification of the two band intensities representing the exact distribution between the two topoisomers is difficult (since ethidium bromide will intercalate into untwisted DNA with a different efficiency than overtwisted DNA (2)), we can nonetheless draw important conclusions with regard to the helical pitch of our minicircles under the cyclization conditions.

To support our conclusions regarding the relative twist sustained within each minicircle, we show that the observed Bal-31 sensitivity and the topoisomer distributions of our minicircles are entirely consistent with predicted twist values. In Table S2, we consider the implications of the 104 bp linear DNA being a semi-integral number of helical turns (in this case, we choose 9.5 turns for simplicity, although the true value is expected to fall within the range of 9.4-9.6 turns). We neglect any effects of twist-bend coupling (which would be expected to untwist the helix as the minicircle is formed) or changes to ionic strength. If 104 bp of DNA with our specific sequence was exactly equal to 9.5 helical turns, the corresponding helical pitch would be equal to 10.95 bp/turn. Since cyclization occurs at

65°C, we can then account for the effects on helical pitch that accompany a drop in temperature (e.g., as the solution is brought to room temperature). Given the temperature sensitivity of DNA (DNA is untwisted by 0.0105 angular degrees per bp per degree Celsius of temperature increase (3)), the room temperature helical pitch of the 104 bp DNA sequence is then calculated to be 10.79 bp/turn. This value for helical pitch is consistent with what is predicted from sequence-dependent twist values reported in the literature (1), which when applied to our minicircle sequences yields an expected value of 10.76 bp/turn. In Table S3, we calculate the residual twist that would remain within each minicircle (cyclized at 65°C) after equilibrating to room temperature. As can be seen in the table, the predicted Bal-31 sensitivities match our experimental observations. Therefore, by considering only the formation of the two 104 bp topoisomers, we can show that our interpretation of the Bal-31 data is reasonable. However, we wish to stress that the residual twist within each minicircle is sensitive to the local environment, and consequently this discussion is intended only to note that our conclusions regarding the *relative* differences in the residual twist sustained within each minicircle template are consistent with the established literature once sequence- and temperature-dependent effects are taken into consideration.

1. Kabsch, W., C. Sanders, and E. N. Trifonov. 1982. The ten helical twist angles of B-DNA. Nucleic Acids Research 10: 1097-1104.
2. McClendon, A. K., A. C. Rodriguez, and N. Osheroff. 2005. Human topoisomerase II rapidly relaxes positively supercoiled DNA. The Journal of Biological Chemistry 280: 39337-39345.
3. Duguet, M. 1993. The helical repeat of DNA at high temperature. Nucleic Acids Research 21: 463-468.

Size of Topoisomer:	104	Bp
Helical Turns:	9.5	turns
Helical Pitch:	10.95	bp/turn
Cyclization Temp:	65	°C
Final Temp (T_f):	20	°C
Temp Sensitivity:	-0.0105	angular deg's/°C/bp
Helical Pitch (@ T_f):	10.79	bp/turn

Table S2 Predicted Helical Pitch at Room Temperature.

Minicircle Size *bp*	Helical Turns -	ΔTw @ 65°C *degrees*	ΔTw @ 20°C *degrees*	Predicted Bal-31 Sensitivity
100	9.13	-48.46	-95.71	*Sensitive*
101	9.23	-81.35	-129.07	*Sensitive*
102	9.32	-114.23	-162.43	*Sensitive*
103	9.41	-147.12	-195.78	*Sensitive*
104	9.50	-180.00	-229.14	*Sensitive*
105	9.59	147.12	97.50	*Insensitive*
106	9.68	114.23	64.15	*Insensitive*
107	9.77	81.35	30.79	*Insensitive*
108	9.87	48.46	-2.57	*Sensitive*

Table S3 Predicted Bal-31 Sensitivity.

Molecular Beacon Design

2′-o-methyl RNA molecular beacons (Sigma Life Science, The Woodlands, TX) were designed to include a 21 bp recognition loop (complementary to the RNA sequence produced upon transcription of the underlined portion of minicircle sequences listed in the Supporting Material) and a 5 bp self-complementary stem sequences (underlined below). A hexachlorofluorescein (HEX) fluorescent dye was labeled at the 5′ end, and a Black Hole Quencher 1 (BHQ1) was attached to the 3′ end:

HEX – CGAGCATGACATCGGGGGTATCGAAAGCTCG – BHQ1

Transcription Kinetics

A simplified kinetic equation describing the transcription of DNA by T7RNAP can be written:

$$E_{fr} + D \underset{k_{bind}^{-1}}{\overset{k_{bind}}{\rightleftarrows}} E_{cl} \cdot D \underset{k_{isom}^{-1}}{\overset{k_{isom}}{\rightleftarrows}} E_{op} \cdot D \underset{k_{init}^{-1}}{\overset{k_{init}}{\rightleftarrows}} E_{in} \cdot D \cdot R_n + NTP_{n=1-12} \xrightarrow[k_{for}]{} E_{el} \cdot D \cdot R_{12+n} + NTP \xrightarrow[k_{diss}]{} E_{frx} + D + R$$

where E_{fr} = free T7RNAP, E_{cl} = T7RNAP (closed complex), E_{op} = T7RNAP (open complex), E_{in} = initiating T7RNAP, E_{el} = elongating T7RNAP, E_{frx} = heparin inactivated T7RNAP, NTP = nucleotide triphosphates, D = DNA template, R = RNA transcript, k_{bind} = DNA binding rate constant, k_{isom} = rate constant describing isomerization into open complex, k_{init} = rate constant for initiation (includes abortive cycling), k_{cat} = elongation catalytic rate constant, and k_{diss} = dissociation rate constant.

Since our assay is specific to the elongation phase of transcription, this model must be refined to:

$$\begin{array}{c} E_{frx} \\ \uparrow k_{diss(stalled)} \\ E_{fr} + D \xrightarrow[k_{for}]{} E_{st} \cdot D \cdot R_{20} \xrightarrow[k_{cat}]{} E_{el} \cdot D \cdot R_{20+n} + NTP \xrightarrow[k_{diss}]{} E_{frx} + D + R \end{array}$$

where $k_{diss(stalled)}$ describes the rate at which stalled T7RNAP dissociated from the template prior to the addition of UTP and k_{for} is a pseudo first order rate constant describing the formation of stalled ECs (E_{st}) after the synthesis of the first 20 nucleotides, and is therefore the overall apparent rate constant resulting from the kinetics governing template binding (k_{bind}), open complex formation (k_{isom}), and initiation (k_{init}).

Molecular Beacon Fluorescence Detection

Fluorescence was measured using an AMINCO-Bowman Series 2 luminescence spectrometer (Thermo Spectronic, Rochester, NY), equipped with an external photomultiplier tube module. All reaction solutions, 65 µl in volume, were excited with 535 nm light in a 50 µl Sub-Micro Quartz Fluorometer Cell (16.50F-Q-10/Z15, Starna Cells, Inc., Atascadero, CA), and the emission from the cuvette was first filtered with a longpass filter (OG570, Schott, Inc., Elmsford, NY).

Transcription Elongation Assays

The fluorescence signal from molecular beacons hybridized to target RNA in solution was experimentally determined to scale as 0.4085 AU per nM of hybridized molecular beacon (see Fig. S3). The number of nucleotides transcribed was calculated from the fluorescence data by determining the concentration of hybridized beacon with the above calibration factor and multiplying the result by the number of nucleotides in the transcript to synthesize an additional beacon target sequence (i.e. - the nucleotides transcribed in a single round of the minicircle template, either 100 bp, 106 bp, or 108 bp). This value was then normalized by the number of T7 RNAP experimentally determined to occupy the template after template and heparin incubations in order to account for the template-specific rates of EC formation and EC stability (described below).

A nonlinear least squares fitting routine was implemented using MATLAB (The MathWorks, Natick, MA) to fit the time-dependent fluorescence data (N, the number of nucleotides transcribed per elongating T7 RNAP) to the solution to the first-order differential equation describing the reaction kinetics,

$$N(t) = P \times \left(1 - e^{-k_{diss} t}\right) \qquad \text{Eq. S1}$$

where the amplitude of the rising exponential (P) is equal to the transcription processivity (the number of nucleotides synthesized per T7 RNAP on average before dissociation) and k_{diss} is the dissociation rate of the enzyme from the template (in s^{-1}). The elongation velocity, k_{cat}, is equal to the maximal rate of transcription and is given by the value of the first derivative of Eq. S1 at t = 0,

$$\frac{dN(t)}{dt} = P \times k_{diss} \times e^{-k_{diss} t} \rightarrow k_{cat} = \frac{dN(0)}{dt} = P \times k_{diss} \qquad \text{Eq. S2}$$

Elongation Complex Formation Assays

Concentrations and buffer conditions were identical to transcription elongation assays, with the following modifications. T7 RNAP was incubated with the template for varying periods of time prior to the addition of heparin. Heparin was added and, as above, the solution was incubated for 5 minutes prior to UTP addition. In all cases, transcription was allowed to proceed for 10 minutes before transcription was terminated by heat inactivation of T7 RNAP. The time-dependent occupancy data (O_f, in percentage of template occupied after EC formation) were fit to the solution to the first-order differential equation describing the reaction kinetics,

$$O_f(t) = 1 - e^{-k_{for} t} \qquad \text{Eq. S3}$$

where k_{for} is the rate of elongation complex formation.

Elongation Complex Stability Assays

Experimental conditions were identical to transcription elongation assays, with the following modifications. T7 RNAP was incubated with the template for 10 minutes prior to the addition of heparin. Heparin was then added and the solution was incubated for varying periods of time prior to UTP addition. In all cases, transcription was allowed to proceed for 10 minutes before transcription was terminated by heat inactivation of T7 RNAP. The time-dependent occupancy data (O_s, in percentage of template remaining occupied by stable ECs after heparin incubation) were fit to,

$$O_s(t) = e^{-k_{diss(stalled)}t} \quad \text{Eq. S4}$$

where $k_{diss(stalled)}$ is the dissociation rate of stalled ECs from the template (which is inversely related to EC stability).

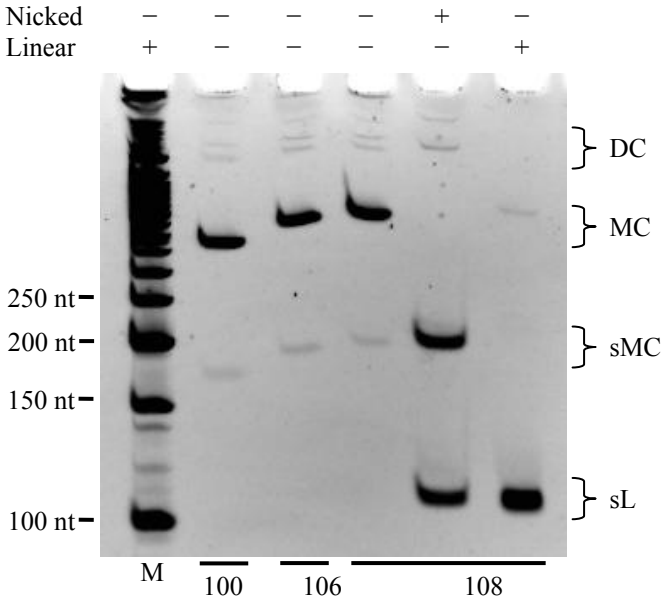

Figure S1 Minicircle Template Purity. Following exonuclease treatment (digestion of all linear DNA species), the minicircle template preparations were analyzed by denaturing PAGE (10% polyacrylamide, 7M urea, 1x TBE, 30V/cm), followed by staining with ethidium bromide. Quantification of the contaminating species (mostly double-stranded, circular dimers and single-stranded, circular monomers) reveals that they comprise ~1% of the exonuclease-resistant ligation products. DC: circular dimers (+ and – topoisomers), MC: circular monomers, sMC: circular, single-stranded monomers (form V DNA), sL: linear, single-stranded monomers, M: DNA reference ladder. Nicked: minicircles treated with the enzyme Nt.BbvCI, which will nick only one strand of the double-stranded minicircle (which will thereafter denature into a single-stranded circular and linear species). Linear: linear DNA ladder (M, leftmost lane) or EcoRV linearized minicircle (108, rightmost lane).

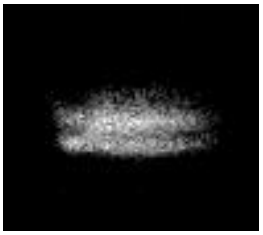

Figure S2 104 bp Minicircle Topoisomers. The minicircles were analyzed by denaturing PAGE (10% polyacrylamide, 7M urea, 1x TBE, 30V/cm), followed by staining with ethidium bromide.

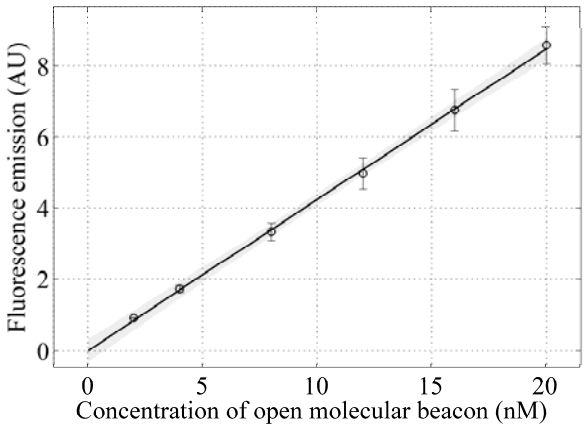

Figure S3 Molecular Beacon Calibration. To relate the measured fluorescence intensity to a concentration of hybridized molecular beacon, a know concentration of molecular beacons were incubated with complementary oligonucleotide targets. The fluorescence intensity was then measured using a luminescence spectrometer and external photomultiplier tube module. The buffer conditions, temperature, and photomultiplier tube voltage, were identical to those used in all experiments. From the resulting data, a linear fit yielded a slope of 0.4085 AU per nM of hybridized molecular beacon.