REGULATING GENE EXPRESSION THROUGH DNA MECHANICS: TIGHTLY LOOPED DNA REPRESSES TRANSCRIPTION

by

Troy Albert Lionberger

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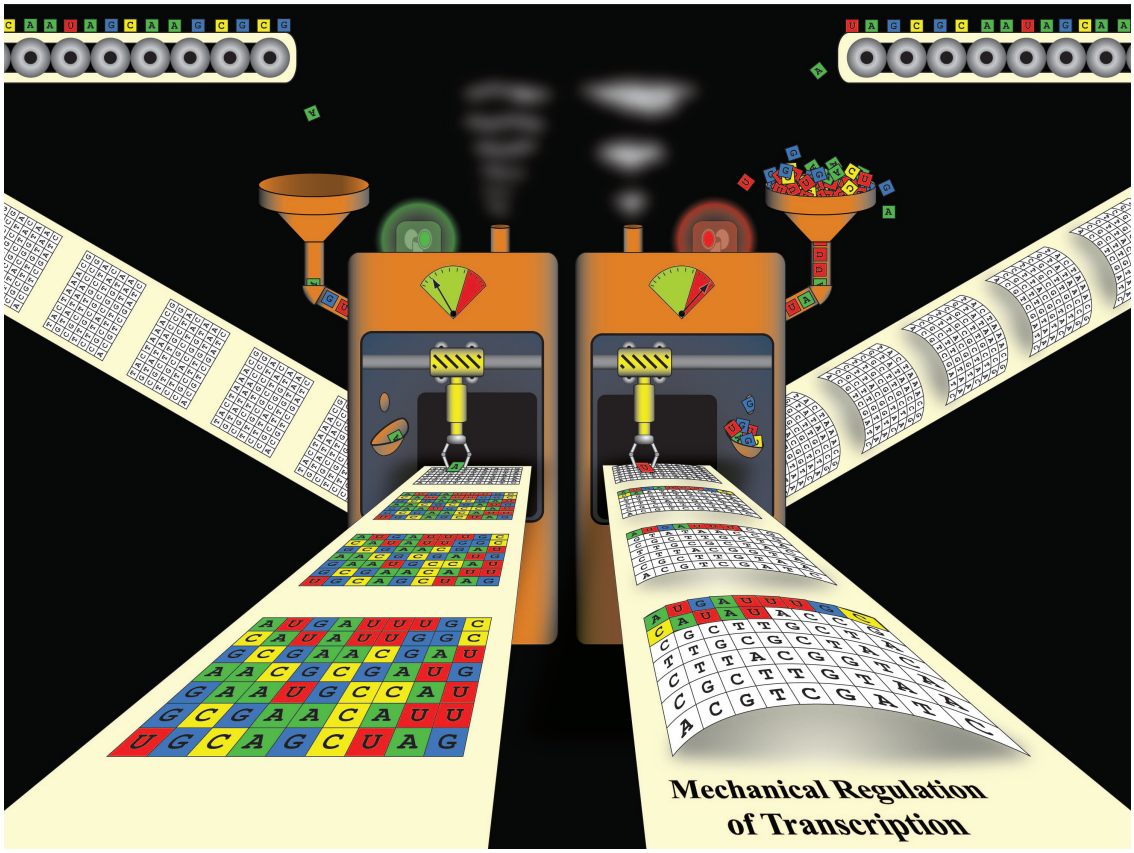
Doctoral Committee:

Professor Edgar Meyhöfer, Chair
Professor Noel C Perkins
Professor Nils G Walter
Associate Professor Katsuo Kurabayashi
Associate Professor Kristen J Verhey



Cell biologists *understand a system when they can define protein function in the context of its cellular environment.*

Molecular biologists *understand a system when they can relate protein function to the underlying molecular structure.*

Molecular biophysicists *understand a system when they have predictive power over and a physical understanding of the structure-function relationship on a molecular level.*

To Diane – You made graduate school
one of the best experiences of my life.
Thank you.

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CHAPTER 1

Introduction

The regulation of gene expression is critical to healthy cell function, which inherently requires tight control over the copy number of proteins within the cell as well as their distribution within complex spatial and temporal gradients. Efficient control of gene expression is not only central to the differentiation of specific cell types and tissues, but also to adequately responding to developmental changes, environmental stresses, and external stimuli. A general paradigm for how gene expression can be controlled through the regulation of transcription by RNA polymerase (RNAP) was first derived from work on bacteria [1]. According to this general model, gene regulatory proteins “switch” the transcription of individual genes on and off by modulating RNAP activity. Some of the earliest studies of gene regulation focused on the role of repressor proteins. The widely accepted model for repression endorses the general paradigm that a gene located downstream of a DNA repressor binding site will be repressed if a repressor protein recognizes and binds this site [2]. Support for this model was provided by the identification of repressor protein binding sites immediately upstream of genes near the gene promoter (the DNA sequence recognized as a binding site by RNAP) [3]. Interestingly, many repressors (λ phage repressor,

Salmonella phage P22 repressor, *eve* in Drosophila, and the repressors for the *ara*, *gal*, *lac*, *deo*, *trp*, *gln*, and *mer* operons in *E. coli*) have since been shown to bind to bipartite operators and consequently deform the intervening DNA into highly stressed, looped topologies [4-5]. In these systems, the looped DNA often contains the promoter sequence for RNAP [6-8]. Consequently, the promoter extends free in solution away from the repressor, and is not necessarily prevented on steric grounds from being bound by RNAP. In fact, DNA that is looped by repressors in *E. coli* has been shown to be not only capable of being bound by RNAP, but in some cases is actually bound by the polymerase with higher affinity [9].

1.1 Evidence for the template-mediated, mechanical regulation of transcription

While the concept of "mechanical" repression of RNAP activity may seem new to many within the biological community, there in fact exists a body of work either postulating the existence of such a mechanism (emanating largely from unresolved peculiarities or anomalies in the data) or attempting to directly demonstrate and characterize the relationship between DNA mechanics and transcription [10-23]. Here, I present a brief synopsis of the relevant literature from which emerges my central hypothesis. This discussion is intended to (1) serve as a high-level summary of the observations I interpret as suggestive of a role for DNA mechanics in directly regulating transcription, and (2) highlight outstanding questions regarding the mechanics of DNA. As will become clear from the following examples, a central problem emerges from the literature: all of

the evidence in the literature supporting a relationship between transcription by RNAP and highly bent and twisted DNA emanates from work performed with supercoiled DNA. The need to address this very complex, experimental shortcoming serves as the primary motivation for the present work.

The lactose repressor paradox

The series of experiments performed by the Müller-Hill group characterizing the *lac* repressor (LacR) provide perhaps the most compelling biological evidence that mechanical stress on the level of the DNA template regulates transcription (see Fig. 1). Krämer *et al.* initially reported electron microscopy data that LacR binds to two operator sequences in DNA and forms looped topologies [24]. By systematically varying the distance between the two operators, the authors provided evidence that LacR formed loops with increasing frequency as the DNA between the operators was increased from 74 bp to 535 bp. This observation can be explained, at least in part, by experimental evidence from Shore and Baldwin showing that the probability of two points along a single DNA molecule to randomly diffuse into close proximity is largest if their spacing is around 500 bp [25]. It was suggested that this particular spacing was optimal because it was, in essence, the best compromise between the exponentially increasing stiffness of DNA as it becomes shorter and the increasing diffusional search radius required as DNA becomes larger.

The probability of loop formation by LacR now being characterized, attention eventually turned to understanding the efficiency of LacR to repress transcription as a function of loop size. The study by Müller *et al.* utilized an *in vivo* approach to quantify the relative ability of LacR to repress a gene as the distance between two *lac* operators was varied from 57.5 bp to 1493.5 bp [20].

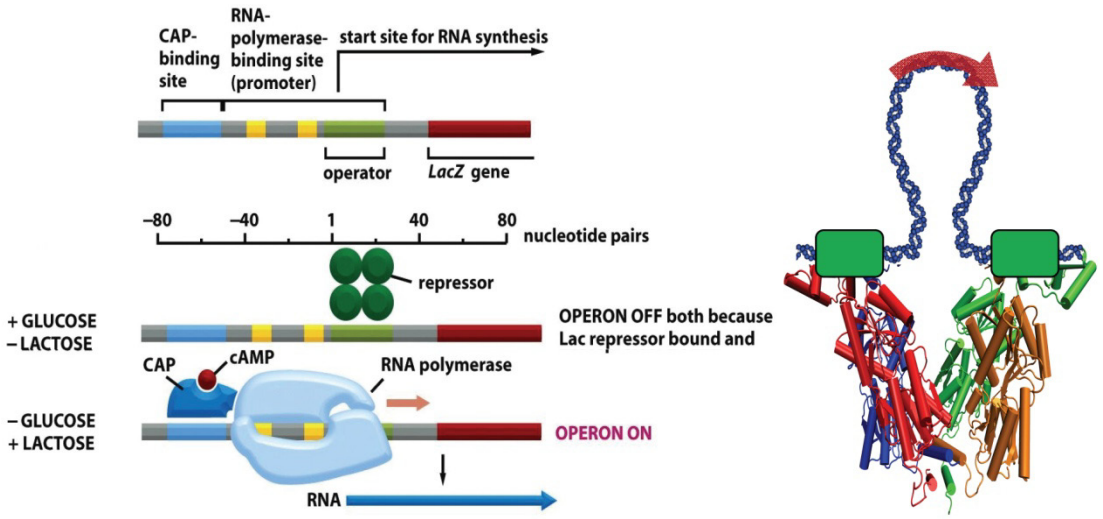

Figure 1. The Lactose (*lac*) Operon. **(a)** The *lac* operon is switched on in the presence of lactose and the absence of glucose. In the presence of the *lac* repressor protein (LacR), transcription is prevented from ensuing. A critical, missing component of this regulatory system is that LacR is a tetrameric protein that binds simultaneously to two operators. Not shown in this figure (adapted from [26]) but critical to our understanding of the molecular mechanism by which *E. coli* RNAP is repressed, is that a second operator site lies immediately upstream of the CAP-binding site. **(b)** The resulting loop of DNA (shown here docked to LacR [27]) is required for the operon to be switched off.

Surprisingly, it was found that the repression was maximal when the inter-operator distance was 70.5 bp. This construct demonstrated repression levels approximately 20-fold higher than in the constructs with inter-operator distances around 500 bp. In the context of the conventional paradigm for repression where repression is simply due to the binding of a repressor protein to its respective binding site, this result appears to directly contradict the imaging study by Krämer

et al. That is, there is an apparent conflict between the conclusions that the repressor binds more often when the inter-operator distance is around 500 bp, yet the ensuing repression from this loop-repressor complex is many times weaker than when the inter-operator distance is around 70 bp. These paradoxical results can be reconciled by a revised model of transcriptional repression by loop-forming repressors that includes, as a component of repressor action, consideration of the mechanical stress imparted to the DNA template by the repressor-induced looping.

As would be predicted by this revised general model for loop-forming repressors, a number of loop-forming repressor systems have been implicated in repressing transcription through the mechanical stress imparted onto the DNA template. Choy *et al.*, for example, sought to demonstrate that transcription repression induced by the loop-forming galactose repressor (GalR) was, at least in part, attributed to the torsional inflexibility of the DNA within a GalR-induced loop [23]. The authors attempted to demonstrate the important role played by looped DNA (as opposed to a particular repressor protein) by replacing the two GalR operator binding sites with LacR operators, and successfully verified that the *gal* operon could be repressed by LacR. Though certainly an important first step in attempting to address the role played by DNA mechanics in gene regulation, this study (like the LacR study by Müller *et al.*) failed to conclusively demonstrate that the mechanical state of the DNA template was causal to the ensuing repression because a known repressor protein was always present

during transcription. Therefore, the direct role played by the repressor protein could not be excluded as a possible cause of the observed repression.

Divergent promoter systems: Gene-specific regulation mediated by transcription-induced torsional stress

The ability of mechanically perturbed DNA to regulate gene expression is readily apparent in divergent (or bidirectional) promoter systems, where promoters of two adjacent genes are oriented in opposite directions and appear within a few hundred bases of each other. The close proximity of the promoters results in a regulatory phenomenon known as transcriptional coupling, in which transcription of one gene directly regulates the neighboring gene. The coupling between adjacent genes originates from the ability of actively transcribing RNAP molecules to generate upstream negative torsional stress [28-33]. The subsequent mechanical strain imparted to the DNA structure of the upstream promoter in turn modulates the expression of the closely spaced, divergently oriented gene [34-35], though the molecular mechanism(s) underlying this behavior remain unclear. Importantly, transcriptional coupling occurs *without* the participation of regulatory proteins, and thus presents a powerful example of DNA mechanics directly regulating transcription by RNAP. It should be noted for purposes of this discussion that regulating transcription through the relative placement of transcriptionally coupled gene pairs is hardly uncommon in nature: up to 40% of the *E. coli* genome [36-37] and 10% of the human genome [38-39] is arranged in such a manner. In both cases, the expression of the adjacent

genes has been demonstrated to be statistically correlated, thus potentially implicating mechanical stress in DNA as an evolutionarily conserved regulatory mechanism [36]. However, our ability to elucidate the molecular mechanism underlying transcriptional coupling is significantly hindered by the fact that the mechanical state of the DNA has so far only been defined in terms of the *supercoiling* generated during the transcription of adjacent genes. As will be discussed shortly, this experimental shortcoming leads to ambiguity regarding the bending and torsional stresses sustained within the DNA template. Consequently, it remains unclear what mechanical conditions actually persist during transcriptional coupling. This clarity appears quite necessary to understanding the role of mechanics in coupled promoter systems, as we have yet to understand how divergent promoters can be repressive in some genes systems and yet stimulatory in others [36]. Presumably, these differences would be better understood if the bending and twist applied to DNA could be precisely controlled prior to the characterization of RNAP activity.

The relationship between transcription and supercoiling

Supercoiling has long been known to be capable of influencing global levels of gene expression [40]. Though supercoiling under physiological conditions is tightly controlled by topoisomerases and typically maintained in a mildly negative state [41-43], the global levels of superhelicity are known to be a potent control mechanism employed by cells to regulate gene expression in response to environmental challenges [44-49]. In addition, it is now known that

essential metabolic pathways are in fact coordinated by changes in supercoiling [50]. In light of the relationship between supercoiling and gene expression, it is not surprising that many RNAPs have been identified, such as *E. coli* RNAP [40, 51-54] and bacteriophage T7 RNAP [55-56], that are specifically affected by the superhelicity of the DNA substrate. In the case of *E. coli* RNAP, it has been shown that the kinetics of open complex formation increases by as much as 150 times under conditions where the DNA template sustains optimal levels of supercoiling [57]. Furthermore, a single molecule, magnetic trapping assay has been used to demonstrate that negative supercoiling enhances transcription by *E. coli* RNAP [58]. However, while these studies do provide direct evidence that there is some, mechanical aspect of the DNA template that influences RNAP activity, the molecular mechanism underlying the dependence of gene expression on the superhelicity of DNA remains elusive due to experimental limitations inherently present in any study that utilizes supercoiling as a measure of the mechanical state of DNA.

1.2 The central problem: Supercoiling is a poorly defined mechanical state

The tertiary structure of DNA arising from superhelicity was initially discovered as a result of the unexpected appearance of both a relaxed (nicked) and more compacted (supercoiled) species within a population of sequence-identical circular DNA molecules [59]. Supercoiling would become one of the earliest and most widely studied mechanical properties of DNA once it became clear that the dependence of the electrophoretic mobility of DNA on tertiary

structure enabled separation of circular DNA species according to their relative differences in superhelicity [41, 60-61]. The number of supercoils (mathematically described as “writhe”) and the torsional state of DNA (“twist”) was theorized to be related by a conserved geometrical parameter known as the “linking number,” defined simply as the total number of twists in a circular helix. The linking number was correctly predicted to be exactly equal to the sum of twist and writhe and is constant within closed, circular DNA molecules [62]. Importantly, although the *linking number* is never changed within a closed domain, the *twist* and *writhe* are allowed to vary so long as any changes to the twist are directly compensated for by changes in the writhe. While twist can be simply thought of as the geometrical parameter reflecting of torsional stress within DNA, writhe is complicated by the fact that it is inherently composed of a combination of both torsional and bending stress, the magnitudes of which are highly dependent on the geometry of the writhe (supercoiling) that is formed [21, 63-64].

One important difference between twist and writhe is that supercoiling is often a large-scale phenomenon, typically distributed over thousands of base pairs of DNA. Given that the length scale over which DNA-binding proteins interact with DNA is typically below 100 base pairs, it is not likely that supercoiling *per se* is the mechanical characteristic of DNA that influences these protein interactions. Instead, the magnitudes of bending and twisting on smaller regions of the DNA (say, below 100 bp) are likely to be more relevant to the molecular level interactions of interest to biologists (albeit, these bending and twisting stresses may arise as a direct result of supercoiling within the DNA).

While all previous studies attempting to characterize the torsional state of DNA have done so by measuring the superhelicity, interpretations of the results from these studies are complicated by the fact that, in reality, both torsional and bending stresses appear within supercoiled DNA (the magnitudes of which are never actually measured). The torsional stress is assumed to be related to the supercoiling by the assumption that any change in supercoiling must be related to changes in twist, while the bending stresses are typically ignored completely. Clearly, these assumptions are problematic given that it is not known how a change in the torsional state of DNA in solution is *partitioned* between writhe (which contributes to bending stress) and twist (the torsional stress remaining after writhe is formed).

Complicating the matter further, it has been shown that (1) the efficiency with which supercoiling is formed is dependent on the length of DNA [65-67], (2) there is a critical value of torsional stress that can be sustained within DNA before supercoiling is actually formed [68-72], and (3) the torsional stress within a supercoil is directly related to the pitch of the superhelical structure [21, 63-64]. Consequently, without information regarding the torsional loading conditions prior to the onset of supercoiling or the geometric details of the supercoil that is formed, **it is not possible to definitively ascertain the torsional and bending stresses within supercoiled DNA**. While this conclusion may appear at first to be a relatively minor or tangential detail of the mechanics of supercoiled DNA, it is in fact crucial to understanding how the mechanical state affects transcription. The underlying importance of this point becomes clear when one considers that it

is theoretically possible to relieve all torsional stress within a supercoiled, circular DNA template by simply altering the geometry of the superhelical structure (for example, by forming a solenoidal supercoil). Therefore, given the inherent ambiguity regarding the bending and torsional stress states within supercoiled DNA, all relationships previously drawn between supercoiling and transcription are technically not capable of distinguishing between the effects of bending and twisting DNA on RNAP activity. This important limitation underscores the pressing need for experimental techniques that are capable of precisely controlling the bending and torsional stress of DNA during transcription by RNAP.

1.3 T7 RNA polymerase (T7RNAP) as a model system for studying the role of DNA mechanics on transcriptional repression

In light of how little is actually known about the molecular mechanism underlying the relationship between RNAP activity and DNA mechanics, the choice of model system is not trivial. Given the use of looped DNA by regulatory proteins in organisms ranging from bacteriophages to eukaryotes, it is reasonable (if not called for) to test my hypothesis in general terms. That is, if DNA looping is causal to repression in otherwise unrelated regulatory systems, the most conservative approach to demonstrating that mechanical stress can directly repress transcription would be to intentionally choose a model RNAP that is *not* known to be naturally repressed by loop-forming repressors. The chosen RNAP should also be functionally robust and display transcriptional activities common to other RNAPs. In addition, a reductionistic approach to quantifying the

effects of template bending on RNAP activity dictates that transcriptional competency should not depend on the assembly of a multi-subunit polymerase complex. I therefore sought to design an assay capable of quantifying the transcription activity of bacteriophage T7 RNA polymerase (T7RNAP), a widely studied model RNAP that is remarkably robust, as indicated by its ability to transcribe through a variety of topological challenges in the transcription template [73]. Importantly, T7RNAP has only one known repressor, T7 lysozyme, which does not loop DNA. In addition, T7RNAP has been shown to transcribe through loop-forming repressors during transcription elongation [74], thus satisfying the experimental specification of choosing a model RNAP that stands the highest likelihood of not being repressed by looped DNA.

1.4 Methodological challenges for characterizing the biophysical properties of T7RNAP during transcription of bent and twisted DNA templates

The most detailed level of understanding of any molecular system is arguably derived from single molecule observations. By monitoring the activity of an individual molecule (such as T7RNAP), it is possible to observe enzymatic processes that are normally buried within the average (ensemble) behavior that typically characterized using conventional methods. Ensemble measurements inherently suffer from the fact that the observation is limited to a large population of molecules in solution, often making it very challenging to characterize behaviors that occur either infrequently or over very short time scales. Elucidating the chemomechanical properties of transcription by single RNAP's

has been attempted by several single molecule studies employing a wide range of sophisticated biophysical techniques, including optical [75-89] and magnetic trapping [58, 90-91], FRET [92-97], tethered particle motion [98], time-resolved fluorescence localization [99-100], and fluorescence lifetime trajectories [101]. Many of these studies have focused specifically on the elongation phase of transcription, largely owing to experimentally advantageous stability of transcription elongation compared to the relatively unstable and short-lived pre-elongation phases, which include promoter binding, open complex formation, initiation, and abortive cycling. However, these techniques have thus far failed to prove capable of controllably bending DNA with the degree of curvature typically encountered in physiologically relevant contexts (for example, the ~100bp loops formed by loop-forming repressors).

The principle limitation with all current force spectroscopy-based methods is that the nanoscale physical properties of DNA must necessarily be interfaced (and interrogated) with microscopic structures and surfaces several orders of magnitude larger than the characteristic dimensions of the DNA. In order to be manipulated optically or magnetically, for example, DNA is typically tethered between a fixed (or reference) position and a free bead that is capable of being manipulated by the trapping system. The reference end of the DNA is typically anchored on one end to a fixed reference position, such as a modified glass surface or a spherical bead restrained by either a glass capillary or an optical trap with high stiffness. The geometric constraints imposed by current techniques capable of manipulating the structure of DNA preclude the ability to controllably

bend DNA with degrees of curvature many times smaller than the structures used to impart force into the DNA. The only technique currently capable of bending DNA, which utilizing four independently moveable optical traps [102], is not currently able to precisely prescribe the magnitude and position of the curvature relative to DNA elements of interest for DNA-protein studies.

CHAPTER 2

Controlling the Bending and Torsional Stress in DNA Templates

In order to comprehensively understand how gene expression is controlled through the regulation of transcription, it is necessary to discern the underlying mechanisms utilized by regulatory proteins at the molecular level. As previously discussed, many gene repressors bind to two closely spaced sites on DNA, forcing the DNA template to adopt a highly bent, looped structure sustaining significant mechanical stresses. Often, as with the lactose (*lac*) repressor in *Escherichia coli* [20, 24] and the even-skipped (*eve*) repressor in *Drosophila melanogaster* [103], the promoter sequence that is recognized and bound by RNAP is located within these repression loops (Fig. 2, left). Curiously, unlike repression mechanisms involving the competitive binding of a repressor and RNAP for a single binding site, repressor-induced looping of the promoter leaves the RNAP binding site exposed and, in some cases, still able to be bound by RNAP [104]. While it has been established that many loop-forming repressors depend on the formation of DNA loops to effectively regulate RNAP activity [7, 105], it is unclear if DNA looping is itself causal to repression. Several repressor studies have posited that the mechanical state of the looped DNA is responsible for the induced repression [20, 23]. Yet, a direct observation of the ability of

mechanically stressed templates to independently repress transcription has not been made, largely owing to the lack of an assay capable of quantifying the transcriptional competency of an RNAP from DNA templates sustaining well-defined levels of mechanical stress in the absence of DNA-binding proteins.

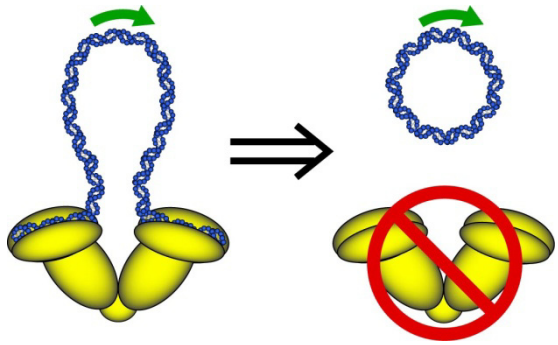

Figure 2. Towards the Direct Observation of the Effects of DNA Mechanics on Transcription. Repressor-induced, looped DNA topologies containing a highly bent promoter (green arrow) are recreated with DNA minicircles, eliminating any influence on transcription by the repressor.

2.1 Controlling the bending curvature of transcription templates

The ability to stably maintain high degrees of bending and torsion in transcription templates is fundamental to addressing the relationship between mechanical stress and RNAP activity. This requisite condition can be satisfied by the generation of circular DNA, where geometrical constraints necessitate that bending and torsional stresses resulting from ring closure be distributed along the DNA and will be sustained so long as the double-stranded helix remains intact. Ideally, DNA minicircles would be generated that replicate the bending curvature expected in loop-forming repressor systems, such that transcription can be directly measured in a repressor-free context. As a first attempt to understanding the effects of mechanical stress on transcription, minicircle templates on the order of 100 bp in size were designed in order to impart

biologically relevant bending stresses onto the DNA that were comparable to those encountered in many known loop-forming repressor systems [4-5, 106] (Fig. 2, right). Importantly, the bending and twist sustained within a minicircle template are defined only by the number of base pairs in the circular DNA and are applied in the absence of DNA-binding proteins. Furthermore, and in contrast to previous transcription assays using torsionally stressed, supercoiled plasmids, modest levels of residual twist within the sub-persistence length DNA minicircles are not theoretically expected to be relieved by supercoiling [65-67]. By using DNA minicircles that encode a RNAP-specific promoter sequence in a transcription assay, any observed differences in transcription activity (relative to transcription from unstressed DNA) must necessarily be attributed to the mechanical properties of the DNA template.

The bending stresses that are imparted onto DNA and stabilized by repressor proteins (and thus intended to be sustained within DNA minicircle templates) are significant. Since 100 bp DNA minicircles do not occur in nature, the only option available to create the minicircle templates is to ligate the free ends of 100 bp linear DNA together using a DNA ligase. Displaying the physical characteristics of a semi-flexible polymer, DNA is governed by mechanical laws that dictate that the bending stiffness decays exponentially with length along the DNA. The characteristic length scale of the exponentially decaying bending stiffness of a semi-flexible is often described by the persistence length, formally defined as the length over which the correlation of two tangent vectors along the polymer is lost. For DNA, the bending persistence length is on the order of 150

bp and the torsional persistence length is roughly 225 bp. Consequently, and in contrast to the circularization of linear DNA longer than the persistence length, circularization of 100 bp of DNA must contend with the bending stiffness of DNA.

Existing theory provides a useful framework through which we may be made aware of potential experimental issues associated with circularizing DNA that is on the order of 100 bp in length. According to the worm-like chain model developed by Kratky and Porod [107] (the currently accepted model describing the governing mechanics of DNA), the energy required to bend a double-stranded 100 bp DNA into a minicircle is more than $30k_BT$ (Fig. 3). If the only available source of energy is from the thermal environment, such an energetically unfavorable process would occur so infrequently as to render the behavior virtually unobservable (see [108]). As a point of reference, we are informed from statistical mechanics that for a thermally-driven process described by the Boltzmann distribution, the probability of thermal energy spontaneously providing $30k_BT$ of energy is 9×10^{-14}. Together, these theoretical considerations

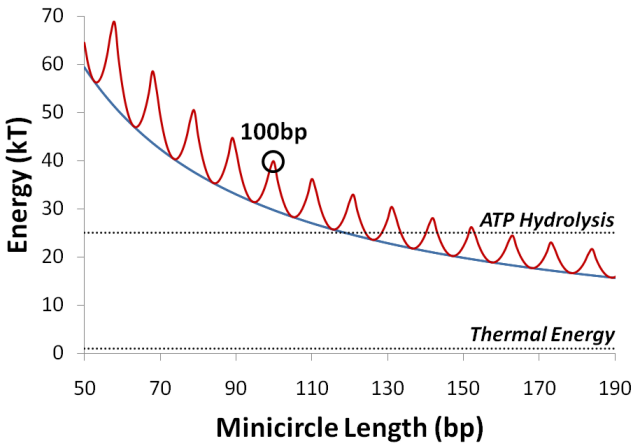

Figure 3. Formation of DNA Minicircles from Linear DNA is Energetically Unfavorable. Plotted is the energy required to bend DNA (blue) into a circular conformation, as a function of length. Also shown is the additional energy required to twist the ends into proper alignment for ligation (red). Any twist (regardless of sign) will necessarily increase the energy required for minicircle formation. $40k_BT$ is required to form a 100 bp minicircle (circle), significantly more than would be even theoretically available from ATP hydrolysis.

tell us that a 100 bp linear DNA in the presence of DNA ligase is, on energetic grounds alone, less likely to form circular monomers than the more energetically favorable, higher-order concatemers, such as circular dimers, trimers, etc.

Experimental attempts to circularize short linear DNA molecules support this conclusion. One of the earliest attempts to circularize sub-persistence length DNA was performed by Hodges-Garcia *et al.*, who concluded that small minicircles could not be produced using conventional ligation-mediated ring closure techniques without the addition of the histone-like protein HU [109]. Presumably, as a nonspecific DNA-binding protein known to significantly bend linear DNA upon binding, HU was able to significantly reduce the energetic cost associated with ring closure by introducing flexibility into the DNA [110]. These conclusions were generally accepted by the DNA mechanics community until a controversial study by Cloutier and Widom proposed that the bending and twisting flexibility of short fragments of linear DNA was actually much higher than is predicted by the worm-like chain model [111-112]. A follow-up study by Du and Vologodskii challenged this claim [108], and it has since been shown that the Cloutier and Widom study was fundamentally flawed by the use of high concentrations of T4 DNA ligase during circularization [113]. DNA ligase, it became clear, was able to nonspecifically bind DNA and introduce bends into DNA, akin to HU though to a much lesser extent. Clearly, the conventional ligation-mediated ring closure of linear DNA is significantly hindered on energetic grounds for sub-persistence length DNA. Therefore, the preparation of sufficient quantities of DNA minicircles to use as transcription templates for RNAP requires

the development of an alternative approach to circularizing sub-persistence length DNA.

2.2 Ligation-assisted minicircle accumulation

Two strategies have recently been developed that are able to overcome the energetic limitations of circularizing sub-persistence length DNA. The first is a method that was developed by Shroff *et al.* to study the entropic stiffness of single stranded DNA, and relies on the hybridization of a single-stranded linear DNA to a complementary single-stranded DNA minicircle [114-115]. Though not specifically designed by the authors to prepare double-stranded DNA minicircles, the Shroff *et al.* method is theoretically capable of producing a singly-nicked minicircle, which could then be covalently closed by DNA ligation. However, this method is not appropriate for producing preparative quantities of DNA minicircles for two principle reasons: (1) it uses an inefficient single-stranded DNA ligase (the availability of which is limited) to form the single-stranded DNA minicircle, and (2) it relies on synthetic single-stranded DNA oligonucleotides as a starting substrate. DNA oligonucleotides are problematic because they are both limited in size (typically ≤ 100 bp) and are produced with questionable, length-dependent sequence fidelity. Ideally, double-stranded DNA minicircles would be generated from linear DNA that was either purified directly from bacteria or amplified by high-fidelity PCR.

The second strategy to overcoming the energetic cost of bending double-stranded DNA is the ligation-assisted minicircle accumulation (LAMA) method

developed by Du *et al.* [116]. Like the Shroff *et al.* method, LAMA exploits the intrinsic flexibility of single-stranded DNA and the free energy from hybridization of complementary DNA to generate double-stranded DNA minicircles (Fig. 4). Briefly, two linear DNA sequences of the size of the minicircle are designed such that they can be melted and reannealed, either reforming the original linear DNA or instead producing a circular species with two single-stranded nicks. By using a thermophilic DNA ligase capable of maintaining activity at the melting and reannealing temperatures, the nicked minicircles are ligated into closed minicircles which precludes further melting. However, the linear DNA will still be capable of melting and reannealing in the next temperature cycle. While the probability of minicircle formation (i.e.- thermodynamic stability) is lower than reformation of linear DNA, the reaction can be repeated an arbitrary number of

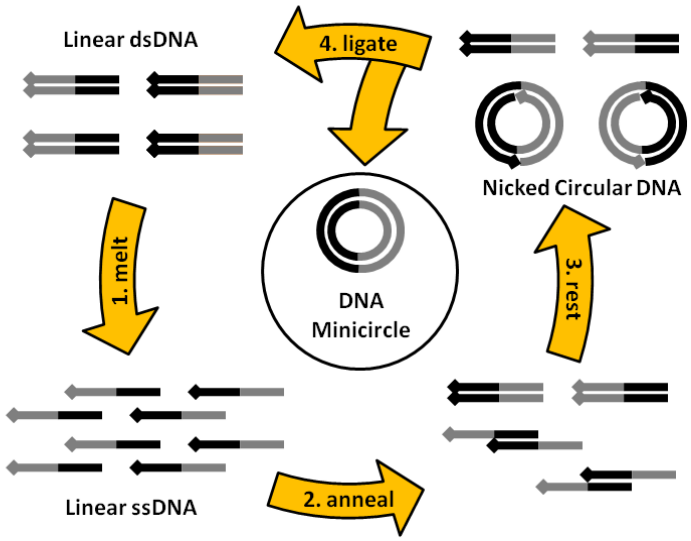

Figure 4. Overview of the Ligation-Assisted Minicircle Accumulation Method. Intact, double-stranded DNA minicircles are created by (1) melting two species of linear DNA where the first half of one is complementary to the second half of the other, (2) annealing to form a mixture of homogeneous and heterogeneous linear annealing species, (3) further annealing to allow heterogeneous species to form double stranded, doubly nicked minicircles, (4) ligation of the nicks by a thermophilic DNA ligase to form intact minicircle templates.

times to accumulate double-stranded minicircle products. Linear DNA remaining upon the conclusion of the thermocycling ligation reaction is digested by DNA exonucleases that are specific to digesting linear DNA and thus have no effect on circular DNA. The LAMA method has proven capable of producing double-stranded minicircles as small as 64 bp.

Although the Du *et al.* method was not specifically designed to be used as a preparative method, it nonetheless offers a powerful solution to efficiently producing DNA minicircles of arbitrary size, and perhaps more importantly, is easily scalable. Circular DNA templates 100 bp, 106 bp, and 108 bp in length were designed and the LAMA method was modified to produce nearly homogeneous solutions of minicircle templates, as confirmed by gel electrophoresis (Fig. 5a) and cryo-electron microscopy (Fig. 5b). All three minicircles experience varying levels of torsional stress that arises as a result of forming closed circles from linear DNA that is not an integral number of helical turns in length. By incubating each species with BAL 31 nuclease, a single-stranded DNA nuclease known to specifically digest untwisted circular DNA [117], the relative levels of torsional stress within the minicircles were qualitatively determined (Fig. 5c). The 100 bp minicircle (denoted henceforth as 100c) was quickly digested, indicating that it was the most highly untwisted template, whereas the 106 bp minicircle (106c) proved resistant to BAL 31, demonstrating that it is either not twisted or moderately overtwisted. The 108 bp minicircle (108c) is sensitive to BAL 31, but less so than 100c, and so is only mildly untwisted.

2.3 Experimental complications associated with quantifying the absolute magnitude of DNA twist

Ascertaining the absolute value of twist within the minicircle templates is not a trivial matter (for review, see [118]). The conventional approximation for the helical pitch of DNA ranges in the literature from 10.3 - 10.5 bp/turn [118-120]. For studies using long (and hence torsionally flexible) lengths of DNA, this average value may be an appropriate first-order approximation. However, for

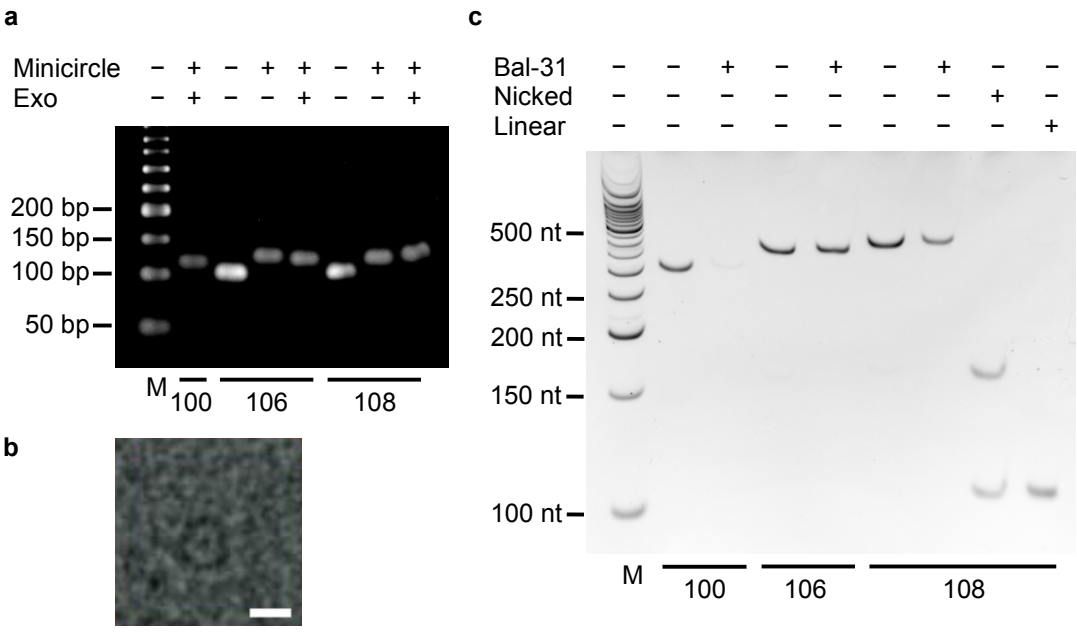

Figure 5. Analysis of DNA Minicircle Transcription Templates. **(a)** Native agarose gel electrophoresis of DNA (3% agarose, 0.5x TBE, 10 V/cm) of the specified length (100 bp, 106 bp, 108 bp), either as linear DNA prior to cyclization into minicircle templates or immediately following the cyclization procedure. Minicircle products were resistant to a prolonged incubation with Exonucleases I and III ("Exo"), revealing the presence of a circular DNA species. **(b)** Cryo-EM image of 100 bp minicircle confirms that cyclization procedure generates circular monomers (white scale bar = 10 nm). **(c)** Denaturing PAGE of minicircle templates (10% polyacrylamide, 7 M urea, 1x TBE, 30 V/cm) reveals that the 100 bp minicircle is highly untwisted (and hence quickly digested by BAL 31), the 106 bp minicircle is not untwisted (resistant to BAL 31), and the 108 bp minicircle is moderately untwisted (modestly digested). Minicircle templates were incubated with 0.015 U/µl for 15 minutes at 30°C. Singly nicked and linearized 108 bp minicircles provide references for linear and circular single-stranded DNA species under denaturing conditions.

short (sub-persistence) lengths of DNA, the physical description of DNA as a polymer with uniform mechanical properties begins to fail. That is, there naturally exists some yet-undefined size scale below which DNA must be treated as a anisotropic, composite-like structure, where the mechanical properties are dependent on not only the individual nucleotides, but also their respective order along the DNA. In the case of the helical pitch (upon which the residual DNA twist within DNA minicircles is dependent), for example, a strong dependence on the sequence has been well documented in the literature [121-122]. In order to begin to address the consequences of the particular sequence used to create the three minicircles, the sequence-dependent twist values reported in the literature were used to calculate the residual twist theoretically predicted to exist within the three minicircle templates that have been produced (Table 1). As is readily apparent from this calculation, 100c is predicted to be highly untwisted, 106c to be moderately overtwisted, and 108c to be slightly untwisted. These predictions are well-aligned with the relative magnitudes and signs of the twist qualitatively derived from the observed susceptibility to digestion by BAL 31 nuclease (Fig. 5c).

Minicircle Size	100bp	106bp	108bp
Helical Turns	9	10	10
Twist	-100°	+50°	-10°

Table 1. Theoretical Predictions of Residual Twist in DNA Minicircles. Values have been calculated for the particular DNA sequences used to form DNA minicircles using the experimentally derived, sequence-dependent values for helical pitch [122].

However, these theoretical expectations must be tempered by the significant complications underlying any attempt to provide quantitative values for

the absolute magnitude of DNA twist. The only method currently available that is capable of determining the residual twist retained within a circular DNA species relies on DNA cyclization [25, 118]. Previous attempts to quantify DNA twist have circularized a known length of linear DNA with DNA ligase and then quantified the efficiency of circularization by calculating the fraction of all products formed that are monomeric circular species. By repeating this process as the length of DNA is sequentially increased by one base pair, a periodicity in the circularization efficiency is immediately apparent, which arises as a direct result of the helical pitch of the helix. That is, if the linear DNA is bent such that the two free ends are in sufficient proximity to each other to be ligated, but then are additionally required to twist into the proper alignment before for ligation can occur, the energy required to ligate the free ends together increases (or conversely, the efficiency of forming monomeric circular species decreases). Only by determining the periodicity of the cyclization efficiency (as a function of the length of the starting linear DNA) and then the relative placement of a given circular species can the absolute value of twist be determined. Complicating the interpretation of this measurement, the helical pitch is known to strongly depend on both the temperature and the ionic strength of the surrounding medium [120]. Therefore, determining the absolute twist from circularization efficiency measurements with an appreciable level of confidence requires that ligation-mediated ring closure take place in the buffer and at the temperature that will be used in all subsequent experiments. Unfortunately, the buffer conditions and temperature required for

ligation are often not easily adapted to other systems (e.g.- DNA ligase will not function in buffers without magnesium and are temperature sensitive).

CHAPTER 3

Single Molecule Characterization of Minicircle DNA Topology

As previously discussed, 100 bp, 106 bp, and 108 bp DNA minicircles have been efficiently produced as monomeric circular species (see Chapter 2). These minicircles were further shown to experience varying magnitudes and signs of torisonal stress despite marginal differences in the levels of bending stress (owing to a maximal change in sequence length, and hence bending curvature, of only 8%). However, it remains unclear how the circular DNA structure and topology within the minicircle templates are affected by the significant mechanical stresses that are sustained. Two possible consequences of bending and torsional stress, in particular, must be addressed before the DNA minicircles can be considered to be transcription templates with well-characterized mechanical properties: (1) the formation of noncanonical DNA structures, and (2) the presence of significant levels of supercoiling. The formation of noncanonical structures is especially problematic in light of evidence that torsional stress in DNA can be relieved by both DNA denaturation [116-117] and structural rearrangements of DNA into alternative conformations that are thermodynamically favored under conditions of mechanical stress. Alternative stress-induced conformations include the left-handed helical Z-form [123-126],

the triple-stranded H-form [127], and the base-flipped P-form [128-130]. Similarly, bending stress has been suggested to be relieved by DNA kinking [116]. If stress-induced kinking were to occur within the minicircle templates, for example, observations of transcriptional repression may be misinterpreted as directly related to strained (but continuous) DNA instead of simply being a reflection of the structural discontinuity introduced by unstacked bases within DNA. It is already known from digestion by the single-stranded DNA nuclease BAL 31 that 100c and 108c must partially denature with some observable frequency (Fig. 5c). What is not yet clear is the extent to which partial denaturation (or other stress-induced transitions) affect the mechanical properties of the DNA within the minicircles or change the overall topology.

3.1 Observing single DNA minicircles by cryo-electron microscopy

As an attempt to directly characterize the topological consequences of the bending and torsional stress sustained within the minicircle templates, I sought to observe the DNA topology of single minicircles using cryo-electron microscopy (EM). Cryo-EM has the distinct advantage of allowing biological specimens to be observed in nearly physiological conditions. Briefly, DNA samples in an appropriate buffer are applied to a holey carbon film grid, briefly blotted to remove most of the sample (leaving only a very thin surface layer that is roughly 50nm in thickness), then rapidly cooled to -160°C by plunging the blotted grid into liquid ethane. Under conditions where the rates of cooling are on the order of megakelvins per second, the buffer freezes so rapidly that ice crystals are

kinetically prevented forming and the biological sample is frozen within an amorphous, glass-like medium, a process known as cryo-vitrification. Vitrification enables interrogation of biological structures using transmission electron microscopy without having to contend with the optical aberrations that would otherwise be introduced by grain boundaries from ice crystals. For imaging DNA samples, the buffer conditions prior to vitrification are typically varied in the concentration of divalent cations (such as magnesium), such that comparisons can be made between the DNA conformation under conditions where the electrostatic repulsion of the negatively charged phosphate backbone is either strongly or weakly shielded.

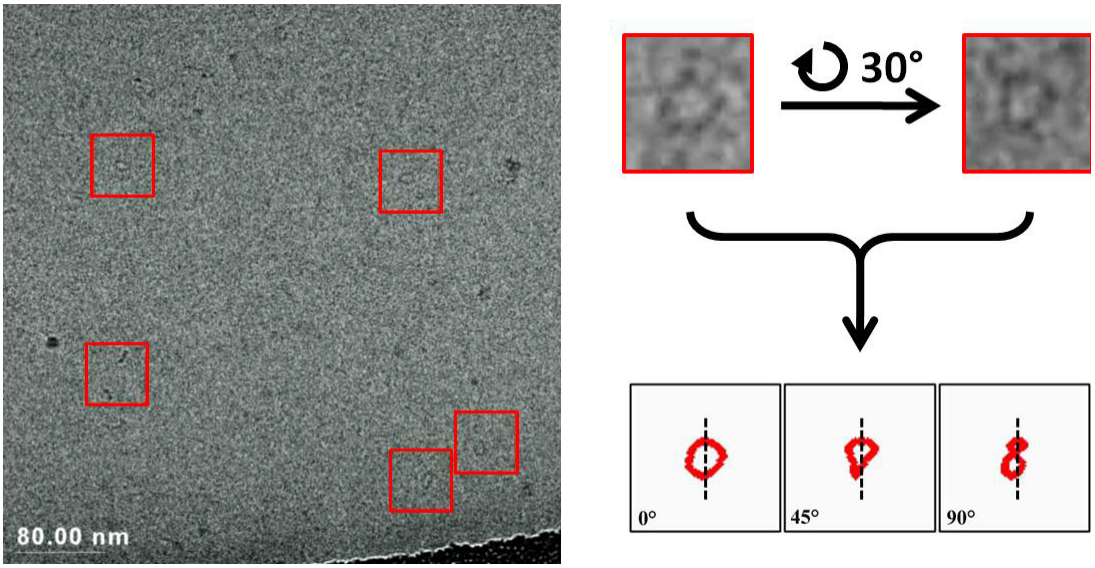

Figure 6. Single Molecule Observations of DNA Minicircle Topology Using Cryo-Electron Microscopy (Cryo-EM). **(left)** Cryo-EM stereo images were made by imaging single minicircles before and after rotating the sample by 30°. **(right)** From the image pairs, 3D reconstructions are calculated for each minicircle, shown here rotated successively about the vertical axis (black dashed line).

Following the methodology developed by Stasiak and colleagues to image small circular DNA [131-132], cryo-EM was successfully used to image and

quantitatively characterize the three DNA minicircles that were designed to serve as transcription templates (Fig. 6). After sample preparation and cryo-vitrification, single DNA minicircles were identified in the field of view and imaged onto a CCD camera (Fig. 6, left). The specimen holder was then tilted by 30° and the minicircles were reimaged (Fig. 6, right). Given the known angle of tilt between the stereo pair of images, the 3D minicircle topology can be reconstructed using an algorithm developed specifically for cryo-EM observations of DNA [133].

3.2 Characterizing the topology of DNA minicircles from 3D reconstructions of stereo image pairs

The three-dimensional space curves describing the topology of the DNA minicircles (Fig. 7) reveal several notable features. It is immediately apparent from the galleries of reconstructed topologies that thermal energy is sufficient to overwhelm the inherent mechanical rigidity of the minicircles. That is, despite the high bending stiffness of the sub-persistence length DNA, the considerable influence of the thermal environment gives rise to observed bending behaviors that are relatively uncorrelated between minicircles of the same type. If, in the

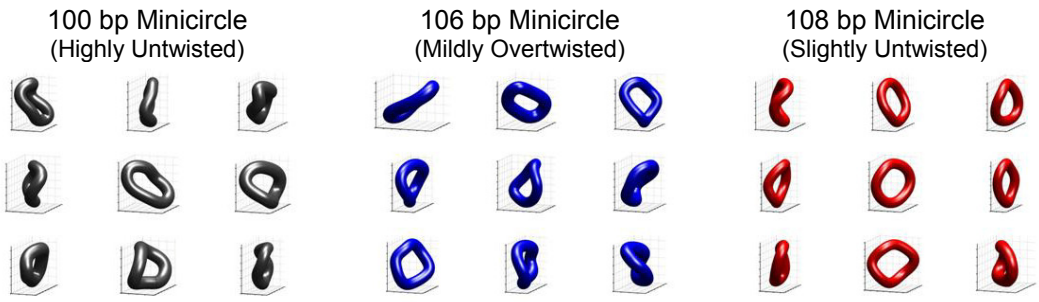

Figure 7. 3D Reconstructions of DNA Minicircles. Nine reconstructions from each minicircle species are shown (black: 100c, blue: 106c, red: 108c).

extreme case, thermal energy was significantly lower than the energy required to bend the DNA templates (e.g.- at temperatures approaching absolute zero), it would be expected that all reconstructed topologies for a given minicircle species would be identical. The single observed topology, in this case, would simply reflect any sequence-dependent intrinsic curvature that is present in the given DNA sequence. From the cryo-EM observations, it is clearly not the case that a substantial fraction of DNA minicircles on the order of 100 bp in size adopt a single, preferential topology. It is furthermore evident that each 3D minicircle reconstruction displays significant topological differences that are attributed to the influence of thermal energy.

3.3 Implications for stress-induced helical destabilization within DNA minicircles

Aside from highlighting the influence of thermal energy on the minicircle topology, the 3D reconstructions also provide information regarding the possible formation of sites of enhanced flexibility as a topological consequence of bending and torsional stress (e.g.- DNA undergoing a stress-induced transition into a preferred noncanonical conformation). While the resolution of cryo-EM is not sufficient to describe the atomic details of the DNA structure (and hence is not capable of providing direct evidence of the appearance of alternative DNA structures), the reconstructed topology does provide insight into the overall mechanical state of the template under significant bending and torsional conditions. For example, if a single site along the DNA template transitioned into an alternative configuration (such as kinked or denatured DNA, or other

noncanonical, hyper-flexible forms of DNA), then the mechanical properties of this site are likewise expected to change. It is reasonable on energetic grounds alone to predict that any transition of DNA into alternative structures must do so in order to relieve mechanical strain energy.

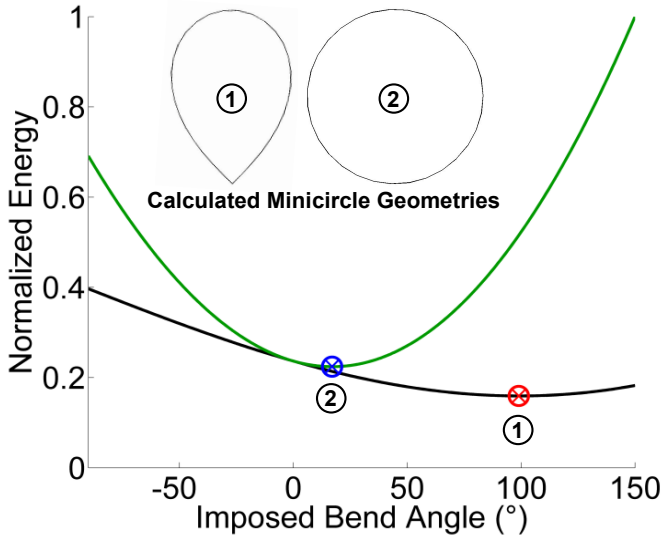

Figure 8. Simulation of the Energetic Cost to Bend a 100 bp DNA Minicircle at a Single Site. The results highlighted are: **(1)** the DNA transition site becomes infinitely flexible (black line), in which case the expected bend angle is 99° (red circle), **(2)** the bending stiffness of the transition site in a 21 node minicircle is unchanged (green line), in which case 17° is expected (blue circle).

It is unlikely that any transition *increases* the mechanical stiffness (and hence bending or torsional energy) at the transition site. Therefore, as a number of experimental studies have concluded [123-130, 134-136], any stress-induced transition is expected to necessarily introduce flexibility into the DNA.

In order to develop some intuitive understanding for the possible topological consequences of structural transitions in DNA that increase DNA flexibility, let us consider what happens if a single site of enhanced flexibility appears within a sub-persistence length DNA minicircle (Fig. 8). To both motivate this discussion and to illustrate the conclusions quantitatively, an elastic rod model has been used to theoretically predict the energetic cost of bending a single site in the DNA, considering cases where the site becomes infinitely

flexible and where the bending stiffness remains unchanged. The simulation begins by first approximating a 100 bp DNA minicircle by 21 rigid segments joined by linear, elastic springs with known bending stiffness (k). An angular constraint (θ_c) is then imposed at one node in this spring system, and the remaining bend angles (θ) are determined using the imposed boundary condition. The energetic cost (E) for the imposed angular constraint is then calculated from Hooke's Law:

$$E = \frac{1}{2}\sum k \cdot \theta_n^{\,2} \qquad \text{(Eq. 1)}$$

For a perfectly circular, intact DNA template a global energetic minimum occurs at a bending angle of 17° (which is a function of the number of nodes representing the circular topology; this bending angle approaches 0° as the length of the rigid segments decreases) (Fig. 8, blue circle). The quadratic energy potential, plotted as a function of bending angle (Fig. 8, dashed line), illustrates the energetic cost to bend one site within an intact DNA minicircle. In the limit where a single site within the template becomes infinitely flexible, a teardrop-like geometry would form and the bending angle at the site of flexibility would be equal to 99° (Fig. 8, red circle). In addition, the quadratic nature of the energetic potential becomes significantly shallower, which suggests that the teardrop-like structure is more likely than the intact minicircle to explore a larger range of bending angles under the influence of thermal energy.

To compare the simulated data to the 3D reconstructions created from cryo-EM stereo image pairs (Fig. 7), the curvature at 21 points along each experimentally derived space curve was calculated and compiled into curvature distributions for each species of minicircle (Fig. 9). As can be readily seen from the distributions, 100c and 106c show the most similarities. By applying the nonparametric Kolmogorov-Smirnov (K-S) test to the curvature distributions, the 100c and 106c minicircles are statistically drawn from the same distribution with 95% confidence. This conclusion is supportive of the theoretical predictions for the relative levels of twist, which suggest that 100c and 106c should sustain the highest levels of under- and overtwist, respectively (Table 1). A noteworthy observation from the curvature distributions is that the bending predicted to occur in response to a site of enhanced flexibility (99°, see Fig. 8) is not observed. However, this could be attributed to a number of factors, ranging from reconstruction errors filtering out sharp bending to the fact that sites of finite flexibility (as opposed to the infinite flexibility considered in the simulation) will bend DNA to a lesser extent than is predicted from theory for a planar minicircle

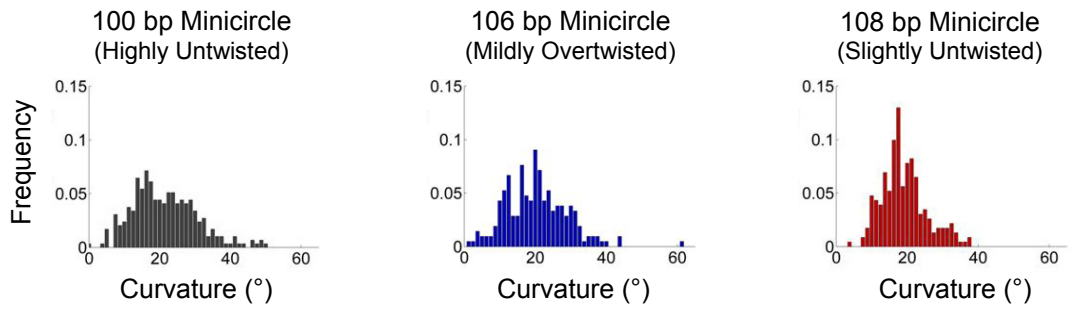

Figure 9. Curvature Analysis of DNA Minicircle Reconstructions. From the 3D space curves defining the best-fit solution to the stereo pairs of images collected by cryo-EM, the bending curvature was calculated from 21 points along each reconstruction. Shown are the compiled probability distributions from the observations (colored as in Fig. 7).

with a perfectly flexible kink (Fig. 8).

Minicircle Species	100c	106c	108c
Average Bend Angle	22.5°	20.3°	19.1°
Standard Deviation	9.5°	8.6°	6.3°
Sample Size	14	10	11

Table 2. Curvatures Calculated from 3D Reconstructions of DNA Minicircles. The average and standard deviation of bend angles was calculated for the three DNA minicircle species at 21 nodes along each 3D space curve reconstructed from the cryo-EM data.

Since the identification of a single point along the minicircle that appears to be kinked (i.e.- displays high curvature resulting from stress-induced helical destabilization) is prone to reconstruction error, it is perhaps more appropriate to attempt to infer DNA kinking from the other "signatures" of enhanced flexibility. To illustrate this point, consider how the curvature of the intact (non-kinked) portion of the minicircle might react to the appearance of a single, hyperflexible site. Using the elastic rod model previously described (Fig. 8) as a first-order approximation, the bending curvature distributions have been calculated for a perfectly planar minicircle in which a single site of flexibility is introduced and kinked by some known angle (Fig. 10). To account for the possibility that a "real" DNA kink is likely not to be infinitely flexible, the kinking angle was varied from 0° (approximating a perfectly circular topology, i.e.- the stiffness of kinked and intact DNA are equal) to 100° (roughly equal to the previously predicted bending angle for an infinitely flexible kink, see Fig. 8).

On energetic grounds, it is expected that if a kink does appear within a minicircle (for which out-of-plane bending is ignored), the kinking angle should lie somewhere between 0° and 100°, depending on the flexibility of the kink. Beginning with the perfectly circular 0° case, only a single bending angle (17°) is

represented in the curvature distribution (reflecting the unchanging curvature along a smooth circle that is only represented by 21 nodes). As the kinking angle is increased, it is apparent that the bending angles for the remaining intact minicircle DNA gradually broadens and follows a roughly uniform distribution. Within these curvature distributions, the maximum and minimum angles appear with elevated frequencies because in the teardrop-like structure, the DNA nearest the kink is relatively straight (i.e.- the minimum curvature) while the DNA farthest from it smoothly approximates a circle (i.e.- the maximum curvature). At 100°, the curvature distribution spans virtually the entire range from 0° to 25°.

Relating the simulated curvature distributions to the experimentally-derived distributions (Fig. 7) is not trivial, largely owing to the "real-world" nature of sub-persistence length DNA that could be experiencing sequence-dependent curvature, anisotropic bending and torsional stiffness, or other unexpected mechanical features. Nonetheless, one important conclusion does emerge: the simulation suggests that kinking is not stably maintained in any of the three minicircle species that have been observed. The curvature distributions shown in Figure 10 suggest that if the DNA

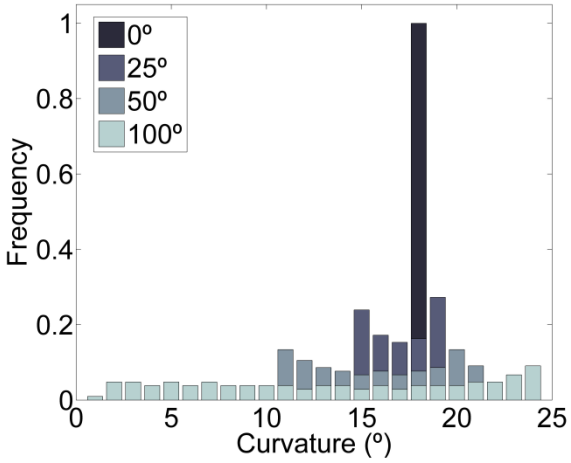

Figure 10. Simulated Curvature Distributions for a Planar Minicircle as a Function of the Bend Angle Introduced by a Single Kink. Shown are four overlaid curvature distributions for a 100 bp DNA minicircle in which a single site is kinked by 0°, 25°, 50°, and 100°. Note that the distributions only include the non-kinked DNA, and do not include the angle from the kink.

bending stress within DNA minicircles is partially relieved by DNA kinking (or more generally, any structural transition that leads to enhanced flexibility), it is expected that the curvature distribution would reveal this indirectly through the curvature distribution of the non-kinked DNA. That is, the mechanical deformation of the intact DNA is expected to naturally respond to a site of enhanced flexibility, resulting in the appearance of a weighted uniform curvature distribution (as shown in Fig. 10). However, the three experimental distributions are clearly not uniformly distributed, nor do they reveal elevated frequencies of occurrence by the maximal and minimal bending angles. These observations are therefore interpreted as evidence in support of the conclusion that kinking does not occur.

It should be noted that the primary assumption of the simulation (the minicircle remains planar) is supported by many previous experimental studies that conclude that sub-persistence length, circular DNA is expected to experience virtually no out-of-plane bending under the levels of torsional stress maximally allowed within the minicircle templates (±½ turns of twist, owing to the use of ligation-mediated ring closure [116]) [65-67]. In apparent contrast to this assumption, many of the individual 3D reconstructions of minicircles (from which the curvature distributions were derived) clearly appear to experience out-of-plane bending. However, it must be remembered that the out-of-plane bending captured by the rapid freezing of the sample during cryo-vitrification is presumably induced only by thermal energy and is therefore short-lived, as evidenced by the fact that out-of-plane bending shows virtually no correlation

between samples imaged under identical conditions. Regardless, even if the simulation did consider the out-of-plane bending curvature during kinking, the conclusions are not expected to fundamentally change. That is, a weighted uniform distribution would still be anticipated to result from kinking, though the absolute values of the bending angles will naturally be different than the in-plane case. Therefore, the simplifying assumptions in the numerical simulation approximating DNA kinking appear to be reasonable.

3.4 Implications for DNA supercoiling within small minicircles

The 3D reconstructions also provide information regarding the DNA supercoiling present within the DNA minicircles in response to the differences in torsional stress sustained within the three minicircle species. DNA supercoiling is quantified mathematically by the scalar quantity known as writhe, formally defined as the average number of signed crossings of a space curve observed from all possible viewing angles [137]. Within a closed topological domain (such as a DNA minicircle), the sum of the writhe and twist (collectively known as the linking number) is conserved. Therefore, any twist applied to DNA can be theoretically converted into writhe, governed only by the underlying energetic costs associated with the conversion. Since writhe is fundamentally related to the bending, and the bending persistence length (stiffness) of DNA is roughly ⅔ that of its torsional counterpart, it is possible that torsional stress can be relieved by forming more energetically favorable bending (writhe). However, it is a striking feature of the 3D reconstructions that despite the rather significant differences in

the predicted twist sustained within the DNA minicircles, supercoiling (writhe) is notably absent.

Minicircle Species	100c	106c	108c
Average Writhe	0.017	0.018	0.009
Standard Deviation	0.072	0.063	0.028
Sample Size	14	10	11

Table 3. Writhe Calculated from 3D Reconstructions of DNA Minicircles. Following the methodology detailed in [137], the average and standard deviation of writhe was calculated for the three DNA minicircle species that were observed by cryo-EM.

The calculated writhe for the three species of DNA minicircle that have been observed by cryo-EM are shown in Table 3. As is immediately apparent, the average writhe is very close to zero, indicative of the minicircles remaining, on average, close to planar. In all cases, the standard deviation of the writhe is at least three times larger than the mean. To gain some theoretical perspective on the significance of these values, an elastic rod model has been developed to describe the thermal fluctuations of small DNA minicircles [138-139]. Extending this model to describe the average writhe, $\langle Wr \rangle$, and variance of writhe, σ_w^2, of a thermally oscillating minicircle [66-67], given the number of base pairs (N), ratio of torsional to bending rigidity (Ω = 1.4), bending persistence length (a = 50nm), separation between adjacent base pairs (Δs^o = 0.34nm), and linking number (ΔL_k), it has been shown that:

$$\langle Wr \rangle = \frac{3\,\Omega\,N}{8\,\pi^2}\left(\frac{\Delta s^o}{a}\right)\Delta L_k \qquad \text{(Eq. 2)}$$

$$\sigma_w^2 = \frac{3\,N^2}{32\,\pi^4}\left(\frac{\Delta s^o}{a}\right)^2 \qquad \text{(Eq. 3)}$$

From these two equations, it is immediately apparent that the average writhe, but not the variance, is directly proportional to both the number of base

pairs in a minicircle and the linking number (which in this case, given low magnitudes of writhe, can be thought of as the residual twist remaining within a minicircle template). However, as previously discussed, the absolute magnitude of twist is not precisely known for the three minicircles observed (see Chapter 2.3). Nonetheless, upper limits on the residual twist can be reasonably imposed by the use of ligation-mediated cyclization of a nicked circular species to form the minicircles. That is, it is unlikely that the twist within the minicircles can be larger than 180° because it is energetically unfavorable to ligate a nick that requires more than one-half turn to bring the free ends into proper registration (in this case, the nick would be energetically favored to rotate in the opposite direction, requiring less twist to bring the free ends close enough for ligation to occur). Therefore, if we are to consider the range of possible linking numbers that could plausibly be represented, and account for the known sign of the twist detected by BAL 31 (Fig. 5), then the average writhe could be at most -0.018 for 100c, +0.019 for 106c, and -0.020 for 108c (Table 4). The standard deviation of writhe (σ_w), on the other hand, is predicted to be 0.021 for 100c, 0.022 for 106c, and 0.023 for 108c, regardless of the twist sustained within the minicircle. The uncoupling of σ_w from the linking number is reflective of the expectation that the variance of the writhe over time will be driven only by the influence of the thermal environment.

Minicircle Size	100 bp	106 bp	108 bp
Average Writhe	[0,-0.018]	[0,+0.019]	[0,-0.020]
Standard Deviation	0.021	0.022	0.023

Table 4. Theoretically Predicted Writhe in DNA Minicircles. The range of possible values for the average writhe and the standard deviation was calculated for each minicircle, as described in [66].

It is apparent upon inspection that the averages and standard deviations calculated from the experimental data (Table 3) depart significantly from theory (Table 4) in several cases. For example, the average values for writhe for 100c and 108c (which should be negative according to the BAL 31 data, which demonstrates that negative twist is sustained in each) are actually positive. In addition, the standard deviations observed in the cryo-EM data for 100c and 106c are roughly three times the maximum values predicted from theory. However, before drawing conclusions from these observations, we must consider the two primary experimental limitations with the cryo-EM data: (1) errors arising from 3D reconstruction, and (2) small numbers of observations. The calculated means and standard deviations of the writhe are undoubtedly prone to some level of experimental error, which is expected to arise largely as a result of reconstructing 3D space curves from noisy stereo image pairs. In addition, any error is likely to be exaggerated by the relatively few numbers of observations. Unfortunately, however, there is currently no positive control that can be easily implemented to quantify the expected levels of error, and imaging single DNA minicircles that are 10nm in diameter straddles the lower limits of resolution using cryo-EM. Therefore, any confidence in the conclusions drawn from this study should be tempered accordingly.

Since writhe is defined as the number of times the projection of a space curve crosses itself (as viewed from all perspectives), and each 3D reconstruction is composed of 21 points (nodes) along each minicircle topology, errors in calculating the writhe are potentially enhanced by the independent

localization error for each point along the reconstructed space curve. It should be noted that the same reconstruction errors are also expected to decrease confidence in the bending curvature calculated at individual nodes, but as previously discussed, regions of elevated flexibility (i.e.- high curvature owing to helical destabilization) are expected to be revealed by the curvature distributions, not the curvature at an individual node (see Chapter 3.3). Since the reconstruction at every node will be subjected to the same fundamental sources of error, the curvature distributions are expected to be less influenced by error than either the writhe or the curvature at individual nodes.

3.5 Discussion and conclusions

Presented here are the first direct, single molecule observations of the topological effects of torsional stress on sub-persistence length circular DNA. Despite the largely unavoidable sources of experimental error, several major conclusions emerge from the cryo-EM observations. First, the experimental data lend support to theoretical predictions that sub-persistence length DNA minicircles will remain planar on average, even while sustaining moderate levels of torsional stress. Second, despite the observation that untwisted minicircles will be prone to localized base pair melting (as assayed by BAL 31 nuclease), the helically destabilized regions appear not to contribute significantly to the observed curvature. This conclusion is bolstered by the comparison of the curvature distribution between the most overtwisted (106c) and most untwisted (100c) minicircle, which are statistically indistinguishable (as determined by the

Kolmogorov-Smirnov test). Since the curvature distribution of a highly untwisted (100c) minicircle known to experience local helical destabilization appears no different than an overtwisted (106c) minicircle, it is concluded that the observed bending curvatures within these two minicircles do not arise from destabilization of the helical structure. Adding to this argument is the observation that the slightly untwisted minicircle (108c) minicircle, which has proven susceptible to BAL 31 digestion (indicative of helical destabilization), displays less bending curvature than either the 100c and 106c species.

One important consideration in the interpretation of the curvature and writhe data is that the two are not entirely uncorrelated. That is, while it is possible for a DNA minicircle to experience bending in the absence of writhe (e.g.- a circle can be deformed into an ellipse while remaining planar), writhe cannot occur without a corresponding increase in curvature. Therefore, the observation that the most torsionally stressed minicircles (100c and 106c) appear to display comparable levels of curvature and standard deviations of writhe is not coincidental. The standard deviation of writhe, it should be remembered, is a statistical representation of the fluctuations in the out-of-plane bending (attributed directly to writhe) as the minicircles are deformed by the influence of thermal environment. Therefore, minicircle species that are observed to have a greater standard deviation of writhe will be naturally expected to experience elevated levels of curvature. This correlation is evident in the analysis of the 3D reconstructions from the cryo-EM data of DNA minicircles (Tables 2 and 3). Therefore, the elevated levels of bending observed in the highly untwisted (100c)

and overtwisted (106c) minicircles can at least partially be explained by a likewise increase in the standard deviations of writhe (which are both roughly three times that of the 108c species). Taken together, these conclusions also lend support for the possibility that 108c sustains the least torsional stress of the three minicircles tested. This particular conclusion is highly relevant for any future interpretations of transcription data from these minicircle templates since the BAL 31 susceptibility data is incapable of determining if 106c is more overtwisted than 108c is untwisted (which now appears to be the case).

The conclusions from this imaging study also shed light on the use of BAL 31 nuclease, particularly in regards to recent suggestions that BAL 31 is capable of specifically recognizing kinked DNA in minicircles [116]. In an attempt to characterize the mechanical threshold beyond which bending DNA will induce kinks to form, Du *et al.* created a library of DNA minicircles of varying sizes and used BAL 31 digestion to assay for kink formation. However, BAL 31 nuclease is widely accepted to be a single-stranded DNA nuclease [140], and has not been reported prior to this study to possess the ability to specifically digest kinked DNA. The ability of BAL 31 to recognize single-stranded DNA has proven capable of degrading the weakly hydrogen-bonded or fully denatured (single-stranded) regions within untwisted DNA [117]. Indeed, Du *et al.* report that the minicircles that are predicted to be untwisted are also susceptible to BAL 31 digestion. However, the authors appear to over-interpret the nuclease data to be specifically indicative of DNA kinking. While it is reasonable to hypothesize that kinked DNA will be susceptible to BAL 31 nuclease, and it is equally plausible

that the 64 bp minicircles have in fact exceeded the bending threshold required to kink DNA, the authors speculate that the susceptibility of a 100 bp minicircle to BAL 31 is evidence that it also kinks. However, the cryo-EM data presented here strongly suggest otherwise, instead favoring the interpretation that the negative torsional stress within 100 bp minicircles simply denatures local regions of the minicircle. This melted DNA can be recognized and digested by BAL 31, but in contrast to the expected mechanical features of a kink, does not appear to significantly enhance the flexibility of the DNA. The cryo-EM data clearly show the 100 bp and 106 bp minicircles (which are susceptible and resistant to BAL 31, respectively) display comparable topological features (curvature and writhe), and do not support the conclusion of Du *et al.* that 100 bp minicircles are kinked. Therefore, while the use of BAL 31 to assay for kink formation may be appropriate, the cryo-EM data presented here strongly suggest that BAL 31 nuclease susceptibility alone is not sufficient evidence for DNA kinking and should be cautiously interpreted without supporting, complementary structural data.

With regards to the intended use of DNA minicircles as transcription templates, perhaps the most important conclusion from the direct observation of single DNA minicircles by cryo-EM is that sub-persistence length, circular DNA can be designed to sustain well-defined levels of bending and torsional stress. By not appreciably relieving either bending stress (by transitioning into hyperflexible conformations) or torsional stress (through the generation of writhe), DNA minicircles on the order of 100 bp in size can be effectively used to control and

maintain the mechanical stress state of a DNA template prior to transcription by RNA polymerase. However, two limitations to using minicircle templates as a general solution to controlling the stress state should be noted. First, while these observations describe the minicircle templates prior to transcription, the mechanical state *during* transcription remains uncharacterized. Therefore, it is possible that the changes to the mechanical state of DNA during transcription (e.g.- untwisting the DNA to form an open complex, bending the DNA at the RNAP binding site, etc.) could exceed the energetic barrier to form undesirable structural features within the template. Second, the size of the minicircle template matters. Larger minicircles will inevitably enter a region in which supercoiling can be tolerated, and smaller minicircles will eventually form noncanonical structures, such as the DNA kinks reported by Du *et al.* [116]. These experimental considerations emphasize the need to perform structural characterizations akin to the present imaging study before DNA minicircles are used in assays attempting to relate the mechanical features of DNA to enzyme function.

CHAPTER 4

Transcription of Highly Bent DNA

The broad hypothesis providing the central motivation for this work is that mechanical stress, and in particular bending stress, applied to the DNA template is sufficient to repress transcription by RNA polymerase (RNAP). However, the regulation of RNAP activity can occur at several kinetic checkpoints during the multistep transcription cycle. Productive transcription requires the sequential progression of RNAP through six evolutionarily conserved phases of transcription: promoter recognition, DNA template binding (closed complex formation), isomerization (open complex formation), initiation, elongation, and termination [26, 141]. Transcription initiation, elongation, and termination are can be treated as irreversible reactions, though initiation suffers from a tendency to undergo a behavior that is unique among RNAP's known as abortive cycling. Abortive cycling involves the polymerase prematurely terminating and restarting transcription from the transcription start site, and arises from the thermodynamic stability of the nascent RNA transcript when it is hybridized to the DNA strand used by RNAP as a complementary sequence template [97, 142-143]. Premature termination during abortive cycling occurs when the reannealing of the nontemplate, single-stranded DNA (which is sequence-identical to the RNA

transcript) fails to displace the transcript from the template strand. Consequently, a phenomenon known as DNA “scrunching” occurs in which the polymerase continues to transcribe downstream DNA while maintaining contact with the promoter [90, 93]. DNA scrunching will continue until the transcribing RNAP either prematurely aborts transcription (when the RNA transcript fails to displace from the template DNA) or transitions to elongation (when the 5′ end of the nascent RNA successfully follows the transcript exit tunnel within the RNAP structure [144]). Beyond the transcription of a RNAP-dependent number of base pairs, the nascent transcript exits the polymerase and a significant conformational rearrangement occurs within the RNAP. This rearrangement significantly stabilizes the nucleoprotein complex and signals the beginning of the highly processive elongation phase of transcription. The following study focuses specifically on the effects of mechanically stressed DNA on the elongation phase of transcription.

4.1 Experimental limitations of conventional transcription techniques call for the development of a more sensitive assay

The general approach used to characterize the effects of highly bent DNA on transcription was to challenge RNAP to utilize DNA minicircles as transcription templates and quantify any differences in the observed elongation behavior (relative to wild-type RNAP activity). As previously discussed, the single-subunit bacteriophage T7 RNAP was chosen as a model system (see Chapter 1.3), and DNA minicircle templates were therefore designed to include the 17 base pair

consensus sequence for the T7RNAP Φ10 promoter (TAATACGACTCACTATAGGGAG, underlined bases are the initially transcribed bases) [145]. However, minicircle templates on the order of 100 bp are technically challenging to produce (see Chapter 2.1), and so unlike most other transcription studies, assays characterizing transcription from minicircle templates will be inherently limited by the concentrations of the DNA template. This experimental limitation poses a significant problem with respect to the compatibility of techniques commonly used to measure transcription from circular DNA templates.

Typically, transcription assays are performed in the presence of radioactively labeled [γ-^{32}P]GTP (resulting in 5′ labeling of transcripts with a single radioactive phosphorus), which is followed by separation of the transcripts by molecular weight using gel electrophoresis under denaturing conditions (typically, the gel includes high concentrations of either urea or formaldehyde). The most sensitive method available to observe transcription involves defining a transcription sequence that allows the RNAP to transition into the elongation phase utilizing only three of the four available ribonucleotide triphosphates (NTP's). This predesigned sequence ends with RNAP requiring the fourth NTP, which is intentionally made available only as dideoxyribonucleotide triphosphates (ddNTP's) [55]. Consequently, since ddNTP's interrupt transcript elongation, all transcripts produced from elongation will be identical in size and can then be separated by denaturing gel electrophoresis to produce high contrast bands. However, this method inherently suffers from two primary issues: (1) the ddNTP

is expected to significantly repress the rate of nucleotide incorporation (the K_m for dNTP's by T7RNAP is roughly 100-fold larger than for NTP's [146]), thus precluding the quantification of elongation velocity under physiological conditions, and (2) the dependence on transcription of short transcripts prevents characterization of the inherent RNAP elongation complex processivity (or alternatively, the dissociation rate) from circular templates.

Characterizing the unhindered elongation behavior of T7RNAP, including elongation velocity and processivity, requires the ability to quantify transcript production from T7RNAP under (near) physiological conditions. The transcripts that will be produced from circular DNA templates during the reaction will therefore be both very long (for highly processive polymerases such as T7RNAP) and variable in length (owing to the uncoordinated termination of individual polymerases from the template, which is governed only by the dissociation kinetics of the enzyme). Consequently, the electrophoretic separation of transcripts will result in a "smear" that directly reflects the size distribution of the transcripts [147-149]. Enzyme kinetics predicts that any well-behaved, processive RNAP where termination can be described by a single dissociation rate will yield an exponential distribution of product lengths. However, due to the finite separation range of conventional gel electrophoresis and the highly processive nature of elongation by T7RNAP (which can easily transcribe more than 30,000 nucleotides before dissociating [150]), the characteristic processivity is difficult to measure in an absolute, quantitative sense. Instead, the distribution of long RNA products produced from circular DNA templates is typically only

used to qualitatively compare the processivity of RNAP's as a function of some variable of interest. Though the smear method does present an improvement over the use of short defined sequences and ddNTP-induced termination, the broad distribution of transcript lengths requires that very high concentrations of DNA template be used. Therefore, in light of the fact that minicircle transcription assays are inherently limited by the concentration of the template, use of gel electrophoresis-based methods are at present incompatible with characterizing T7RNAP transcription elongation from minicircle templates.

4.2 A novel solution: A molecular beacon-based minicircle transcription assay

In order to characterizing the effects of template mechanics on RNAP activity, a robust method capable of quantifying transcription from small minicircles is required. Given the relative difficulty in producing homogenous solutions of minicircle templates, the method must also be sensitive enough to measure transcription from nanomolar concentrations of template (roughly three orders of magnitude lower than conventional techniques). Perhaps the more pressing challenge, however, is for the assay to be capable of measuring transcripts of varying size without the loss in sensitivity that would inherently accompany techniques which size sort transcripts, such as gel electrophoresis. Molecular beacons (MB's) present an ideal solution to these experimental limitations. MB's are single-stranded oligonucleotides labeled on one end with a fluorescent dye and on the other with a quencher that prevents fluorescence emission from the dye when the two labels are in close proximity (Fig. 11). MB's

are designed such that 4-6 bases on each end are complementary and capable of hybridizing, forming a stem-loop structure that positions the quencher close enough to the fluorophore to render the beacon effectively non-fluorescent. The 20-30 bases that are looped by the hybridization of the complementary stem-forming ends include a recognition sequence that remains single-stranded and capable of hybridizing to a complementary, single-stranded target sequence (e.g.- an RNA transcript). Hybridization of the MB's to a target sequence in solution mechanically stiffens the looped region as it becomes double-stranded DNA, forcing the stem to melt and displace the quencher from the fluorescent dye. Consequently, MB's can report the presence of a target RNA sequence by emitting a fluorescence intensity that is proportional to the concentration of target molecules in the solution.

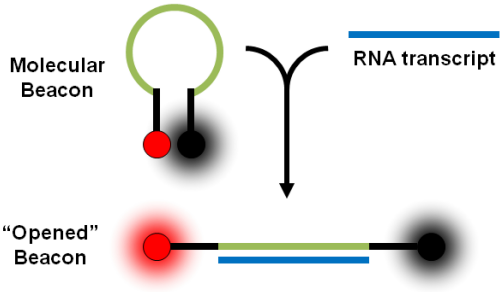

Figure 11. Molecular Beacon Methodology. Molecular beacons are stem-loop forming oligonucleotides that are end-labeled with a fluorophore (red) and a quencher (black). The close proximity of the fluorophore to the quencher makes the beacon effectively not fluorescent until a complementary RNA transcript (blue) hybridizes with the recognition loop (green).

MB's are particularly advantageous in the context of a minicircle transcription assay because they are capable of faithfully reporting the total number of times the target sequence was transcribed by RNAP, irrespective of the total size of the transcript. This unique characteristic of MB's naturally accounts for the fact that T7RNAP is able to transcribe a DNA minicircle (and hence transcribe through a target sequence) multiple times before dissociating

from the template. Unlike the conventional methods discussed earlier, MB's are capable of quantizing the transcription signal, effectively reporting the number of times the T7RNAP completes an entire round of transcription from a given circular template. So long as the polymerase makes many rounds on the template, the number of nucleotides can therefore be calculated by multiplying the measured concentration of fluorescing MB's by the number of nucleotides transcribed per complete round of the minicircle template (i.e.- the number of base pairs in the minicircle). The simplifying assumption that the T7RNAP makes many rounds on a template accounts for the fact that the number of nucleotides transcribed prior to transcription of the first target sequence in a minicircle template will be slightly less than the number of base pairs in the minicircle (though the data could be normalized accordingly in cases where few rounds are made by T7RNAP on average).

One important caveat regarding the use of molecular beacons, however, is that MB's synthesized entirely of unmodified, single-stranded DNA can be nonspecifically recognized by T7RNAP, resulting in errant transcription of the MB sequence itself (leading in turn to a background level of transcription, even in the absence of template) [151]. This errant transcription signal proved intolerable given (what proved to be) the greatly reduced transcription by T7RNAP from minicircle templates. To improve the sensitivity of the assay, MB's were synthesized such that every base was methylated at the 2′ position (2′-O-methyl-MB's). The 2′ methylation of the ribose moiety has been previously demonstrated to prevent recognition by T7RNAP while not interfering with the hybridization of

RNA transcripts to the recognition loop [151]. Using 2′-O-methyl-MB's (henceforth simply referred to as MB's), the activity of T7RNAP from minicircle templates can be characterized virtually free of background transcription.

To characterize the dependence of T7RNAP activity on DNA template mechanics, the observations were specifically directed to the elongation phase of transcription by employing a well-established technique utilizing heparin (Fig. 12). Heparin is a potent inhibitor of T7RNAP binding to DNA, and thus restricts all observed transcription activity to T7RNAP that are already bound to the template (such as elongation complexes). The minicircle templates were specifically designed such that the first 20 bases could be transcribed in the presence of only ATP, CTP, and GTP (Fig. 13a). Since T7RNAP transitions into a stable elongation complex beyond the synthesis of 12 nucleotides of RNA, and the polymerase is allowed to transcribe 20 nucleotides in the minicircle templates, the stable complex will stall when it encounters a base in the template DNA for which the required complementary NTP (UTP) is not available. Consequently, elongation complexes can be preloaded onto a given minicircle template by first

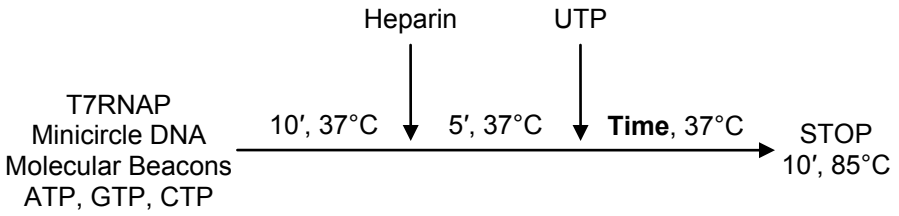

Figure 12. Experimental Procedure for Quantifying T7RNAP Elongation. Stalled T7RNAP elongation complexes were preloaded onto minicircle DNA templates during a 10′ incubation in the absence of UTP. Heparin was then added and incubated with the stalled elongation complexes for 5′. Transcription elongation from stalled T7RNAP remaining associated with the template was initiated by the addition of UTP and allowed to continue for a variable amount of time.

incubating T7RNAP with the template in the absence of UTP (Fig. 13b). Then, following the preloading incubation, the addition of heparin (Fig. 13c) will effectively restrict all free T7RNAP from binding the template. Finally, adding UTP to the solution will initiate transcription from the stalled elongation complexes (Fig. 13d). As a result, transcription will ensue only from polymerases that had successfully transitioned into a stable elongation complex during the preloading incubation.

Transcription activity was quantified from the fluorescence emission resulting from hybridization of MB's to a specific target sequence within the synthesized transcript (Fig. 13e). This highly sensitive measure of transcription activity is especially advantageous in this assay because it is desirable to use low concentrations of template, particularly given the challenge to produce large quantities of a template that is energetically unfavorable to form. The resulting fluorescence accumulates over time, owing to the high stability of the hybridized transcript-beacon complex, and serves to report the number of complete rounds made by an elongating T7RNAP transcribing a given minicircle template (Fig. 13f).

Elongation-specific transcription assays make possible the observation of several T7RNAP behaviors of interest. The magnitude of the fluorescence signal reflects both the number of transcribing polymerases and the average elongation velocity from a given transcription template. Since all elongating polymerases are prohibited from rebinding the template in the presence of heparin, the accumulation of fluorescence over time is governed by the kinetics for a single

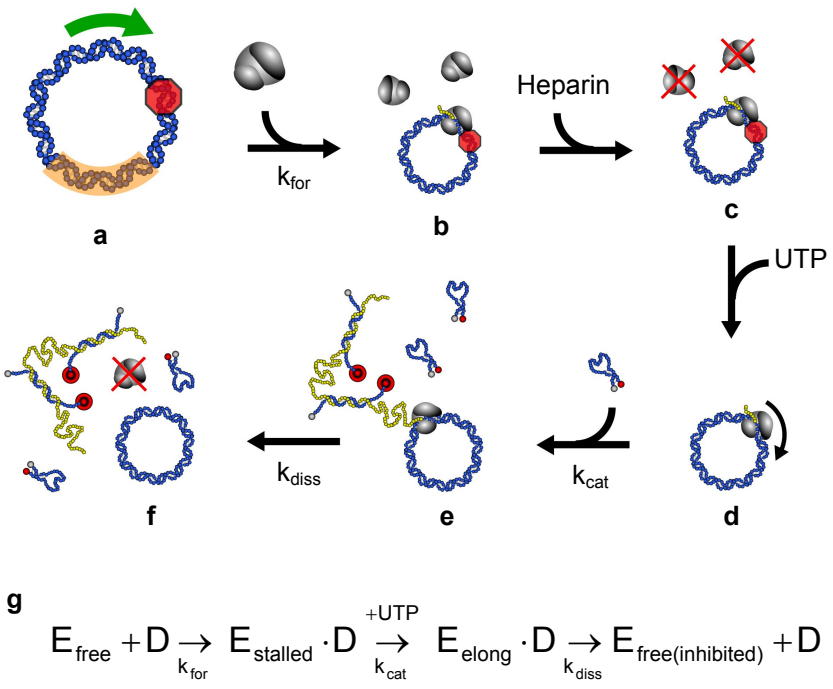

Figure 13. Overview of the Minicircle Transcription Assay. **(a)** The minicircle template contains a T7RNAP promoter sequence, a site at which T7RNAP elongation complexes will stall in the absence of UTP (red octagon), and a target sequence (orange line) from which the corresponding RNA transcript will hybridize to a designed molecular beacon sequence. **(b)** T7RNAP ("E_{free}") is incubated with the DNA template ("D") without UTP, and forms stalled elongation complexes ("$E_{stalled}$") at some rate of complex formation (k_{for}). **(c)** Heparin addition inactivates unbound T7RNAP. **(d)** UTP addition initiates transcription from active elongation complexes ("E_{elong}"), which synthesize RNA at the elongation velocity (k_{cat}). **(e)** Molecular beacons hybridize to target transcript sequence. **(f)** Elongating T7RNAP detach from the template at some rate of dissociation (k_{diss}). **(g)** A simplified kinetic model governing T7RNAP behavior.

step, irreversible reaction. The fluorescence signal is therefore expected to increase exponentially in time to saturation and can be easily fitted to a single exponential function. From the fitted exponential function, the average elongation velocity (k_{cat}, Fig. 13g) will be equal to the maximal slope (i.e.- the first derivative when time is equal to zero), the polymerase dissociation rate from the template (k_{diss}, Fig. 13g) will be equal to the fitted exponential rate constant, and the average processivity of the enzyme (the number of nucleotides transcribed before dissociation) will be linearly related to the amplitude (see Appendix).

4.3 Repression of transcription elongation as a function of the mechanical state of the DNA template

Using the MB-based minicircle transcription assay, the effects of bending stress on transcription by T7RNAP have been directly quantified using three highly bent minicircle templates, the highly untwisted 100c, overtwisted 106c, and modestly untwisted 108c DNA minicircles (refer to Chapter 2). These three minicircle templates were specifically designed with the expectation that the variations in the residual twist sustained within each species of minicircle would reveal any relationship existing between transcription and the torsional state of DNA. If the transcription activity from the three templates did not differ significantly, then torsional stress could be effectively ruled out as the causal factor in any observed repression. To begin the discussion of the results, let us

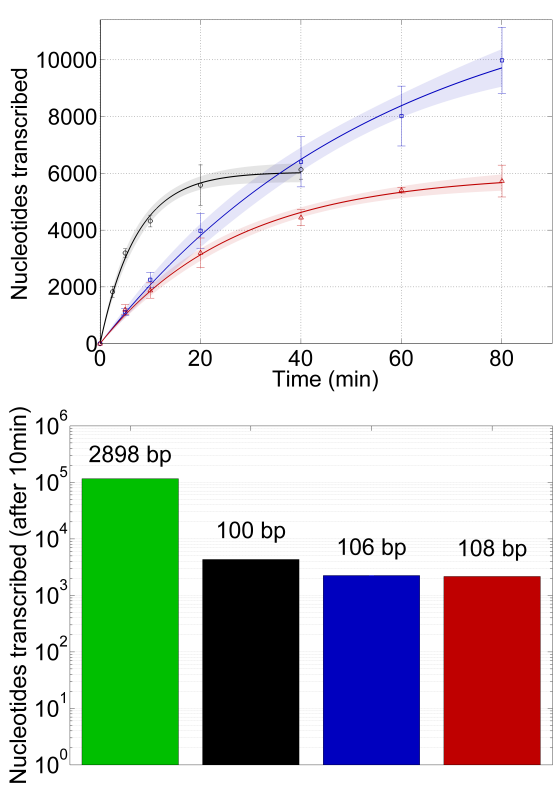

Figure 14. Transcription Elongation of T7RNAP on Highly Bent DNA Templates. **(a)** Transcription from the moderately overtwisted template (106c, blue squares) is roughly 5 times slower than from the most untwisted template (100c, black circles), but is also more processive. The moderately untwisted template (108c, red triangles), by comparison, demonstrates an elongation velocity comparable to 106c, but the processivity of 100c. **(b)** The number of nucleotides transcribed over a 10 minute period from each of three highly bent templates (each of which sustains various levels of twist) is significantly lower compared to an unstressed 2898 bp circular plasmid (green bar).

first consider T7RNAP activity from the overtwisted (106c) minicircle. By fitting the observed data to a single exponential function, T7RNAP demonstrated a k_{cat} of 3.8 nt/s and an overall processivity of 12,900 nt (Fig. 14a, blue squares), both of which are significantly below literature values from linear template DNA (k_{cat} ~ 230 nt/s [152-153], processivity > 30,000 nt [150]). Considering next the case where the DNA minicircle template sustains modest levels of untwist (108c), the calculated value of k_{cat} was almost identical to the overtwisted (106c) minicircle (3.7 nt/s), but the processivity was reduced more than twofold to 6,000 nt (Fig. 14a, red triangles). Interestingly, as the template is further untwisted, the processivity remained constant (6,000 nt), but k_{cat} increased nearly fourfold to 13.9 nt/s (Fig. 14a, black circles). While these data support a clear relationship between T7RNAP behavior and template twist, transcription from these minicircle templates remains in all cases significantly reduced compared to wild-type T7RNAP activity. As a positive control, transcription by T7RNAP of a 2898 bp circular template was compared after 10 minutes to transcription from the minicircle templates. The large, supercoiled circular plasmid was expected to provide a comparison to a DNA template that is expected to experience no significant bending stresses beyond those induced by thermal energy (Fig. 14b). Relative to transcription from the large plasmid, T7RNAP elongation is significantly repressed on highly bent minicircle templates: k_{cat} is reduced by at least 15 times and the processivity declines by at least 9 times (Table 5).

The data unequivocally demonstrate that bending stress applied to DNA is sufficient to repress transcription of an otherwise competent transcription

template, and suggest that torsional stress also plays a role in influencing the kinetics of elongation by T7RNAP. The modulation of k_{cat} due to the torsional state of the DNA can be put into context when one considers that the promoter must be untwisted by T7RNAP during the isomerization phase of transcription, where the polymerase accesses the nucleotide bases [154]. As a direct consequence of this step, the DNA flanking the open complex is necessarily overtwisted (owing to the boundary conditions imposed by a closed circular DNA topology). In a closed domain such as a circular template, this torsional stress will be maintained within the template during transcription, and T7RNAP will be required to transcribe the template against this torsional load. However, if the DNA template already sustained some level of untwist before open complex formation, the overtwist generated by the polymerase during open complex formation can be partially alleviated. Therefore, the restorative effects of DNA untwist on k_{cat} can be interpreted as reflecting the relief of the torsional load encountered by T7RNAP as it transcribes the circular DNA. However, in the context of the molecular mechanism underlying transcription by T7RNAP, it remains unclear at present why the processivity appears to positively correlate with overtwisting the DNA template.

4.4 The stability of T7 RNA polymerase elongation complexes as a function of template mechanics

The stability of the T7RNAP elongation complex has previously been measured by challenging stalled complexes to timed incubations with heparin

[155]. It is expected that a first order rate constant can describe the dissociation rate of the stalled elongation complex (k_{stall}). It is therefore expected that k_{stall} will depend particularly on the time over which stalled complexes are incubated with heparin prior to the initiation of transcription. In one closely related study, Mentesana *et al.* used a heparin challenge assay (Fig. 15) to demonstrate that the stability of stalled elongation complexes depends not only on the position along the template at which T7RNAP is stalled, but also on the template mechanics. The authors report that T7RNAP is less stable on supercoiled templates than linear templates, raising the possibility that highly bent (and controllably twisted) minicircle templates might also affect the stability of stalled T7RNAP elongation complexes.

To directly test whether T7RNAP elongation complex stability was dependent on template mechanics, the previously described transcription assay was modified to report the fraction of minicircle templates remaining occupied by T7RNAP elongation complexes as a function of heparin incubation time (Fig. 15). Briefly, T7RNAP was first incubated with minicircle templates in the absence of

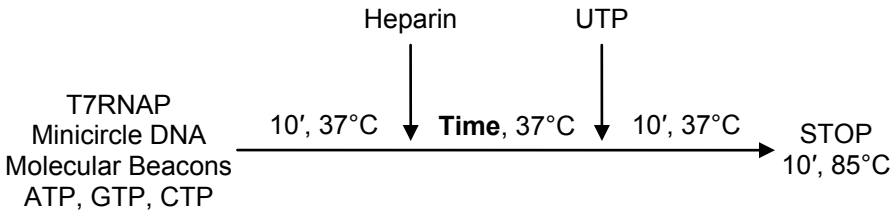

Figure 15. Experimental Procedure for Quantifying T7RNAP Elongation Complex Stability. Stalled T7RNAP elongation complexes were preloaded onto minicircle DNA templates during a 10′ incubation in the absence of UTP. Heparin was then added and incubated with the stalled elongation complexes for a variable duration of time. Transcription elongation from stalled T7RNAP remaining associated with the template was initiated by the addition of UTP and allowed to continue for 10′.

UTP for a fixed period of time and then subjected to a variable incubation period with heparin. Following the addition of UTP, all stalled elongation complexes remaining associated with template were transcribed under identical conditions. The resulting signal, in this case, is expected to be dependent on the time over which stalled elongation complexes were incubated with heparin. Since the fraction of templates occupied by stalled elongation complexes is expected to decline exponentially with time, the signal arising from transcription by the elongation complexes remaining on the template should behave likewise. Therefore, by fitting a single exponential to the molecular beacon fluorescence intensity as a function of incubation time with heparin, the first order rate constant describing the dissociation of elongation complexes from the template (k_{stall}) can be calculated directly. To quantitatively compare the kinetics of elongation complex stability, the half-life ($t_{½}$) of bound elongation complexes in the presence of heparin is typically used. However, k_{stall} will be used as the convention of choice, and is inversely related to the half-life used in previous studies, according to the relationship:

$$k_{stall} = \ln(2) \times t_{½}^{-1} \qquad \text{(Eq. 4)}$$

Applying now the heparin challenge to stalled T7RNAP that had been preloaded onto highly bent minicircle templates (Fig. 16), it is immediately apparent that the stability of T7RNAP elongation complexes is not strongly dependent on the torsional state of the template. The characteristic rate constants (k_{stall}) calculated from the exponential fits to the data range from

2.5×10^{-3} to 3.6×10^{-3} s^{-1}, but any differences in k_{stall} are negated by the range over which 95% confidence in the fitting can be ensured. However, though the three cases of torsional stress that were tested do not show statistically significant differences in the calculated values of k_{stall}, the 95% confidence intervals for the exponential fits to the data (as illustrated by the shaded regions in Figure 16) do reveal one interesting trend: overtwisting the DNA template appears to decrease the stability of T7RNAP.

The apparent trend observed in the heparin challenge data offers an interesting comparison to the previous work by Mentesana *et al.* [155]. The two primary conclusions that emerged from the Mentesana *et al.* study were: (1) the half-life ($t_{½}$) for elongation complexes stalled on supercoiled plasmid DNA in the presence of heparin was roughly 9 minutes, and (2) elongation complexes were roughly three times more stable on linear DNA than on supercoiled DNA. To compare the measured half-life with the dissociation rates calculated in the present study, Equation 3 reveals that the off-rate (k_{stall}) measured in the Mentesana *et al.* study is approximately 1.3×10^{-3} s^{-1}. From this value, the elongation complexes

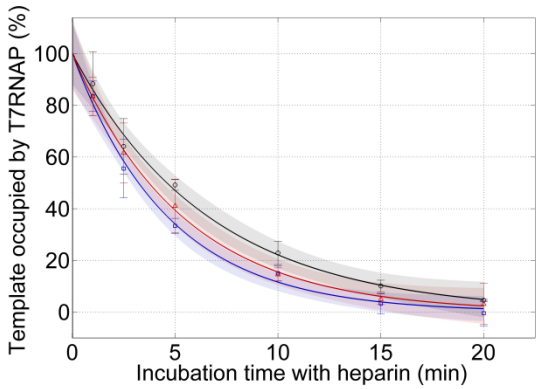

Figure 16. Elongation Complex Stability as a Function of Torsional Stress in the DNA Template. T7RNAP elongation complex stability was measured by varying the duration of time stalled T7RNAP elongation complexes were incubated with heparin. The characteristic rate constants for the exponentials fitted to data from the three minicircles (100c, black circles; 108c, red triangles; 106c, blue squares) reflect an overall apparent rate constant describing the heparin induced dissociation rate.

challenged by heparin in the previous work appear between two- and threefold more stable than T7RNAP elongation complexes formed on the minicircle templates used in the present study. The direct comparison between these two studies may at first appear to be of questionable significance given that the present work uses a heparin concentration that is 50 times higher than in the Mentesana *et al.* study. However, it has previously been shown, that the stability of stalled elongation complexes on linear DNA is virtually independent of the concentration of heparin [156], though it should be noted that the validity of this assumption remains untested for T7RNAP bound to templates sustaining significant mechanical stress.

The observed trend in the T7RNAP elongation complex stability on minicircle templates sustaining varying levels of torsional stress supports the hypothesis that untwisting DNA stabilizes the elongation complex. Given the expectation that T7RNAP will necessarily overtwist the DNA minicircles during open complex formation (see Chapter 4.3), this conclusion suggests a role for DNA mechanics in modulating the elongation complex stability. In cases where T7RNAP is required to form a stalled elongation complex on minicircle templates that already sustain some degree of overtwist, the torsional load experienced by the stalled T7RNAP will be greater than when it is bound to untwisted templates. Therefore, since the stall sequences within the minicircle templates are identical, the only energetic differences between the three cases arise from the torsional and bending stress sustained within the templates. Also, since the three minicircles are expected to experience much larger variations in residual twist

than in bending, bending can reasonably be eliminated from consideration. The interpretation of the data that now emerges supports a model in which the greater torsional load encountered by T7RNAP elongation complexes bound to overtwisted minicircle templates enhances the dissociation of the stalled polymerase from the template. While appearing to directly contradict the conclusion from Mentesana *et al.* that the elongation complex is less stable on negatively twisted, supercoiled DNA, it should be remembered that the comparison in the Mentesana *et al.* study was made between supercoiled and linear DNA. Therefore, given that linear DNA is expected to relieve any torsional load encountered by the polymerase (by free rotation of the DNA flanking the T7RNAP binding site), the proposed model does not necessarily contradict previous findings since a comparison to linear DNA was beyond the scope of the present study.

4.5 Pre-elongation phases of transcription are modulated by template mechanics

In order to quantitatively measure the rate at which T7RNAP forms stable elongation complexes, the MB-based transcription assay was modified to report transcription from templates as a function of the time that T7RNAP and DNA minicircle templates were incubated in the absence of UTP prior to the addition of heparin (Fig. 17). Since elongation complex formation is fundamentally determined by the kinetics of promoter recognition and binding, open complex formation, and transcription initiation, but the MB-based assay is only capable of reporting transcription elongation, a pseudo first order rate constant, k_{for} (Fig.

13g), was defined to quantitatively describe the kinetics of all pre-elongation phases of transcription [86]. k_{for} can be determined by varying the time T7RNAP is incubated with template DNA prior to the addition of heparin (Fig. 13b-c) and measuring the concentration of hybridized beacon resulting from otherwise identical transcription conditions. Consequently, the resulting signal will be directly proportional to the number of elongation complexes formed at the time of heparin addition. A single exponential fit of the fluorescence signal as a function of incubation time yields an apparent rate constant (k_{for}) describing the formation of stalled T7RNAP elongation complexes given a single rate-limiting step. By applying relaxation kinetics [157] to previously reported rates of T7RNAP binding and transcription initiation [158] and taking into account the concentrations of T7RNAP and template used in our assay, k_{for} is predicted to be on the order of 0.20 s^{-1} for the pre-elongation kinetics of T7RNAP from linear (unstressed) DNA templates (for derivation, see Appendix).

Given the dominant role of bending in repressing T7RNAP elongation, it was hypothesized that tightly bending DNA would also dominate the repression

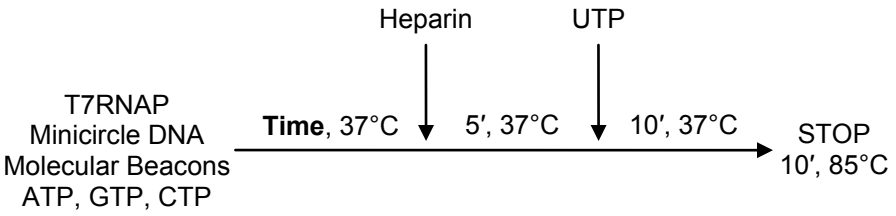

Figure 17. Experimental Procedure for Quantifying T7RNAP Elongation Complex Formation. Stalled T7RNAP elongation complexes were preloaded onto minicircle DNA templates a variable amount of time in the absence of UTP. Heparin was then added and incubated with the stalled elongation complexes for 5′. Transcription elongation from stalled T7RNAP remaining associated with the template was initiated by the addition of UTP and allowed to continue for 10′.

behavior in the pre-elongation phases of transcription. Also, given the expectation that open complex formation overtwists the DNA minicircle template, it was expected progressively higher levels of untwist in the template (such as in 100c and 108c) would relieve the torsional load acting against the polymerase during these early phases and thus increase k_{for} as the template is further untwisted. Surprisingly, however, k_{for} is reduced for the highly untwisted (100c, 2.0×10^{-3} s^{-1}) and overtwisted (106c, 2.4×10^{-3} s^{-1}) minicircle templates, compared to the mildly untwisted 108c (4.8×10^{-3} s^{-1}) (Fig. 18). These results suggest that there is an optimal level of negative torsional stress, above or below which elongation complex formation is retarded. In attempting to interpret these data, the conclusions must be limited to the cumulative effects of mechanical stress on all pre-elongation phases because the molecular beacon assay is specifically designed to monitor elongation and is thus incapable of directly addressing any specific pre-elongation phase. Nonetheless, a clear influence of torsional stress on the rate of elongation complex formation is observed. Despite the clear influence of residual twist within the DNA template, however, the observed rates of elongation complex formation are all highly

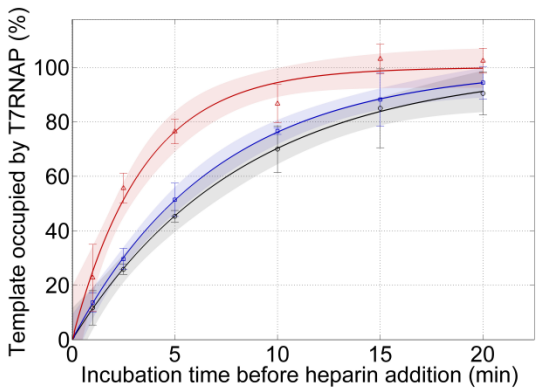

Figure 18. Elongation Complex Formation of T7RNAP on DNA Minicircle Templates. Occupancy of template with T7RNAP elongation complexes is measured as a function of incubation time prior to the addition of heparin. The characteristic rate constants for the exponentials fitted to data from the three minicircles (100c, black circles; 108c, red triangles; 106c, blue squares) reflect an overall apparent rate constant describing transcription initiation.

repressed compared to behavior predicted from previously reported kinetic data. This particular observation demonstrates that mechanically stressed DNA is capable of repressing some (or all) pre-elongation phases of transcription, and further suggests that bending is largely responsible for the observed repression of elongation complex formation.

The observed rates of elongation complex formation sheds light on a recently published experimental concern regarding the use of nucleotide stalled elongation complexes. It has been previously shown that when T7RNAP is stalled more that 12 bp downstream of the transcription start site, the promoter is sufficiently accessible to allow a second T7RNAP to bind and independently initiate transcription [159]. If the first polymerase is stalled between 12 and 20 bp from the start site, the second T7RNAP is unable to successfully transition into a stable elongation complex and is incapable of displacing the stalled, downstream polymerase. Since the transcription assay used in the present study relies on stalling T7RNAP 20 bp downstream of the start site, it may at first appear possible for a second polymerase to simultaneously transcribe the minicircle template. However, it has previously been shown that T7RNAP initiation complexes that have not yet transitioned into more stable elongation complexes will be readily displaced from the template in the presence of heparin [155]. Furthermore, the prolonged incubation of stalled elongation complexes with heparin has been shown to displace more than 50% of the preloaded, stalled elongation complexes (see Chapter 4.4). It is therefore considered unlikely that unstable initiation complexes could remain associated with the template under

such stringent conditions. This conclusion is further supported by the observation that the elongation complex formation data is well-described by a single exponential function, which mathematically represents the expected solution for a kinetic reaction described by a single, bimolecular rate constant (in this case, describing the binding of a single T7RNAP to a DNA template).

4.6 Discussion and conclusions

The data presented here constitute the first direct observation of the ability of transcription to be specifically repressed by high levels of bending stress applied to the DNA template. Previous work relating to the role of DNA mechanics in transcription regulation has focused largely on the effects of DNA supercoiling, but as discussed elsewhere (see Chapter 1.2), DNA supercoiling is a poorly defined state of mechanical stress. The studies discussed here present a marked advance over the use of supercoiled DNA in transcription studies. By using DNA templates that stably maintain predefined and well-behaved levels of bending and twist, the effects of each form of mechanical stress on transcription can now be directly quantified. Indeed, the high levels of bending stress within DNA minicircle templates on the order of 100 bp in size have been specifically demonstrated to be capable of significantly repressing transcription elongation by T7RNAP, one of the most robust model RNAP's. In particular, the elongation complex formation and stability, as well as elongation velocity and processivity, of T7RNAP have demonstrated strong dependencies on the mechanical state of the template (Table 5).

Size (bp)	Twist	k_{for} ($\times 10^{-3}$ s^{-1})	k_{stall} ($\times 10^{-3}$ s^{-1})	k_{cat} ($nt \cdot s^{-1}$)	k_{diss} ($\times 10^{-3}$ s^{-1})	Processivity ($\times 10^{3}$ nt)
100	--	2.0 (1.3, 4.1)	2.5 (1.8, 4.2)	13.9 (11.2, 17.6)	2.3 (2.0, 2.8)	6.0 (5.7, 6.4)
106	+	2.4 (1.8, 3.6)	3.6 (2.8, 5.0)	3.8 (2.1, 7.5)	0.3 (0.2, 0.5)	12.9 (10.0, 15.8)
108	-	4.8 (3.4, 8.0)	3.1 (2.3,4.9)	3.7 (2.9, 4.8)	0.6 (0.5,0.8)	6.0 (5.5, 6.4)
2,898	**n/a**	**~200***	**1.3†**	**>194**	**n/a**	**>115**

Table 5. Transcription by T7RNAP is Sensitive to Template Mechanics. Mean values for k_{for}, k_{stall}, k_{cat}, k_{diss}, and processivity are determined from the best-fit exponential function fitted to data; the 95% confidence intervals from the fits are shown for each parameter in parentheses. *Calculated from [158]. †From [155] (comparable conditions, except that the heparin concentration in [155] was 50 times lower than used in the present study).

In order to thoroughly understand the origin of the observed transcriptional repression, however, we require additional data that is not currently available. It is not yet clear, for example, how possible secondary effects of the template mechanics dynamically affect transcription by T7RNAP. For example, it remains possible that sequence-dependent bending stiffness or intrinsic curvature could be capable of "locking" the conformation of the minicircle template and prevent the free rotation about the helical axis that would be allowed in that case of a perfectly isotropic, circular DNA. The locked conformation could consequently present the promoter sequence on one, unchanging face of the DNA double helix. Depending on the energy required to rotate the presented face into more a favorable orientation, T7RNAP may be required to bind to and initiation transcription on a template whose orientation is bent in a nonideal orientation. On the other hand, it is also possible that the DNA duplex melting that accompanies the formation of an open complex is capable of relieving any preferential orientation of the template.

Another additional piece of data that is required to understand the origin of the observed repression of transcription from DNA minicircle templates is the dynamic interaction between the polymerase and the template itself. As a DNA minicircle is transcribed, two modes of polymerase translocation are possible: (1) the DNA template could remain in a fixed orientation, in which case the RNAP would be required to spiral about the helical axis of the DNA, or (2) the DNA template could be allowed to rotate about its helical axis, thus allowing T7RNAP to always remain bound to the outer edge of the circular template (in this case, the template would be required to "roll in place" about the helical axis as transcription ensues). It is expected from previous work that the increased drag of the nascent transcript will eventually prevent T7RNAP from undergoing rotation about the DNA [30], but since no previous transcription study has employed circular DNA templates that were significantly below the persistence length, it is not yet clear if this model for transcription holds true for DNA minicircle templates.

The present interpretation of the transcription data is that the T7RNAP remains associated to the outer edge of the minicircle template, and in this orientation, responds to the mechanical load that is applied by the template mechanics. While the above discussion of possible secondary considerations clarifies the limits of our knowledge regarding the structure-function relationship underpinning the observed template-induced repression, the discussion in no way detracts from the overall conclusion from this study: mechanically stressed DNA can significantly repress transcription. Nevertheless, the discussion does

highlight the pressing need to develop a more detailed understanding of the molecular mechanism governing the observed repression of transcription by highly stressed DNA templates. In addition, since the use of a MB-based transcription assay precludes a more detailed characterization of pre-elongation kinetics, there also exists a need to develop more specialized assays that are more appropriate for the observation of other, specifically targeted phases of transcription.

CHAPTER 5

Conclusion

The broad aim of this work was to test the hypothesis that highly bent DNA is capable of repressing transcription by RNA polymerase (RNAP). Challenging this hypothesis, however, was limited by the inability of conventional methods to prescribe controlled levels of bending stress within a DNA transcription template. To address these experimental limitations, a method was developed to produce circular DNA transcription templates on the order of 100 bp in size that stably maintain bending curvatures that are comparable to the bends introduced into DNA by many regulatory proteins. Three DNA minicircle templates were produced (100 bp, 106 bp, and 108 bp in size), each sustaining varying levels of torsional stress but comparable levels of bending curvature. The topological features of the minicircle templates were directly observed by cryo-electron microscopy and shown to sufficiently resist the formation of any stress-induced structural transitions that alter the mechanical properties of the DNA template. To quantify the ability of RNAP to utilize highly stressed DNA minicircle templates, a novel fluorescence-based assay was developed to specifically measure the elongation kinetics of bacteriophage T7 RNA polymerase (T7RNAP). This highly sensitive transcription assay has been used to

quantitatively characterize the enzyme kinetics governing the elongation complex formation, stalled elongation complex stability, transcription elongation velocity, and processivity of T7RNAP. By challenging T7RNAP to transcribe small circular DNA templates sustaining varying degrees of over- and undertwist, it has been demonstrated that simply looping the DNA template is sufficient to strongly repress the activity of T7RNAP. In addition, while the bending stresses sustained within the minicircle templates tested appear to dominate the observed repression, the torsional mechanics of DNA have also been shown to strongly influence the elongation velocity and processivity of T7RNAP. These findings constitute the first direct experimental evidence of the bending mechanics of DNA playing a direct role in transcriptional regulation.

In the context of previous observations of a relationship between DNA mechanics and transcription, the results presented herein constitute a significant advance in our understanding of the molecular mechanisms underlying template-induced modulation of RNAP activity. To understand and discuss the significance of the findings, it is important to review past work that first provided insight into the influence of the mechanical state of DNA on the regulation of transcription. It has long been known that RNAP activity is dependent on the mechanical state of the DNA template. Since the observation that transcriptional activity was enhanced from DNA templates that were negatively supercoiled [40], many other studies have attempted to understand the influence of the mechanical state of DNA on transcription [51, 54-55, 58]. Important first steps have been made towards elucidating the structure-function relationship between template

mechanics and RNAP activity. One illustrative example of such a study was performed by Gopal *et al.*, who successfully identified the thumb subdomain of T7RNAP as being especially sensitive to the degree of DNA supercoiling [55]. However, as was previously discussed (see Chapter 1.2), the use of supercoiled DNA in transcription studies presents significant and unavoidable complications in interpreting the data on a mechanistic level. The dynamic and uncontrolled repartitioning of bending and torsional energy within supercoiled DNA [137] greatly hinders attempts to elucidate the functional relationship between RNAP and highly bent and twisted templates. In addition, since the bending and torsional stresses within supercoiled DNA are exclusively determined by the geometrical parameters of the DNA supercoils [62], the mechanical state of the DNA cannot be defined without detailed knowledge of the tertiary structure. Previous transcription studies utilizing supercoiled DNA lack geometrical descriptions of the supercoiled DNA, and therefore cannot ascertain the bending and torsional stresses sustained by the DNA template at the site of RNAP activity. Since it is not possible in these cases to accurately determine the dependence of transcription on the twist and bending sustained within the DNA template, conclusions from these studies must therefore be restricted to the qualitative demonstration that negative torsional stress is sufficient to enhance transcription.

The dependence of RNAP activity on DNA bending has been investigated in a number of studies using intrinsically curved DNA sequences inserted within the transcription template [17, 19, 160-162]. These studies have shown that

stress-free curvature within the DNA template can modulate transcription, but they do not address the effects of bending stress *applied* to the template (a condition more biologically relevant to understanding the role of tightly looped DNA induced by DNA-binding proteins). Perhaps the most direct attempt to address the effect of forcibly bending DNA on transcription was performed by TenHarmsel and Biggin [163], who used a 245 bp circular DNA template to stably maintain bending stresses during *in vitro* transcription by *Drosophila* RNA polymerase II (RNAP II) in an attempt to demonstrate that the loop formed by the *eve* repressor was causal to transcriptional repression in this system. The authors reported that overall transcription was repressed from their circular templates, and attributed the decline in RNAP activity to the inhibition of binding of the general transcription factor TFIID (the binding of which was presumably directly repressed by the bending curvature imparted to the DNA template). Importantly, the authors presented DNase I footprinting data suggesting that the bending direction of the TFIID binding site within the circular template was expected to be different than the bending direction induced by TFIID. In light of previous evidence that the orientation of bent DNA is capable of modulating the binding affinity of proteins known to bend DNA [164], it remains unclear if TFIID binding was dependent on the template bending stress itself or simply the bending orientation. Presumably, this unresolved issue could be addressed if circular templates were created such that the TFIID binding site was somehow bent in an orientation that was favored by TFIID.

As a positive control for transcription factor binding, TenHarmsel and Biggin used the TATA-binding protein (TBP), a subunit of the multiprotein TFIID complex, which proved capable of readily binding to the circular template. However, even in this case, transcript production by RNAP II remained highly repressed, indicating that template bending is capable of interfering with some other step(s) in the kinetic pathway of transcription. It remains unclear if transcription from circular templates using TFIID shares the unidentified rate-limiting step when TBP is used, or put another way, if the observed repression is only modestly attributed to template-induced TFIID binding inhibition. It is easily possible, for example, that TFIID binding in actuality plays only a minor role in the overall observed repression, especially given that the binding study performed by the authors failed to quantify the kinetics of TFIID binding.

The results reported by TenHarmsel and Biggin provide strong evidence of the regulatory role played by the mechanical state of DNA. Yet this work also highlights the need to employ more detailed, reductionistic approaches using precisely characterized DNA templates to determine the underlying molecular interactions between RNAP and mechanically stressed DNA (and thus identifying the mechanistic basis of template-induced repression). Taken together, the observation that DNA bending can repress the pre-elongation phases of transcription in both eukaryotic RNAP II (shown by TenHarmsel and Biggin) and an evolutionarily unrelated bacteriophage RNAP (present work) suggest that DNA bending may be a common mechanism capable of regulating gene expression among otherwise unrelated RNAP systems.

If the bending mechanics of DNA indeed serve as a ubiquitous regulatory mechanism, then previous studies of regulatory proteins that form tightly bent loops within the DNA template should be consistent with this overarching framework. As one illustrative example, let us consider again the well-studied lactose repressor (LacR). It has been generally accepted that loop formation primarily serves to locally enhance repressor binding [5, 141, 165-166]. However, as previously discussed (see Chapter 1.1), it has also been shown that DNA loops are formed by LacR with lower probability as the loops become smaller [24, 167]. A model of repression in which RNAP activity is simply dependent on the presence of a bound repressor predicts, in this case, that LacR-induced repression will necessarily be lower when the loops are smaller because LacR is binding less frequently. However, it has been shown that the overall repression of *E. coli* RNAP actually *increases* as LacR forms smaller loops of DNA [20, 105]. As suggested by the Müller-Hill group, this apparent paradox can be resolved by including, as a component of repression, the mechanical stress within the loop [20]. In this revised model, the seemingly contradictory LacR data can be easily reconciled: as the repressor loops become smaller and more energetically costly to bend, the kinetic barrier for LacR to form these loops increases; but once tight loops are formed, RNAP is more efficiently repressed by the significant LacR-induced bending stresses sustained within the loop. Indeed, the observation of transcriptional repression arising from RNAP utilization of highly bent minicircle templates supports this model, albeit with an unrelated RNAP.

It has also been suggested that loop-forming repressors inhibit open complex formation by stabilizing the torsional inflexibility of the looped DNA [23, 54]. Applying this model to the DNA minicircle transcription system, it would be expected that transcriptional repression would be largely relieved as templates sustain increasing levels of untwist. Though torsional stress is observed to be capable of directly modulating elongation velocity, processivity, and pre-elongation kinetics, these effects are modest in comparison to the apparent influence of bending on the activity of T7RNAP. Therefore, in contrast to the model proposed by previous work [23, 54], the data suggest that bending plays a dominant role in the repression of transcription by RNAP's from DNA templates that are tightly looped by loop-forming repressors. While the elongation kinetics have been shown to be capable of being repressed by DNA bending, it must be noted that a more detailed study is needed before the relationship between DNA bending and polymerase activity during the pre-elongation phases of transcription can be defined. That is, the minicircle transcription assay described here has being specifically designed to quantify transcription elongation and is therefore not capable of discriminating between early phases of transcription, including promoter binding, open complex formation, and initiation.

The molecular beacon-based minicircle transcription assay unequivocally demonstrates that tight looping of an otherwise competent transcription template can repress transcription elongation by T7RNAP. The data support a model by which targeted genes can be specifically regulated at the DNA level through local mechanical stresses within the template. From this model emerges the possibility

that regulatory proteins that are known to significantly deform DNA serve primarily to induce and maintain a stressed DNA topology, which in turn directly modulates the activity of RNAP. Given the important role that the mechanical and topological properties of DNA inherently play in all known transcription systems, it will now be of critical importance to determine the extent to which template-induced repression serves as an evolutionarily conserved mechanism common to otherwise unrelated regulatory systems.

5.1 Future directions

The work described herein, though primarily intending to serve as a conclusive demonstration of the ability of highly bent DNA to repress transcription, leaves many questions unanswered regarding the structure-function relationship underpinning template-induced repression. Now that a clear relationship has been shown to exist, there exists a compelling need for more detailed studies that are capable of complementing the present work with additional insights into the molecular mechanism(s) by which stressed DNA templates interact with RNAP. Here, the most pressing issues are briefly outlined for clarity.

(1) *The binding orientation of T7RNAP elongation complexes to minicircle DNA templates*. To provide further insight into the interpretation of the data from the transcription of DNA minicircles by T7RNAP, the binding orientation of the polymerase relative to the minicircle curvature must be determined. Few techniques are available that would enable the physical

characterization of T7RNAP binding to the minicircle template, but perhaps the most appropriate technique meeting the required experimental specifications is conventional transmission electron microscopy (TEM). By preloading T7RNAP onto minicircle templates in the absence of UTP, the nucleoprotein complex could be readily applied to a specimen grid, incubated with a heavy metal staining solution, and imaged by TEM. It is expected that the observation of stalled elongation complexes bound to minicircle templates would provide insight into the role of the binding orientation of T7RNAP on highly bent templates. If TEM proves capable characterizing the binding orientation of T7RNAP, the method is likely to enable the iterative design of minicircle templates (for example, by intentionally encoding sequence-dependent curvature) to controllably position T7RNAP relative to the bending orientation of the template.

(2) *The effects of template stress on pre-elongation phases of transcription by T7RNAP*. Given the fundamental limitations of the molecular beacon-based minicircle transcription assay, new approaches must be devised to address the effects of template mechanics on promoter binding, open complex formation, and transcription initiation. It is likely, given the limiting concentrations of the minicircle template, that the conventional biochemical methods capable of characterizing these early phases of transcription will not be easily compatible with minicircle transcription assays. Single molecule assays present a powerful alternative that is not

hindered by template-limiting conditions. Several methods have already been devised that may prove capable of allowing for the quantitative characterization of transcription initiation [58, 86, 90, 93, 97]. The most promising of these methods to accomplish this aim is to monitor the FRET interaction between a donor fluorophore conjugated to an RNAP and an acceptor fluorophore at the leading or trailing edge of a DNA template [93, 97]. Such an assay could provide powerful new information regarding the initiation and abortive cycling behavior of T7RNAP, but does require that minicircles be designed to include a specifically positioned fluorophore near the promoter site. Fluorescently labeling minicircle templates, however, is unfortunately beyond the current capabilities of the LAMA method used to produce minicircle templates, and therefore would require the procedure to be modified.

(3) *The relationship between the T7RNAP structure and template-induced repression*. Owing to the fact that the transcription community has thus far lacked the ability to controllably prescribe tight bends in sub-persistence length DNA, it has not been previously possible to test the transcriptional competency of an RNAP from DNA templates sustaining well-characterized mechanical stresses. Consequently, very little is actually known about the structural elements of the polymerase structure that are specifically affected by template stress. It is therefore quite appropriate to apply a common approach from molecular genetics (gene mutagenesis) to T7RNAP, using the current minicircle transcription assay to identify mutant

polymerases that display either repressed or enhanced transcription activity from mechanically stressed templates. By then correlating the transcriptional behavior of mutants of interest to their respective gene mutations, it is expected that the subdomains within the polymerase structure that are responding specifically to template mechanics would become apparent. It would then be possible to identify structural homologues of this structural subdomain in otherwise unrelated RNAP's.

(4) *The extent to which template-induced repression is employed in unrelated regulatory systems*. Though previous experimental data strongly suggests that other model RNAP's can be likewise repressed by mechanically stressed DNA templates, this supposition must be challenged directly. Quantifying the effects of DNA bending on other RNAP systems is perhaps most readily accomplished by modifying the current minicircle transcription assay to employ minicircle templates encode a different RNAP-specific promoter sequence.

APPENDIX

Materials and Methods

1. Minicircle Template Synthesis

Sufficient quantities of minicircle templates were created by modifying the ligation-assisted minicircle accumulation technique previously described [116]. 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. Upon ligation, 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. 12-15μg of each product was then added to *Taq* DNA ligase, 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.

Substrates for the ligation-assisted minicircle accumulation technique were prepared by inserting the 100 bp, 106 bp, or 108 bp minicircle sequences 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). 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).

2. Molecular Beacon Design

2′-o-methyl RNA molecular beacons (Sigma Life Science) were designed to include a 21 bp recognition loop (complementary to the RNA sequence produced upon transcription of the underlined portion of the previously listed minicircle sequences) 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

3. Transcription Elongation Assays

0.5nM of minicircle template was incubated with 8.5nM T7RNAP for 10 minutes in transcription buffer (40mM Tris-acetate, 10mM Mg-acetate, 1mM each of ATP, GTP, and CTP, 20nM 2′-o-methyl-RNA molecular beacons, 0.05%v/v Tween-20, pH 8.0). 5mg/ml heparin was added to inactivate free T7RNAP, and

incubated for 5 minutes prior to the initiation of transcription with the addition of 1mM UTP. The transcription reaction was terminated by heat inactivation of the polymerase (and destruction of secondary structure in the RNA transcript) at 80°C for 10 minutes. The solution was then slowly cooled at a rate of -0.1°C/s to allow molecular beacons to hybridize to RNA target sequences present.

Fluorescence was measured using an Aminco-Bowman Series 2 (AB2) luminescence spectrometer (Thermo Spectronic), equipped with an external photomultiplier tube module. All reaction solutions, 65µl in volume, were excited with 535 nm light in a 50µl Sub-Micro Quartz Fluorometer Cell (16.50F-Q-10/Z15, Starna Cells, Inc.), and the emission from the cuvette was first filtered with a longpass filter (OG570, Schott). The fluorescence signal from molecular beacons hybridized to target RNA in solution was experimentally determined to scale as 0.458 AU per nM of hybridized molecular beacon. 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 a(n) (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 T7RNAP experimentally determined to occupy the template after incubation for 10 minutes to normalize the elongation rate of T7RNAP enzyme molecules.

A nonlinear least squares fitting routine was implemented using MATLAB (The MathWorks, Natick, MA) to fit the time-dependent fluorescence data (N, in

units of nucleotides) to the solution to the first-order differential equation describing the reaction kinetics,

$$N(t) = P \cdot \left(1 - e^{-k_{diss} \cdot t}\right) \qquad \text{(Eq. A1)}$$

where the amplitude of the rising exponential (P) is equal to the transcription processivity (the nucleotides synthesized per enzyme on average) 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. 1 at t = 0,

$$\frac{dN(t)}{dt} = P \cdot k_{diss} \cdot e^{-k_{diss} \cdot t} \rightarrow k_{cat} = \frac{dN(0)}{dt} = P \cdot k_{diss} \qquad \text{(Eq. A2)}$$

4. Elongation Complex Formation Assays

Concentrations and buffer conditions were identical to transcription elongation assays, with the following modifications. T7RNAP 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 T7RNAP.

The time-dependent occupancy data (O, in percentage of template occupied) was fit to the solution to the first-order differential equation describing the reaction kinetics,

$$O(t) = 1 - e^{-k_{for} \cdot t} \quad \text{(Eq. A3)}$$

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

5. Minicircle Template Sequences

Templates were designed to include the following features:

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 elongation complexes at +20 site until UTP is present.

6.) MB Sequence: sequence from which transcribed RNA will hybridize to 2'-o-methyl-RNA molecular beacons.

Minicircle size (bp)	
100	…ATCGGATCCTAATACGACTCACTATA**GGGAG**ACCACAACCAC **CACC**TGAATTCCAGCTGTAAGAAACCATTTCGATACCCCCGATG TCATCCTCAGCGAT…
106	…ATCGGATCCTAATACGACTCACTATA**GGGAG**ACCACAACCAC **CACC**TctgGAATTCCAGCTGTAAGAAACCAtgcTTTCGATACCCCC GATGTCATCCTCAGCGAT…
108	…ATCGGATCCTAATACGACTCACTATA**GGGAG**ACCACAACCAC **CACC**TctgaGAATTCCAGCTGTAAGAAACCAtgcaTTTCGATACCCC CGATGTCATCCTCAGCGAT…
Note: lower case letters are sequences inserted to change the size of the minicircle template.	

6. 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 the designed assay is specific to the elongation phase of transcription, this model must be refined to:

$$E_{fr} + D \underset{k_{for}}{\rightarrow} E_{st} \cdot D \cdot R_{20} \underset{k_{cat}}{\rightarrow} E_{el} \cdot D \cdot R_{20+n} + NTP \underset{k_{diss}}{\rightarrow} E_{frx} + D + R$$

where k_{for} is a pseudo first order rate constant describing the formation of stalled elongation complexes (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}).

To predict from published work what values of k_{for} can be expected for unrepressed T7RNAP behavior, it is necessary to determine kinetic rate constants for the pre-elongation steps composing k_{for}. Decomposing k_{for} into a bimolecular step (binding) defined by the rate constants k_1 and k_{-1}, and a unimolecular step (isomerization, initiation, abortive transcription) defined by k_2, the general kinetic equation can be expressed as:

$$E + D \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} E_1 \cdot D \underset{k_2}{\rightarrow} E_2 \cdot D$$

where E_1 = bound T7RNAP and E_2 = stalled T7RNAP elongation complexes. Describing initiation kinetics, Ujvari and Martin [158] found that k_1 = 0.039 $nM^{-1}s^{-1}$, k_{-1} = 0.48 s^{-1}, and k_2 = 0.47 s^{-1}. In order to predict the expected k_{for} given these values, relaxation kinetics is employed to express the apparent rate of formation of $E_2 \cdot D$ in terms of two apparent first order rate constants, one

reflective of the bimolecular step ($k_{bimolecular}$) and one of the unimolecular step ($k_{unimolecular}$). From [157], these rates are given by:

$$k_{bimolecular} = k_{-1} + k_{+1}([E] + [D])$$

$$k_{unimolecular} = \frac{k_2}{1 + \frac{k_{-1}/k_{+1}}{[E] + [D]}}$$

Substituting the concentrations of DNA template ([D] = 0.5 nM) and T7RNAP ([E] = 8.5 nM) employed in our transcription assays, we find that $k_{bimolecular}$ = 0.83 s^{-1} and $k_{unimolecular}$ = 0.20 s^{-1}. The rate-limiting step, therefore, is expected to occur in the unimolecular step (including isomerization, initiation, and abortive transcription).

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