Dynamics of Single DNA Looping and Cleavage by Sau3AI and Effect of Tension Applied to the DNA

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ABSTRACT Looping and cleavage of single DNA molecules by the two-site restriction endonuclease Sau3AI were measured with optical tweezers. A DNA template containing many recognition sites was used, permitting loop sizes from ~10 to 10,000 basepairs. At high enzyme concentration, cleavage events were detected within 5 s and nearly all molecules were cleaved within 5 min. Activity decreased ~10-fold as the DNA tension was increased from 0.03 to 0.7 pN. Substituting Ca^{2+} for Mg^{2+} blocked cleavage, permitting measurement of stable loops. At low tension, the initial rates of cleavage and looping were similar (~0.025 s^{-1} at 0.1 pN), suggesting that looping is rate limiting. Short loops formed more rapidly than long loops. The optimum size decreased from ~250 to 45 basepairs and the average number of loops (in 1 min) from 4.2 to 0.75 as tension was increased from 0.03 to 0.7 pN. No looping was detected at 5 pN. These findings are in qualitative agreement with recent theoretical predictions considering only DNA mechanics, but we observed weaker suppression with tension and smaller loop sizes. Our results suggest that the span and elasticity of the protein complex, nesting of loops, and protein-induced DNA bending and wrapping play an important role.

INTRODUCTION

DNA looping, which occurs in many fundamental biological processes such as DNA transcription, recombination, and repair, facilitates interactions of multiple proteins bound at distant sites on a DNA molecule (1–8). Looping permits a greater number of proteins beyond just those at neighboring sites to be involved in the regulation of a process. Localization of a protein at one site also increases the effective concentration of that protein at the second site, increases net affinity, and provides higher specificity through the redundancy in sequence recognition (1,9,10).

A number of restriction endonucleases (REases) requiring interaction at two sites for efficient cleavage activity have been found to operate by DNA looping, and these constitute a convenient model system for studying this process (11–16). Recently we used single DNA manipulation to study cleavage and looping by many different one-site and two-site REases (17,18). We found that 5 pN of tension strongly inhibited all of the two-site enzymes while having virtually no effect on the one-site enzymes.

Here, we report in-depth studies of a particular two-site enzyme, Sau3AI, which is a popular 4-nucleotide cutter for the construction of library clones. We found it to be well suited for measurements due to its high activity. Looping of DNA by this enzyme has been directly imaged by electron microscopy (12). We characterized the dependence of DNA cleavage and looping on enzyme concentration, incubation time, and applied tension. Sau3AI recognizes the short, palindromic sequence GATC and we used a DNA template containing 55 recognition sites, permitting a quasicontinuum of possible loop sizes from ~10 to 10,000 basepairs (bp). Cleavage was measured in the standard reaction buffer, and stable DNA looping was measured by substituting Ca^{2+} for Mg^{2+}, which facilitates specific binding while blocking cleavage (12). Forced loop disruption after a variable incubation time allowed us to characterize the rate of looping, distribution of loop sizes, and binding strengths of loops. Two recent theoretical studies have considered the effect of DNA tension on loop formation, and our data provide the first opportunity to compare experimental results with the predictions (19,20).

METHODS

Sau3AI was obtained from New England Biolabs (NEB; Beverly, MA), and one unit is defined as the amount of enzyme required to digest 1 μg of λ DNA in 1 h at 37°C in a total reaction volume of 50 μl. The enzyme was diluted in the recommended reaction buffer (10 mM Bis-Tris-Propane-HCl, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.0) for cleaving experiments, and $CaCl_2$ was substituted for $MgCl_2$ for looping experiments.

The DNA construct was prepared by ligating a digoxygenin (DIG)-labeled polymerase chain reaction (PCR) fragment (4282 bp) to a 10,845-bp biotin-end-labeled restriction fragment of pBACe3.6 (Children's Hospital of Oakland Research Institute). The PCR fragment was generated by amplification of a sequence from pFastBac HT-b (Invitrogen, Carlsbad, CA) using the primers 5′-GTGGTATGGCTGATTATGATC and 5′GCAGCCTGAATGGCGAATGG and was labeled by incorporation of 20 μM of dUTP-11-DIG (Roche, Indianapolis, IN) and 200 μM each of dATP, dCTP, dGTP, dTTP in the PCR. This fragment is thus labeled along its full length with DIG and serves as a handle. It usually binds along its full length to the anti-DIG microsphere, such that the sites in this section are usually not available for looping. The 10,845-bp fragment was produced by digesting pBACe3.6 with BsrGI (NEB) and labeled using the Klenow fragment of *Escherichia coli* DNA polymerase I, exo^- (NEB) to incorporate dATP-14-biotin (Invitrogen). Both fragments were purified (Qiagen, Valencia, CA; PCR purification kit) and digested with XhoI (NEB). To isolate the desired products the samples were separated by gel electrophoresis and purified using a gel extraction kit (Qiagen). The two fragments were then ligated using T4 DNA ligase (NEB).

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Bacteriophage phiX174 DNA, used as a negative control template, was purchased from NEB and was labeled by digesting with XhoI and end labeling with dATP-14-biotin (Invitrogen). The DNA was then digested with StuI, purified using the Promega (Madison, WI) Wizard DNA clean-up kit, and end labeled with dUTP-11-DIG.

Streptavidin coated microspheres (200 μl of 0.5% w/v, 2.2-μm diameter; Spherotech, Libertyville, IL) were washed by twice centrifuging at 10,000 $\times g$ and resuspending in 200 μl of phosphate buffered saline (PBS) pH 7.4 (Fisher Scientific, Loughborough, Leicestershire, UK) and 0.1 mg/ml bovine serum albumin (BSA) (NEB). A total of 5 μl of diluted DNA (~10–100 ng/μl) was mixed with 5 μl of microspheres and incubated for ~45 min at room temperature on a slowly rotating mixer; 5–10 μl of these microspheres were diluted in 0.5 ml of PBS and loaded into a 1-ml syringe for injection into the sample chamber. Protein G coated microspheres (200 μl of 0.5% w/v, 2.8-μm diameter, Spherotech) were washed in the same manner and resuspended in 20 μl PBS, and 5 μl 200 μg/ml of anti-DIG (Roche) was added. The microspheres were incubated on the mixer for ~45 min and then washed three more times and resuspended in 20 μl PBS. Finally, 5 μl of the microspheres were diluted in 1 ml of PBS and loaded into a syringe for injection into the sample chamber.

Our optical tweezers instrument has been described previously (21). In brief, the anti-DIG coated microsphere was held by a micropipette while the microsphere carrying the DNA was trapped with the optical tweezers. The DNA tension was monitored at 100 Hz. The two types of microspheres were brought into proximity such that the DIG-labeled end of one DNA molecule bound to the anti-DIG coated bead, forming a single DNA tether between them. All measurements were done at room temperature (~20°C).

RESULTS

Detection of single DNA cleavage events

Our experimental technique is shown schematically in Fig. 1. A single DNA molecule was held stretched at an end-to-end extension of ~95% of the DNA contour length, corresponding to a tension of 5 pN, which inhibited cleavage. The enzyme solution was then flowed into the sample chamber, and the DNA molecule was quickly relaxed to a specified extension (corresponding to a desired tension) for a specified incubation time. Cleavage was monitored by testing for the presence of the DNA tether by quickly separating the microspheres. If the molecule was cleaved, the measured force remained zero as the microspheres were separated.

We first investigated what range of concentrations and incubation times would be needed to observe enzyme activity. As shown in Fig. 2 *A* cleavage was observed over concentrations ranging from 0.04 to 200 units/ml when using incubation times ranging from 30 s to 5 min. These measurements were made with the DNA held at a fractional extension of 50% (~0.1-pN tension) and were repeated with

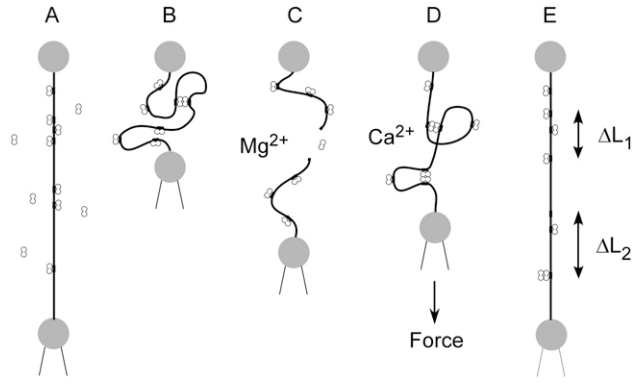

FIGURE 1 Schematic illustration of single DNA molecule looping and cleavage measurements. (*A*) A single DNA molecule was held stretched between two microspheres using optical tweezers (*top*) and a positioned micropipette (*bottom*). Sau3AI was introduced while the DNA was held stretched with a tension of 5 pN. (*B*) The molecule was quickly relaxed to a specified extension, corresponding to lower tension, whereupon looping could occur. (*C*) In a solution containing 10 mM Mg^{2+}, the molecule was rapidly cleaved, which was detected by separating the microspheres. (*D*) When Ca^{2+} was substituted for Mg^{2+} cleavage was inhibited and loop formation was detected. (*E*) After a variable incubation time, loops were disrupted by stretching the DNA, permitting measurement of loop sizes (e.g., ΔL_1 and ΔL_2) and disruption forces.

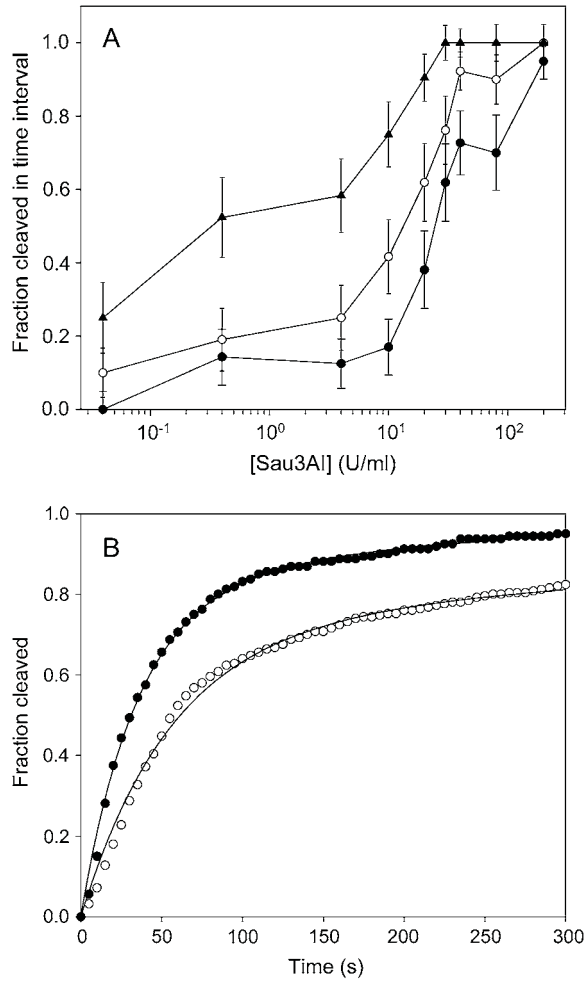

FIGURE 2 (*A*) Dependence of DNA cleavage on Sau3AI concentration for incubation times of 30 s (●), 60 s (○), and 300 s (▲) with a DNA tension of 0.1 pN. The fraction of molecules cleaved was determined for ~25 trials at each condition. (*B*) DNA cleavage versus incubation time with 40 units/ml (●) or 10 units/ml (○) Sau3AI and 0.1 pN DNA tension. The lines are fits to saturating double exponentials.

~25 molecules at each condition. Higher resolution measurements were made by quickly testing for the tethered molecule every 5 s and repeating the measurement with ~200 molecules. As shown in Fig. 2 *B* the majority of molecules were cleaved in ~5 min at 40 units/ml and a DNA tension of 0.1 pN. These data are not well described by a single saturating exponential, as would be anticipated for a single-step, single-pathway reaction. Such simple behavior might be expected, for example, if looping was the sole rate-limiting step and there was only one possible loop that could form. Rather, the data were better fit by a sum of saturating exponentials, suggesting multiple timescales. We attribute this behavior to the wide spectrum of possible loop sizes that could form on the DNA template and the fact that (as will be presented below) short loops form significantly faster than long loops, leading to a spectrum of possible timescales for loop formation. When fit to a double saturating exponential, the characteristic timescales were ~56 and 690 s with 10 units/ml Sau3AI and decreased to ~35 and 230 s with 40 units/ml. That a fourfold increase in enzyme concentration produced only a two- to threefold increase in reaction rate suggests that we are near the high concentration limit where enzyme binding is not rate limiting. Due to the need to carry out many repeated trials, we chose to use 40 units/ml and incubation times ranging 10–300 s in most of the measurements.

Dependence of cleavage on DNA tension

To quantify the effect of tension on cleavage, we chose an incubation time of 30 s so that the fraction of molecules cleaved was <100%. As the tension was increased from 0.06 to ~0.7 pN, the activity decreased exponentially and the magnitude of the decrease was ~10-fold at 0.7 pN (Fig. 3). Measurements done at 2.5 and 5 pN indicated only one and zero cleavage events, respectively, in N ~30 trials. The experimental trend is thus in qualitative agreement with the exponential inhibition of looping predicted by theory, although a 10-fold inhibition is predicted to occur at a somewhat smaller force (~0.1 pN) for an optimum-sized teardrop-shaped loop (~500 bp) (19,20). In our experiment, a quasicontinuum of loop sizes is possible. Theory predicts that the degree of inhibition by tension should decrease with decreasing loop size, suggesting that our loops are predominantly smaller than anticipated for an optimally sized teardrop loop. Indeed, as will be presented below, we find much shorter loops than are predicted considering only DNA mechanics.

Detection of stable DNA loops

To study DNA looping directly, measurements were again carried out as illustrated in Fig. 1 but with Ca^{2+} substituted for Mg^{2+} in the reaction buffer. The molecules were incubated for a specified time, and the DNA was then stretched at a rate of 150 nm/s to assess loop formation. If the DNA remained tethered after reaching a tension of 60 pN, it was relaxed and the incubation and stretching were repeated. If the tether detached from the microspheres, which typically occurred after 1–10 stretch cycles, the enzyme solution was drained from the sample chamber, a new DNA molecule was tethered, and a new aliquot of enzyme solution was introduced. Measurements were repeated ~200 times at each tension and incubation time to accumulate statistics on loop formation.

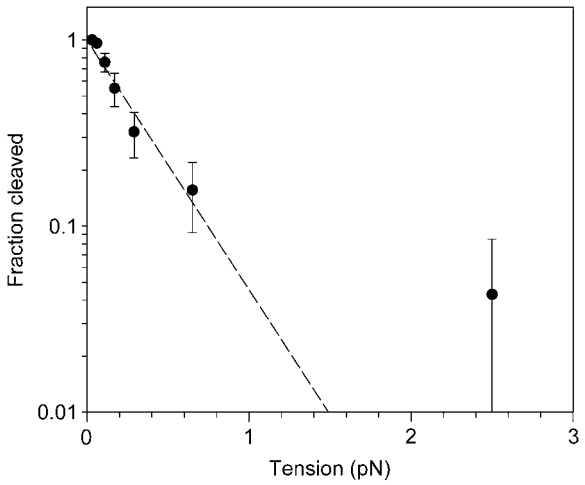

FIGURE 3 Dependence of cleavage on applied tension. Activity is reported as fraction of DNA molecules cleaved in 30 s. The error bars are calculated as the standard deviation of the binomial distribution $(p(1-p)/N)^{1/2}$, where p is the probability of a molecule being cut and N is the number of trials ($N \sim 30$ for each point). The dashed line is an exponential decay fit to the data, indicating a 1/e point at ~0.3 pN.

Typical force-extension data sets are shown in Fig. 4. Before introducing the enzyme the measured elasticity was as expected for a single, naked DNA molecule (22). After incubation with the enzyme the DNA tether was often shortened by a variable length, consistent with loop formation. Upon stretching we recorded sudden drops in the measured force, each followed by a steady increase in tension. These ''sawteeth'' indicate events in which sequestered lengths of DNA are suddenly released, consistent with the disruption of the individual DNA loops. Analysis of these events yields the number of loops formed and the disruption force and DNA length change associated with each loop. The observed length changes were consistent with the possible loop sizes given the known separations of recognition sites on the DNA templates.

Four different control experiments were carried out (50 trials each). First, DNA was stretched in the reaction buffers with no enzyme added to confirm that there were no non-enzyme-specific interactions. Second, DNA was incubated with several one-site REases (BstNI, HaeIII, and MspI) with many recognition sites on the template, and no events were observed. Third, Sau3AI was tested on a template containing

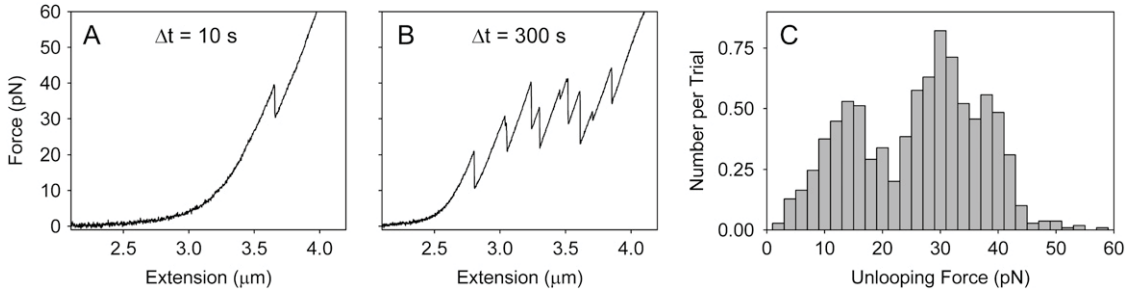

FIGURE 4 (*A* and *B*) Typical force-extension data sets after incubation of DNA and 40 units/ml Sau3AI in a buffer containing Ca^{2+} for 10 s or 300 s. The DNA was stretched at a rate of 150 nm/s, and the sharp drops indicate unlooping events. (*C*) Measured distribution of loop disruption forces.

no recognition sites (bacteriophage phiX174 DNA), and no events were observed.

Loop disruption forces ranged from ~3 to 60 pN with a mean of 25 pN and standard deviation of 11 pN (Fig. 4 *C*). This range of forces is similar to that measured for disruption of other protein-DNA complexes by optical tweezers, such as in our previous study of nucleosome unraveling (21). Interestingly, the distribution of disruption forces for the loops was bimodal. We can rule out that the protein-protein and protein-DNA interactions have substantially different strengths, because if this were the case the weaker unbinding events would be more frequent, whereas the opposite was observed. Rather this finding suggests that individual complexes may have heterogeneous binding modes or that the binding energy landscape contains multiple barriers (23).

Frequency of looping

The number of loops formed in a single DNA molecule can be directly tabulated in our experiment by counting the disruption events in each force-extension data set. The mean number of loops formed versus time is plotted in Fig. 5 *A* (at 40 units/ml Sau3AI and 0.1 pN DNA tension). Fewer loops were formed than the total number possible ($N_{sites}/2 = 27$), and the loops were essentially irreversible on the timescale of the experiment—thus our measurements report on the initial kinetics of loop formation. On average, ~5 loops were formed in 5 min and a clear decrease in the rate of loop formation was seen after ~1 min. Such a decrease is expected due to an overall depletion of available sites and, in particular, the depletion of nearby sites that form loops more rapidly (as will be shown below). Progressive reduction in the slack in the DNA due to loop formation would also contribute to the decrease in looping rate.

At low tension the initial rates of cleavage and looping were similar (~0.025 s^{-1} at 0.1 pN), suggesting that looping is rate limiting under these conditions. Unexpectedly, however, loop formation was not as strongly inhibited as cleavage as the tension was increased. For example, at a tension of 0.3 pN, ~60% of molecules formed one or more loops in 1 min but only ~20% of molecules were cleaved in the same time period. One possible explanation for this finding is that cleavage experiments were done with Mg^{2+} and looping experiments were done with Ca^{2+}, and it is possible that stable loop formation may occur more readily in Ca^{2+}. However, although one may expect an overall dependence on the species of divalent cation, it seems unlikely that the form of the tension dependence would vary with species. Rather, we interpret this result as indicating that cleavage of the looped complex is perturbed by tension. The tension results in stress applied directly across the complex and this may partly inhibit the cleavage reaction. Although cleavage by one-site enzymes was shown to be affected only by much higher

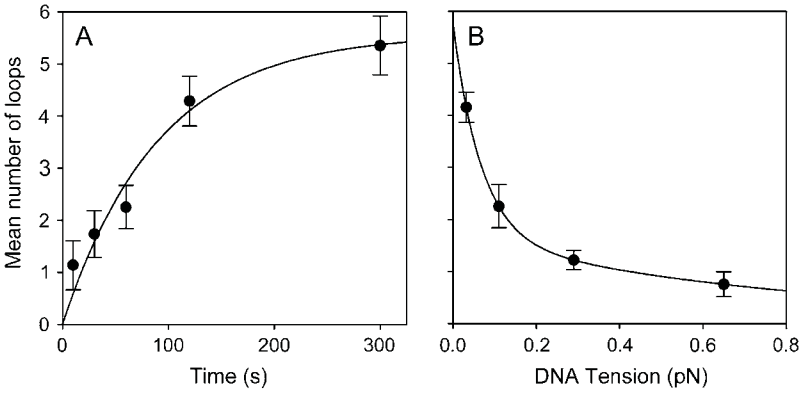

FIGURE 5 (*A*) Mean number of loops per molecule formed versus incubation time with 40 units/ml Sau3AI and a DNA tension of 0.1 pN. (*B*) Mean number of loops formed versus DNA tension after a 1-min incubation time with 40 units/ml Sau3AI.

tensions (~20–40 pN), these enzymes are only affected indirectly by tension through inhibition of protein-binding-induced DNA bending (17,24). In our experiments, complete suppression of both cleavage and looping was observed at a tension of 5 pN.

The probability of looping decreased sharply with tension (Fig. 5 *B*) but not as sharply as the predicted exponential dependence. Detailed comparisons with the theoretical predictions are given in the discussion section. As discussed in further detail below, this finding suggests that higher-order protein-specific effects not considered in full detail by the theories play an important role in looping.

Distribution of loop sizes

Although loops formed with Sau3AI have been previously imaged by electron microscopy (12), these experiments were done with a specific fragment having two sites separated by 272 bp, and the dynamics of looping and effect of site separation were not studied. Moreover, the dependence of looping properties on DNA tension has not been systematically examined for any system. Although many theoretical models have predicted the dependence of the probability of loop formation on loop size, little experimental data are available for comparison (19,20,25–30). An advantage of our method is that loops are measured directly and loop size distributions are obtained from measurements repeated on an ensemble of complexes.

The separations between recognition sites on the DNA template dictate the sizes of loops that can form in our experiment. Due to our use of a long DNA template with 55 binding sites, the distribution of possible loops is quasicontinuous. Comparisons between measured and possible loop sizes are shown in Figs. 6 and 7. Although the distribution of possible loop sizes is nearly continuous and flat over the range from 0 to 3000 bp, the measured distributions are strongly skewed toward shorter loops, a finding consistent with the expectation that long loops are entropically unfavorable. On the other hand, we observed many loops shorter than the persistence length of DNA (~150 bp), which is striking given that such small loops are predicted to be unlikely in classical DNA looping theories due to the bending rigidity of DNA. Detected events in our experiment ranged from as small as 7 bp to as large as ~2700 bp. Our resolution in detecting small loops was not limited by instrument resolution (~5 bp) but by the distribution of sites in the DNA template (only a few pairs of sites were separated by <10 bp). Our findings clearly show that loops substantially smaller than the persistence length are readily formed with this enzyme.

As the incubation time was increased from 10 to 300 s, the loop size distribution shifted only a small amount (Fig. 6, *C–G*). The mean size increased from 220 bp and reached a plateau of 300 bp after ~1 min (Fig. 7 *E*). The most prominent feature was that the height of the distribution grew, reflecting the increase in the total number of loops. In contrast, the size distribution shifted more dramatically with increasing DNA tension (Fig. 7, *A–D*). As the tension was increased from 0.03 to 0.7 pN, the distributions narrowed and the mean size decreased from ~430 to 140 bp (Fig. 7 *F*). The fraction of long loops was reduced; at 0.7 pN no loops longer than 600 bp were observed, whereas at 0.03 pN ~¼ of the loops were longer than 600 bp. The fraction of very short loops also increased; only ~5% of loops were shorter than 60 bp at 0.03 pN, whereas that fraction was ~40% at 0.7 pN. This dramatic shift to shorter loops is in qualitative accord with recent theoretical predictions (19).

Normalized distributions

To estimate the inherent probability distributions corrected for the influence of the DNA template, we normalized the number of measured events in each bin by the number of possible pairs of sites having corresponding separations (Fig. 8). The maximum number of loops that can form in a given molecule is equal to $N_{\text{sites}}/2$, truncated to the nearest integer, but the number of different possible loops in an ensemble of measurements equals the number of combinations of pairs of sites $N_{\text{pairs}} = N_{\text{sites}}(N_{\text{sites}} - 1)/2$. We note that fine-scale modulations in looping activity at 5-bp intervals are often expected due to phasing with respect to the helical pitch of the DNA (2). Here such modulation would be expected to average out within our length bins as they are of much larger width. Moreover, we would not expect much helical modulation since our template does not have site separations corresponding to every multiple of 5 bp.

The observation of an optimum loop size is in accord with the theoretical expectation that very small loops are unfavorable due to the bending rigidity of DNA, whereas very large loops are entropically unfavorable. Our size distributions are in closer agreement with predictions of a model (19) that postulates a sharp 90° kink at the apex of the loop than with predictions of the classical worm-like chain (WLC) model with an ideal teardrop geometry. At 0.7-pN tension, however, the most frequent loop size shifted to less than 60 bp, which is even substantially smaller than the ~100-bp optimum predicted by this 90° kink model. With this shift in loop size we also observe much lower inhibition of loop formation by tension than predicted theoretically. A fair number of loops formed at all tensions and incubation times were very short (<60 bp) compared with any of the theoretical predictions.

DISCUSSION

Comparisons with other DNA looping systems

Transcription factors

Examples of DNA looping interactions that promote or repress transcription are found in both prokaryotes and

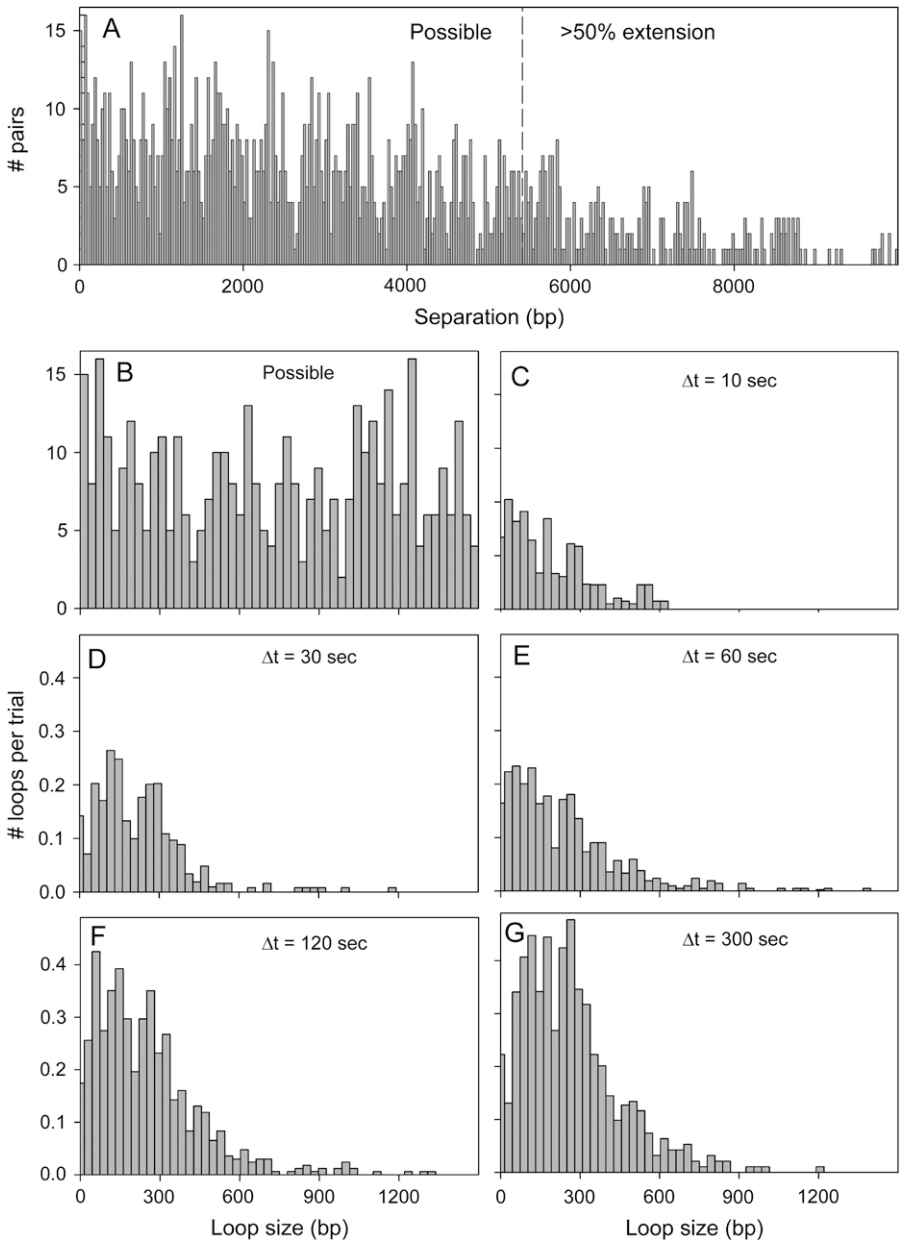

FIGURE 6 Distribution of loop sizes. (*A* and *B*) Possible loop sizes calculated given the positions of the recognition sites on the DNA template. The vertical dashed line indicates loop sizes which would be completely inhibited at DNA extensions >50% (0.1 pN tension). The distribution in *B* uses the same bp axis as those in plots *C*–*G*. (*C*–*G*) Measured distribution versus incubation time, with 40 units/ml Sau3AI and a DNA tension of 0.1 pN.

eukaryotes and involve stretches of DNA ranging from thousands of bp to <100 bp (1). Examples of short loop systems in *E. coli* include a 92-bp stretch in the lactose operon, a 93-bp stretch in *N*-acetylglucosamine operon, a 113-bp stretch in the galactose operon, and a 211-bp stretch in arabinose operon (31–34). Systematic experiments varying intersite distances in the *lac* operator indicate that repression was maximized for a 71-bp loop length and still ample for a 58-bp loop (32). In the araBAD system no lower limit to the spacing was observed, suggesting that the protein complex itself has significant flexibility (31). Notably, in these in vivo experiments, short loop formation may be facilitated by the presence of polyamines and histone-like DNA binding proteins in the cell that act to compact the DNA.

Single-molecule studies

A number of simplified in vitro studies of DNA looping have been performed. Finzi and Gelles developed an elegant tethered particle assay to measure the single DNA looping transitions induced by lactose repressor on a DNA template with a 305-bp site separation (35). Characteristic timescales for loop formation ranged from 5 to 80 s, which is similar to that measured in our experiments with Sau3AI. A magnetic tweezers assay was recently used to observe looping by galactose repressor on negatively supercoiled DNA as facilitated by the DNA binding protein HU (36). In these experiments a characteristic timescale for loop formation of ~20 s was measured with ~1 pN of tension applied to the DNA.

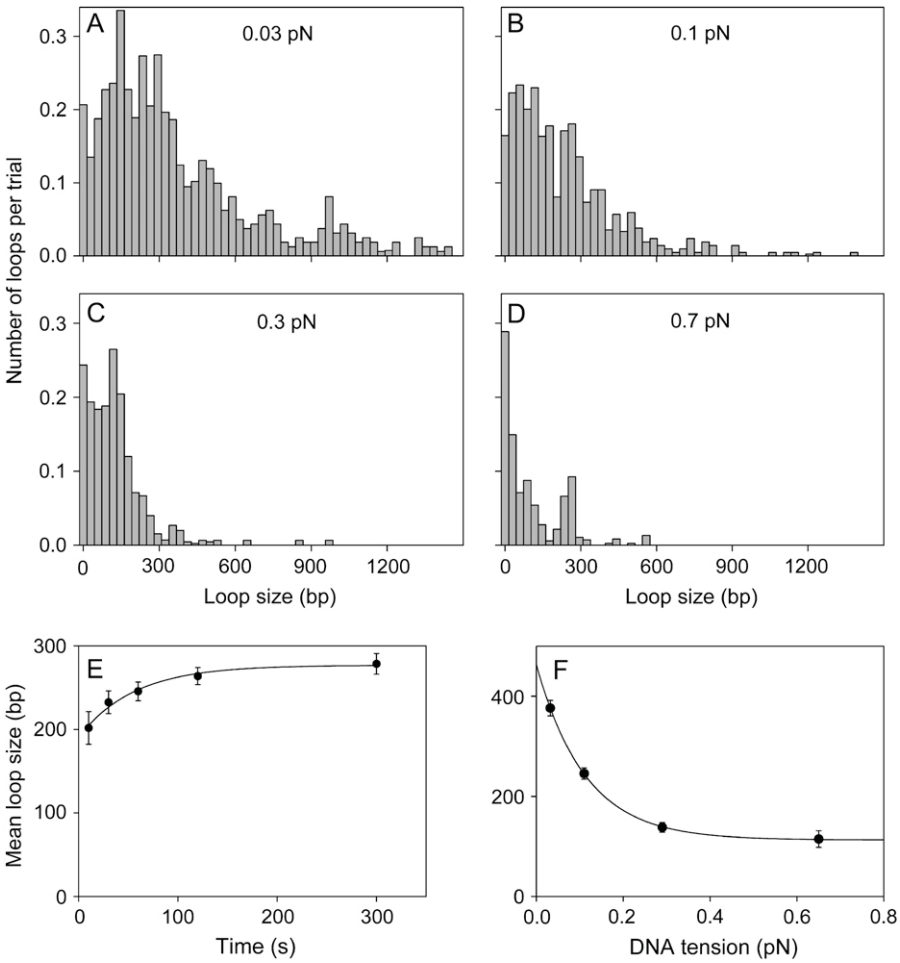

FIGURE 7 Distribution of loop sizes. (*A–D*) Measured distribution versus applied DNA tension, with 40 units/ml Sau3AI and an incubation time of 1 min. (*E*) Mean loop size versus incubation time. (*F*) Mean loop size versus DNA tension.

Two-site restriction endonucleases

There have also been studies of several different two-site REases. Of particular interest are studies of the cleavage activity of *Eco*RII with site separations varying from 5 to 952 bp (37). The highest activity was observed with a 10-bp spacing, which likely results in a complex in which the two subunits are bound adjacent to each other in full contact with the DNA rather than a complex in which the DNA is looped out away from the protein and through the solution. However, significant activity with *Eco*RII was also observed for separations in the range from 21 to 191 bp, in accord with our findings. The activity with *Eco*RII extrapolated to zero at ~1000 bp. In our experiment larger loops, up to ~2700 bp, were detected but very infrequently. Looping with BspMI has recently been studied using magnetic tweezers, and loop sizes ranging from ~90 to 1500 bp were detected, but size statistics were not presented (15). Looping with NaeI and NarI was recently studied using a tethered particle assay with templates having fixed site spacings of 455 bp and 305 bp, yielding characteristic looping times of ~10 and ~40 s, respectively (16). Finally, looping with *NgoMIV* has been detected with a 160-bp site spacing by fluorescent resonance energy transfer measurements (38).

Comparisons with theoretical models

Smaller loop sizes

Both of the published models for tension-dependent DNA looping are based on the simplifying assumption that looping occurs by thermal fluctuations that are governed only by the mechanics of the DNA molecule (19,20). In both cases tension is predicted to strongly suppress loop formation. Neglecting bending energy of the DNA, the probability of forming a loop of size ΔL by thermal fluctuations against an applied tension F is expected to be proportional to $\exp(-F\Delta L/kT)$. Therefore, tension is predicted to shift the size distribution toward lower ΔL. On the other hand, bending rigidity is incorporated via use of the WLC model and this penalizes the formation of loops substantially shorter than the persistence length (~150 bp). The net effect is that the most probable size for an ideal teardrop-shaped loop is predicted to decrease from ~500 bp at zero tension to ~225 bp at 0.5 pN. Sankararaman and Marko (19) have also

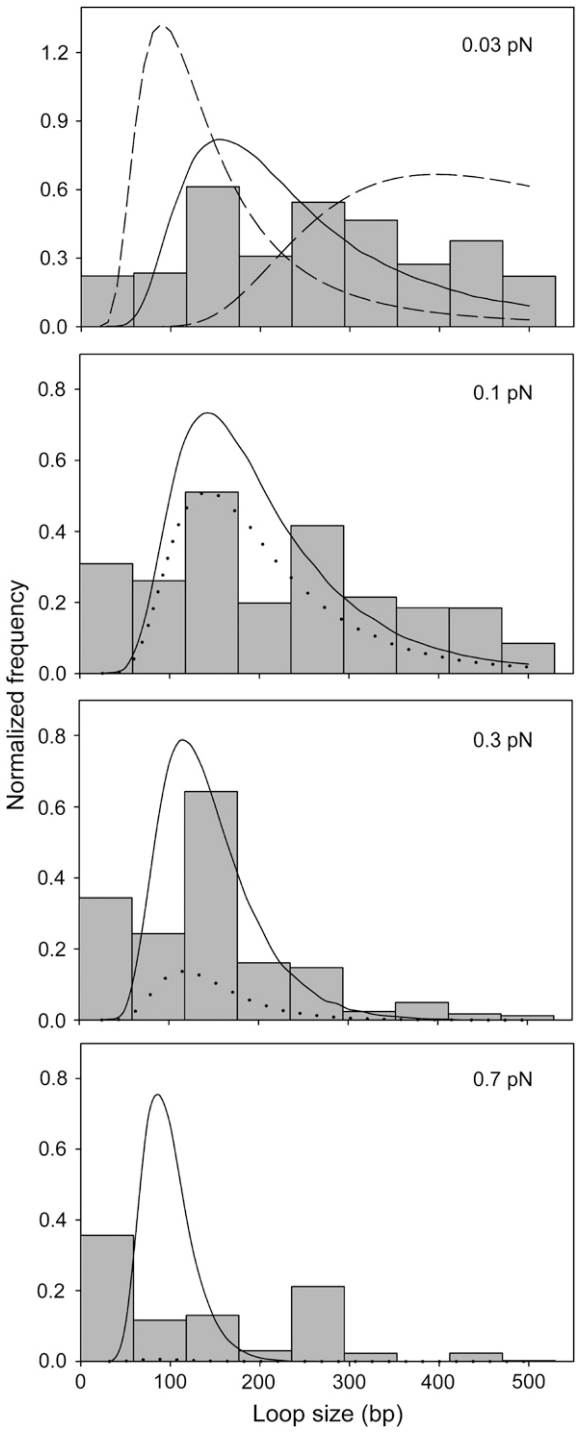

FIGURE 8 Normalized distributions of loop sizes versus DNA tension after incubation with 40 units/ml Sau3AI for 1 min. The histograms were normalized by dividing the number of events (per molecule) in each bin by the number of available pairs of sites on the DNA template having separations in the same range. The dashed lines in the top panel are the tension-free probability distributions from Sankararaman and Marko (19) for the 90° kink (*left*) and teardrop model (*right*). The solid lines are the predicted distributions for the 90° kink model at similar tension values (0.04, 0.15, 0.3, and 0.7 pN). These distributions were scaled to have the same area as the observed distributions. The dotted lines are the predicted distributions scaled to maintain their predicted magnitudes relative to the 0.03 pN case.

considered the effect on the probability of looping of a fixed 90° kink at the apex of the loop and predicted that this would reduce the inhibitory effect of tension and shift the loop size distribution to even smaller values.

As our template allows for a quasicontinuum of possible loop sizes, our situation is somewhat similar to the case of ''nonspecific'' loops considered by Sankararaman and Marko (19). The measured optimum loop size is plotted versus incubation time and DNA tension in Fig. 9, *A* and *B*. The dependence on incubation time was very weak, whereas a sharp decrease in size was observed with increasing tension. This finding is in qualitative, but not quantitative, agreement with the predicted trend. At zero tension the observed optimum size (~155 bp) is much shorter than what is predicted by classical WLC models (~500 bp for the ideal teardrop geometry) and is in closer agreement with the 90°-kink model, which predicts an optimum size of 110 bp. We note that the possible loop geometries with Sau3AI are not known. However, even compared with the kink model, we observed a greater reduction in size with increasing tension. The optimum size dropped from 155 bp to less than 50 bp at 0.7 pN, whereas the model predicts a drop from 110 at zero tension to ~85 bp at 0.7 pN. The theoretical calculation for tensioned DNA with a 90° kink actually predicts an optimum loop size of ~155 bp at 0.03 pN, which agrees well with our data but differs with the prediction of the zero tension theory. On the other hand, the degree of inhibition was actually closer to that predicted for the teardrop geometry.

Mechanisms for small loops

In some cases, such as with the one-site REase EcoRV, protein binding can induce sharp bends in DNA (39). Whether Sau3AI induces DNA bending is not known, but it is certainly possible. The 90° kink model in Sankararamen and Marko (19) was proposed to model protein-induced bending. In our experiment, however, binding could presumably only occur inside a loop if additional recognition sites existed between the pair of sites in question, and this is not very likely for closely spaced sites. However, it seems quite possible that protein-induced bends at the closure point of the loop, rather than in the interior, could facilitate the formation of short loops.

Recent cyclization experiments with DNA molecules shorter than 100 bp provided evidence for spontaneous kinking of DNA (40). Following this report, a number of researchers have proposed models for spontaneous kinks, which could possibly form by mechanisms such as localized strand separation, facilitating formation of very short loops (41–43). However, this possibility is controversial, as other experiments and calculations suggest that spontaneous kinks would be very rare and thus unlikely to occur between closely spaced sites in our experiment (43).

A number of possible effects besides protein-induced or spontaneous DNA kinking could be considered in an attempt

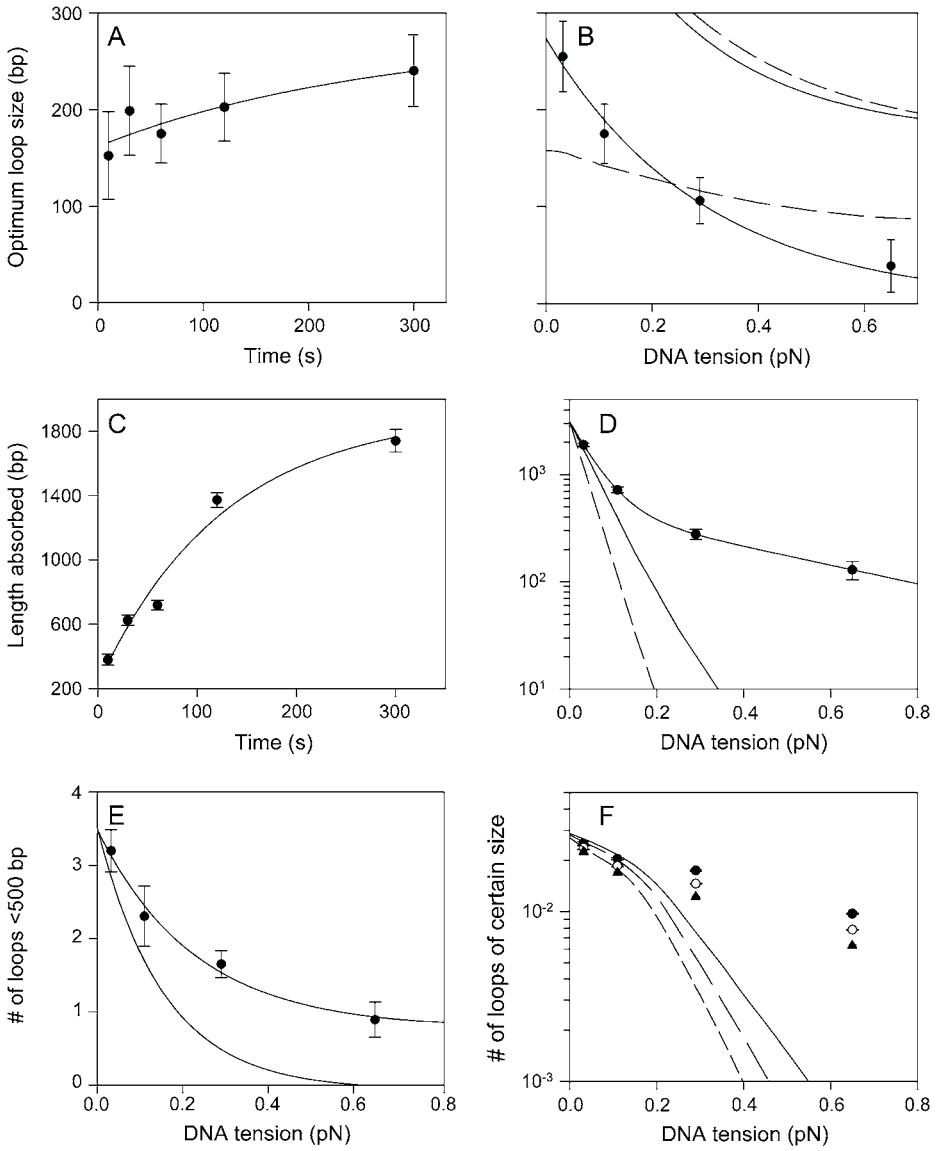

FIGURE 9 Further data analysis and comparisons with theoretical predictions. (*A*) Optimum loop size versus incubation time. The solid line is a fit to a saturating exponential. (*B*) Optimum loop size versus DNA tension. The lower solid line is a fit to a decaying exponential. The lower dashed line is the prediction for the 90° kink model, and the upper dashed line is the prediction for the teardrop model in Sankararaman and Marko (19). The upper solid line is the prediction from Blumber et al. (20). (*C*) Mean length of DNA absorbed into loops versus incubation time. The fit line is an offset saturating exponential. (*D*) Mean length absorbed in 1 min versus DNA tension. The upper solid line is a fit to a decaying double exponential. The lower solid line is the prediction for the 90° kink model, and the lower dashed line is the prediction for the teardrop model in Sankararaman and Marko (19). The predictions were normalized to the experimental value extrapolated to zero tension. (*E*) Mean number of loops shorter than 500 bp versus incubation time (40 units/ml Sau3AI and 0.1-pN DNA tension) calculated from the normalized distributions (Fig. 7). The dashed line is a fit to a decaying exponential. The lower line is the theoretical prediction calculated by integrating the probability distributions for the 90° kink model from 0 to 500 bp and normalizing to the experimental value extrapolated to zero tension. (*F*) Mean number of loops of size 100 bp (●), 125 bp (○), and 150 bp (▲). The lines are the theoretical predictions for 90° kink model: 100 bp (*solid line*), 125 bp (*long dashes*), and 150 bp (*short dashes*), each normalized to the experimental value extrapolated to zero tension.

to reconcile the loop sizes with predictions of classical models of DNA mechanics. First, the persistence length could be shorter than the often assumed value of 150 bp. Values as low as ~120 bp have been reported in solutions containing divalent cations like those used in this study (44). However, this difference is not of sufficient magnitude to account for the discrepancy. Second, the protein complex also has a finite span (estimated to be of the order ~10–30 bp), which would reduce the necessary bending of the DNA (27) and also lead to an underestimation in the measured loop sizes (since the extension measured before unlooping would include this span). However, these effects are not of sufficient magnitude to reconcile the very small loops we observe.

Additionally, multiple loops can form in our experiment and it has recently been predicted that loop rearrangement entropy would result in slightly smaller loop sizes (29). Moreover, nested loops (loops within loops) may occur and these would yield measured events of size equal to the length of DNA sequestered between one Sau3AI dimer and the next, rather than the full length of DNA between the pairs of sites. In fact, we find some evidence for such effects. If looping was completely random and loops were independent of each other, the number of loops per molecule would be expected to follow Poisson statistics. Systematic deviation from this behavior was observed (Fig. 10, *A* and *B*). The variance was wider than expected, particularly at low tension where multiple loops often form. This suggests cooperativity in multiple loop formation. Such cooperativity could arise because the formation of one loop would tend to bring other pairs of sites into closer proximity, thus facilitating the formation of nested loops. Such behavior is not merely a

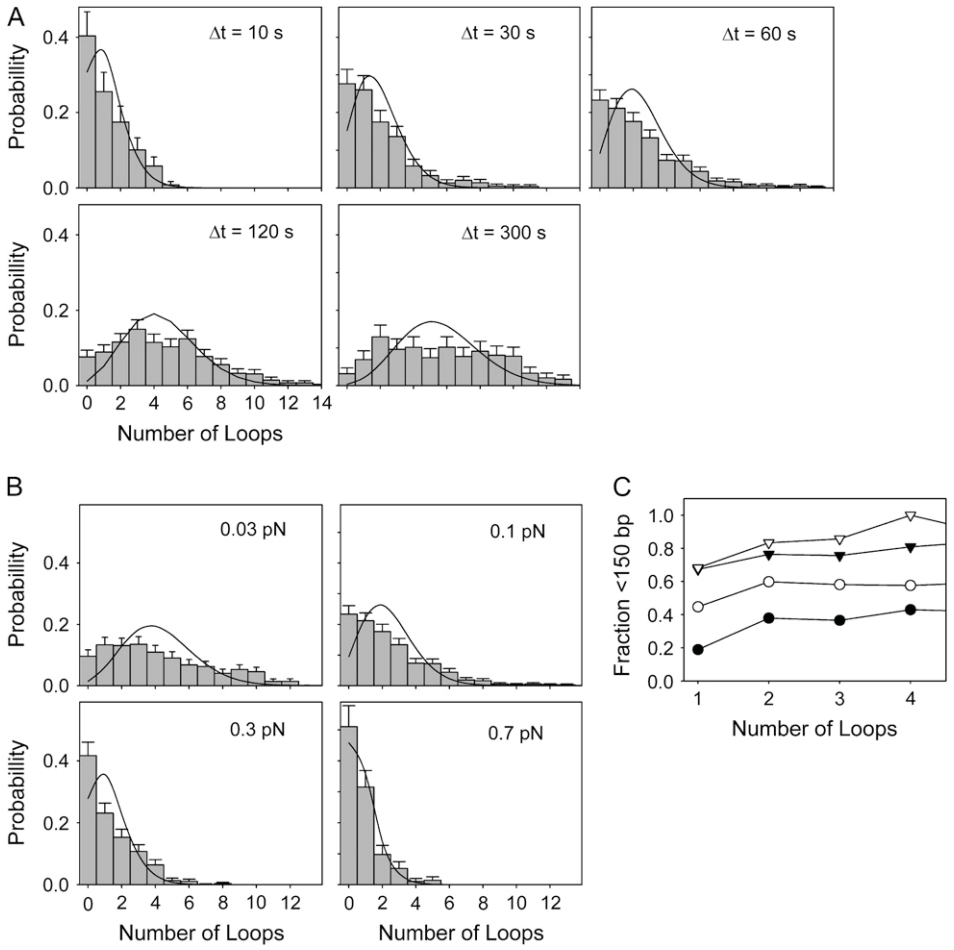

FIGURE 10 (*A*) Normalized probability distributions of numbers of loops per trial versus incubation time. The lines indicate Poisson distributions having means equal to the experimental means. (*B*) Number distributions versus DNA tension with the lines calculated as in *A*. (*C*) Fraction of loops of size <1 persistence length (150 bp) in the subset of molecules which formed a certain number of loops (*N*). The symbol representations are 0.03 pN (●), 0.1 pN (○), 0.3 pN (▼), and 0.7 pN (▽).

curiosity as transcription factors such as RXR have been shown to act by looping sites that are nested between promotor and enhancer sites (45). Additional evidence for such behavior in our experiment is that the fraction of small loops (<150 bp) is greater for molecules having two or more loops than it is for those having only one loop (Fig. 10 *C*). On the other hand, the influence of this effect is also somewhat limited because the mean number of loops formed in our experiments ranged from 4 to 5 at low tension to <1 at 0.7 pN. A significant fraction of small loops was measured in molecules that formed only one loop at all tension levels, which means that an alternative explanation for these loops is still needed.

Size and flexibility of the protein complex may play an important role in facilitating the formation of such short loops (1,30,46). In the case of the smallest loops we observed (<30 bp), we imagine that the DNA is wrapped across the surface of the enzyme complex, akin to how DNA is wrapped in the nucleosome, rather than looping freely through the solution. We suspect that these effects, in combination with the effects of protein span and potential induced bending of the DNA at the closure point of the loop, must explain the deviation of our findings from the predictions of classical WLC theories.

Lower inhibition of looping by tension

In Fig. 9 *E*, we compare the observed frequency of loops versus applied tension with the total looping probability predicted by Sankararaman and Marko (19). This comparison was made by integrating the area under the predicted probability functions over the range calculated (0–500 bp) and scaling the result so that it matched the mean number of observed loops in the limit of zero tension. As with the other metrics discussed above, this comparison again reveals lower than predicted inhibition of looping by tension. Sankararaman and Marko also calculated the rate of absorption of length of DNA into loops. In our experiment the initial rate was ~35 bp/s and the total length absorbed saturated at ~2000 bp after 5 min (with 40 units/ml Sau3AI and 0.1 pN DNA tension). Significantly less reduction in the length absorption rate with tension was observed than predicted. On the other hand, at 0.11 pN DNA tension and assuming a binding energy of 10 kT, the multiple-loop entropic compression theory of

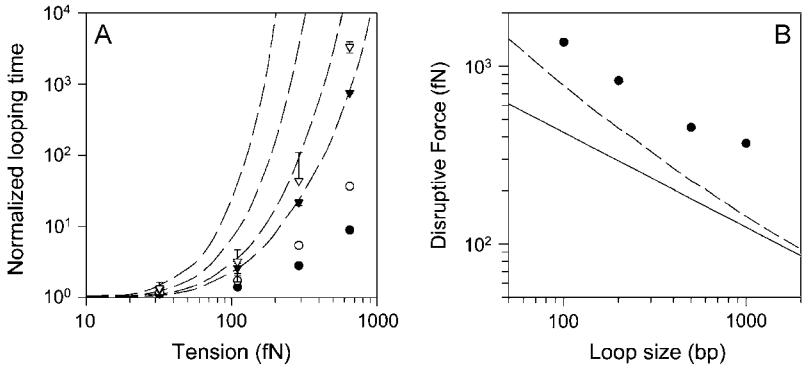

FIGURE 11 Comparisons with theoretical predictions in Blumberg et al. (20). (*A*) Relative looping time measured as a function of DNA tension for loop sizes of 100 bp (●), 200 bp (○), 500 bp (▼), and 1000 bp (∇). The dashed lines are the predicted results for hairpin loops of 1000 bp, 500 bp, 200 bp, and 100 bp (*left* to *right*). (*B*) Disruptive tension as defined in Blumberg et al. (20) versus loop size (●). The solid line indicates the value predicted for a hairpin loop and the dashed line that predicted for a circular loop geometry.

Sankararaman and Marko predicts ~14% ''loop coverage'', a value close to the ~18% fraction we observed (29).

We also compared our results with the theoretical predictions of Blumberg et al. (20) (Fig. 11). By applying the thermodynamic expression for detailed balance, they calculated free energy differences between looped and unlooped DNA within a two-state WLC model and estimated the time required to form a loop under tension relative to the time at zero tension. They considered two different extremes of loop geometry. The antiparallel geometry corresponds to a ''hairpin'', with maximum bending of the DNA exiting the loop, whereas the parallel geometry is ''circular'', with no bending of the DNA exiting the loop. As Sau3AI binds a palindromic recognition sequence, it could exhibit either or both of these extremes of geometry, or something in between. Consistent with the trends discussed above, this metric shows that looping time dramatically increases with tension, but to a much lower degree than predicted. Whereas a 100-fold increase is predicted for a 200-bp loop at 0.3 pN, we observed only a ~5.5-fold increase. This difference may be partly explained by the fact that our template has 27 pairs of sites, whereas the theory calculates the probability of a single looping event of specified length. Such an explanation cannot, however, reconcile the less drastic dependence on tension, which is most certainly associated with the occurrence of smaller than predicted loop sizes.

Blumberg et al. (20) define a ''disruptive force'' as the DNA tension needed to increase the mean looping time by a factor of 100, a change that would have a clear biological effect in the *lac* repressor system. Although we find a systematically higher disruptive force, the dependence on loop size was similar to that predicted, and the predictions for the hairpin loop were in closer agreement with our findings than those for the parallel loop (Fig. 11 *B*). Interestingly, close inspection of the electron microscopy data on looped DNA-Sau3AI complexes does appear to reveal a hairpin geometry in many images (12).

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REFERENCES

1. Schleif, R. 1992. DNA looping. *Annu. Rev. Biochem.* 61:199–223.
2. Dunn, T. M., S. Hahn, S. Ogden, and R. F. Schleif. 1984. An operator at −280 base pairs that is required for repression of araBAD operon promoter: addition of DNA helical turns between the operator and promoter cyclically hinders repression. *Proc. Natl. Acad. Sci. USA.* 81: 5017–5020.
3. Mukherjee, S., H. Erickson, and D. Bastia. 1988. Enhancer origin interaction in plasmid-R6K involves a DNA loop mediated by initiator protein. *Cell.* 52:375–383.
4. Su, W., T. Middleton, B. Sugden, and H. Echols. 1991. DNA looping between the origin of replication of Epstein-Barr virus and its enhancer site: stabilization of an origin complex with Epstein-Barr nuclear antigen 1. *Proc. Natl. Acad. Sci. USA.* 88:10870–10874.
5. Oehler, S., M. Amouyal, P. Kolkhof, B. von Wilcken-Bergmann, and B. Muller-Hill. 1994. Quality and position of the three lac operators of E. coli define efficiency of repression. *EMBO J.* 13:3348–3355.
6. Weiner, B. M., and N. Kleckner. 1994. Chromosome pairing via multiple interstitial interactions before and during meiosis in yeast. *Cell.* 77:977–991.
7. Blackwood, E. M., and J. T. Kadonaga. 1998. Going the distance: a current view of enhancer action. *Science.* 281:60–63.
8. Chen, Y., and P. A. Rice. 2003. New insight into site-specific recombination from Flp recombinase-DNA structures. *Annu. Rev. Biophys. Biomol. Struct.* 32:135–159.
9. Vilar, J. M., and S. Leibler. 2003. DNA looping and physical constraints on transcription regulation. *J. Mol. Biol.* 331:981–989.
10. Buchler, N. E., U. Gerland, and T. Hwa. 2003. On schemes of combinatorial transcription logic. *Proc. Natl. Acad. Sci. USA.* 100:5136–5141.
11. Kruger, D. H., G. J. Barcak, M. Reuter, and H. O. Smith. 1988. EcoRII can be activated to cleave refractory DNA recognition sites. *Nucleic Acids Res.* 16:3997–4008.
12. Friedhoff, P., R. Lurz, G. Luder, and A. Pingoud. 2001. Sau3AI, a monomeric type II restriction endonuclease that dimerizes on the DNA and thereby induces DNA loops. *J. Biol. Chem.* 276:23581–23588.
13. Mucke, M., D. H. Kruger, and M. Reuter. 2003. Diversity of type II restriction endonucleases that require two DNA recognition sites. *Nucleic Acids Res.* 31:6079–6084.
14. Halford, S. E., A. J. Welsh, and M. D. Szczelkun. 2004. Enzyme-mediated DNA looping. *Annu. Rev. Biophys. Biomol. Struct.* 33:1–24.
15. Yan, J., D. Skoko, and J. F. Marko. 2004. Near-field-magnetic-tweezer manipulation of single DNA molecules. *Phys. Rev. E.* 70:011905.

16. van den Broek, B., F. Vanzi, D. Normanno, F. S. Pavone, and G. J. Wuite. 2006. Real-time observation of DNA looping dynamics of type IIE restriction enzymes NaeI and NarI. *Nucleic Acids Res.* 34:167–174.

17. Gemmen, G. J., R. Millin, and D. E. Smith. 2006. Tension-dependent DNA cleavage by restriction endonucleases: two-site enzymes are ''switched off'' at low force. *Proc. Natl. Acad. Sci. USA.* 103:11555–11560.

18. Gemmen, G. J., R. Millin, and D. E. Smith. 2006. DNA looping by two-site restriction endonucleases: heterogeneous probability distributions for loop size and unbinding force. *Nucleic Acids Res.* 34: 2864–2877.

19. Sankararaman, S., and J. F. Marko. 2005. Formation of loops in DNA under tension. *Phys. Rev. E.* 71:021911.

20. Blumberg, S., A. V. Tkachenko, and J. C. Meiners. 2005. Disruption of protein-mediated DNA looping by tension in the substrate DNA. *Biophys. J.* 88:1692–1701.

21. Gemmen, G. J., R. Sim, K. A. Haushalter, P. C. Ke, J. T. Kadonaga, and D. E. Smith. 2005. Forced unraveling of nucleosomes assembled on heterogeneous DNA using core histones, NAP-1, and ACF. *J. Mol. Biol.* 351:89–99.

22. Bustamante, C., S. B. Smith, J. Liphardt, and D. Smith. 2000. Single-molecule studies of DNA mechanics. *Curr. Opin. Struct. Biol.* 10: 279–285.

23. Derenyi, I., D. Bartolo, and A. Ajdari. 2004. Effects of intermediate bound states in dynamic force spectroscopy. *Biophys. J.* 86:1263–1269.

24. van den Broek, B., M. C. Noom, and G. J. Wuite. 2005. DNA-tension dependence of restriction enzyme activity reveals mechanochemical properties of the reaction pathway. *Nucleic Acids Res.* 33:2676–2684.

25. Merlitz, H., K. Rippe, K. V. Klenin, and J. Langowski. 1998. Looping dynamics of linear DNA molecules and the effect of DNA curvature: a study by Brownian dynamics simulation. *Biophys. J.* 74:773–779.

26. Rippe, K., P. H. von Hippel, and J. Langowski. 1995. Action at a distance: DNA-looping and initiation of transcription. *Trends Biochem. Sci.* 20:500–506.

27. Rippe, K. 2001. Making contacts on a nucleic acid polymer. *Trends Biochem. Sci.* 26:733–740.

28. Yan, J., R. Kawamura, and J. F. Marko. 2005. Statistics of loop formation along double helix DNAs. *Phys Rev E.* 71:061905.

29. Sankararaman, S., and J. F. Marko. 2005. Entropic compression of interacting DNA loops. *Phys. Rev. Lett.* 95:078104.

30. Zhang, Y., A. E. McEwen, D. M. Crothers, and S. D. Levene. 2006. Statistical-mechanical theory of DNA looping. *Biophys. J.* 90:1903–1912.

31. Lee, D. H., and R. F. Schleif. 1989. In vivo DNA loops in araCBAD: size limits and helical repeat. *Proc. Natl. Acad. Sci. USA.* 86:476–480.

32. Muller, J., S. Oehler, and B. Muller-Hill. 1996. Repression of lac promoter as a function of distance, phase and quality of an auxiliary lac operator. *J. Mol. Biol.* 257:21–29.

33. Plumbridge, J., and A. Kolb. 1998. DNA bending and expression of the divergent nagE-B operons. *Nucleic Acids Res.* 26:1254–1260.

34. Geanacopoulos, M., G. Vasmatzis, V. B. Zhurkin, and S. Adhya. 2001. Gal repressosome contains an antiparallel DNA loop. *Nat. Struct. Biol.* 8:432–436.

35. Finzi, L., and J. Gelles. 1995. Measurement of lactose repressor-mediated loop formation and breakdown in single DNA molecules. *Science.* 267:378–380.

36. Lia, G., D. Bensimon, V. Croquette, J. F. Allemand, D. Dunlap, D. E. Lewis, S. Adhya, and L. Finzi. 2003. Supercoiling and denaturation in Gal repressor/heat unstable nucleoid protein (HU)-mediated DNA looping. *Proc. Natl. Acad. Sci. USA.* 100:11373–11377.

37. Reuter, M., D. Kupper, A. Meisel, C. Schroeder, and D. H. Kruger. 1998. Cooperative binding properties of restriction endonuclease EcoRII with DNA recognition sites. *J. Biol. Chem.* 273:8294–8300.

38. Katiliene, Z., E. Katilius, and N. W. Woodbury. 2003. Single molecule detection of DNA looping by NgoMIV restriction endonuclease. *Biophys. J.* 84:4053–4061.

39. Winkler, F. K., D. W. Banner, C. Oefner, D. Tsernoglou, R. S. Brown, S. P. Heathman, R. K. Bryan, P. D. Martin, K. Petratos, and K. S. Wilson. 1993. The crystal structure of EcoRV endonuclease and of its complexes with cognate and non-cognate DNA fragments. *EMBO J.* 12:1781–1795.

40. Cloutier, T. E., and J. Widom. 2004. Spontaneous sharp bending of double-stranded DNA. *Mol. Cell.* 14:355–362.

41. Yan, J., and J. F. Marko. 2004. Localized single-stranded bubble mechanism for cyclization of short double helix DNA. *Phys. Rev. Lett.* 93:108108.

42. Wiggins, P. A., R. Phillips, and P. C. Nelson. 2005. Exact theory of kinkable elastic polymers. *Phys Rev E.* 71:021909.

43. Du, Q., C. Smith, N. Shiffeldrim, M. Vologodskaia, and A. Vologodskii. 2005. Cyclization of short DNA fragments and bending fluctuations of the double helix. *Proc. Natl. Acad. Sci. USA.* 102:5397–5402.

44. Wang, M. D., H. Yin, R. Landick, J. Gelles, and S. M. Block. 1997. Stretching DNA with optical tweezers. *Biophys. J.* 72:1335–1346.

45. Yasmin, R., K. T. Yeung, R. H. Chung, M. E. Gaczynska, P. A. Osmulski, and N. Noy. 2004. DNA-looping by RXR tetramers permits transcriptional regulation ''at a distance''. *J. Mol. Biol.* 343: 327–338.

46. Villa, E., A. Balaeff, and K. Schulten. 2005. Structural dynamics of the lac repressor-DNA complex revealed by a multiscale simulation. *Proc. Natl. Acad. Sci. USA.* 102:6783–6788.