Forcing Chromatin*

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Recent years have witnessed the emergence of single-molecule methods, a powerful set of tools that allow the study of individual biological macromolecules or complexes thereof, one at a time, often in real time. What follows is a brief overview of the single-molecule approaches already applied to investigating chromatin fiber structure and dynamics: the atomic force microscope, optical tweezers, and magnetic tweezers. The first results are highly encouraging, and although the field is still in its infancy, its potential to provide insights unachievable so far cannot be overstated.

The Chromatin Fiber and DNA Accessibility

The DNA in the eukaryotic cell nucleus is organized as chromatin, a nucleoprotein structure in which small basic proteins, histones, form globular cores around which between 102 and 168 bp of DNA form left-handed superhelical turns (1–4). These particles, termed nucleosomes, are spaced along the DNA at certain distances, with the length of interconnecting, linker DNA varying according to cell or tissue type. These nucleosomal arrays are visualized, when deposited from low ionic strength buffers, as beads-on-a-string structures in electron (1) and AFM5 micrographs (5, for some recent examples see Refs 6–8). Increasing the ionic strength leads to compaction of the nucleosomal arrays until they reach dimensions compatible with the dimensions of the cell nucleus (typically, ~1 meter of DNA is packed into the confines of a nucleus only several micrometers in diameter). These further levels of compaction involve additional proteins, both linker histones (so termed because of their binding to the linker DNA, outside of the nucleosome particle) and non-histone proteins. Understanding chromatin structure and dynamics is of paramount importance to understanding processes requiring access to the DNA template, such as transcription, replication, recombination, and repair.

For the DNA to be accessible to the enzymatic machineries involved in all these processes, the compacted chromatin fiber has to undergo unraveling (9), followed by temporary removal of histones from the DNA in the immediate chromatin region involved. Regulation of gene activity at the level of transcription initiation is believed to also involve some kind of dynamic alterations to the structure (chromatin remodeling, see Ref. 10) so as to allow binding of sequence-specific transcription factors to their recognition sequences.

The emergence of single-molecule methods (11, 12) has provided a powerful set of tools to approach chromatin structure and dynamics in an unprecedented way, allowing real-time observations on the behavior of individual chromatin fibers and assessing the variability among individual representatives of a fiber population. The majority of the single-molecule chromatin work has been done using AFM, both for visualization and micromanipulation, but recently several other single-molecule techniques have proved useful: optical tweezers, magnetic tweezers, and flow fields. These methods allow investigation of the mechanical properties of chromatin DNA in real time in response to application of force, thus closely mimicking the cellular environment in which macromolecules constantly experience forces of different magnitudes and origins. The molecular machineries involved in DNA transactions push and pull on the DNA, denature the double helix, introduce bends and kinks, in other words apply forces to the double helix in the chromatin context. What is the behavior of the chromatin fiber in such circumstances? Here we will discuss the first meaningful results obtained upon mechanically manipulating single chromatin fibers, comparing the different techniques in terms of their capabilities and limitations.

Chromatin Assembly Under Applied Force

Chromatin assembly in vivo takes place massively during DNA replication; in addition, nucleosomes have to assemble in the wake of the transcriptional machinery because the transcribing RNA polymerase removes nucleosomes in its way (by itself or with the help of other factors). The naked DNA stretches behind the polymerase have to quickly reform chromatin so that the roles chromatin plays in compacting the DNA and regulating its functions are restored. It is essential to realize that this reformation of nucleosomes in the wake of RNA polymerase (probably in the wake of other DNA-tracking enzymes as well) is a process that takes place while the DNA molecule is still under tension as a result of the pulling exerted by the stationary RNA polymerase (13) on the transcribed DNA. Both RNA and DNA polymerases have been shown to be among the strongest molecular motors, developing forces of up to 30–40 pN (14, 15). If the forces measured in vitro are also developed in vivo, then the question arises whether the DNA under tension can be assembled into nucleosomes and what the force dependence of the assembly process is.

The group of Viovy in France has used videomicroscopy experiments in which a single λ-DNA molecule (48.5 kbp, 16.4 μm contour length) was attached at one end to a glass surface, the other end being free so that it could be stretched by a flow (Fig. 1a) (16). The assembly of chromatin on such single DNA molecules was achieved by flowing in of Xenopus or Drosophila cell-free extracts; the shortening of the molecules as a result of the assembly was observed by real-time fluorescence microscopy (DNA was fluorescently labeled by intercalation of YOYO-1). There was a clear dependence of the rate of chromatin assembly on the shear rate, hence on the force applied to the DNA. Assembly could proceed, albeit at a much reduced rate, up to forces of 12 pN.

Similar findings have been reported by using an optical tweezers/flow set-up to follow chromatin assembly on a single λ-DNA molecule driven by Xenopus egg extract (17). In this study, a single DNA molecule was suspended between two micron-sized beads, one held by a pipette and the other in an optical trap (Fig. 1b). Because the presence of cell debris in the extract precluded the use of the optical trap for force measurements, the optical trap was switched off during the assembly, and the instrument was used in the laminar flow mode. Forces were estimated using either the parameters of the laminar flow (Stokes’ law) or by measuring the Brownian motion of the freely suspended bead. The addition of the extract led to visible shortening of the distance between the two beads, reflecting chromatin formation. The kinetics was strongly dependent on the applied force, with complete inhibition of assembly at forces exceeding 10 pN.

A more detailed study of single chromatin fiber assembly has been carried out using magnetic tweezers (18), the kind of instrument pioneered in the laboratories of David Benchimol and Vincent Croquette in Paris (19). In these experiments, the λ-DNA molecule is suspended between a glass surface and a micron-sized magnetic bead, and assembly is driven by the addition of a solution of purified histone octamers and nucleosome assembly factor (Fig. 1c). The force on the bead can be manipulated by changing the distance between the bead and an external magnet, and the assembly can be

1 The abbreviation used is: AFM, atomic force microscope.

2 This minireview will be reprinted in the 2003 Minireview Compendium, which will be available in January, 2004.

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followed by real-time recording of the movement of the bead on a videoscreen.

An example assembly curve is presented in Fig. 2a. In this experiment assembly was performed at 1.3 pN applied to the bead, i.e., to the DNA tether. The initial fast assembly gradually slowed down, as expected from the diminishing length of free DNA available for nucleosome assembly. The overall dependence of the rate of assembly on the force agrees with earlier results (16, 17). In addition, the instrumental set-up allowed controlled adjustment of the force during a single round of assembly by changing the distance between the external magnet and the bead. The rheostat control of the force clearly revealed (i) that higher forces did slow the assembly down (Fig. 2b), and (ii) that the response of the system to the applied tension was instantaneous. As soon as the applied force was changed, there was a corresponding change in the assembly rate. Finally, the data gave a clear indication of the reversibility of the assembly process, as illustrated in the blow-up of a portion of the assembly curve (Fig. 2a). Note that the general trend of DNA shortening resulting from nucleosome formation is interrupted by occasional lengthening of the DNA tether, presumably reflecting nucleosome disassembly. The dynamic equilibrium between an assembled and a disassembled state could be easily shifted toward assembly at low forces and disassembly at high forces (for further details and discussion, see Ref. 18).

**Disassembly of Chromatin Fibers under Force**

The application of mechanical stretching to chromatin DNA is expected to lead to chromatin disassembly, i.e., dissociation of histone octamers from the DNA. The force required for this dissociation reflects nucleosomal strength and depends on the number, kinds, and strength of interactions that need to be broken during dissociation. Chromatin fiber disassembly under applied force has been studied successfully so far only with optical tweezers.

Earlier attempts to use the AFM for mechanically stretching chromatin fibers have run into a rather unexpected artifact. Long native chromatin fibers isolated from chicken erythrocyte chromatin fibers (19) were deposited on mica or glass surfaces and pulled with the AFM tip (20, 21). In such stretching experiments the scanning of the sample in the x and y direction needed for imaging was disabled, and the cantilever-mounted tip was allowed to move only in the z direction, i.e., upwards and downwards, away and toward the surface.

When the AFM tip is pushed into the sample, it may attach to a fiber by nonspecific adsorption; upon retraction, it stretches the fiber and force-extension curves are recorded (Fig. 3, a and b).
These curves exhibited a sawtooth pattern, similar to the patterns obtained upon stretching of multidomain proteins like titin (22) or tenascin (23) (Fig. 3c). Each of the peaks in these patterns arises because the initial entropic stretching of the polymer chain, accompanied by a gradual build-up of tension in the molecule, is followed by enthalpic changes in the internal organization (unraveling) of a certain individually folded domain. As a result of the unraveling of a domain the chain elongates in a jump, which in turn leads to an abrupt fall in the tension of the molecule (Fig. 3d). Successive unraveling events of individual domains in a multidomain polypeptide chain lead to the appearance of multiple peaks in the force-extension curve, hence the sawtooth pattern.

The force-extension curves of both chromatin fibers extracted from cells and fibers reconstituted in vitro exhibited the multipeak appearance (Fig. 3b), but a closer look at the distances between individual peaks made it clear that the sought after unraveling of individual nucleosomes as a result of mechanical stretching did not occur, despite the relatively high forces applied, in the range of 300–600 pN. Control experiments with glutaraldehyde-fixed fibers (in which unraveling is precluded because of the fixation) and analysis of AFM images of the fibers being stretched suggested that the jumps in the force curves corresponded to removal of successive intact nucleosomes from the glass surface, followed by stretching of the naked DNA between the nucleosomes attached to the tip and the surface. Earlier work on stretching pieces of naked double-helical DNA with optical tweezers (24), optical fibers (25), or AFM (26, 27) has demonstrated a structural transition from the B-form DNA to the so-called S (stretched)-form, in which DNA was extended to ~2-fold over its original contour length. The force causing the B-to-S transition was measured to be ~70 pN in both the optical tweezers and optical fibers experiments and ~120 pN in the AFM work. Despite the apparent inconsistency in these numbers and the lack of agreement about the nature of the structural changes occurring during the "overstretching transition" (for further discussion, see Refs. 28–30), it is clear that the forces applied in the chromatin stretching experiments (20, 21) were by far exceeding those needed for the B-to-S transition; thus, the scenario explaining the observed sawtooth pattern by a succession of nu-

**Fig. 4.** a, schematic of the optical tweezers experiments of Bennink et al. (31). Chromatin assembly is shown on a single λ-DNA molecule (left-hand panels), followed by stretching of the assembled fiber (right-hand panels). b, view of an enlarged portion of the force-extension curve, with discrete relaxation events clearly distinguishable. The length increments between two opening events are indicated as $\Delta l$. The values for $\Delta l$ are quantized at 65 nm (for further analysis of the original data, see Ref. 18), reflecting unraveling of one or more nucleosomes at a time.

**Fig. 5.** a, schematic of the experimental approach of Brower-Toland et al. (32). Preassembled, fully saturated nucleosomal arrays containing 17 positioned nucleosomes were mechanically stretched by moving the coverslip at a constant velocity relative to the bead; the position of the bead was kept constant by modulating the light intensity of the trapping laser (velocity clamp mode of operation). b, a representative force curve: the low force range and high force range are designated, with discrete opening events seen only in the high force range (redrawn or modified from Ref. 32).
cleosomes popping off from the surface followed by stretching of linker DNA seems highly likely.

Three papers have reported results on stretching individual chromatin fibers with optical tweezers, consistently measuring nucleosomal strength in the range of 20–40 pN. Cui and Bustamante (31) stretched isolated chicken erythrocyte fibers, Bennink et al. (32) pulled on fibers directly reconstituted in the flow cell from λ-DNA and purified histones with the help of Xenopus extracts (Fig. 4a), and Brower-Toland et al. (33) used preassembled nucleosomal arrays containing 17 nucleosomes as their stretching substrate (Fig. 5a). Up to 20 pN, the fibers underwent reversible stretching, but applying stretching forces above 20 pN led to irreversible alterations, interpreted in terms of removal of histone octamers from the fibers with recovery of the mechanical properties of naked DNA.

The high speed of data acquisition in the Bennink et al. (32) experiments allowed recording of force curves in which discrete, sudden drops in force could be observed upon fiber stretching, reflecting discrete opening events in fiber structure (Fig. 4b). These opening events were quantized at increments in fiber lengths of ~65 nm and were attributed to unwrapping of individual nucleosomal particles. The high resolution data allowed the first direct measurements of the forces needed to unspool individual nucleosomes: these forces ranged between 20 and 40 pN. As Fig. 5b shows, the forces to unravel the 17 individual nucleosomes in the preassembled nucleosomal array (33) were exactly in the same range.

One important difference in the results reported by Bennink et al. (32) and Brower-Toland et al. (33) lies in the way the nucleosome yielded to the applied tension. The step size with which the chromatin fiber elongated at each opening event was ~65 nm in Ref. 32; this was interpreted to mean that the entire nucleosomal DNA unwrapped from around the histone octamer in a single dissociation event. Contrary to that, the step size in the Brower-Toland et al. (33) work was only about ~25 nm. A model was proposed in which the dissociation process occurred in steps, determined by points of stronger contact between the histone octamer and the DNA that served as roadblocks to unraveling. It was suggested that the application of low forces led initially to unwrapping of the ends of the nucleosomal DNA from the octamer until positions +4 and −4 of the DNA superhelicity were reached and that the application of higher forces (Fig. 5b) caused disruption of the +4 and −4 DNA/protein contacts, which led to unraveling of the remaining DNA. A jump. The peaks in the force curves corresponded only to this second step in the unraveling of individual particles, whereas the first step had no recognizable signature related to nucleosomes. The fiber lengthened in a continuous manner in the low force regime of stretching. The way nucleosomes unraveled in the two types of experiments can be, at least in part, explained by the much different rates of load application (for further discussion on this and some related points, see Refs. 29 and 34).

It may be important to mention here that the mechanical forces needed to unravel individual nucleosomes are in the same range as the stall forces developed by RNA and DNA polymerases (14, 15), the enzymes that encounter nucleosomes while reading the information in the DNA. This may mean that the polymerases are by themselves capable of removing the nucleosomes in their way.

Conclusions

This brief overview of the single-molecule approaches to understanding chromatin fiber structure and dynamics convincingly shows the potential these methods have for providing insights unachievable until now. The application of these methods to chromatin studies is still in its infancy but would undoubtedly constitute the research focus of more and more laboratories in the future.

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