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Do Femtonewton Forces Affect Genetic Function? A Review

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Abstract. Protein-Mediated DNA looping is intricately related to gene expression. Therefore any mechanical constraint that disrupts loop formation can play a significant role in gene regulation. Polymer physics models predict that less than a piconewton of force may be sufficient to prevent the formation of DNA loops. Thus, it appears that tension can act as a molecular switch that controls the much larger forces associated with the processive motion of RNA polymerase. Since RNAP can exert forces over 20 pN before it stalls, a 'substrate tension switch' could offer a force advantage of two orders of magnitude. Evidence for such a mechanism is seen in recent *in vitro* micromanipulation experiments. In this article we provide new perspective on existing theory and experimental data on DNA looping *in vitro* and *in vivo*. We elaborate on the connection between tension and a variety of other intracellular mechanical constraints including sequence specific curvature and supercoiling. In the process, we emphasize that the richness and versatility of DNA mechanics opens up a whole new paradigm of gene regulation to explore.

Key words: DNA, Mechanics, Looping, Tension.

Abbreviations: Wormlike Chain (WLC), Sankararaman and Marko Model (SM), Blumberg, Tkachenko and Meiners Model (BTM), Shimada and Yamakawa Model (SY)

Introduction

The importance of mechanical forces for the regulation of biological processes has been known for more than a century on a macroscopic level. While these early studies focused on tissue- and organ-level processes such as bone growth [1], it has become increasingly clear that mechanical forces also matter on the cellular scale. They can affect cell growth, differentiation, motility signal transduction and gene expression [2,3]. As such, aberrant cellular responses to mechanical signals have been implicated in a variety of diseases [4], including pulmonary disease [2], cardiac disease [5,6] and cancer development [7]. Therefore, the question of how a mechanical stimulus on the outside of the cell is transduced into biological function is an area of great current interest. Mechano-sensitive ion channels

provide one well-known example of a mechanotransduction mechanism [8], but it appears that mechano-sensitive signaling often requires the communication of the mechanical stimulus to deep within the cell [3]. In fact, experiments have shown that the eukaryotic cytoskeleton can efficiently transport mechanical stress from its attachment points on the cell membrane to the nuclear membrane [9]. This poses the intriguing possibility that mechanical tension in the nucleus might lead to a genomic response. In this article we discuss the possibility that the conformational response of DNA to relatively weak tension can alter protein-DNA interactions and thereby affect transcriptional regulation. The core of this argument is based upon recent theoretical arguments suggesting that sub-piconewton tension has a dramatic effect on the rate of protein-mediated DNA loop formation.

We first review theoretical arguments and *in vitro* experimental evidence concerning the disruptive effect of tension on DNA looping. We then consider the more complicated situation of DNA looping *in vivo* and consider whether or not it is likely that femtonewton forces play a significant role in gene regulation. In the context of DNA looping *in vivo*, we will reflect on how other mechanical constraints such as supercoiling, and sequence-induced curvature can provide stability against tension in the substrate DNA.

Force Scale of Protein-DNA Interactions

Over the past decade, the response of DNA-protein interactions to mechanical tension has received increasing attention and recent experiments provide insight into how mechanical stimuli might affect genomic response [10,11]. For instance, Wang *et al*. have shown that over 20 pN of force is needed to completely stall prokaryotic RNA polymerase [12] and Brower-Toland et al discovered that nucleosomes reversibly detach from DNA with tension under 50 pN [13]. These observations have been described by the general concept of mechanochemistry, which considers the alteration of a reaction's energy landscape as a result of an applied force [14].

For protein processes involving a single binding event, the reaction energy and the length scale over which this reaction occurs determine the force scale for mechano-chemical effects. Binding enthalpies of several k_BT (4.1 pN-nm) and sub-nanometer length interaction distances suggest forces of the order of 10 pN are needed to directly affect protein activity. However, for genomic protein complexes involving multiple binding sites on ds-DNA, a different force scale is relevant. In particular, 80 fN which is the ratio of k_BT to the persistence length of DNA (50 nm) is sufficient to stretch a DNA coil *in vitro* to approximately 50% of its nominal contour length [15]. This small force underscores the inherent flexibility of DNA and suggests that forces on the100 fN scale can impede the formation of complexes that involve multiple DNA binding sites.

PROTEIN-MEDIATED DNA LOOPING

Protein-mediated DNA looping is perhaps the simplest example for demonstrating the potential significance of femtonewton forces on genomic function. These loops form when two sites on a single DNA molecule are connected via a linker protein or multi-protein complex. Looping in prokaryotes often involves a single regulatory protein that binds to two distant but specific operator sites. Typical examples of looping systems include the *lac*, *gal*, and *ara* operons in *E. coli* [16]. Meanwhile, in eukaryotes, transcription is often initiated by transient looping between a distantly bound transcription factor and a complex of transcription factors at the promoter [17]. By providing a framework for a network of DNA-protein interactions, DNA looping provides a versatile gene regulatory mechanism. In addition, DNA looping provides a cooperative mechanism that can amplify the action of a small number of proteins, either by increasing the effective concentration of the protein or by providing a scaffold for building a larger protein complex [18].

From a physics perspective, DNA looping depends on the thermal fluctuations of the substrate DNA bringing distant operator sites into close proximity (see Figure 1). It has been shown in a considerable body of theoretical [19] and experimental [20,21] work that these thermal fluctuations of the substrate DNA are exquisitely sensitive to mechanical tension on the aforementioned natural force scale of 80fN for ds-DNA. Therefore, it is possible that transcriptional control through protein-mediated DNA looping is equally susceptible to such femtonewton forces.

Marko and Siggia considered the effects of tension on DNA looping by using a two-state statistical mechanics model of DNA looping [22]. In their model, loop formation is thought of as an all-or-none phenomenon in which binding of a looping

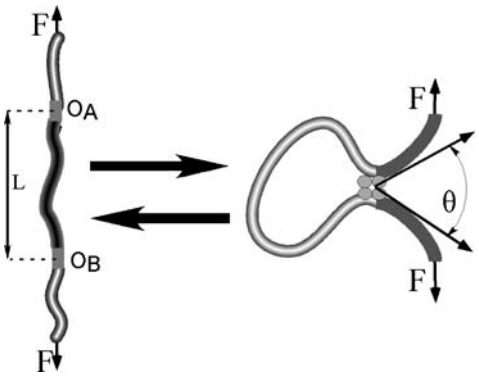

Figure 1. Two-State Model of Protein-Mediated DNA looping under tension
DNA looping under tension is characterized by the inter-operator distance (L), the applied tension (F) and the opening angle (θ). Tension tends to drive the equilibrium toward the unlooped state, particularly for large inter-operator distances.

protein coincides with the immediate storage of DNA of length, l, inside the loop. In this manner, a free energy, μ, can be assigned for loop formation in the absence of tension. Then, assuming looping is favorable in the absence of tension and that entropic effects can be ignored, the amount of tension needed to make looping unfavorable is $f^* = \mu/l$. For $\mu = 20\ k_B T$ (typical for protein-DNA interactions including the lacR-DNA interaction) and an $l = 50$ nm (150 bp) the critical force, f^*, is equal to 1.6 pN.

Recently, two models have been published that have extended the preceding results to include a more detailed consideration of the geometry of protein-DNA interactions and entropic contributions due to the flexibility of DNA. One model was developed by Sankararaman and Marko [23] and the other by Blumberg, Tkachenko, and Meiners [24] (subsequently denoted by SM and BTM, respectively). Both the SM and BTM models consider how the opening angle (called the kink angle by BTM) affects the response of DNA looping to tension. However, the SM model fixes the opening angle by minimizing the loop energy, whereas the BTM model considers the opening angle as fixed by the looping protein (see Figure 2).

The SM model explicitly calculates the looping probability based on the worm-like chain (WLC) description of DNA [25]. The SM model considers two possible loop geometries. The first is a teardrop geometry that is designed to minimize the bending strain of a WLC loop [26]. The second includes a 90° kink that can be induced by a DNA bending protein. SM present results for the case in which loop locations are localized by specific operator sites and for the case of non-specific looping locations. The results indicate that tensions under five hundred femtonewtons can have a significant impact on looping probability. This effect is particularly pronounced for the formation of non-specifically located, non-kinked loops.

Like the other models, the BTM model of DNA looping employs a two-state statistical mechanics description. However, the BTM model differs in that it focuses

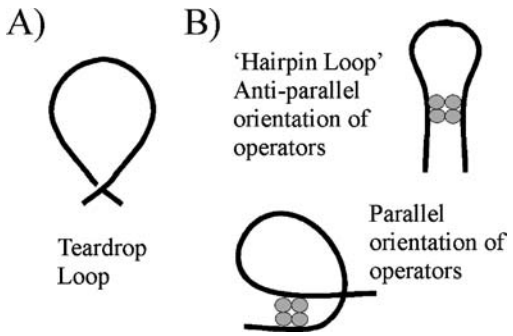

Figure 2. Comparison of Loop Geometries
A) Sankararaman and Marko's model of looping [23] focuses on loop geometries that minimize strain energy. These loops adopt teardrop shapes. B) In Blumberg, Tkachenko and Meiners model of looping [24], the looping geometry is dictated by the linker protein(s). Two example configurations are shown.

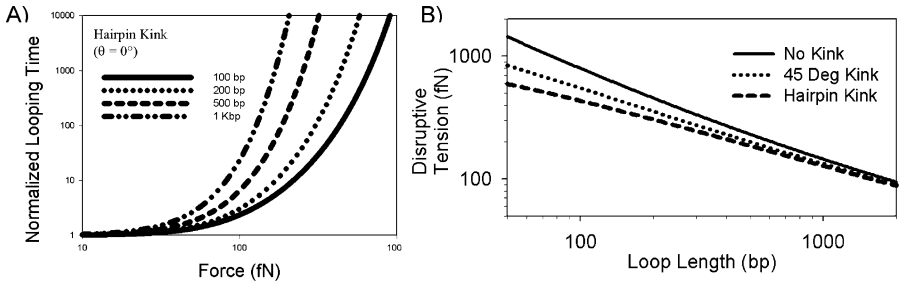

Figure 3. Effect of tension on DNA looping
A) *Normalized loop formation time vs. tension* for hairpin configuration of operators as calculated by the BTM model [24]. The legend indicates loop sizes. The normalized loop formation time is defined so that it is unity when tension is zero. In this way the specific effect of tension is isolated. B) *Disruptive tension vs. loop size*. The disruptive tension is defined as the tension that increases looping time by a factor of one hundred. Each line represents a different opening angle as indicated by the legend and defined in [24].

on the normalized loop formation time. This quantity, which is defined to be unity in the absence of tension, is a function of the tension, loop size and opening angle induced by the linker protein. Importantly, the normalized loop formation time does not depend on the specific details of the protein-DNA interface or loop energy. Thus the specific effects of tension can be isolated. Like SM, BTM find that subpiconewton tension can have a significant effect on the rate of loop formation. BTM also finds that for loops shorter than 500 bp the value of the opening angle can affect the degree to which tension disrupts looping. Hairpin looping configurations are particularly sensitive to tension (see Figure 3).

There are a couple of noteworthy shortcomings of the aforementioned models. For one, they do not explicitly consider the effect of torsional strain. The extent to which torsion affects looping depends on the helical flexibility of the linker protein, sequence dependent curvature of the inter-operator DNA and twist-induced conformational changes of the substrate DNA. Given the complex response of DNA to torsion, a quantitative prediction of its effect on looping is difficult to obtain. However, a few qualitative statements can be made. For instance, it is unlikely that torsion can effect the helical operator alignment by twist alone [24]. Rather the combined effects of twist and writhe need to be considered. A second shortcoming of the preceding models is that sequence specific effects such as intrinsic curvature or heterogeneous bending modulus are not considered. As demonstrated by experiments by Cloutier and Widom [27], this shortcoming of isotropic WLC models is particularly problematic for short strands of DNA. The effects of sequence heterogeneity are likely to be more evident when theory is used to compute the absolute looping probability rather than the relative effects of tension. Recent theory suggests that inclusion of hinge-like effects does indeed effect the response looping to tension, although not by a significant amount [28].

Due to its experimental and theoretical accessibility, protein-mediated DNA loop formation is an appealing system for investigating the effects of tension on DNA conformation and consequent alteration of biochemical activity. However, there are other protein-DNA interactions which may be affected by tension. For instance, Rudnic and Bruinsma have used the WLC model for DNA to describe how there could be a cooperative elastic coupling between two DNA bending proteins [29]. Cooperative binding arises in their model when two binding sites for a DNA bending protein are located close to one another. Then if DNA is under tension and only one bending protein is bound, the resulting kink creates unfavorable strain. However, binding of a second bending protein can negate this inhibitory effect and tension thereby promotes cooperative binding.

Despite alternative mechanisms whereby tension may effect protein-DNA interactions, the subsequent sections continue to focus on protein-mediated DNA looping. This may seem a narrow view to take, but the range of properties expressed by this simple and analytically tractable system will be generalizable to many protein-DNA systems. The commonality of the substrate DNA for the binding proteins means that these proteins may interact with each other through the deformation of the DNA. Transmission of mechanical tension in DNA would open up additional possibilities for novel distant interactions, and allow, for instance, bending proteins to also have allosteric-like interactions with DNA looping proteins. It is important to note that while such mechanical interactions through the DNA exist from a physical point of view their relevance in natural biological systems have not been established unambiguously yet.

TENSION SWITCH

To underscore the potential role tension can play in gene regulation, consider the well-known example of the lactose operon in *E. coli*. In this case, lac repressor (lacR) can form loops of 401 or 92 bp, each of which suppresses transcription [30]. The BTM model predicts that a tension of 270 and 840 fN increases the looping time by a factor of 100 for the large and small loops respectively (As suggested by crystallographic data, a kink angle of 154° is assumed [31]). Because the absence of lacR looping effectively prevents transcriptional repression, a 100-fold increase in loop formation time is comparable to the 70-fold transcriptional enhancement obtained by removal of both secondary operator sites [30]. This demonstrates that sub-piconewton forces may be sufficient to prevent protein mediated DNA loop formation and raises the intriguing possibility that weak mechanical forces act as a switch for gene expression. This effect is particularly striking when compared to the much larger forces associated with the processive motion of RNA polymerase. Since RNAP can exert forces over 20 pN before it stalls [12] a 'substrate tension switch' could theoretically offer control-signal strength amplification, or gain, of a couple orders of magnitude.

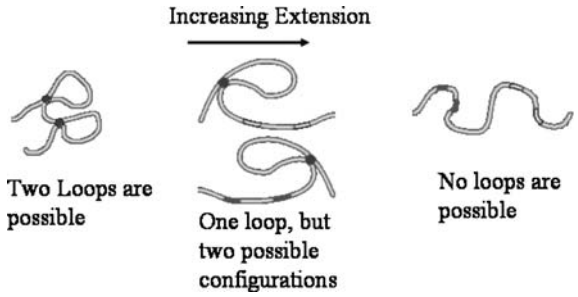

Figure 4. Multiple loop systems offers the possibility for a mechanically controlled, stochastic switch

For two consecutive pairs of looping operators, multiple configurations are possible. As the substrate DNA is extended, the number of loops that can be formed decreases. For moderate extensions, the system might act as a stochastic switch in which only one loop can form.

Mechanical Tension may Create Additional Regulatory Mechanisms

While we have focused on a single loop, the effect of tension on multiple loop systems offers additional novelty. The stochastic nature of multiple loop systems and the sensitive response to tension may provide a new paradigm for combinatorial control of DNA conformation and subsequent gene regulation. A simple, yet intriguing example of how tension may affect DNA looping combinatorics is illustrated in Figure 4. In this figure, two consecutive pairs of looping operators are found in a DNA strand. Each pair of operators binds to a different looping protein. If no mechanical constraints are present, then both loops can form. However, if the ends of the DNA are partially extended via fixed attachment points, then some tension will be present in the strand. If the tension is small enough such that one loop can form, then this will cause an increase in the tension of the substrate DNA. Consequently the ability of the second loop to form will be drastically suppressed. If the separation of the DNA ends is sufficiently large, then no loops can form at all.

If one links the formation of DNA loops to the control of an individual gene, then there are two interesting features of the theoretical system described in Figure 4. First, the modulation of end-to-end-extension allows the possibility to switch between three different regulatory states. Second, there is a region of moderate extension for which one or another gene is turned off, but not both. This results in an ‘exclusive or’ type of stochastic switch. To provide a quantitative example, the BTM model can be modified for constant extension (rather than constant force) by specifying that no external work is done by the tension. Then, consider a DNA strand of length 2.5 kbp containing two pairs of operators that is stretched to a constant extension of 425 nm (corresponding to 50% extension when no loops are formed). If each operator pair can form a 500 bp loop with parallel operator orientation, then according to the BTM model the looping time for a single loop will be a factor of three greater than the tension-free time. This is not a significant

increase. However, once one loop has formed, the resulting relative extension will be 62.5% and would have to increase to 83% for a second loop to occur. Thus the looping time for the second loop would be 800 times the tension-free looping time, significantly diminishing the probability of both loops occurring simultaneously.

The versatility of tension-controlled looping dovetails nicely with the concept of combinatorial regulation of transcription [32]. For example, the multiple binding sites for the SpGCF1 protein within the cis-regulatory regions of sea urchin genes provide a potential example of how multiple looping and associated combinatorial regulation may affect development. In particular, SpGFC1 is thought to bring distant domains into immediate proximity in order to facilitate additional transcription factor interactions [33]. If, as suggested by tensegrity models [3], mechanical stress is coupled to the stochastically driven differentiation patterns that emerge in a developing organism, then the comparatively simple multiple loop system discussed above can be used as a building block for modeling more complex behaviors such as might be expected of SpGFC1 and other multimerizing transcription factors.

This view of combinatorial looping schemes and response to mechanical constraints also provides new perspective on the lac operon. The wild-type lac operon contains one primary operator, O_1, and two weaker, auxiliary operators [30]. The stronger of the two auxiliary operators, O_2, is 401 bp from the primary operator and the weaker operator, O_3, is 92 bp away. While elimination of both operators significantly decreases looping probability *in vivo*, elimination of just one of the auxiliary operators does not have an apparent impact [30]. A natural question to ask then is why there are two auxiliary operators and not just one. One possibility is that the O_3 operator evolved in response to a need for an operator pair that was more stable than O_1–O_2 to the effects of tension or other constraints such as supercoiling. Thus it could be that *in vivo*, O_1–O_2 binding is preferred in some circumstances and O_1–O_3 binding is preferred in other circumstances. The presence of tension in vivo would break the near-equivalence of these two loops and thus reduce some of the perceived redundancy. While there is, of course, no *a priori* reason to think that this property of the DNA sequence is not due to chance – it is nonetheless a possibility that should not be ignored.

In Vitro Experiments

The femtonewton force scale has become increasingly accessible as new instrumentation to apply, measure and calibrate such small forces has been developed [21,34]. In particular, single molecule micromanipulation experiments provide ways to discern whether mechanical control of loop formation, and ultimately transcription, can be controlled through tension in the substrate DNA. Finzi and Gelles pioneered the observation of single molecule DNA looping by visualizing microspheres tethered to a glass cover slip by DNA containing two lacR operator sites. [35] DNA loop formation and breakdown was observed by measuring changes in the Brownian motion of the microspheres when lacR was present.

In recent years a number of optical and magnetic techniques have been developed for applying force and torsion on single molecules [36–38]. These advances have led to preliminary experimental data concerning the relationship between loop formation rate and mechanical tension. For instance, Yan *et al*. observed lambda DNA as it was looped at multiple locations by the restriction endonuclease BspM1 [39]. Then they used magnetic tweezers to show that a force of 1.75 pN is sufficient to recover the original length of DNA. This experiment provides an upper limit for the tension that is needed to drastically reduce the looping probability and supports Mark and Siggia's original consideration of the effect of tension on looping [22].

A second relevant micromechanical measurement was recently reported by Lia *et al*. [40]. They used magnetic tweezers to measure loop formation rates in a DNA fragment containing operators that bind the galactose repressor (galR). Their setup allowed looping to be observed under tension and torsional stress. They found that the looping rate was notably reduced when a tension of 1.32 pN was applied. Meanwhile, if the DNA was subjected to a negative twist of $\sigma = -0.03$ and heat unstable nucleoid protein (HU) was present, then looping could be observed when a linear tension of 0.88 pN was applied. In these experiments, the inter-operator spacing was 113 bp. At first glance, Lia *et al*.'s observation that looping could be easily observed when a tension of .88 pN was applied contradicts the aforementioned theory which suggests that this level of tension should drastically reduce the loop formation rate. However, there are a couple of factors that help resolve this discrepancy. First, the ability to observe looping at .88 pN is consistent with the possibility that the loop formation rate has been drastically reduced provided that the tension-free looping rate is much faster. In fact, Lia *et al*. argue that while they observed a loop formation time of 17 s, the stress free loop formation time is 0.1 ms. In addition, the required presence of HU affects the interpretation of Lia et al's in the context of the SM and BTM theories. By introducing a bend in the inter-operator DNA, HU decreases the effective stress-free distance between the two operator binding sites. According to the SM and BTM theories, the shorter effective distance implies an increased stability to the effect of tension. Thus the notion that sub-piconewton forces can prevent loop formation is still valid in as much as it establishes that under such tension mechanical stabilization through DNA-bending proteins is required to achieve looping. The preceding analysis suggests that if tension is not applied to a galR looping construct, then it may be possible for looping to occur in the absence of HU. Such observations have indeed been made in galR constructs that contain a 31–105 bp insert [41].

Future experiments will hopefully provide additional quantitative data to test the SM and BTM theories. Specifically, it would be useful to measure how inter-operator spacing and looping geometry affects the response to tension. To further understand the galR looping and the stability provided by HU, it would be particularly informative to discern how tension affects looping for various values for inter-operator spacing in the presence and absence of a bending protein. Besides

testing the quantitative predictions of the aforementioned theories, future experiments will hopefully address the potential biological significance by demonstrating a direct link between sub-piconewton tension and gene regulation. Some progress towards this goal has already been made. The ability to monitor transcription at a single molecule level was initially demonstrated for DNA tethered to a coverslip [42]. More recent experiments have shown how the velocity of RNAP is affected by an opposing force acting on RNAP itself [12,43]. However, it appears that experimental demonstration of a direct link between sub-piconewton tension control of DNA looping and transcription has yet to be made.

Tension in Vivo

The significance of theoretical and *in vitro* experimental analysis concerning the effect of tension on DNA looping hinges on whether DNA is stretched in vivo. Unfortunately, it appears that direct measurements of tension *in vivo* have yet to be made. However, some predictions can be made.

From a physics perspective, since DNA is confined within a cell, it cannot adopt an unconstrained Gaussian coil conformation. This suggests a lower limit for the constitutive tension equal to the magnitude of entropic thermal forces, $f_c = 80$ fN. Meanwhile, micromechanical experiments provide an indirect estimate of an upper bound for the constitutive tension. For instance, Strick *et al.* determined that intertwined plectonemes generated by negatively supercoiled DNA are pulled apart with .45 piconewtons of force [44]. In addition, Poirier and Marko argue that less than a piconewton of tension per chromatin strand is sufficient to disrupt the global structure of mitotic chromosomes [45]. Thus in order for DNA to maintain its topological form via supercoiling or large-scale chromosomal organization, these two experiments suggest that less than a piconewton of constitutive tension is present in the cell over large genomic distances.

Further indirect evidence that the constitutive level of tension is less than a piconewton comes from applying the BTM model to experiments that indirectly probe the intracellular mechanical environment. For instance, the experiments of Ringrose *et al.* [46] investigated looping via (FLP) recombination *in vitro* and *in vivo*. Their results provide evidence that ideal loop size for FLP activity is shorter *in vivo* than *in vitro*. They interpreted their results by calculating an 'effective persistence length' of 27nm for DNA *in vivo*, which is about half of the well-established persistence length of 53nm. In contrast, if we assume that the main difference between the *in vitro* and *in vivo* looping is that DNA *in vivo* is under tension, then we can use analysis of the effect of tension to explain their results in a way that is consistent with the wormlike chain model of DNA mechanics. In particular, when compared to the BTM model, their finding of an ideal loop size of 200 bp suggests an *in vivo* tension of 580 fN (See Figure 5 and ref [24]). There are many other possible explanations for the differences including structural changes caused by protein-induced DNA bending or supercoiling. These considerations will

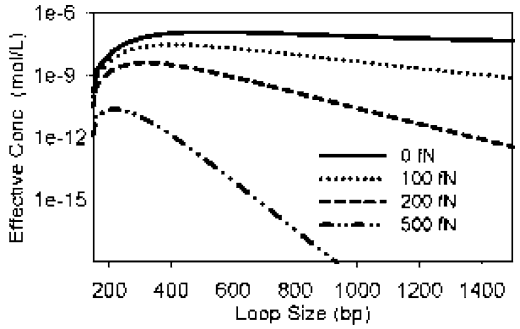

Figure 5. Effective concentration as a function of loop size and substrate tension
Effective concentration vs loop size for different values of applied tension as determined by the BTM model [24]. The effective concentration is a measurement of the likelihood for two unlooped operators to come into contact. Short loops have enthalpic limitations, while long loops have significant entropic costs. The ideal loop size is defined as the inter-operator spacing that maximizes the effective concentration. The legend indicates the tension applied to the substrate DNA.

tend to compact DNA and make it easier for small loops to form [47]. Thus the 580 fN value can be considered as an upper limit measurement for the amount of tension *in vivo*.

Since theory predicts that loop formation is significantly affected by piconewton level tension, it seems appropriate that constitutive *in vivo* tension does not exceed a piconewton. Otherwise, it would be hard for loops to form at all and their biological significance would be undermined.

While it appears unlikely that genomes are under constant piconewton tension, there are many reasons to believe that DNA is subjected to bursts of significant tension. Under appropriate conditions, protein-DNA interactions and molecular motors can provide strong sources of force. RecA alters DNA conformation [48], and its ability to resist forces as high as 100 pN [49] provides one example. The 20 pN stall force of RNA Polymerase is another example [12]. Moreover, DNA and many of its interactions with proteins are well suited to deal with such large forces. For instance it takes 60 pN of tension to induce a structural transition in DNA, [50,51] and nucleosomes can reversibly reassemble after being subjected to 50 pN forces [13].

As reviewed by Thanbichler *et al.* [52] for prokaryotes and Misteli [53] for eukaryotes, the mechanical architecture of the cell is quite elaborate. Dynamic changes of cellular architecture may provide a source of mechanical strain. For instance, it has been proposed by Dworkin and Losick that movement of RNA polymerase (RNAP) is often blocked by macromolecular obstacles [54]. If this is the case then transcription would generate strain as RNAP reels in its transcript. In fact, this mechanism has been postulated to be important in helping to segregate daughter chromosomes in bacterial replication, particularly because the transcription of

highly expressed genes tends to be directed away from the origin of replication. Thus it is possible that during cell division, DNA is under substantial tension and that this in turn effects gene regulation.

Another potential source of tension *in vivo* is the possibility of extensive DNA-cytoskeletal attachments. Any kind of passive connections to an underlying mechanical scaffold would result in tension on the order of the 80 fN entropic force generated by thermal fluctuation alone. In theory, cell growth, cell division, or migration of internal components could subsequently separate attachment points and tension on the order of piconewtons might develop. Thus, it is interesting to note that there is evidence that a contiguous molecular scaffold for mitotic chromosomes does not exist [55] and there is widespread speculation about the extent of a nuclear cytoskeleton in eukaryotes [56,57].

The preceding sources of DNA tension originate from intracellular mechanisms. However, as mentioned in the introduction, it has been proposed by Ingber that extracellular force can be transferred intro eukaryote nuclei and that this is an important means of transforming mechanical signals into biochemical outcomes [3]. It remains to be seen how this tensegrity model of mechanotransduction can be reconciled with aforementioned critiques of the extent of a nuclear scaffold [56,57]. It is possible that very few cytoskeletal contacts are needed for effective force transfer. Alternatively, force transduction within nuclei could occur through indirect means such as molecular crowding in response to nuclear membrane deformation.

Mechanisms that Provide Mechanical Stability

The preceding discussion suggests that the constitutive level of tension that DNA is subjected to is low, but there may be transient periods when piconewton level tension is common. Given the disruptive effect of tension on DNA looping, it is natural to ask whether looping could occur at all during periods of increased tension. In addition, large loops are particularly sensitive to tension and may be difficult to form even if the level of constitutive tension is under 100 fN (The BTM model suggests that just 150 fN of tension is needed to increase the loop formation time of 1 kbp loops by 100). However, as discussed below there are a number of compensatory mechanisms that may allow loop formation to occur at levels of tension higher than that predicted by the SM or BTM models.

Stability via smaller loops

To quantitatively describe how the kinetics of protein-mediated DNA loop formation is affected by inter-operator spacing, the simple yet instructive concept of an effective local operator concentration can be employed. In this model, loop formation is treated as a bimolecular reaction between a protein that is bound to one operator site and a second unbound operator site on the same DNA molecule. If it

is assumed that the loop formation rate is limited by the diffusion of one operator to the other, the reaction rate is proportional to the local concentration of one operator at the location of the second operator. To compute this local operator concentration as a function of inter-operator separation for unconstrained DNA, Shimada and Yamakawa (SY) use the WLC model to derive an interpolative formula that quantifies a balance between the bending energy that is required to bring the two operator sites in contact and the volume that is accessible to the diffusing operator [58]. For short DNA loops the bending energy dominates, while for long loops the entropic cost of a large accessible volume is the limiting factor. There is a loop length that represents a balance between these opposing effects and maximizes the effective concentration. We term this length the 'ideal loop size'. In an unconstrained environment the SY model predicts an ideal loop size of 500 base pairs, a value that has been confirmed in in-vitro measurements of DNA cyclization [59] and FLP recombination [46]. To determine how the effective operator concentration is affected by tension the SY expression for effective concentration is divided by the normalized looping time of the BTM model. The results are shown in Figure 5 which plots the effective concentration versus loop length for different values of applied tension.

Figure 5 shows that as the tension is increased, the effective concentration decreases because it becomes harder for the operator sites to make contact. Moreover, tension causes the effective concentration peak to shift to the left and therefore decreases the ideal loop size. Quantitatively, the BTM model predicts the ideal loop size is 530, 320 and 160 bp for tensions of 0, 200 and 1000 fN respectively [24].

As a caveat, the preceding analysis applies for idealized WLCs which have a uniform, isotropic bending modulus and no intrinsic curvature. However, the WLC is known to be problematic for short strands of DNA and this can alter the predicted looping probabilities. For instance, sequence-specific curvature or bends induced by proteins can significantly enhance the ease of loop formation for small inter-operator sequences [47]. The analysis of ideal loop size is further complicated by the possibility that DNA has spontaneous bending instability that heightens the loop formation of small loops, a viewpoint that is motivated by recent experiments by Cloutier and Widom [27] and analyzed by several groups [60–62]. The need for helical-operator alignment and subsequent 10.5 bp periodicity in looping probability [63,64] is an additional consideration that is often ignored in theoretical analysis of effective concentration. However, all the additional considerations that take into account deviations from the isotropic WLC approximation do not change the premise that ideal loop size is determined by balancing the effects of entropy and bending energy. Thus the key result that ideal loop size decreases as continuous tension is applied still holds.

Assuming DNA is subject to tension *in vivo*, consideration of effective concentration suggests that small loops are more appropriate for the complex mechanical environment of a living cell [24]. The need for small loops may be particularly important in the eukaryotic nucleus because of the extensive interplay between

chromatin structure and gene regulation [65]. Indeed, eukaryotic RNAP often loops with activators that are less than 200 bp away [66], which is shorter than the ideal loop size seen in a force free environment.

Stability via temporal dynamics

The theory and the single molecule *in vitro* experiments that have been described pertain to tension that is applied at a constant level. However, this contrasts markedly with the complex mechanical environment of a living cell. Because of the almost exponential relationship between loop formation time and applied tension, the looping probability will increase markedly if there is a period of time for which DNA is under less stress. That is, if there is significant variation of tension with time, then the effective 'disruptive force for DNA looping' will be less than the average applied tension. As a quantitative example, a Poisson distribution of tension with mean 1pN and standard deviation of 320 fN would yield an identical looping rate for a 100 bp hairpin loop as a continuous tension of 620 fN (according to the BTM model). Equivalently, the loop formation of a 100 bp hairpin loop with a fluctuating tension of 1 ± 0.32 pN in the substrate DNA would be forty times more probable than if a constant tension of 1 pN was applied. The concept that temporal dynamics provides stability to looping processes has been well described by Vilar and Leibler with regard to fluctuations of linker protein concentrations and transcriptional noise (i.e. fluctuations in RNAP activity) [67].

Stability via favorable kinetics

The theories we have considered are based on equilibrium statistical mechanics. As such they do not take kinetic factors into direct account. However there are non-equilibrium strategies that can be employed that either makes it easier for loops to form or make it harder for them to break apart. In these cases, loop formation and associated biology become more stable to the effects of tension.

One example of how assembly can be stabilized pertains to lacR looping. Balaeff *et al*. have postulated that there are two stable loop sizes involving lacR, 76 bp and 86bp [68]. The latter loop size involves CAP binding. So it seems possible that initially a 76 bp loop forms and then CAP binding induces a conformational change that results in an 86 bp loop. As discussed above, the smaller loop size protects against the effect of tension. Then as the loop is enlarged, additional protein-DNA interactions stabilize the looped structure. In this case, the loop formation process is potentially enhanced by incremental changes in structure that provide stability against external mechanical constraints.

In contrast to mechanisms that enhance the kinetics of assembly, eukaryotic nucleosomes provide an example of how existing DNA-protein interactions can be shielded from disruptive effects of tension. Nucleosomes represent a unique example of DNA looping in that instead of there being two distinct DNA operators that cause looping, there is fairly continuous contact between histone proteins that

form the nucleosomes and the DNA that is looped out. This has two effects. For one, nucleosome assembly is more stable to the effects of tension because DNA wrapping around a nucleosome can be thought of as the formation of a series of very small loops rather than one larger 146 bp loop. Second, once a nucleosome is formed, there is a large kinetic barrier that prevents its disassociation. Kulic and Schiessel discuss how kinetic barriers arise from the particular 'spool' geometry of nucleosomes [69]. Furthermore, by occupying the interior of a nucleosome the histone proteins may provide a steric barrier for unpeeling via force. As noted by Marko and Poirier, these kinetic barriers make it difficult to interpret micromanipulation experiments that measure nucleosome disassociation as a function of tension [49]. For instance Brower-Toland *et al*. [13] find that nucleosomes tend to pop off at tensions around 20 pN. While this experiment represents a significant advance in measurement technique, it is an order of magnitude larger than that predicted by thermodynamic analysis and suggests that this type of pulling experiment is conducted far from thermal equilibrium. Interestingly, nucleosome release *in vivo* can be facilitated by other mechanical means such as positive supercoiling [70–72]. Thus while tension may effect the overall probability of nucleosome formation, other mechanical factors may be more influential in transitioning between equilibrium probability distributions.

Stability via protein induced bends or sequence specific curvature

The effects of DNA bending or sequence specific curvature are not straightforward. For instance, several researchers have shown that different sequence curvature can result in different topologies for lacR and SfiI endonuclease loops [73,74]. In general, curvature and bending bring operators closer to each other than they would otherwise be for a straight DNA segment. This decreases the effective operator distance and thus stabilizes looping against the disruptive effects of tension. As discussed earlier, the stabilizing effect of HU bending is relevant in the context of Lia *et al*.'s observations of galR looping in the presence of .88 pN of tension [40], particularly in trying to estimate the loop formation rate in the absence of tension [24].

The effects of sequence induced curvature and bending have been studied by a number of groups [75–77] in the context of force-free looping. Meanwhile, the SM model confirms that when loops have a protein-induced kink in their midline, they are less sensitive to tension [23]. However, the SM model does not consider the complications of helical-operator alignment. This latter constraint refers to how the phase of a bend affects whether operators sites are oriented towards the inside or outside of the bend. The consequent effect on strain energy is what drives the alternative topologies seen in the lacR and SfiI endonuclease experiments. It appears more theoretical and experimental research on the effects of bending would be useful. A full description of how sequence specific curvature and tension interrelate may necessitate computational methods, such as provided by rod mechanics simulations [78].

The Role of Supercoiling

Supercoiling has long been considered a paradigm for genetic control by substrate mechanochemistry. Nonetheless, many questions involving the underlying mechanics remain open, and partial answers have been surrounded by some controversy. Generally, it is assumed that a large increase in looping rate has to follow from the increase in local concentration of an operator site at another operator site if both are incorporated into a plectoneme. In its simplest form, the argument is that in supercoiled DNA the DNA is confined by steric hindrance to a one-dimensional reptative motion along its contour length. The concomitant reduction in the number of free parameters that a random walk must scan to get a desired result is thought to necessarily improve the kinetics of that process. For a visual comparison of these diffusive modes refer to Figure 6.

Theoretic work by Marko and Siggia in 1994 suggested that the view outlined above is incorrect [79]. The 1-D reptation of strands within a plectoneme is correlated over much longer distances than simple 3-D diffusion of an unconstrained fiber. Indeed, the 1-D motion of the DNA in its tube-like constrained environment is almost fully correlated over the size of the plectonemic domain. This plectoneme domain size is typically determined by the length scale on which the plectoneme branches spontaneously, which is typically of the order of several kilobases. This means that much larger pieces of DNA must be shifted by Brownian forces in a plectoneme to juxtapose two distant operator sites compared to the 3-D case. The corresponding increase in drag forces leads to slower operator diffusion. To model loop formation in supercoiled DNA, the process can be regarded as linear diffusion to capture. Using such a model, Marko and Siggia found the reaction time of two sites in a typical plectoneme versus two sites in a random coil with separation

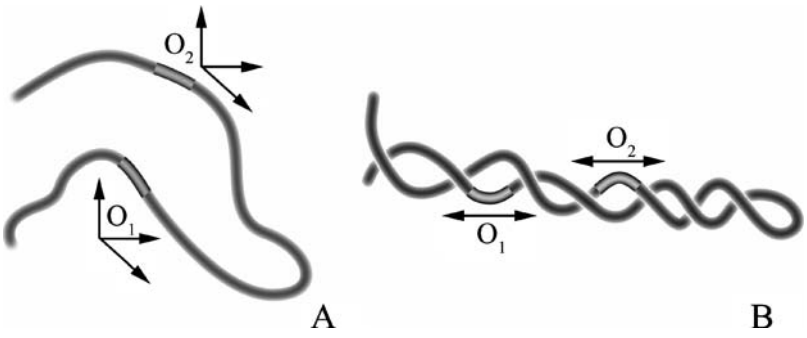

Figure 6. 3-Dimensional versus 1-dimensional diffusion for operator juxtaposition
A) In the absence of supercoiling, a DNA strand will fluctuate in all three dimensions, meaning that the process of operator juxtaposition will follow 3D diffusion kinetics. B) In the presence of supercoiling DNA may form interwound plectonemic domains. In this case, the motion of the DNA is highly restricted and the relative motion of two operators can be characterized by 1D diffusion along the contour length.

greater than a few persistence lengths vastly favored the random coil. Indeed, for the reaction rate to favor a slithering mode with an operator separation of 450 bp, the size of the plectonemic domain would have to be less than the operator separation itself. A long plectoneme, however, can be considered a flexible polymer in its own right with stiffness double that of a single strand, and a separation between sites on average half that of an unfolded chain. In this plectoneme bending and self-encounter mode, Marko and Siggia find that these factors approximately cancel out, and the loop formation rate is nearly that of the unconstrained chain. For two widely separated operators that lie in different plectoneme branches, loop formation rates are enhanced by the increased compactness of the intervening polymer. Marko and Siggia, find that for distances over 50 kbp this effect accounts for a reduction in the reaction time by a factor of two, which is still quite small.

Simulation work published by Jian, Schlick, and Vologodskii in 1998 suggested a different model for the role of supercoiling in an enhanced rate of site juxtaposition [80]. Their work suggests that the interwound domains of DNA in a supercoiled plasmid do not exist stably and slither past each other along their long axes, so much as fluctuate wildly, colliding frequently and rapidly changing overall conformation subject to their geometric constraints. Thus site juxtaposition and loop formation occurs through rapid three-dimensional diffusion in a smaller volume than the un-supercoiled plasmid would occupy. As a result, a strong increase of loop formation rates was observed for supercoiled DNA. It is important to note that these simulations were conducted at levels of electrostatic shielding significantly below what would be expected from the ion concentrations in a live cell (10 mM Na^+ compared to 200 mM Na^+ and K^+). Using more realistic assumptions for the electrostatic interactions, the same group published new data in 2001 that significantly extended their previous results in a surprising way [81]. When the DNA was not so aggressively self-avoiding it did form plectonemes, and site juxtaposition was primarily through the mode of reptation. Furthermore, they did not observe an enhancement of the rate of site juxtaposition as a function of supercoiling under these conditions.

Important considerations for all of these models are the initial conditions. A common initial condition is that of a random initial conformation, followed by diffusion, terminated by capture at an absorptive barrier. More biologically relevant, however, is an initial condition of very close operator apposition, as it would occur right after the breakdown of an existing loop. In this case, immediate recapture contributes significantly to the loop formation rate, and the likelihood of one operator "escaping" another and traversing a great distance as measured by contour length is greatly favored by more dimensions accessible to motion. Similar arguments have been put forward to defeat the notion of 1-D scanning by diffusible DNA binding proteins as speeding their searching for operator sites [82].

Embleton, Vologodskii and Halford conducted an experiment to further shed light on this issue: Using a system of competitive loop formation in a plasmid by the restriction endonuclease SfiI, they showed that the equilibrium abundance for

a small 410 bp loop was greater than that of a large 2 kbp loop [83]. When the initial rate for loop formation was probed in the same system, however, only a small difference in the rate of loop capture was found for loops of differing sizes, suggesting a non-trivial dependence on the stability of loops in supercoiled DNA as a function of length.

Supercoiling is also intimately related to the femtonewton force domain that is the primary subject of this paper. By providing a context for understanding the biological significance of tension, the SM and BTM models provide a unique perspective on DNA supercoiling. Single molecule experiments show that when negatively supercoiled DNA is stretched with forces below .45 pN, the plectonemes remain coiled while the solenoidal domains are stretched [44]. Below this critical force, the plectoneme branches are isolated from the effects of tension and two operators that are normally located within plectonemes can align themselves just as easily as if no tension were present. In the case of positively supercoiled DNA, it appears that 3 pN of tension is required to pull apart plectonemes. The higher critical force provides additional mechanical stability. Supercoiling may therefore be able to protect loop formation against the disruptive effects of tension (see Figure 7). In addition sequence dependent localization of plectoneme ends [84–86] could potentially augment the likelihood that two operators will be insulated from tension.

It is also worth considering, when considering femtonewton forces on DNA, their angular equivalents, i.e. the dynamical relationships between torques and angular strain (over- and under-twist) about the longitudinal axis of the DNA. These have been probed experimentally both *in vitro* and *in vivo* by a number of ingenious methods. Importantly, promoter elements that sensitize and de-sensitize a promoter to local twisting have been identified [87]. The question of the physical behavior of regulatory supercoiling again brings up the role of the RNA polymerases. Simple topological reasoning indicates that, from the point of view of DNA, RNA polymerase is spiraling about it as it transcribes, whereas from the point of view of RNA polymerase, DNA is spiraling around, corkscrewing through its active site.

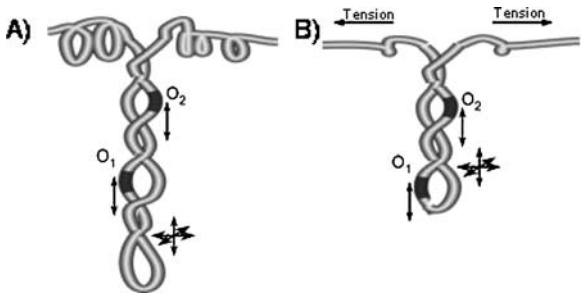

Figure 7. Supercoiling provides mechanical stability
A) In the absence of tension, twisted DNA forms plectonemes (braided regions) and solenoids (spring regions). B) In the presence of tension, the solenoidal regions become extended, but the plectonemic regions retain their topological form.

Newtons third law requires that, regardless of which is "stationary," reaction forces exist between these two bodies, and biochemistry has revealed many proteins exist as the agents of, and to dissipate this torque. The rapidity and degree to which a wave of torsional strain (and thus stress) can propagate down a DNA fiber is largely independent of the conformation of the DNA in between (in dramatic counterpoint to linear displacements), and has an effective decay distance of on the order of a kilobase *in vivo* [88]. Combine this with the presence of torque-sensitive promoters, and it is clear that merely the action of transcribing a gene has an effect on the genes in the neighborhood of that gene, as has been suggested experimentally [89]. This sort of dynamical role for supercoiling continues to be a rich area for study, and is a fundamentally different paradigm than the more topologically motivated view that is commonly held, though they are not mutually exclusive.

Conclusion

Theoretical arguments based on well-established principles of polymer physics provide sound evidence that sub-piconewton forces can affect DNA looping in vitro. Preliminary data from micromechanical measurements supports the notion that mechanical factors, including tension, affect the rate of DNA looping. In principle, a link between tension and DNA looping would imply that gene transcription can be modified by a sub-piconewton force switch. However, it is hard to discern whether this type of gene regulatory mechanism is present *in vivo*. On the one hand, molecular motors and DNA-protein interactions are capable of exerting significant force on DNA. On the other hand, DNA appears to be sufficiently detached from mechanical intracellular scaffolds and is therefore not exposed to significant stretching forces. In addition, there are many compensatory mechanisms that can be used inside a cell to insulate DNA looping against the disruptive effects of tension, including sequence-specific curvature, supercoiling and favorable kinetic schemes. Thus, future research into the mechanics of DNA looping is expected to yield interesting results either because it will demonstrate how mechanical factors can directly affect gene regulation or because it will clarify how biochemical activity is buffered against the effects of intracellular mechanics. We conclude by emphasizing that DNA looping is a single example of the importance of mechanics in molecular biology and we hope that future studies will provide new insights into the intricate relationship between molecular biology and intracellular mechanics.

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