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Forced Unraveling of Nucleosomes Assembled on Heterogeneous DNA Using Core Histones, NAP-1, and ACF

Gregory J. Gemmen[1], Ronald Sim[1], Karl A. Haushalter[2]†, Pu Chun Ke[1]† James T. Kadonaga[2] and Douglas E. Smith[1]*

[1]*Physics Department, University of California San Diego, 9500 Gilman La Jolla, CA 92093-0379, USA*

[2]*Section of Molecular Biology University of California San Diego, 9500 Gilman La Jolla, CA 92093-0346, USA*

**Corresponding author*

Periodic arrays of nucleosomes were assembled on heterogeneous DNA using core histones, the histone chaperone NAP-1, and ATP-dependent chromatin assembly and remodeling factor (ACF). The mechanical properties of these complexes were interrogated by stretching them with optical tweezers. Abrupt events releasing ∼55–95 base-pairs of DNA, attributable to the non-equilibrium unraveling of individual nucleosomes, were frequently observed. This finding is comparable with a previous observation of 72–80 bp unraveling events for nucleosomes assembled by salt dialysis on a repeating sea urchin 5 S RNA positioning element, but the unraveling force varied over a wider range (∼5–65 pN, with the majority of events at lower force). Because ACF assembles nucleosomes uniformly on heterogeneous DNA sequences, as in native chromatin, we attribute this variation to a dependence of the unraveling force on the DNA sequence within individual nucleosomes. The mean force increased from 24 pN to 31 pN as NaCl was decreased from 100 mM to 5 mM. Spontaneous DNA re-wrapping events were occasionally observed in real time during force relaxation. The observed wide variations in the dynamic force needed to unravel individual nucleosomes and the occurrences of sudden DNA re-wrapping events may have an important regulatory influence on DNA-directed nuclear processes, such as the binding of transcription factors and the movement of polymerase complexes on chromatin.

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Introduction

In the nucleus of eukaryotic cells, DNA is assembled with proteins into a periodic, highly folded complex referred to as chromatin.[1] This compaction allows human cells to fit roughly two meters of DNA into a nucleus that is only a few microns in diameter. The basic repeating unit of chromatin, the nucleosome, consists of 147 bp of DNA wrapped in ∼1.7 superhelical turns around a protein octamer that consists of two copies each of histones H2A, H2B, H3, and H4. Fourteen points of contact between the DNA and octamer define the wrapping path.[2] The length of DNA in the nucleosome core is conserved among eukaryotes, although nucleosomal repeat lengths vary from ∼165 bp to 230 bp. The histone octamer is shaped as a cylinder of diameter ∼11 nm and thickness ∼8 nm containing many positively charged residues, whereas DNA is a negatively charged semi-flexible polymer of diameter ∼2 nm and persistence length ∼50 nm. Thus, the wrapping of DNA around the histone octamer involves competition between electrostatic adhesion and resistance to bending.

Chromatin is a dynamic structure that alters its properties and composition during the cell cycle

Present addresses: K. A. Haushalter, Chemistry and Biology Departments, Harvey Mudd College, Claremont, CA 91711, USA; P. C. Ke, Department of Physics and Astronomy, Clemson University, Clemson, SC 29634, USA.

Abbreviations used: ACF, ATP-dependent chromatin assembly and remodeling factor; NAP-1, nucleosome assembly protein 1; WLC, worm-like chain.

E-mail address of the corresponding author: des@physics.ucsd.edu

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and in response to external signals. Modulation of DNA–histone interactions allows cells to regulate processes such as replication, transcription, and repair through dynamic tuning of the structure and accessibility of chromatin.[3] Access to genetic information can require partial or full unwrapping of DNA from the histone octamer, which may occur by a variety of mechanisms. Thermal fluctuations can transiently expose portions of the nucleosomal DNA,[4] remodeling enzymes can actively reposition nucleosomes,[5] and chemical modification of the N-terminal tails of histones can modulate DNA accessibility.[6] Additionally, DNA sequence affects nucleosome stability.[4] Investigating the forces needed to unravel chromatin complexes will provide important insights into the remodeling, unwrapping, and rewrapping required by fundamental biochemical processes. For instance, DNA and RNA polymerases have been shown capable of exerting transient forces up to ~40 pN,[7–9] and it is important to understand the effects that these high forces can have on nucleosomes.

Several recent studies have employed single-molecule manipulation techniques to probe chromatin structure. In pioneering work, Cui & Bustamante[10] stretched chromatin fibers extracted from chicken erythrocytes using optical tweezers. Their studies revealed a "decondensation" transition at a force of ~5 pN that was interpreted as disruption of inter-nucleosomal interactions. Above ~20 pN a second, irreversible transition was observed and interpreted as being due to disruption of nucleosomes, although discrete unraveling events were not observed. Subsequent studies have focused on *in vitro* assembly of histone–DNA complexes. Rapid compaction of DNA was observed upon flowing concentrated *Xenopus* egg extracts or protein solutions past single tethered DNA molecules.[11–13] Upon stretching such complexes assembled *in situ*, Bennink *et al.* observed abrupt lengthening events frequently releasing 382 bp and 191 bp of DNA, which were attributed to unraveling of nucleosomes.[12] Subsequently, however, Brower-Toland *et al.* obtained a different result when using complexes pre-assembled in bulk by salt-dialysis using core histones.[14] An engineered DNA template containing periodic repeats of the sea urchin 5 S RNA positioning sequence was used to create well-defined arrays.[15] When stretching these complexes, continuous lengthening was observed up to ~20 pN, followed by a discrete series of unraveling events each releasing ~80 bp of DNA. These events were proposed to be due to disruption of strong interactions at ±40 bp from the dyad axis in the nucleosome structure.[2] Alternatively, it has recently been proposed that such unraveling events could be explained on a physical basis without invoking specific interactions.[16]

To shed further light on the mechanics of nucleosomes, we report here on the optical tweezers stretching of single nucleosomal arrays (Figure 1(a)) assembled on a heterogeneous DNA sequence using core histones, nucleosome assembly protein 1 (NAP-1), and ATP-dependent chromatin assembly and remodeling factor (ACF).[17,18] In this assembly method, NAP-1 acts as a chaperone that delivers histones to the DNA template and prevents non-specific aggregation, while ACF couples the energy of ATP hydrolysis to the processive deposition of highly periodic arrays of nucleosomes.[19] An advantage of this method is that it yields extended periodic arrays in non-repetitive DNA, as in native chromatin.[20] The use of heterogeneous DNA and ACF-catalyzed chromatin assembly may be expected to lead to differences in behavior compared with nucleosome reconstitution on tandemly repeated positioning elements by salt dialysis methodology. ATP-dependent assembly factors assemble nucleosomes processively along any DNA template[21] without any apparent bias towards sequences having higher equilibrium affinities. For example, nucleosomes that are assembled onto a repeating 5 S rRNA positioning sequence with *Drosophila* S-190 extracts are not positioned in register with the 5 S rRNA sequences (M. Pazin & J.T.K., unpublished observations).

Periodic assembly of nucleosomes on a wide variety of sequences is much closer to the actual situation in native chromatin than that obtained using salt dialysis and repeating positioning elements. Nucleosome assembly using a defined set of purified proteins also has advantages over assembly using cell extracts since the protein composition is precisely controlled. For these reasons, this method of assembly has proven useful for producing well-characterized nucleosome arrays for *in vitro* studies of fundamental processes such as transcription, replication, and recombination.[22–24]

Results

Characterization of complexes

After assembling chromatin in bulk under standard conditions, we assessed the sample by partial micrococcal nuclease digestion (Figure 1(b)). Digestion yielded at least four resolvable bands spaced ~168 bp apart, consistent with the expected nucleosome repeat length for arrays lacking the linker histone H1.[18] This ~168 bp repeat length includes 147 bp wrapped around the histone octomer core (the nucleosome core particle) and ~21 bp of linker DNA. Because the DNA template is linear, it was not possible to use a supercoiling assay to quantify the extent of nucleosome assembly precisely. As noted previously, chromatin that is assembled with linear DNA tends to exhibit less extensive nucleosomal periodicity than chromatin assembled with closed circular DNA.[18] It is apparent, however, that these assembly reactions yield periodic nucleosome arrays that contain sufficient numbers of nucleosomes for our analysis. Also, as ACF assembles nucleosomes processively,

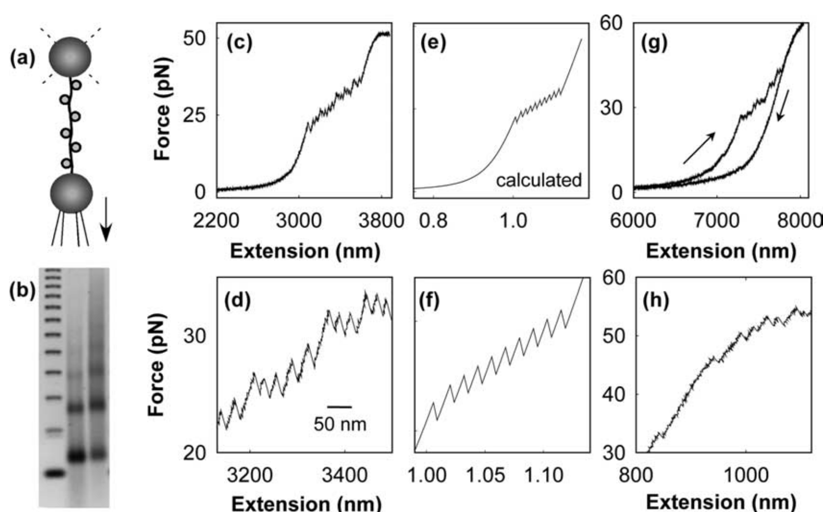

Figure 1. (a) Schematic diagram of an individual complex stretched between a microsphere held by optical tweezers (top) and a microsphere held by a micropipette (bottom). (b) Agarose gel electrophoresis of samples partially digested by micrococcal nuclease. Lane 1 contains 123 bp ladder. Lanes 2 and 3 contain samples digested by 2× and 1× dilutions of micrococcal nuclease. (c) Force-extension measurement of a complex in 5 mM NaCl, showing a series of nucleosome unraveling events. Prior to reaching the full naked DNA extension of ∼8.1 μm, the DNA overstretches at ∼54 pN, the expected value for 5 mM NaCl. Further stretching could not be done, since this complex detached from the microspheres. (d) Zoom of (c) showing the unraveling events. (e) Calculated force *versus* fractional extension for an extensible worm-like chain (WLC) in series with a trap of stiffness 0.17 pN/nm. Good agreement with the events in (c) was obtained by calculating events with 25 nm increases in the contour length (ten unraveling events spaced 40 nm apart are shown). A persistence length of 20 nm and a stretch modulus of 840 pN, average for these complexes, were used in these calculations. These calculated data were used to verify our method of determination of the length of DNA released in unraveling events. (f) Zoom of (e). (g) Data recorded in 100 mM NaCl, showing a stretching cycle, unraveling events, and a relaxation displaying hysteresis. In this example the fiber reaches nearly the expected naked DNA length. The DNA begins to overstretch at ∼63 pN, as expected in 100 mM NaCl. (h) Another data set recorded in 5 mM NaCl showing nucleosome unraveling events occurring into the overstretching transition.

partially assembled arrays contain sections of periodic nucleosomes similar to those present in a given section of a fully assembled array. Importantly, the nuclease digestion results presented here are for the same sample used in the optical tweezers, insuring that the complexes being interrogated contain properly formed nucleosomes. Such pre-characterization of the same complexes was not possible in several previous studies where complexes were assembled *in situ*.[11–13]

Analysis of complexes assembled using ACF and NAP-1 by sucrose gradient sedimentation indicates that while core histones remain bound, NAP-1 dissociates after assembly (D. Fyodorov & J.T.K., unpublished observations). As the microspheres are washed upon injection into the flow chamber, NAP-1 would not be present during measurements. On the other hand, a significant proportion of ACF appears to remain bound to the newly assembled chromatin and may therefore still be present during our measurements. However, ACF is only present in catalytic quantities (∼1 ACF molecule per 50 nucleosome lengths of DNA) and is not active for chromatin assembly or remodeling due to our exchange into a buffer lacking ATP. Therefore, we expect that ATP-dependent chromatin remodeling by ACF would not occur during the unraveling measurements.

Nucleosome unraveling

Complexes were stretched by moving the microsphere on the micropipette in 0.75 nm steps at 100 Hz. This stretching caused the force applied across individual nucleosomes to ramp as high as ∼65 pN on a time scale of ∼10 seconds, which we have chosen to be similar to the time it takes the RNA polymerase II complex to transcribe through a nucleosome *in vivo*.[25,26] Initially, we observed a non-linear increase in force (Figure 1(c)). Such behavior is characteristic of polymer elasticity in general.[27] The most striking departure from this simple behavior was the occurrence of runs of small sawteeth in the force-extension data (Figure 1(d)), corresponding to sudden lengthening events (Figure 1(e) and (f)). As each event releases <147 bp of DNA and occurs in <10 ms, which is one to two orders of magnitude less than the average time between events, we attribute these to the unraveling of individual nucleosomes. Variations in the events will be discussed below.

While unraveling events were identifiable throughout all data sets, significant heterogeneity in the behavior of different complexes was observed. They displayed different initial degrees of compaction and elastic behavior. Initial lengths ranged from almost zero up to the full length of the DNA template. Similar heterogeneity was reported in studies of native chromatin.[10] We attribute these variations to differences in the degree of assembly of complexes, partial adsorption to the microspheres, partial unraveling prior to and during tethering, and lack of complete unraveling of some complexes at even the highest applied forces.

As seen in Figure 1(c) and (g), a force plateau appears at ∼54 pN (in 5 mM NaCl) and ∼63 pN (in 100 mM NaCl), characteristic of the "overstretching transition" of double-stranded (ds) DNA.[28] The

observation of this transition indicates that the elasticity in this high force regime is dominated by sections of naked DNA exposed by unraveled nucleosomes, plus any remaining linker DNA. As shown in Figure 1(h), nucleosome unraveling continued to occur into the overstretching transition, a phenomenon not observed in previous studies.[12,14] When complexes were partially overstretched and then relaxed, large hysteresis in force-extension was almost always observed (Figure 1(g)), indicating that many nucleosomes were irreversibly unraveled.

Multiple stretch–relax cycles

In several cases we were able to carry out multiple stretch–relax cycles before the tether broke (Figure 2). Most complexes did not stretch out to the full extension expected of the DNA template. Remarkably, unraveling events occurred even after multiple cycles that partially overstretched the DNA. Such high-force events were not observed in previous studies, where nucleosomes seemed to be irreversibly dissociated upon reaching the overstretching transition.[12,14] Our finding may explain the observation that the elasticity of native chromatin does not return to that of dsDNA after being partially overstretched.[10]

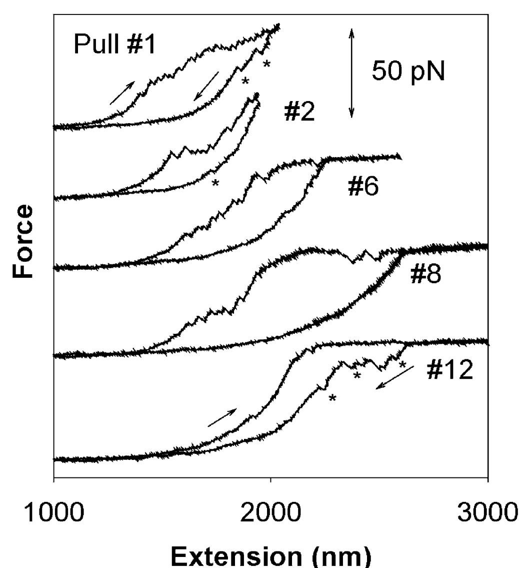

Figure 2. Repeated stretching and relaxing of an initially compact fiber. It remained tethered during 12 stretch–relax cycles and displayed unraveling events and hysteresis even after multiple pulls into the DNA overstretching regime. Selected stretch–relax cycles are indicated by the numbers next to each plot. The plots have been shifted along the force axis for display purposes. The scale bar represents 50 pN. Although the frequency of unraveling events decreased after multiple stretches the tether had not unraveled to the expected DNA length (~8.1 μm). Asterisks indicate spontaneous shortening events observed during force relaxations.

Distribution of unraveling lengths and forces

The peak force (F) and DNA length (ΔL) released were recorded for each event. All resolvable events in all recorded data sets were included in constructing the ΔL distribution. The distribution of ΔL values for the 5 mM NaCl data is shown in Figure 3(a). This distribution was unchanged for additional measurements in 20 mM, 50 mM, and 100 mM NaCl (Figure 3(b)). A single peak ranging from ~55 bp to 95 bp, having a maximum at ~74 bp, encompassed ~70% of all events. Of the remaining events, two-thirds were >150 bp, too large to be nucleosomes. We attribute these to unbinding of complexes partially adsorbed to the microspheres. Notably, the value of 72–80 bp reported for positioned nucleosomes[14,29] falls in the middle of our peak, whereas the 382 and 191 bp values most frequently observed for complexes assembled *in situ*[12] fall well outside it (<5% of events were within 10 nm of 191 bp). However, more recent higher-resolution measurements report events of both ~176 bp and ~88 bp.[30]

The distribution of nucleosome unraveling forces was broad. It extended from ~5 pN to 65 pN in 100 mM NaCl and shifted to higher force as the NaCl was decreased to 5 mM (Figure 4(a) and (b)),

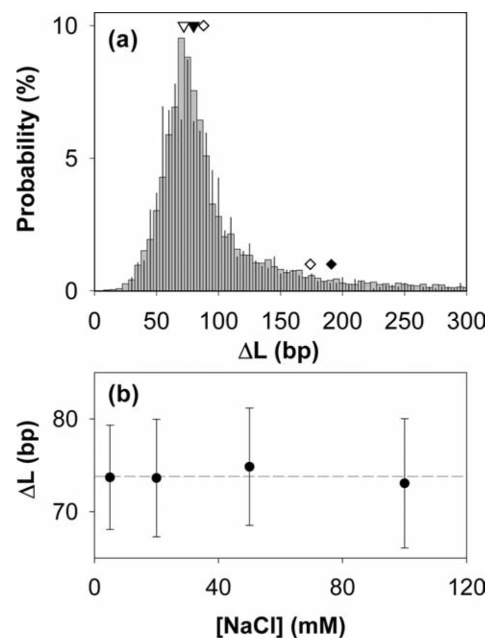

Figure 3. (a) Distribution of DNA lengths (ΔL) released in unraveling events for complexes in 5 mM NaCl. ΔL was determined by measuring the distance along the extension axis from each peak to the following point of equal force. This procedure was validated on simulated data (Figure 1(f)) calculated by using the extensible WLC model.[32,50] Gray bars indicate entire set of results, while thin black needles represent only events that occurred at forces greater than 42 pN (and into overstretch). Previously reported values are indicated by the following symbols: open triangle;[14] filled triangle;[29] filled diamond;[12] open diamonds.[30] (b) Independence of the peak ΔL on NaCl. The ΔL values average to 74(±3) bp.

consistent with the mechanical stability of nucleosomes increasing with decreasing monovalent salt. This effect had not been investigated in previous stretching experiments. The observed range of forces is significantly larger than the ~22–32 pN range reported for positioned nucleosomes.[14] In both cases, the forces are higher than the value of ~2 pN predicted from equilibrium theory,[31] suggesting that unraveling is a non-equilibrium process. The distribution of ΔL values for high force events ($F \geqq 42$ pN) was the same as for all events (Figure 3), confirming that they are consistent with nucleosome unraveling.

Spontaneous rewrapping

Although most nucleosomes appeared to unravel irreversibly, abrupt shortening events were sometimes observed in real time during relaxation of the tethered complex (Figure 2). Shortening events following overstretching of naked DNA have been observed previously and are due to re-annealing of partly melted DNA.[28] However, we observed shortening events after complexes were stretched to a force below the overstretching force, where melting hysteresis is not observed. We therefore attribute these events to spontaneous rewrapping of DNA onto octamers that were not completely dissociated during stretching. Real time observation of re-wrapping events has not been reported previously although evidence of re-wrapping was obtained by observing unraveling following repeated stretching of fully unraveled complexes (extending to the naked DNA length).[14]

Shortening events were rare compared with unraveling events. Only 73 were observed in 366 relaxation measurements. The mean decrease in tether length was 34 nm ($\sigma = 15$ nm), not far from the ΔL value observed during unraveling. Event forces ranged from 7 pN to 63 pN (mean = 37 pN, $\sigma = 15$ pN), similar to those observed for unraveling. The mean waiting time for re-wrapping from the beginning of relaxation was 2.1 seconds ($\sigma = 1.3$ seconds), although the distribution did not follow a single exponential decay as would be expected for a single step process in steady state. Rather, the distribution was peaked at ~1.7 seconds, consistent with re-wrapping kinetics being force-dependent.

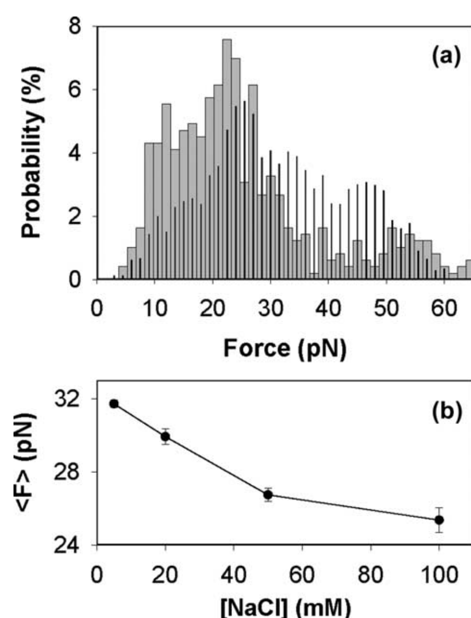

Figure 4. (a) Distribution of unraveling forces in 100 mM NaCl (gray bars; mean = 25 pN, standard deviation $\sigma = 16$ pN, $n = 498$ events) and 5 mM NaCl (black needles; mean = 32 pN, $\sigma = 14$ pN, $n = 4305$). In many trials the DNA tether broke before the measurement was complete, so in order to minimize a low force bias only trials that reached $F > 40$ pN were included. The small peak in the distribution at high force is attributed to two effects. First, the force-extension curve begins to flatten ~5 pN before the overstretch plateau, resulting in a decreased loading rate that biases some events towards higher force. There is not a peak at the overstretch force plateau because the majority of complexes completely detach before the full overstretching plateau is reached. Second, at high force there is occasionally unbinding of portions of complexes partially adsorbed to the microspheres, also biasing some events towards higher force. (b) Average force *versus* salt.

Elasticity of complexes

Sections of force-extension data without unraveling events were analyzed to determine the elastic properties. Native chromatin folds into a higher order structure in 100 mM NaCl.[1] However, factors that promote folding, such as linker histones and divalent cations, were not included in our experiments. We found no evidence of the decondensation transition observed by Cui & Bustamante, suggesting that our complexes are unfolded (or unfolded at a force too low to resolve). This observation agrees with the findings for positioned nucleosomes.[14]

Good fits to force-extension data were obtained using the extensible worm-like chain (WLC) model with persistence length (P), stretch modulus (S), and contour length (L) as parameters.[32] Full packaging of DNA into H1-free nucleosome arrays is expected to result in a ~sevenfold compaction, but verification of this expectation was not possible due to incomplete assembly as well as partial adsorption of complexes to the microspheres. Here, we analyzed the elasticity of complexes exhibiting ~5% to 50% of the DNA length. Distributions of P and S are given in Figure 5. In 5 mM NaCl, the mean value of P was 10 nm ($\sigma =$ 11 nm, $n = 359$ fits), which is substantially smaller than the ~50–80 nm values reported for naked DNA[33–35] and 30 nm value reported for native chromatin.[10] The mean value of S, on the other hand, was 840 pN ($\sigma = 690$ pN, $n = 396$), which is within the ~770–1000 pN range reported for naked DNA.[33–35] It is quite different, however, from the value of 5 pN reported for native chromatin.[10] As the NaCl was increased from 5 mM to 100 mM, the mean value of P shifted up to 18 nm ($\sigma = 18$ nm,

$n=146$), implying higher extensibility at low force. The mean S dropped slightly to 770 pN ($\sigma=370$, $n=95$), which is slightly smaller than the reported values of 1000–1350 pN for naked DNA.

Discussion

Unraveling length

The nucleosome core contains 147 bp of DNA wrapped ∼1.7 turns around the core histone octamer, and hence the observed 55–95 bp events do not correspond to the entire nucleosome unraveling in a single step. This finding is consistent with the proposed mechanism that the flanking DNA (i.e. the DNA at the ends of the nucleosome) peels off continuously at low force, whereas most of the inner turn (∼80 bp) is released abruptly at higher force.[14] More recent studies investigating the role of histone tails in forced nucleosome unraveling also report a comparable value of 72(± 4) bp for the inner turn.[29] These values also agree with theoretical predictions of Kulíc & Schiessel for an elastic rod wrapped with uniform adhesion on a cylindrical spool. Their calculations show that the first turn can be peeled off easily, but removal of the final turn *via* stretching presents a significant kinetic barrier. This property is attributed to the spool having to tilt and the DNA having to bend before complete unraveling can occur.[16]

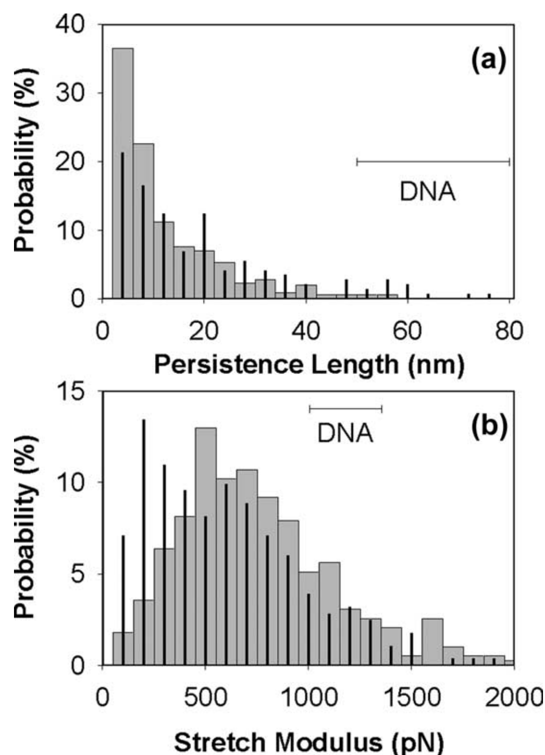

Figure 5. Persistence length (P) and stretch modulus (S) were obtained by fitting force-extension data to the extensible WLC model. The following expression, valid in the range of forces covered, was used: $x/L=1-0.5(kT/FP)^{1/2}+F/S$, where x/L is fractional extension, kT is the thermal energy, F is force.[32] (a) Histogram of persistence length values for 100 mM (gray bars) and 5 mM NaCl (black needles). The scale bar indicates the range for naked DNA.[34,35] As P measures extensibility at low force, only sections of data extending below 5 pN and spanning at least 3 pN (the average span was 14 pN) were fit ($N=359$ fits). All sections of data satisfying these criteria, whether occurring before, between, or following observed unraveling events were included. Sections following unraveling events in one stretch cycle were included because multiple stretching cycles usually revealed many additional unraveling events, as shown in Figure 2. (b) Histogram of stretch modulus (S) values for 100 mM (gray bars) and 5 mM NaCl (black needles). The scale bar indicates the range of reported values for naked DNA.[34,35] As S measures extensibility at high force, only sections extending above 20 pN and spanning at least 5 pN (the average span was 18 pN) were fit ($N=396$).

In comparison to our findings, events most frequently releasing 382 bp and 191 bp were observed for complexes assembled *in situ* using extracts.[12] The 191 bp events were attributed to unraveling of individual nucleosomes and the 382 bp events were attributed to unresolved pairs of 191 bp events. One possible reason for this different finding is that the *Xenopus* extracts contain additional proteins, such as HMG and linker histones, that could increase the effective length of wrapped DNA. These complexes were also stretched ∼13 times faster and the measurements also did not appear to have high enough resolution to resolve smaller events. Indeed, more recent, higher resolution measurements indicate events of both ∼176 bp and ∼88 bp,[30] the difference being attributed to nucleosomes either having or not having linker histone B4. Discrete unraveling events were not observed in the initial studies of native chromatin;[10] however, stretching was done in ∼150 bp steps, which were too large to resolve the unraveling of individual nucleosomes.

In situ assembly

A question that should be raised is whether the *in situ* assembly method used in several recent studies[11–13,30] leads to proper nucleosome formation. Isolated DNA molecules, at essentially infinite dilution, are immobilized inside a chamber, and protein solution is flowed in continuously. These solutions contain ∼1 to 100 μg/ml of histones and, in the case of extracts, many other proteins capable of binding to DNA. Therefore, the histone:DNA mass ratio is vastly higher than the ∼1:1 level used in standard bulk assembly reactions and present in native chromatin. While sample characterization assays were performed in these previous studies, these tests were done on different samples than those used in the single molecule experiments.

In our experience using core histones, NAP-1, and ACF, proper assembly *in vitro* only occurs in a narrow range around a ∼1:1 histone to DNA mass

ratio.[17,18] In preliminary work, we experimented with *in situ* assembly on single DNA molecules by flowing in core histones, NAP-1, and ACF at the concentration used in bulk assembly and at a 1/10 dilution of core histones. In both cases we observed rapid compaction, similar to previous findings.[11–13] However, control experiments showed that compaction was ATP-independent, whereas assembly in bulk is known to be ATP-dependent and occur 10–100 times more slowly.[21] Because it is not possible to characterize the single complexes assembled *in situ* using standard bulk assays such as micrococcal nuclease digestion, we believe that they may not be correctly assembled. Assembly at high protein:DNA stoichiometry tends to result in formation of non-specific protein–DNA aggregates rather than well-formed nucleosome arrays.

Variations in unraveling events

The most notable difference between our results and those obtained for positioned nucleosomes formed by salt dialysis[14] is that our unraveling events occurred over a wider force range. In 100 mM NaCl, our measurements revealed unraveling forces ranging from nearly fourfold lower to twofold higher than that observed for nucleosomes assembled on the 5 S RNA positioning element (∼22–32 pN). The largest fraction of our events (52%) occurred at lower forces (<22 pN), whereas 28% fell in the 22–32 pN range and 20% occurred at >32 pN. In both studies the complexes contained only core histones, although different assembly methods and DNA templates were used. That the histones came from different organisms is not expected to cause differences because core histones are highly conserved across eukaryotes. Also, nuclease digestion patterns suggest that different assembly methods result in the same nucleosome core structure.[1] While we used a higher stretching rate (75 *versus* 28 nm/s) this would also not explain our finding a majority of events at lower force, because unraveling force increases with pulling rate.[14,36]

Brower-Toland *et al.* made measurements in 100 mM NaCl plus 1.5 mM $MgCl_2$, whereas we simply used NaCl ranging from 5 mM to 100 mM. We did not include divalent cations because complexes were extremely difficult to tether at higher overall ionic strengths in our experience, presumably because these conditions promote condensation of the nucleosome arrays.[1] Mg^{2+} has been shown to cause increased screening of histone–DNA electrostatic interactions,[37] potentially leading to lower unraveling forces. Therefore, some of our higher-force events could be interpreted as occurring due to the lack of Mg^{2+} in our experiment relative to that of the studies with positioning elements. However, we observe a majority of unraveling events at lower forces. This is the opposite trend of what would be expected if this shift were due to the absence of Mg^{2+}. While bending of certain DNA sequence motifs may be stabilized by divalent cations, potentially favoring stability of some nucleosomes, recent crystallographic studies by Davey & Richmond suggest that "DNA conformation appears to dictate metal binding, as opposed to the converse".[38]

We therefore interpret the higher variability in unraveling force as being due to our use of a non-repetitive, heterogeneous DNA sequence instead of the tandemly repeated 5 S RNA positioning sequence used by Brower-Toland *et al.*[14] The relative affinities of nucleosomes on different DNA sequences have been previously studied in competition assays, revealing that different sequences can have up to ∼4 kcal/mol differences in equilibrium stability.[4,39] Relative to the 5 S RNA positioning element, random DNA has on average only a slightly (∼0.5 kcal/mol) higher free energy, whereas physically selected sequences have been identified with ∼3 kcal/mol lower free energy. This variability may arise from factors such as variations in inherent curvature and/or flexibility of different sequences.[4] During mechanical unraveling, lower force events presumably correspond to lower-affinity sequences and higher force events correspond to higher-affinity sequences, although a quantitative relationship between these equilibrium and non-equilibrium properties remains to be determined. That most events occur at lower force is consistent with λ DNA, which is non-eukaryotic and not naturally incorporated into nucleosomes, behaving largely as a random sequence. That some nucleosomes can unravel at forces as low as ∼5 pN suggests that spontaneous unraveling may sometimes occur, further reconciling the need of cells for chromatin assembly and remodeling enzymes to maintain the integrity of chromatin.

Events that occurred at forces >32 pN also cannot fully be explained by our use of a higher stretching rate compared to the studies of positioned nucleosomes[14] because the expectation value of force is only a logarithmic function of the loading rate.[36] Unraveling events also occurred after multiple cycles of stretching, as mentioned above. Thus, a small fraction of sequences in λ DNA appear to yield more stable nucleosomes than those probed in previous studies. Notably, enzymes such as RNA and DNA polymerases, which must partly unravel nucleosomes, are capable of exerting such high transient forces.[7,8] Based on its high rate of ATP consumption,[21] we suspect ACF is also capable of exerting comparable forces. Although two different sequences from λ DNA were used (see Methods), we did not observe the data from individual complexes to segregate into two distinct groups with differing forces or unraveling lengths, as would be expected if the two DNA sequences were behaving differently. Our interpretation is that both molecules are behaving as essentially random sequences from the point of view of nucleosome unraveling.

The increase in force as NaCl was decreased from 100 mM to 5 mM is consistent with reduced ionic screening, whereas the finding that ΔL is

unchanged is consistent with the nucleosome maintaining its overall structure in this range of salt concentration.[1] Studies of endonuclease accessibility do indicate, however, that the nucleosome is a dynamic structure.[4] Part of the width in our ΔL distribution may therefore be due to nucleosomes being in different structural states at different times. Wider variations in ΔL observed in the re-wrapping events may occur because the canonical nucleosome structure may not always properly reassemble after mechanical disruption, especially as assembly factors such as NAP-1 and ACF were not active.

Potential effect of dilution

The single-molecule experiments reported here and by other groups usually involve a large dilution of complexes. One possible concern, therefore, is the phenomenon of dilution-driven dissociation of nucleosomes.[40–42] While previous studies suggest that a fraction of nucleosomes may dissociate upon dilution, we do not believe this alters our conclusions or biases our measured force distributions. Cotton & Hamkalo reported that dissociation of a fraction of nucleosomes "does not result from special properties of a subset of the nucleosomes",[40] a feature also noted by others.[43] Therefore, dissociation of octamers would simply reduce our overall data collection efficiency. Further, we use relatively low ionic strengths in our experiment. It is well established that high salt can dissociate nucleosomes by shielding the electrostatic attraction between histones and DNA. Indeed, most dilution-driven dissociation experiments in the literature were carried out at [NaCl] > 100 mM. Cotton & Hamkalo reported that dissociation only becomes significant at salt concentrations above 50 mM NaCl and decreases sharply as salt is decreased further. Also, only a fraction of nucleosomes dissociate and even at 150 mM NaCl, this fraction is < 40%. In other dissociation measurements Lilley *et al*. used 150 mM ammonium sulfate and Thåström *et al*. used 200 mM NaCl.[42,44] In contrast, we made measurements spanning a range of lower concentrations (5, 10, 20, and 100 mM NaCl). Further, the literature indicates that the time scale of dilution-driven dissociation is on the order of an hour,[37,42,45,46] whereas we began some of our measurements within minutes after dilution. Being part of an extended array would also contribute to increased nucleosome stability. It has been reported that individual nucleosomes with long DNA (> 155 bp) dissociate more slowly than ones with only 147 bp.[45] The inherent attraction between dissociated histones and the DNA molecule from which they came also creates an effectively increased histone concentration that mitigates the effects of dilution-driven dissociation in extended arrays.

Another issue is whether either dilution and/or mechanical stretching may lead to partial dissociation of the histone octamer, such as dissociation of the H2A/H2B heterodimer, which could possibly provide an alternative explanation for the observation of approximately "half-nucleosome-sized" unraveling events. A recent study comparing the dissociation of radiolabeled histones from mononucleosomes after one hour of dilution and observed unraveling events in both dilute and stabilizing conditions suggest that a 25 nm unraveling length may be a consequence of H2A/H2B dissociation and that a 50 nm event corresponds to unraveling from the full histone octamer.[47] However, histone octamer instability was also considered previously by Brower-Toland *et al*., who found that the 25 nm events are unchanged when histone octamers are crosslinked, implying that these events do correspond to the removal of DNA from whole octamers.[14] Further studies of these events showed measurable differences in unraveling force following cleavage of the tails of H2A/H2B,[29] indicating that these histones were still present despite large dilutions. These findings are consistent with previous studies suggesting that dissociation of core histones from DNA in solutions below 0.75 M NaCl is highly cooperative and without dissociation intermediates.[46] A different explanation for two different unraveling lengths may be the presence or absence of linker histones, as suggested in a recent study in which events of both ~30 nm and ~60 nm were observed when stretching complexes assembled using *Xenopus* extracts containing embryonic linker histone B4.[30]

Conformation of complexes

The lack of a folding transition suggests that our complexes adopt the "beads-on-a-string" or "10 nm fiber" form observed for native chromatin in low salt.[1] For lack of a specific model for chromatin elasticity, we fit our data using the extensible WLC model. A smoothly bending polymer is ascribed a persistence length that characterizes its entropic bending and a stretch modulus to describe enthalpic deformation. Our data fit quite well to this model, with persistence length, stretch modulus, and contour length as fitted parameters. These fits, however, do not necessarily imply that the picture of a smoothly bending polymer accurately portrays the microscopic conformation of the complexes. Regardless of its meaning in the WLC model, a fitted persistence length of 10–20 nm implies that our complexes have a lower extensibility than naked DNA, particularly for fractional extensions < 90%. This property could be due to the force needed to align the bent linker DNA and to peel DNA from the histone octamer. The observed increase in extensibility in 100 mM salt would then be attributable to easier peeling of the DNA due to increased ionic screening. Our finding of a stretch modulus only ~34% smaller than that for naked DNA suggests that, by a fractional extension of 90%, most DNA straightening and peeling has occurred, and sections of linker DNA and unraveled DNA dominate the elasticity.

Conclusions

Our data provide direct evidence that there is significant variability in the non-equilibrium force needed to unravel individual nucleosomes assembled on heterogeneous DNA sequences. From a biological standpoint, this finding suggests that resistance to unraveling could be an important factor in the functioning of DNA-directed processes in the nucleus. Some nucleosomes will be easier to unwrap and reposition than others, and we speculate that this effect could depend on the biological context. For example, in a promoter region of a gene where nucleosomes will be disrupted during binding of transcription factors, nucleosomes may present less resistance. On the other hand, in a region that needs to be transcriptionally repressed, nucleosomes may be more difficult to unravel. The movement of polymerases and helicases is also slowed by the presence of nucleosomes, which need to be partially or wholly unraveled to allow passage of these enzymes.[48,49] The large variation in unraveling resistance forces and occurrence of spontaneous re-wrapping events reported here are expected to have an important influence on these processes.

That approximately half of the DNA in the nucleosome core particle is resistant to mechanical unraveling provides a plausible explanation for how nucleosome stability might be maintained while allowing access of DNA binding proteins. Brower-Toland *et al*.[14] postulated that easy peeling of the outer DNA turn would allow access to this portion, whereas the inner turn is protected by stronger interactions. In contrast, Kulíc & Schiessel[16] argued that no specific features in the nucleosome crystal structure or endonuclease accessibility data suggest a large-enough energy barrier at a specific position. Their calculations suggest that the inner turn is actually bound with lower energy than the outer turn. During protein binding, electrostatic repulsion between the two DNA turns is envisioned to facilitate partial unwrapping of one turn, either inner or outer, while the other turn remains strongly bound to preserve overall stability.

The combination of *in vitro* chromatin assembly methods and optical tweezers manipulation is expected to open a number of future research directions. The dependence of nucleosome unraveling on DNA sequence, histone composition, and solution conditions may be studied. The effects of histone modifications such as acetylation and phosphorylation, which correlate with changes in gene expression, are also of great interest. Very recently, optical tweezers have been used to measure differences in unraveling forces and lengths upon removal or acetylation of histone tails, indicating that the tails influence specific interactions within the nucleosome core.[29] Other future directions include the analysis of factors governing higher-order folding, which may depend on having long, highly periodic nucleosome arrays, and the study of chromosomal proteins such as the linker histone H1. Finally, mechanical manipulation of single chromatin fibers may allow the dynamics of assembly and remodeling enzymes such as ACF to be measured in real time.

Methods

Nucleosome assembly

End-labeled DNA was prepared using *Escherichia coli* DNA polymerase I Klenow fragment to fill in the ends of methyladenine-free λ DNA (NEB) with biotin-dATP and dCTP (Invitrogen). The DNA was then cut by XbaI and purified using a spin column (Promega Wizard DNA clean up kit). A second fill-in was done with digoxygenin-labeled dUTP (Roche), resulting in two fragments of ~8.1 μm. Core histones were purified from *Drosophila* embryos and recombinant NAP-1 and ACF were purified following baculovirus expression in Sf9 cells as described.[18] Nucleosome arrays were assembled exactly as described in the detailed methods paper by Fyodorov *et al.*[18]

Optical tweezers

A diagram of the experimental apparatus, with specific components listed, is shown in Figure 6. A 1064 nm laser beam was attenuated to ~220 mW and focused into the microfluidic chamber. A glass micropipette pulled to a ~1 μm opening held the anti-digoxygenin coated microsphere *via* suction applied using a syringe. The pipette was manipulated relative to the trapped microsphere by a piezoelectric nano-positioning stage. The reported values of molecular "extension" refer to the measured end-to-end distance of the molecule not including the radii of the microspheres. The outgoing trapping beam was collected by a second objective and directed onto a position-sensing detector for force measurements. Force was calibrated by hydrodynamic drag and by overstretching DNA.[28] The trap stiffness was ~0.17 pN/nm.

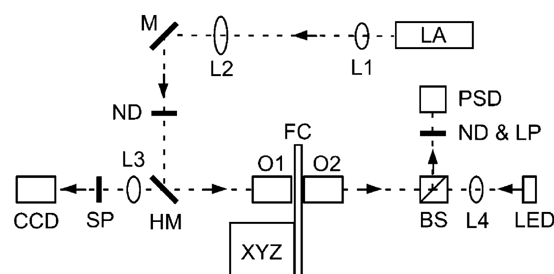

Figure 6. Schematic diagram of the optical tweezers instrument. LA = 1000 mW, 1064 nm Nd:YAG laser (Crystalaser); L1–L4, plano-convex lenses (Thorlabs); M, mirror (Thorlabs); ND, neutral density filter (Edmund Optics); HM, hot mirror (Edmund Optics); O1 and O2, water immersion objectives, 60x, 1.2 NA (Olympus); FC, flow chamber; XYZ, piezoelectric nano-positioning stage (Mad City Labs); BS, beam splitting cube (Newport); LP, long pass filter (Edmund Optics); PSD, position sensing detector (On-Trak); LED, blue light emitting diode (Digi-key); SP, short pass filter (Edmund Optics); CCD, charge coupled device video camera (Watec).

Tethering of complexes

Diluted complexes were incubated with 0.5% 2.2 μm streptavidin microspheres (Spherotech) in 20 mM Tris (pH 7.8), 5 mM NaCl, 1 mM EDTA, and 0.2 mg/ml BSA (to block non-specific binding) for one hour at ∼22 °C. Microspheres were injected into the chamber containing 20 mM Tris (pH 7.8), 1 mM EDTA, and 5–100 mM NaCl, and brought in contact with a 2.8 μm protein G-anti-digoxygenin coated microsphere to form a tether (Figure 1(a)). It should be noted that the nature of the linkages used are such that the DNA is torsionally unconstrained. Measurements were done at ∼22 °C.

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