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The Structure of DNA Overstretched from the 5'5' Ends Differs from the Structure of DNA Overstretched from the 3'3' Ends

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ABSTRACT

It has been suggested that the structure that results when double-stranded DNA is pulled from the 3'3' ends differs from that which results when it is pulled from the 5'5' ends. In this work we demonstrate, using λ phage dsDNA, that the overstretched states do indeed show different properties, suggesting that they correspond to different structures. For 3'3' pulling versus 5'5' pulling, the following differences are observed. (i) The forces at which half of the molecules in the ensemble have made a complete force-induced transition to single stranded DNA are 141 ± 3 pN and 122 ± 4 pN, respectively. (ii) The extension vs. force curve for overstretched DNA has a marked change in slope at 127 ± 3 pN for 3'3' and 110 ± 3 pN for 5'5'. (iii) The hysteresis (H) in the extension vs. force curves at 150 mM NaCl is 0.3 ± 0.8 pN μm for 3'3' versus 13 ± 8 pN for 5'5'. (iv) 3'3' and 5'5' molecules show different changes in hysteresis due to interactions with β-cyclodextrin, a molecule which is known to form stable host-guest complexes with rotated base pairs, and glyoxal that is known to bind stably to unpaired bases. These differences and additional findings are well-accommodated by the corresponding structures predicted on theoretical grounds.

INTRODUCTION

Double-stranded DNA (dsDNA) exists in a variety of different conformations *in vivo*, even in the absence of force. Changes in the conformation of dsDNA can be produced by applied force and torque, both *in vivo* and *in vitro*. Some effects have been explored using single molecule techniques that stretch and / or twist single dsDNA molecules (1-10). Many research groups have shown that if a force of approximately 65 pN is applied to the ends of a long dsDNA, the molecule will undergo an abrupt overstretching transition into a structure having a length-per-base pair that is approximately 1.7 times the length of B-DNA (1-10). It has been proposed that the structure of overstretched DNA plays an important role in the function of proteins whose interaction with dsDNA involves a change in length (11-14).

The overstretching transition has been extensively studied; however, the nature of the overstretched state or states remains in dispute (1-10,15). Theoretical proposals of Lebrun and Lavery (16) have suggested that, because the base pairs in B-DNA are tilted with respect to the backbone, the nature of the overstretched structure would depend upon the ends to which force is applied. Pulling from the two 5' ends of a duplex (hereafter 5'5') should increase base pair tilt; oppositely, pulling from the two 3' ends (3'3') should decrease base pair tilt (Fig. 1A). Though this proposal has a great intuitive attraction, experimental results published previous to this work have not provided unequivocal support for the predicted structural difference (1,2,8-10,17).

In this paper, we apply a constant force to the ends of bacteriophage λ DNA, ~48,000 bp, and demonstrate several consistent differences, all of which are in agreement with

and/or predicted by the overstretched dsDNA structures proposed by Lebrun and Lavery (16).

RESULTS

Background. Pulling experiments were performed using an apparatus, described in detail previously (18), that permits parallel analysis of the effects of force on 200-300 DNA molecules in a single experiment (Fig. 1B). For each molecule in such an ensemble, one end is attached to a fixed surface (glass capillary) while the other end is attached to a magnetic bead. A permanent magnet is then brought close to the surface, resulting in a force that pulls the magnetic bead away from the surface. The direction of the magnetic field is parallel to the direction of the applied force and the axis of the DNA molecule, so the bead is free to rotate.

In the current study, force was exerted on 3'3', 5'5' or 3'5' ends of bacteriophage λ DNA, ~48,000 bp, using a series of constructs in which dsDNA is tethered to the bead and to the surface by one strand terminus at each end, in each case via extravidin linkages to biotinylated single-stranded DNA (ssDNA) spacer linkers (Fig. 1A). The ssDNA spacers are present to ensure that force is applied to only one phosphodiester backbone at each end. Such open single-stranded regions also allow free rotation of the attached dsDNA (19), which prevents the permanent accumulation of torsional stress during the pulling process.

All three types of DNA substrates undergo very similar responses to force: (i) straightening against entropic effects at very low force; (ii) a discrete increase in length to ~1.7 times that of B-DNA at about 65 pN (1, 2); and (iii) the force-induced separation of

the DNA into two complete separate strands ("melting") at higher forces. These similarities are illustrated for representative individual molecules subjected to 3'3' and 5'5' pulling (Fig. 1C, blue and red curves, respectively) and for 3'5'pulling (see SI Fig.6). As illustrated by these data, the forces required for overstretching, the widths of the overstretching transition, and the lengths of the overstretched states are all very similar for all three pulling conditions. We have analyzed hundreds of molecules in each of the three configurations. In 150 mM NaCl, F_{ov}, the force at which the overstretching transition is half complete, is 64.1 ± 3 pN for 3'3' pulling and 65.8 ± 3 pN for 5'5' pulling. F_{ov} for 3'5' pulling in 150 mM NaCl is 65.1 ± 3 pN. The three substrates continue to show very similar extension vs. force curves for increasing forces up through about 80 pN as can be seen in Fig. 1C for 3'3' and 5'5' and in SI Fig.6 for 3'5'. At higher forces, as described below, the extension vs. force curves for each of the three constructs of the overstretched DNA become dissimilar; in particular, the force required to melt the DNA depends on the ends to which the force is applied, as we will discuss in detail below.

3'3' and 5'5' pulling induce force-dependent melting at different forces.

Applying a force to either the 3'3' ends or the 5'5' ends of a dsDNA molecule can result in the force-induced separation of dsDNA into ssDNA strands (above). In the experimental protocol used here, force-induced separation of dsDNA strands into ssDNA results in a loss of linkage of the bead to the surface (Fig. 1C) that is detected as a rapid movement of the bead away from the surface in the direction of the force. To analyze force-induce melting in detail, a set of molecules was first placed under a constant initial force for a time $\Delta T = 1$ second. Force was then increased at 1-second time intervals in force steps of $\Delta F = 4$ pN for forces less than 50 pN and $\Delta F = 2$ pN for higher forces. The

same force-change algorithm was used for all of the pulling techniques. The fraction of beads that have left the surface during each 1-second interval was determined at the end of the interval. Data was only taken while the force was constant.

Figure 2A shows that, as force is increased from 80 to 100 pN, the fraction of magnetic beads that remains attached to the surface by DNA molecules decreases progressively both for 3'3' and 5'5' pulling (blue and red lines, respectively) with both decreases well-described by the same single exponential decay as a function of force (Fig. 2A, dashed line). This is equivalent to an exponential decay as a function of time, if the decay constant is insensitive to force.

The forces at which the losses for the two pulling techniques begin to increase beyond the initial exponential are 125 pN for 3'3' pulling and 110 pN for 5'5' pulling (see arrows on Fig. 2A). We interpret the initial exponential decay as the failure of the biotin terminal linkages that anchor each dsDNA molecule to the surface and its bead, and the subsequent increase in decay to force-induced melting. This interpretation is supported by analysis of bead loss for molecules attached by their 3'5' ends, where beads are released by failure of a tether but not by melting of the DNA (Fig. 2A green line). In this case, bead loss is characterized by a single exponential, indicative of tether failure, until the applied force exceeds 165 pN, a force much greater than that required for melting of DNA pulled from the 3'3' or 5'5' ends. It is possible that the increase in decay rate at 165 pN occurs because the decay rate for the tethers is no longer insensitive to force. Thus, we interpret the data shown in Fig. 2A to mean that molecules bound by their 5'5' ends undergo force-induced melting at lower forces than molecules bound by their 3'3' ends.

To mathematically isolate force-induced melting from effects due to tether decay, the primary data for 3'3' and 5'5' pulling shown in Fig. 2A were multiplied by $\exp[\alpha \text{ force}]$, where $\alpha=0.0255$. A linear plot of the results is shown in Fig. 2B where the lines in the figure are a guide to the eye. At lower forces, where bead release is characterized by a single exponential associated with tether failure, the adjusted value for the fraction of beads remaining is approximately equal to 1 and independent of force; however, when the decay rate increases at higher forces, the adjusted values begin to decrease significantly below 1 and to depend strongly on force. Given these effects, one characterization of the stability of the molecules is the force, $F_{1/2SsDNA}$ at which the number of the remaining beads is equal to half the value that would be expected if the bead loss were due only to the decay of the tethers, i.e. when the adjusted fraction of the beads remaining reaches 0.5 (Fig. 2B). For 3'3' pulling we find that $F_{1/2ssDNA} = 141 \pm 7$ pN (black circles) whereas for 5'5' pulling, $F_{1/2ssDNA} = 123 \pm 4$ pN (gray squares).

3'3' and 5'5' overstretched structures show changes in the slope of the extension vs. force curve at different forces.

Bead loss can be affected by the failure of the biotin-extravidin bonds; therefore, we developed a technique for studying mechanical stability in which the measurement is not affected by the failure of these biotin bonds. As noted above with respect to Fig. 1C, the slopes of the extension vs. force curve for the 3'3' and 5'5'overstretched states are approximately constant for forces up to about 100 pN; however, at higher forces, there is a marked increase in slope. The dotted lines in Fig. 1C show the force, F_{start}, at which this change in slope occurs for each of the two curves shown.

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For purposes of this analysis, a measurement of F_{start} was only considered valid if, at forces above F_{start}, the molecule increased in length by at least 500 nm before the bead separated from the surface. This length change criterion was chosen to make sure that the change in slope is not associated with a failure of one of the several extravidin-biotin linkages, since such failure would result in a distance increase of less than about 5 nm (Fig. 1C). We also note that, at forces between 70 pN and the force at which this change in slope occurs, the time during which we pause at each force is sufficient for the molecule to reach a length that remains constant for time intervals longer than a minute. Thus, the extension vs. force curves represent equilibrium states, or at least states that are metastable for minutes.

F_{start} values were determined for tens of individual molecules. For each total population of molecules, the fraction having an F_{start} value greater than a particular given force was plotted as a function of force (Fig. 3A: dashed vertical and horizontal lines are included for clarity). For 3'3' pulling (black line) this analysis yields a transition force of $F_{1/2Fstart} = 127 \pm 3$ pN whereas for 5'5' pulling (gray line) the transition force is $F_{1/2Fstart} = 110 \pm 3$ pN. As these results are unaffected by tether failure, they provide an additional strong indication that the two overstretched structures are different.

As a further check on the consistency of results from the bead-loss assay versus melting transition analysis, we estimated F_{start} from the melting analysis and compared that with the F_{start} values determined by bead loss. If we assume that most or all of the molecules exhibiting a definable F_{start} also go on to complete the force-induced melting transition before their tethering fails, then F^{*}_{ssDNA}, the force at which the bead separated from the surface reflects the mechanical stability of the DNA; thus, F_{start} defined by bead

loss analysis and F_{start} defined by this transition should correspond. Here the asterisk indicates that these values are obtained for the subset of the molecules with a well defined F_{start}. The fraction of molecules exhibiting an F^*_{ssDNA} greater than a given force is plotted as a function of force as can be seen in Fig. 3B. The forces at which half of the molecules in the sample have undergone a force-induced transition to ssDNA (vertical lines) are $F^*_{1/2\ ssDNA} = 138 \pm 3$ for 3'3' pulling, and $F^*_{1/2\ ssDNA} = 122 \pm 3$ for 5'5' pulling. Importantly, $F^*_{1/2\ ssDNA}$ distributions calculated in this way for the subset of molecules that show a clear F_{start}, are in good agreement with the $F_{1/2\ ssDNA}$ values determined by bead loss analysis, 141 and 123 pN, respectively (above). Thus, these results suggest that (i) the effects due to tether failure were successfully removed from the data shown in Fig. 2B and (ii) F_{start} does signal the onset of force-induced melting. Additional evidence that suggests that F_{start} is the onset of force-induced melting is presented in the supplemental information.

3'3' and 5'5' structures exhibit different dependencies of hysteresis on charge screening by monovalent salt.

In the structures proposed by Lebrun and Lavery (16), the minimum spacing between the backbones is smaller in the 5'5' overstretched structure than in the 3'3' structure. This difference suggests that, in some ionic strength conditions, the repulsion between the phosphate backbones in the 5'5' overstretched structure might be less well screened than the repulsion between the phosphate backbones in the 3'3' overstretched structure. Such a difference in screening may explain why the 5'5' overstretched form melts at a lower force than the 3'3' overstretched structure, as suggested in previous work (20). Earlier work using 3'3' pulling has also shown that if the charge screening is inadequate, DNA does not return efficiently from the overstretched state to the B-form, resulting in a

hysteresis in the extension vs. force curves (21). By extension, if in a particular buffer the 5'5' overstretched state is less well screened than the 3'3' state, then in that buffer the hysteresis for 5'5' should be larger than for 3'3'. In what follows, we describe the results of hysteresis measurements at a range of charge screening conditions, and find that at 150 mM NaCl the 3'3' overstretched structure is indeed more effectively screened than the 5'5' overstretched structure, whereas at 1 M and 20 mM NaCl concentrations we do not observe significant difference in screening.

Typical extension vs. force curves for 3'3' and 5'5' pulling in the different ionic strength conditions are shown in Fig. 4. In all conditions, the curves for all cycles overlap at low forces, suggesting that almost the entire molecule has returned to its original B form at low forces. The hysteresis for 3'3' and 5'5' are similar at high and low salts, 1M and 20 mM NaCl respectively, suggesting that the charge screening is similar in both cases; however, at an intermediate salt concentration of 150 mM NaCl, the hysteresis is much more dramatic for 5'5' pulling than for 3'3' pulling, suggesting that the 3'3' is well screened but the 5'5' structure is not. These results suggest that the effective screening length in 1 M NaCl is smaller than either backbone spacing and that the effective screening length in 20 mM NaCl is larger than either backbone spacing, whereas in 150 mM the effective screening length is larger than the 5'5' backbone spacing but smaller than the 3'3' backbone spacing. This result is consistent with Lebrun and Lavery's prediction that the backbone spacing in the 5'5' structure is narrower than the spacing in the 3'3' structure.

It is possible to quantify the hysteresis, H, in the extension vs. force curves (see SI text). For 150 mM NaCl, the resulting H values are 0.3 ± 0.8 pN μm for 3'3' and 13 ± 8

pN μm for 5'5' pulling. Similarly, the H value for 3'3' pulling in 1 M is 0.5 ± 0.5 pN μm and 44 ± 36 pN μm in 20 mM NaCl. For 5'5' pulling in 1 M NaCl we obtain H = 1.1 ± 1.4 pN μm and 49 ± 30 pN μm in 20 mM NaCl. We note that, while preparation of 3'3' and 5'5' substrates involves two and four ligation steps, respectively, this difference cannot be the basis for hysteresis because 3'3' preparations with two and four ligation steps show the same hysteresis (see SI text).

Hysteresis of 3'3' and 5'5' structures are differentially affected by both cyclodextrin, which binds flipped bases, and glyoxal, which modifies unpaired bases at positions involved in H-bonding

Lebrun and Lavery (16) proposed that the 5'5' structure would have base pairs pulled outwards or flipped whereas the 3'3' structure would not. To test this prediction, we examined the hysteresis in the presence of β-cyclodextrin, a macrocycle that reacts specifically with DNA bases that are extruded from the helix to form a host-inclusion compound (22, 23). We find that, for DNA overstretched from the 5'5' ends in 150 mM NaCl, the difference between the hysteresis in cyclodextrin and the hysteresis without was 2.8 times the standard deviation for hysteresis in the absence of cyclodextrin (above). In contrast, there was no increase in hysteresis for 3'3' pulling in 150 mM NaCl. Similarly, in 100 mM NaCl, where 3'3' pulling is hysteretic, the change in hysteresis due to cyclodextrin was less than the standard deviation in the absence of cyclodextrin.

Rotated bases are also sites of ssDNA; therefore, we examined the effects of glyoxal on hysteresis since glyoxal interacts with unpaired bases, irreversibly modifying chemical positions involved in H-bonding so as to preclude re-pairing (24). Once again the effect of this small molecule on hysteresis depends on the pulling technique: for 5'5' pulling

glyoxal removes the hysteresis, whereas the glyoxal has no effect on the hysteresis for 3'5' pulling. Specifically: Fig. 5A shows that the marked hysteresis for the 5'5' state in 150 mM NaCl is eliminated in the presence of 50 mM glyoxal. In this salt condition, the average hysteresis for 5'5' pulling in 10 mM glyoxal is H = 1.4 ± 2.4 pN μm whereas in the absence of glyoxal the hysteresis was 13 ± 8 pN μm. The hysteresis is removed for glyoxal concentrations down to 10 mM, and at glyoxal concentrations below 10 mM the hysteresis is reduced but not eliminated. In contrast, glyoxal has no effect on the hysteresis for 3'3' pulling, even in 100 mM NaCl where hysteresis is observed: in the absence of glyoxal the hysteresis in 100 mM NaCl is H = 3.1 ± 3 and the hysteresis in the presence of 10 mM glyoxal is H = 3.8 ± 4. Thus, even when the 3'3' overstretched structure shows hysteresis, the glyoxal does not reduce it.

We note that while, under some experimental conditions glyoxal interactions are slow and long times are required to observed the interaction (25), under the conditions in our experiment, glyoxal rapidly binds to some unpaired bases in much less than a minute (SI Fig. 7). Control experiments confirm that if a dsDNA molecule is unzipped in the presence of glyoxal, then glyoxal will maintain permanently open ssDNA. (SI Fig. 7); therefore, unlike the cyclodextrin, glyoxal does maintain open ssDNA even in competition with Watson and Crick pairing.

In sum, small molecules that bind either to rotated base pairs or open DNA change the hysteresis when the DNA is pulled from the 5'5' ends, but do not affect the hysteresis when the same molecules are pulled from the 3'3' ends, suggesting that the two structures are different.

Evidence that overstretching involves a structural change that is distributed along the entire length of the pulled molecule rather than locally near the ends.

In the section above we considered several experiments demonstrating that the structure that results from overstretching dsDNA from the 3'3' ends differs from the structure that results when the same dsDNA is overstretched from the 5'5' ends. One question not answered is whether the structures are different throughout the molecule or only near the ends. The difference between these two possibilities does not affect the conclusion that 3'3' and 5'5' pulling techniques result in different structures, but it could affect detailed interpretations of observed effects. In what follows, we will present evidence that suggests that the force on the bases extends throughout the molecule. Two different types of evidence are considered: 1. the effect of glyoxal or cyclodextrin on the hysteresis for molecules overstretched from the 3'5' ends; 2. comparison of the force required to initiate melting for different pulling techniques with the same sequences at the ends.

First, the two possible situations can be distinguished by comparing the effects of pulling on the 3' and 5' ends of a single strand (3'5' pulling; Fig. 1A) with the effects of 3'3' and 5'5' pulling. If overstretching is confined to regions near the ends, the behavior resulting from 3'5' pulling will be an equal mixture of the effects of 3'3' and 5'5' pulling, with each mode dominating at one of the two ends and no force between base pairs near the middle of the molecule. Thus, if effects are limited to the ends, 3'5' pulling should exhibit hysteresis at 150 mM NaCl due to the structure at the 5' end alone, since the 3' end does not exhibit hysteresis at this NaCl concentration. Similarly, this hysteresis

should be enhanced by cyclodextrin and removed by glyoxal; however, while 3'5'

pulling does exhibit hysteresis, that hysteresis is not significantly affected by either

cyclodextrin or glyoxal. Thus, the structure that results when dsDNA is overstretched

from the 3'5' ends does not consist of a 3' structure at one end and a 5' structure at the

other.

Second, if the force between the base pairs in the molecule were confined to short

sequences near the end, then the initiation of melting would be determined by the short

sequences near the ends of the molecule. Consequently, any molecule should begin to

melt when its weakest end begins to melt regardless of the force applied to the stronger

end. To test this proposition, we compared the F_{start} values for DNA overstretched from

the 3'3' and 5'5' ends with the F_{start} values for 3'5' pulling and for three other pulling

modes in which one or both ends are tethered by both strand termini, rather than by only

one ("3'K, 5'K and KK pulling"; see SI text). We show that the F_{start} values for all of the

pulling techniques are affected by the natures of the attachments at both ends, not just the

weakest end (see SI text); therefore, the force on the bases must extend through the entire

length of the molecule.

DISCUSSION

We have presented evidence that DNA overstretched by 3'3' pulling responds

differently to mechanical force than DNA overstretched by 5'5' pulling, which in turn

implies that the overstretched states have different structures. Further, all of the observed

differences are explained by two features of the models of overstretched DNA proposed

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by Lebrun and Lavery (16): 1. the 5'5' structure has a narrower backbone spacing than the 3'3' structure 2. the 5'5' structure has rotated bases that are not available in 3'3' pulling.

Evidence that the 5'5' overstretched structure has narrower backbone spacing than the 3'3' overstretched structure. To probe for differences in backbone separation, we analyzed the effects of charge screening by analysis of hysteresis as a function of variations in ionic strength. The 3'3' overstretched structure should be better screened than the 5'5' overstretched structure in conditions giving a characteristic screening length that is shorter than the 3'3' phosphate backbone separation but longer than the 5'5' phosphate backbone separation. Correspondingly, in the presence of 150 mM NaCl, the 5'5' overstretched structure exhibits hysteresis while the 3'3' overstretched structure does not. Moreover, both structures are effectively screened (no hysteresis) at 1 M NaCl while neither structure is effectively screened (substantial hysteresis) at 20 mM NaCl, implying that 150 mM NaCl represents a condition of intermediate screening length. These results are consistent with the 5'5' overstretched structure having a narrower backbone separation than the 3'3' structure.

A further prediction of the difference in backbone separation is that, in conditions where the two different structures are differentially screened (i.e. 150 mM NaCl), the 5'5' overstretched structure would melt at a lower force than the 3'3' overstretched structure. We confirm that this is the case using three different assays. The first assay measures the force at which a magnetic bead separates from the surface to which it was bound by a dsDNA molecule, where we made a correction for the bead loss due to a failure of the biotin tethers at the ends of the molecules. The second assay measures the force at which

the extension vs. force curves for the overstretched state exhibit a marked change in slope that precedes force induce melting. The third assay measures the bead loss exclusively for molecules whose extension vs. force curves showed a characteristic change in slope before the beads separated, where this assay did not include any correction for biotin failures.

It is important to compare these results with the two earlier studies that measured the shear force for 3'3' and 5'5' pulling. Recent AFM results have shown that if a high pulling rate is applied to DNA molecules, molecules overstretched from the 3'3' ends are more stable against force-induced melting than molecules overstretched from the 5'5' ends; however, if the pulling rate is lower, no difference is observed (20). A study that separated short DNA sequences using a constant force found that the melting force for 3'3' pulling was the same as for 5'5' pulling. That study showed that the melting force increased with sequence length, but approached an asymptotic value that is below the overstretching force. (26). Both of these previous measurements and the results presented here are all consistent if one assumes that B-form DNA pulled from the 3'3' ends melts at the same force as B-form DNA pulled from the 5'5' ends because the B-form structure is independent of pulling technique, whereas DNA overstretched from the 5'5' ends melts at a lower force than DNA overstretched from the 3'3' ends because the overstretched structures for the different pulling techniques are different, consistent with Lavery's proposal. This interpretation is supported by earlier AFM work showing that while the overstretching force is independent of sequence length and pulling rate (27), the shear force increases with both length and pulling rate (28). Taken together these two sets of

results imply that at high pulling rates DNA will overstretch before it shears and at low pulling rates it will shear before it overstretches.

A second prediction of Lebrun and Lavery is that the 5'5' overstretched structure should produce rotated ("flipped") bases whereas the 3'3' overstretched structure should not. In accord with this prediction, hysteresis analysis was carried out in the presence of cyclodextrin, a molecule that specifically binds rotated bases. The presence of cyclodextrin increases the hysteresis for 5'5' pulling by 2.8 times the standard deviation for the control, whereas the change for 3'3' and 3'5' pulling are within the standard deviation for the controls. In addition, a very strong differential effect is observed for glyoxal, a molecule that interacts with free bases preventing Watson-Crick pairing. We find that the presence of glyoxal eliminates the hysteresis for 5'5' pulling but does not affect the hysteresis for 3'3' or 3'5' pulling. Thus, the results of small molecule binding assays suggest that the 5'5' structure presents rotated bases and that the 3'3' does not.

***In vivo* implications.** The increase in dsDNA length achieved by proteins such as RecA is close to the 1.7 fold extension achieved after overstretching. However, similarity between the overstretched state and the deformation of DNA exerted by certain proteins does not seem to be related in a simple way. It has been recently reported that structure of DNA in a RecA/DNA filament is locally similar to B-DNA within a series of three bases (triplet) and with a larger step in between triplets yielding an overall deformation of 1.5 fold (29). These properties are significantly different from, and not immediately relatable to, the structures resulting from overstretching. Indeed, an earlier mechanical study demonstrated that binding of RecA to overstretched DNA resulted in a reduction of the extension from 1.7 times to 1.5 times (14). Thus, while the forces required for

overstretching are reasonably compatible with biological activity, the details by which those forces exert their effects remain to be established.

MATERIALS AND METHODS

Preparation of dsDNA constructs. The constructs were prepared by hybridizing and ligating the oligonucleotides complementary to each sticky end of lambda phage DNA 0.5 mg/ml (NEB, Beverly, MA) (48502 base pairs). In order to improve the binding to the surfaces, the oligonucleotides included a short single-stranded DNA (ssDNA) spacer with one or several biotin labels at the 3' or 5' ends. Ligations steps were done in the presence of a thermostable DNA Ligase (Ampligase, Epicentre, Madison, WI). These ligation steps also remove nicks that might have been present before ligation. The occurrence of nicks can be detected by analysis of molecules pulled on their 3'5' ends. In this case, force-induced melting leaves one strand tethered between the bead and the surface, unless a nick is present, in which case the bead is released. Similarly, a bead bound to a surface via a DNA molecule attached by an ssDNA tail at one end and a dsDNA tail at the other can only remain bound if there are no nicks in the strand with the ssDNA tail and can return fully to dsDNA only if there are no nicks in either backbone. SI Figs. 6 and 7 confirm that these DNA constructs had no nicks. Finally, a bead bound to a DNA molecule that is fully unzipped can remain bound only if there are no nicks in either backbone and several publications have shown that DNA treated using the techniques described in this paper can be completely unzipped (18,30,31). We also note that prior interpretations of overstretching studies have sometimes suggested that nicks exist and affect measurements (17, 25, 32, 33).

Before each experiment the DNA sample was mixed with a suspension of extravidin coated beads in buffer and incubated on a glass capillary surface for 10 min; the capillaries were always coated with extravidin by overnight adsorption. Superparamagnetic tosylactivated 4.5 μm beads (Dynal, Invitrogen, Carlsbad, CA, USA) were initially modified with extravidin (Sigma-Aldrich, USA) by incubating the beads in a 0.1 mg/ml solution of extravidin in 0.1 M phosphate buffer pH 8.4 at 37ºC overnight.

The measurements were done in PBS buffer (phosphate buffer saline 150 mM NaCl and 10 mM phosphate buffer pH 7.4). Whenever a change in the ionic strength was required, different concentrations of NaCl in phosphate buffer 10 mM pH 7.4 were used.

Acknowledgements

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FIGURE LEGENDS

FIGURE 1 Pulling dsDNA from different ends (A) (1) B-dsDNA; (2) biotin labels (circles) on ssDNA spacer and pulling from 3'3' ends using extravidin molecules (triangles) on the coated surfaces; (3) biotin labels on ssDNA spacer and pulling from 5'5' ends; (4) biotin labels on ssDNA spacers on the same strand allowing for 3'5' pulling. (B) Image of beads tethered to a glass capillary (black surface) at a distance that corresponds to the contour length of bacteriophage lambda. (C) Extension vs. force curves for 3'3' (blue) and 5'5' (red) pulling, where the overstretching force is indicated as F_{ov}. The forces at which the beads separated for particular single lambda phage molecules, F^{*}_{ssDNA} (dashed lines), are shown as well as the forces at which the transition begins, F_{start} (dotted lines).

FIGURE 2 Bead release assay for 3'3', 5'5', and 3'5' pulling. (A) The blue, red, and green curves show data for 3'3', 5'5', and 3'5' pulling in PBS buffer, respectively. The initial fast bead loss is attributable to label failure whereas force-induced melting of already-overstretched DNA starts at higher forces (arrows). The straight line shows a linear fit to the bead loss in the force range between 75 and 100 pN. (B) Data in (A) are multiplied by $\exp(\alpha)$ where $\alpha = 0.0255$ for 3'3' and 5'5'. Vertical dashed lines corresponding to $F_{1/2\ ssDNA}$ are included to characterize the fraction of beads remaining as a function of force for 3'3'and 5'5' pulling.

FIGURE 3 Force dependence for the onset or presumptive completion of force-induced melting for 3'3'and 5'5' pulling in PBS buffer. (A) F_{start} values obtained from the extension vs. force curves that show a clear change in slope. The dashed lines are a guide to the eye. (B) Fraction of molecules from Fig. 2 that have exhibited onset of force-induced melting at/or before the indicated force for 3'3' (black) and 5'5' (grey) pulling regimes. Dashed lines represent a guide to the eye.

FIGURE 4 Stretching several dsDNA single molecules by pulling from different ends. Extension vs. force curves in 10 mM phosphate buffer pH 7.4 and different ionic strength conditions: 1 M, 150 mM, and 20 mM NaCl. In these curves the solid symbols and solid lines correspond to the curve where the force was increased whereas the hollow symbols and dashed lines are for the curves where the force was decreased.

FIGURE 5 Effect of glyoxal on 5'5' and 3'5' pulling. (A) The squares and circles correspond to two different 5'5' molecules measured in 10 and 50 mM glyoxal, respectively, where the closed symbols represent the increasing force curve and the open symbols, the corresponding decreasing force curve. The gray curve with triangles is a control curve for 5'5' pulling in PBS buffer. (B) Overstretching curve of a molecule pulled from the 3'5' ends in 50 mM glyoxal and PBS (the closed symbols represent the increasing force curve and the open symbols, the corresponding decreasing force curve). A typical control curve for 3'5' in PBS is shown with gray triangles.
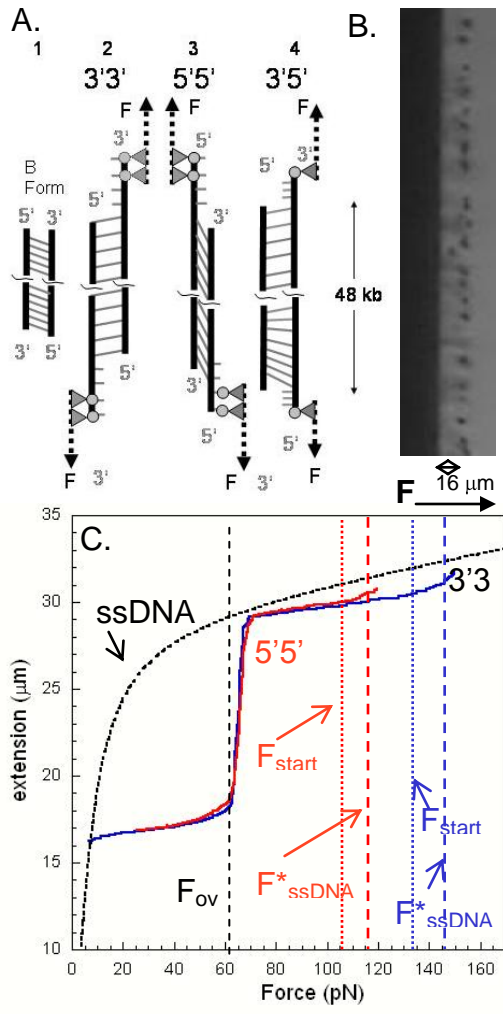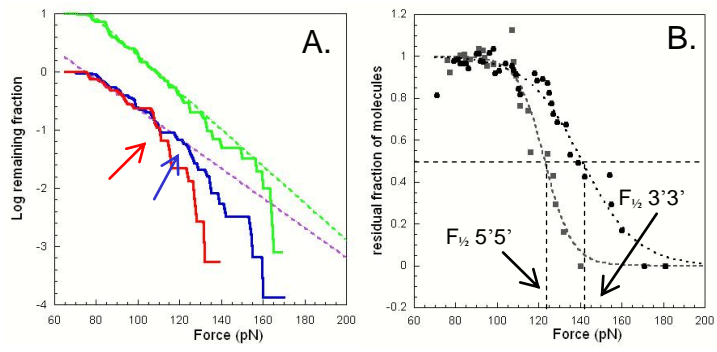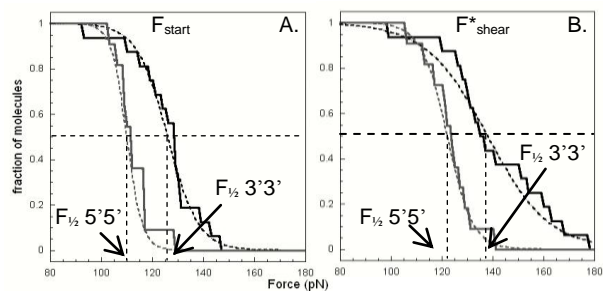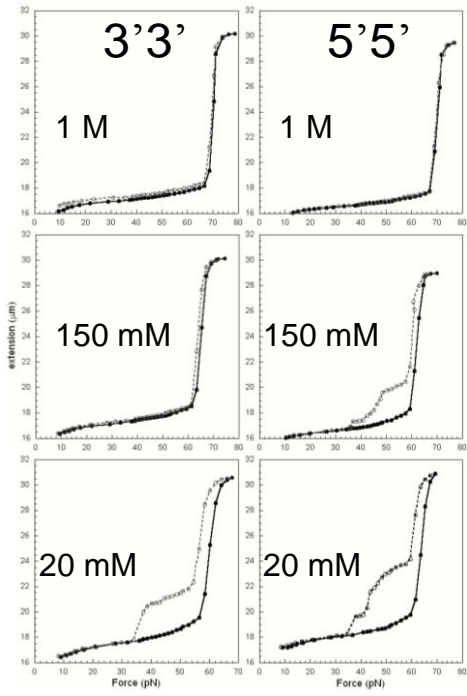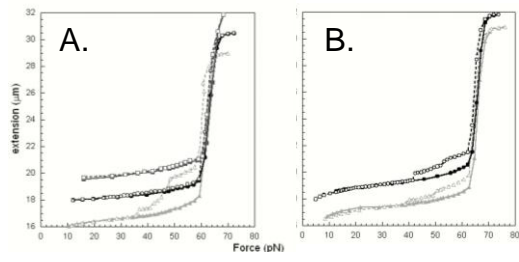
Figure 1

Figure 2

Figure 3

Figure 4

Figure 5