Unfolding individual nucleosomes by stretching single chromatin fibers with optical tweezers

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Single chromatin fibers were assembled directly in the flow cell of an optical tweezers setup. A single λ phage DNA molecule, suspended between two polystyrene beads, was exposed to a *Xenopus laevis* egg extract, leading to chromatin assembly with concomitant apparent shortening of the DNA molecule. Assembly was force-dependent and could not take place at forces exceeding 10 pN. The assembled single chromatin fiber was subjected to stretching by controlled movement of one of the beads with the force generated in the molecule continuously monitored with the second bead trapped in the optical trap. The force displayed discrete, sudden drops upon fiber stretching, reflecting discrete opening events in fiber structure. These opening events were quantized at increments in fiber length of ~65 nm and are attributed to unwrapping of the DNA from around individual histone octamers. Repeated stretching and relaxing of the fiber in the absence of egg extract showed that the loss of histone octamers was irreversible. The forces measured for individual nucleosome disruptions are in the range of 20–40 pN, comparable to forces reported for RNA- and DNA-polymerases.

In the eukaryotic nucleus, processes that use DNA as a template, such as transcription and replication, occur within the context of chromatin. Access to DNA requires disrupting higher-order chromatin structures[1] and unwrapping of the DNA from the histone octamer. The molecular mechanisms underlying these structural transformations remain elusive. The development of an applied tension-based mechanism to remove histones has attracted considerable attention because the transcribing RNA polymerase creates positive supercoiling in front and negative supercoiling in its wake[2]. Polymerase itself is a molecular motor capable of creating forces[3,4] that may, in conjunction with chromatin remodelling factors[5], be involved in histone removal.

We have used an optical tweezers (OT) setup (Fig. 1*a*) to attach a streptavidin-covered polystyrene bead (2.6 μm) to each end of a biotin end-labeled, single λ phage DNA molecule[6]. One bead is held using suction against a glass micropipette tip, which is integrated in a specially designed flow cell (Fig. 1*b*), while the other bead is held in the force-measuring OT. Such a setup can be used to measure force-extension relationships in polymer chains.

Chromatin fiber assembly

After attaching a single DNA molecule between two beads, the original buffer was replaced with diluted *Xenopus laevis* egg extract[7]. This extract, containing core histones and nonhistone proteins but lacking somatic linker histones, assembles nucleosomal arrays on naked DNA molecules[8]. To prevent trapping of cell debris that are occasionally present in the extract, the laser trap was turned off during assembly. The continuous flow of the extract kept the 'free' bead separated from the bead attached to the glass micropipette (Fig. 2*a–c*). Shortly after the introduction

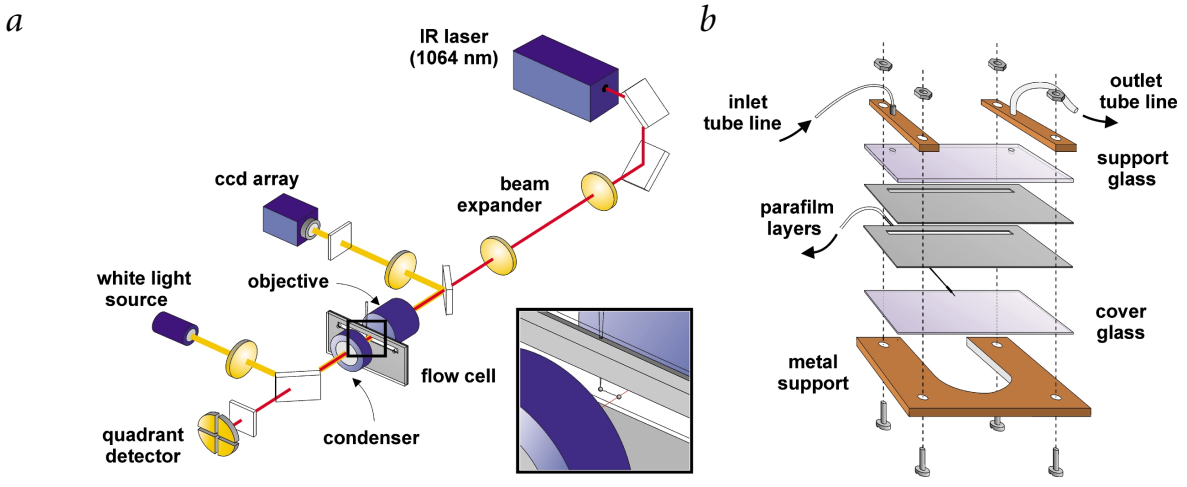

Fig. 1 Experimental setup and the specially designed flow cell. ***a***, Home-built microscope system in which optical tweezers (OT) are introduced. The microscope objective focuses an expanded laser beam to create the OT. A quadrant detector measures the deflection of the laser beam by the trapped polystyrene bead. For small displacements of the bead, the deflection of the beam is proportional to the displacement of the bead from the center of the trap and, therefore, proportional to the force exerted on a DNA molecule attached to it. ***b***, Flow cell design. Two parafilm layers (100 μm thick each) are sandwiched between a microscope support glass and a microscope cover slip (170 μm thick). A 5×50 mm^2 flow channel cut within the parafilm connects through small holes in the support glass to the inlet and outlet tubing. A glass micropipette is inserted with its 1 μm diameter tip in the center of the channel. This pipette holds a polystyrene bead as it is attached to a single DNA molecule, while the other bead is held with the OT (inset Fig. 1*a*). The sandwiched structure is mounted onto a stage that enables three-dimensional motion with respect to the trapped bead.

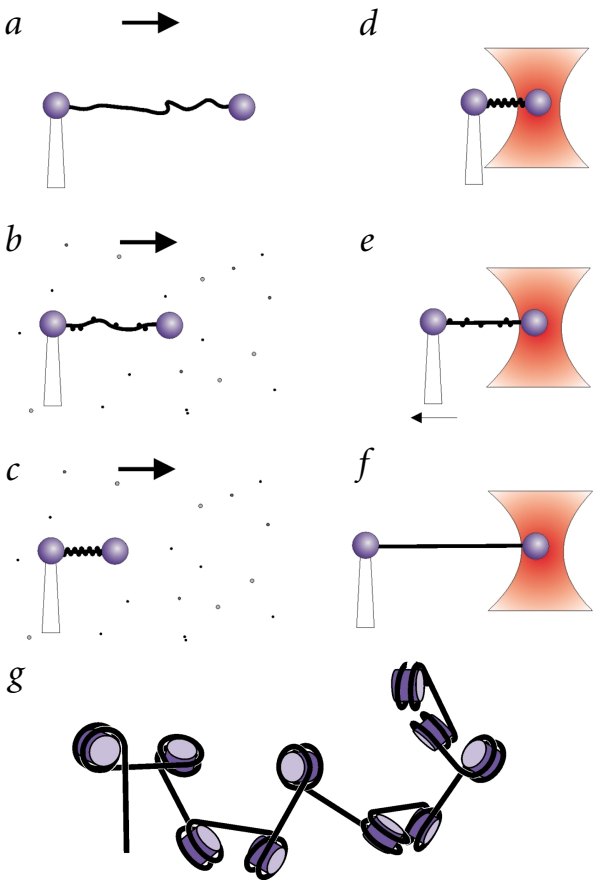

Fig. 2 Schematic representing chromatin assembly on a single λ DNA molecule (*a*–*c*) and stretching of the assembled chromatin fiber (*d*–*f*). ***a***, A single λ DNA molecule suspended between two beads. The arrow indicates the direction of continuous buffer flow. The first bead is held by a glass micropipette using suction. The second bead is maintained downstream from the first one by the drag force. ***b***, *X. laevis* egg extract containing all core histones and numerous nonhistone proteins, but lacking somatic linker histones, is introduced into the flow cell. Histone proteins bind to the single λ DNA molecule, causing its apparent shortening. ***c***, Shortening of the single λ DNA molecule continues and eventually stops. ***d***, To stretch the chromatin fiber, Tris-EDTA buffer replaces the extract, and the second bead is captured using force-measuring OT. ***e***, While continuously monitoring the force, the micropipette is moved away (see arrow) at 1 μm s^{-1} to stretch the chromatin fiber between the two beads, until ***f***, 1.4 times the B-DNA contour length is reached. ***g***, Model of a chromatin fiber with 51–73 bp of linker DNA[30,31]. Each cylinder represents one histone octamer protein core consisting of a core histone H3/H4 tetramer flanked by two core histone H2A/H2B dimers. The DNA is wrapped around each octamer in a left-handed, superhelical fashion.

of the *X. laevis* egg extract, a fast reduction of the distance between the two beads occurred. This apparent shortening of the DNA from 16.4 μm, the contour length of λ DNA, to ~2 μm resulted from the formation of nucleosomes along the DNA molecule.

By controlling the speed of the introduction of the extract into the flow cell, we studied the rate of chromatin assembly at various tensions within the DNA molecule. When the extract was introduced at a relatively high speed with the tension exceeding 10 pN, no apparent shortening was observed, indicating that this tension precluded nucleosome formation. When the tension was reduced to ~5 pN, a slow shortening of the DNA molecule to ~1 nm s^{-1} was observed. Further reduction of the exerted force to ~1 pN resulted in a 160-fold increase in the assembly rate, corresponding to the formation of 2–3 nucleosomes per second. Since nucleosomes will form every 200 bp on average along the DNA molecule with this extract[8], a total number of ~240 nucleosomes is expected. A length reduction from 16.4 μm to ~2 μm yields a shortening of ~60 nm for the formation of a single nucleosome on the λ DNA molecule. Such shortening could correspond to the formation of nucleosomal particles containing two full turns of DNA around each histone octamer (see below).

Force extension curves

Following chromatin assembly, the extract was replaced with Tris-EDTA buffer, and the free bead (Fig. 2*d*) was captured using the optical trap. Care was taken to ensure that forces exceeding 5 pN were not exerted on the chromatin fiber. At this point the micropipette was moved away from the trapped bead at a rate of 1 μm s^{-1}, and the force generated in the chromatin fiber was continuously monitored as its length increased (Fig. 2*e*,*f*).

A typical force extension curve for a reconstituted chromatin fiber is plotted in Fig. 3*b*. For comparison, a force extension curve of the same λ DNA molecule measured before adding the extract is shown (Fig. 3*a*). This curve is typical for a single double-stranded λ DNA molecule[9,10]. The force within the chromatin fiber (Fig. 3*b*) started to increase at extensions of 2–3 μm. When the applied tension reached ~20 pN, a sudden drop in force was observed. This relaxation is indicative of the opening of certain domains within the chromatin structure accompanied by breaking of bonds within the complex. Upon further extension, the force arose to between 20 and 30 pN followed by abrupt drops, again indicating opening events. These structural reorganizations continued until the length of the chromatin fiber approached the contour length of the DNA molecule. From this point onwards, the structure appeared to behave like a DNA molecule without any histones — that is, the B-S transition (overstretching at ~65 pN) of the DNA molecule[9,10] was clearly visible (Fig. 3*b* compared with Fig. 3*a*). The stretching stopped when the length reached ~1.4× the contour length of the B-DNA molecule. Next, the molecule was relaxed at the same speed. The relaxation exhibited a naked DNA-like behavior (Fig. 3*b*).

Discrete drops in the signal can be clearly discriminated upon closer analysis of the force signal during stretching (Fig. 3*c*). Each abrupt drop in force is accompanied by a certain increase in the fiber contour length — that is, the length of the fiber as measured along the axis. Portions of the curve immediately preceding the abrupt drops in force can be described using a worm-like chain model in which entropic as well as intrinsic elasticity is included[11–14]. However, an accurate fit with this model is impossible because only data between 20 and 40 pN are available. Within this force range the entropic contribution to the elasticity is almost negligible, and the force extension relation reduces to $F = (S / L_0)x - S$, where L_0 is the contour length, S the stretch modulus, x the length and F the force on the biopolymer. This expression was used to fit the apparent linear portion of the curve before each drop (the dashed lines in Fig. 3*c*) to obtain the stretch modulus and the contour length of the fiber intermediate occurring at this point of stretching. Using the stretch moduli of several discernible fiber intermediates along the stretch curve and assuming that this parameter does not change significantly upon opening of a single unit, we obtained the contour length of the fiber intermediates for each data point in the force extension curve (Fig. 4*a*).

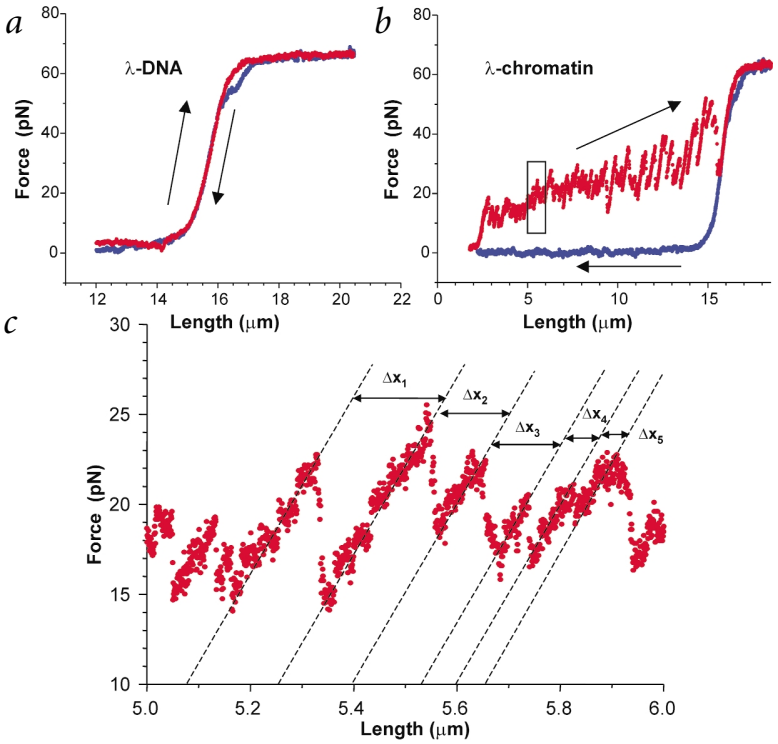

Fig. 3 Stretching chromatin fibers reveals discrete opening events not present during stretching of naked DNA. ***a***, Force extension curve of a single λ DNA molecule in Tris-EDTA buffer. ***b***, Force extension curve of a chromatin fiber assembled on a single λ DNA molecule using the *X. laevis* extract. In experiments with the addition of 0.05% BSA to the buffer solution, the force extension curves were similar. ***c***, More detailed view of the force signal in (*b*). Discrete relaxation events in the fiber can be clearly distinguished. The length increments are indicated as Δx_n.

The opening events are quantized

Having calculated the contour length of the stretched-fiber intermediates, we plotted some typical examples of the changes in contour length as a function of stretching time (Fig. 4*b*). The opening events, seen here as the vertical distances between the horizontal parts of the curves, are quantized with fiber lengthening at each event of ~65 nm or multiples thereof.

Further confirmation of the quantized nature of the opening events can be found by determining a pairwise distance distribution function (PDF)[15,16] of the contour length *versus* time curve (Fig. 4*c*, contour length *versus* time curves in Fig. 4*b*). PDFs were computed by binning contour length differences $(x_j - x_i)$ for all data points $(j > i)$ in a histogram with a bin size of 2.5 nm. Peaks in the frequency distribution histogram are observed at ~65 nm, ~130 nm and ~195 nm, indicating the quantized behavior of the individual opening events. We believe that each discrete, ~65 nm step in the stretching curve represents the unwrapping of the DNA from around a single histone octamer. The quantized steps of ~130 nm, ~195 nm and higher values, represent the simultaneous unraveling of two, three or more nucleosomes. The quantized nature of the disruptions suggests that nucleosome unraveling events are 'all-or-none' under the stretching conditions that we employed.

Successive stretch-relax cycles

To further understand the nature of the opening events, we performed successive stretch-relax cycles on the same fiber (Fig. 5). The experiments were performed slightly differently each time, taking the initial stretch cycle to a different level of stretching — that is, the initial pull was taken to either the final fully stretched stage or, alternatively, was stopped before that stage was reached. We have chosen to present the data from a partial first-cycle stretch that reveals the nature of the opening events (Fig. 5). During the first stretch, taken to an extension of ~11 μm, the major part of the nucleosomal structure unravelled, but some nucleosomes still remained on the DNA. In this second curve, there was no significant increase in force up to 11 μm; from that point on, the remaining nucleosomes were removed. The first and the second stretch cycles add up to a stretch curve taken to the full contour length of DNA (for example, Fig. 3*b*). The third, fourth and fifth stretch cycles show no evidence for any remaining nucleosomal structure, resembling the stretch curve of a naked DNA molecule. This behavior is probably not from DNA that was totally void of any protein because the extract used in these experiments contains a large amount of nonhistone proteins that might bind the DNA molecule without causing any shortening or changing of its elastic behavior. Control chromatin fibers assembled in solution from λ DNA and extract, further purified by sucrose density gradient centrifugation, revealed that many of the nonhistone proteins actually bound to the assembled chromatin fiber (results not shown). Importantly, when the successive pulls were performed in the presence of the extract and when the DNA molecule was given enough time (following the first pull) to reassemble nucleosomes, the subsequent pulls produced curves similar to the original one (data not shown).

The opening events reflect nucleosome unraveling

In preliminary experiments, we measured fiber extension as the concentration of salt in the buffer was increased from 0.15 M to 2 M NaCl with a constant flow force kept at ~5 pN. Under these conditions, we saw a gradual increase in fiber length starting at ~0.8 M NaCl and an abrupt lengthening to a limit length of ~16 μm at ~1.0 M NaCl. These salt concentrations are known to dissociate the core histone H2A/H2B dimers and the core histone H3/H4 tetramers, respectively, from the chromatin fiber[17]. These results suggest that the removal of the histone core proteins, and not other chromatin-bound proteins, causes the steps in our force extension curves.

As recently discussed in detail[18], the nucleosome-chromatin fibers are dynamic structures, with the length of the DNA accommodated by the histone octamer varying anywhere between 100 and 170 base pairs (bp). The partial unwrapping (breathing) of the ends of the DNA from around the protein core, although still preserving the integrity of the particle as a whole, is probably necessary for nucleosomal processes, like transcription, to take place. The relative occupancy of the nucleosomal DNA-length space strongly depends on the environmental conditions; in particular, elevated salt concentrations, as in these experiments, stabilize particles with longer DNA wrapped around the histone octamer.

The presence of proteins that bind at or close to the entry/exit point of the DNA into and out of the nucleosomal particle affect the length of the DNA constrained in the nucleosome. Of the two major protein families, the linker histones and HMG1/2, binding

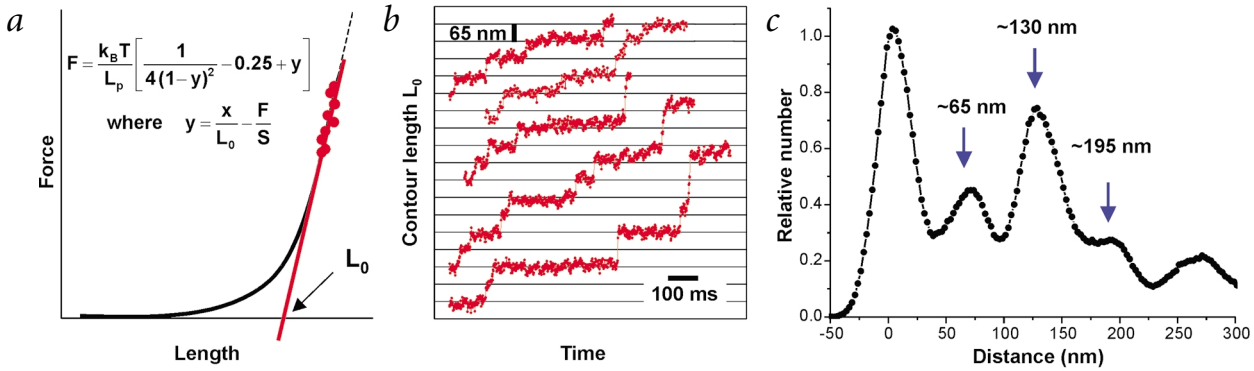

Fig. 4 Disruption events are quantized. ***a***, Schematic presenting how a contour length is calculated for each data point. Each intermediate can be described using a series of two modified worm-like chain functions (expression in figure, see refs 11–14), one describing the free DNA (L_P = 53 nm, S = 1,100 pN) and the other one describing the complete chromatin (L_P = 30 nm, S = 150 pN). In this expression, k_B is the Boltzmann constant; T, the absolute temperature; x, the relative extension of the DNA molecule; F, the exerted force; L_p, the persistence length; and S, the stretch modulus. However, because only data points from 20 to 40 pN were available, an accurate fit using this expression was impossible. Therefore, a linear fit was used in order to determine the contour length of the different intermediates. The slopes of the different portions preceding the abrupt drops in force were determined. Using these slopes and assuming that they change negligibly upon opening of a single nucleosome, a contour length was evaluated for each data point in the force extension curve. Using this procedure, a contour length of the chromatin fiber *versus* time curve was constructed from the force extension curve. ***b***, Short traces of the contour length of the chromatin fiber-stretching intermediates *versus* time. These curves show the discrete and quantized nature of the opening events during the stretch cycle. ***c***, Pairwise distance distribution function (PDF) of the data shown in (*b*). Clearly visible are the peaks at ~65, ~130 and ~195 nm. The histogram illustrates that the observed events are quantized with a unitary step-size of ~65 nm.

at these positions, the somatic linker histones are not present in our extract[19]. The other major proteins capable of locking DNA into two complete turns are the high mobility group (HMG) proteins 1 and 2 (ref. 20) and the embryonic linker histone B4 (ref. 21). We looked for the presence of these proteins in the chromatin assembly extract and found them in abundance (data not shown), in agreement with published data[22]. The binding of such proteins locks the DNA around the histones in a two-turn wrap; however, the trajectory of the nucleosomal DNA ends in such particles differ from those in two-turn particles lacking these proteins[18,23].

The majority of the nucleosomal particles assembled in the *X. laevis* egg extract seem to have about two full turns of DNA. If this is the case, the unraveling of a single nucleosome will lead to an increment in fiber length of ~170 bp (or ~60 nm). These numbers agree with the observed length increments in the stretching curves.

The low force stretching regime

When chromatin was manipulated with forces not exceeding 20 pN (data not shown), the force extension curve showed no significant hysteresis, indicating that chromatin was not unfolding during this stretching regime (see beginning of the stretching curve of Fig. 3*b*; also ref. 24). The elastic properties of the chromatin fiber can be derived from fitting this part of the curve to a worm-like chain model[11–14]. The stretch modulus of chromatin is estimated to be ~150 pN, which is eight-fold lower than that of naked DNA[9].

Discussion

Our measurements show that unwrapping of the DNA from around each histone octamer requires forces between 20 and 40 pN. Results from pulling single chicken erythrocyte chromatin fibers of variable length show irreversible structural transitions at forces of ≥20 pN (ref. 24), in general agreement with the data presented here. That work, using similar methods, observed fiber lengthening with each pull and did not report steps as describe here. These differences could derive from the preparation of the chromatin fibers (these authors used chromatin fibers isolated from cells, and thus contained linker histones) and/or data acquisition rates. Published theoretical calculations predict nucleosome disruption forces to be ~2 pN (ref. 25). One serious limitation in comparing experiment and theory is that the latter applies only under conditions of thermodynamic equilibrium, as stated by the authors themselves. The conditions of our pulling experiments do not meet such criteria because we apply the pulling force at loading rates of ~38 pN s^{-1}.

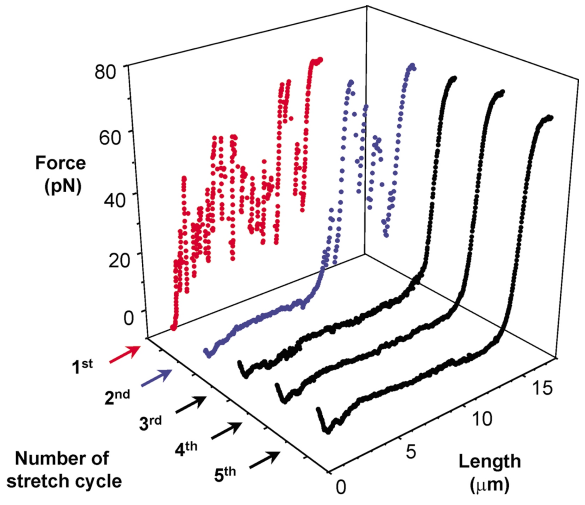

Fig. 5 The unraveling of nucleosomes during stretching is irreversible, as evidenced by performing successive stretch-relax cycles on the same chromatin fiber. In the specific example shown, the first stretch cycle (red curve) was taken to an extension of ~11 μm, at which point the structure was relaxed. All successive stretches were to the fully stretched contour length of the DNA molecule. The second stretch (blue curve) further removed the nucleosomes remaining after the first partial stretch. All other successive stretches (black curves) showed behavior typical of naked DNA, indicating complete removal of nucleosomal structure during the first two stretches.

As recently demonstrated, the rupture forces monitored in pulling experiments are strongly dependent on the pulling rate[26–28]. Once lower loading rates can be applied, the rupture force will probably drop from the observed 20–40 pN. This will have to be confirmed in further experiments. Importantly, the forces needed to break nucleosomes apart are in the range of the forces actually measured for RNA and DNA polymerases[3,4,29]. In OT experiments, *E. coli* RNA polymerase has been demonstrated as the strongest molecular motor described thus far, capable of producing forces of up to 35 pN (ref. 4); a similar force was reported for T7 DNA polymerase[29]. The polymerases may also be assisted by chromatin remodeling factors (for example, SWI/SNF, NURF and CHRAC[5]), which may loosen up the sturdy chromatin structure for easier displacement of histones from the DNA.

Methods

Optical tweezers. Optical tweezers (0.1 pN nm^{-1} trap stiffness) are created using a 1,064 nm laser beam (500 mW, CW, Millennia IR, Spectra Physics) and a water immersion objective lens (100×, 1.2 numerical aperture, Leica).

Extract. Extract[8] from *X. laevis* eggs (12 μl of high speed supernatant) was diluted in 1 ml of assembly buffer (50 mM HEPES-KOH, pH 7.6, 50 mM KCl, 1 mM EDTA and 2 mM β-mercaptoethanol).

Chromatin fiber assembly and stretching. In the chromatin assembly experiments, the tension within the DNA molecule, equal to the drag force exerted on the freely suspended bead in the flow, was calculated using Stokes law: $F = 6\pi\eta rv$, in which η is the viscosity of the buffer solution ($\eta = 1 \times 10^{-3}$ Pa s^{-1}); r, the radius of the bead (r = 1.3 μm); and v, the velocity of the flow. Velocity was determined using videomicroscopy of particles — that is, cell debris — visible in the liquid cell.

Tris-EDTA buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl and 0.01% (w/v) NaN_3) was introduced into the flow cell at a flow rate such that the force exerted on the chromatin fiber suspended between the beads did not exceed 5 pN. While capturing the freely suspended bead, the intensity of the laser establishing the optical trap was slowly increased from zero to its final value.

The deflection signal, which determined the force on the chromatin fiber during manipulation, was sampled at 1.2 kHz. Real-time video analysis of the precise positions of the two beads was used to determine the fiber extension. The rate of extension sampling was limited to a maximum 25 Hz. Because the micropipette bead was moved away at a slow and constant speed, an interpolation algorithm could be used successfully to determine the position of this bead at 1.2 kHz.

Fit of a linear expression to data points to determine the contour length between 20 and 40 pN gave an underestimation of only 4% for the naked DNA and 5% for the chromatin fiber. Therefore, the relative error for each of the length increments as we stretched the chromatin fiber to a DNA molecule will be negligible.

For the salt dissociation experiments, a small mixing chamber was added in front of the flow cell to create a continuous flow in which the [NaCl] changed slowly. Two copper electrodes were inserted into the flow cell to measure the electrical resistance of the buffer. This resistance was calibrated with solutions of known NaCl concentrations and used to monitor the salt concentration within the flow channel in real time.

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