of the RSC complex from the unstained particle images entails combining the information present in this set of projections. This requires precise knowledge of their relative orientations. Evidently, approximate information is provided by comparison with the available stain reconstruction. However, preliminary analysis indicates that the deformation induced by preservation in stain is significant, and this prevents direct use of the stain reconstruction as a meaningful reference for calculation of an improved reconstruction from the unstained particle images. We are currently working to obtain a suitable reference volume, which will allow us to make full use of the information contained in images of unstained RSC particles.

[5] Use of Optical Trapping Techniques to Study Single-Nucleosome Dynamics

By BRENT BROWER-TOLAND and MICHELLE D. WANG

Introduction

Over the past decade, optical trapping techniques have become a standard part of the repertoire of tools available for the study of biological molecules.[1–5] More recently, optical trapping techniques have been applied to the study of chromatin structure and even details of the structure of individual nucleosomes in a chromatin array.[6–8]

The general experimental design of optical trapping experiments with chromatin involves immobilization of one end of a linear DNA molecule on a surface, while the other end of the molecule is attached to a polystyrene microsphere (bead). The microsphere can then be used as a microscopic ''handle'' which can be captured and manipulated by the optical trap (Fig. 1). The optical trap can be used to exert and measure piconewton-scale

[1] K. Svoboda, C. F. Schmidt, B. J. Schnapp, and S. M. Block, *Nature* **21,** 365 (1993).
[2] H. Yin, M. D. Wang, K. Svoboda, R. Landick, J. Gelles, and S. M. Block, *Science* **270,** 1653 (1995).
[3] M. D. Wang, M. J. Schnitzer, H. Yin, R. Landick, J. Gelles, and S. M. Block, *Science* **282,** 902 (1998).
[4] J. Liphardt, B. Onoa, S. B. Smith, I. Tinoco, and C. Bustamante, *Science* **292,** 733 (2001).
[5] S. J. Koch, A. Shundrovsky, B. C. Jantzen, and M. D. Wang, *Biophys. J.* **83,** 1098 (2002).
[6] Y. Cui and C. Bustamante, *Proc. Natl. Acad. Sci. USA* **97,** 127 (2000).
[7] M. L. Bennink, S. H. Leuba, G. H. Leno, J. Zlatanova, B. G. de Grooth, and J. Greve, *Nat. Struct. Biol.* **8,** 606 (2001).
[8] B. Brower-Toland, R. C. Yeh, C. Smith, C. L. Peterson, J. T. Lis, and M. D. Wang, *Proc. Natl. Acad. Sci. USA* **99,** 1960 (2002).

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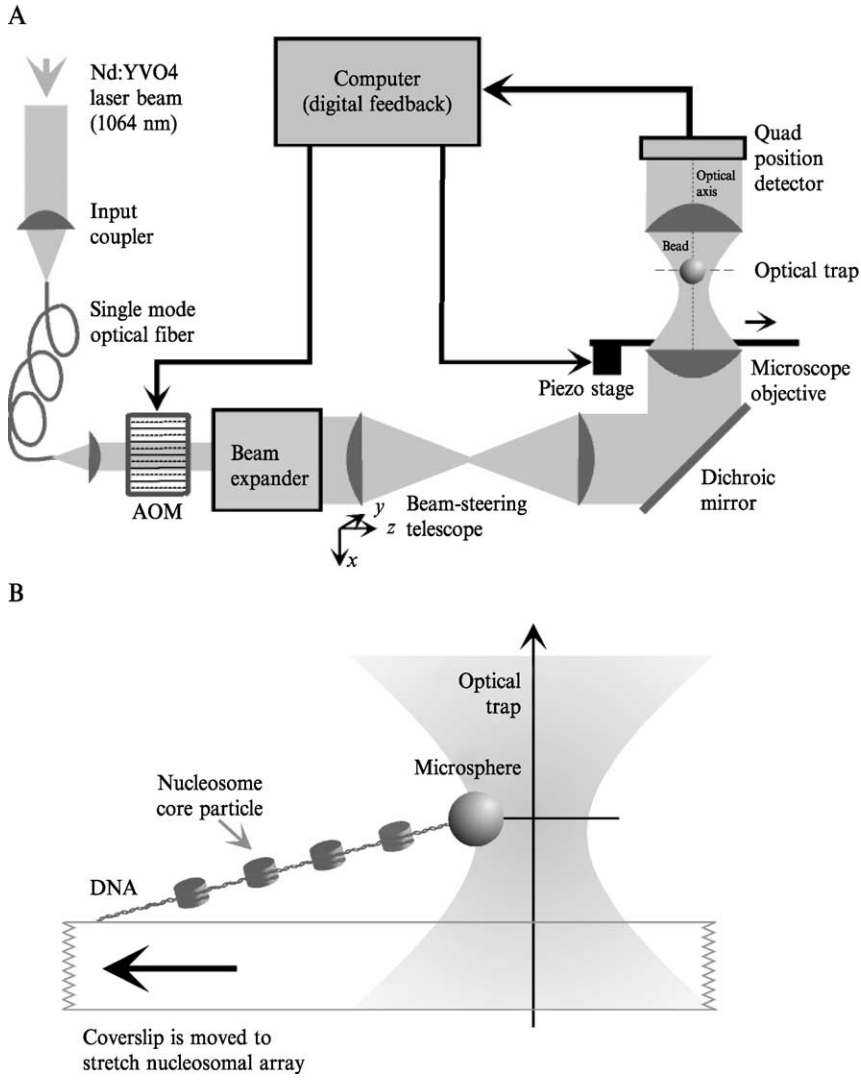

FIG. 1. Instrument and experimental configuration (not to scale). (A) Overall design of the instrument. The drawing is modified from Wang *et al.*[9] (B) Experimental configuration. Under feedback control, a nucleosomal array is stretched between the surface of a microscope coverslip and an optically trapped microsphere.

forces and nanometer-scale displacements on a nucleosomal DNA molecule.

Optical trapping techniques are single-molecule techniques that allow mechanical manipulation of a nucleosomal DNA molecule under physiological solution conditions. Because the sample is immobilized, solution conditions and sample components can actually be varied during the course of experimentation. The experiments can be nondestructive, permitting repeated sampling of the same nucleosomal arrays. An added advantage of the use of an optical trap is the freedom to consider individual nucleosomal structure in the context of a nucleosomal array, rather than on isolated mononucleosomes. These features distinguish optical trapping techniques from others in the repertoire of tools available for the study of chromatin.

In this chapter, we describe the optical trapping system and experimental sample preparation techniques necessary to carry out dynamic structural analysis of individual nucleosomes in nucleosomal arrays. We anticipate that optical trapping experiments will prove valuable in answering questions about chromatin structure that are difficult to access with traditional techniques.

Chromatin Sample Preparation

Preparation of Histones

Owing to the sensitivity of single molecule studies of chromatin, the use of highly purified biochemical components is critical to the success of these experiments. Large quantities of highly purified histone proteins can be prepared from various chromatin samples using standard hydroxyapatite (HAP) chromatography. Starting with washed nuclei from the tissue source of choice, their chromatin content is fragmented by mild MNase digest, and bound to HAP (BioGel HTP, BioRad Laboratories, Hercules, CA) by virtue of their nucleic acid component as previously detailed.[10,11] Linker histone and nonhistone proteins are removed by washing the HAP bed at moderate ionic strengths. Finally, core histone proteins are eluted from the HAP:DNA complex at high ionic strength.

Alternatively, highly purified recombinant histone proteins can be obtained by expression in bacteria, permitting choice and manipulation of primary sequence.[12] This flexibility permits the design of experiments

[9] M. D. Wang, H. Yin, R. Landick, J. Gelles, and S. M. Block, *Biophys. J.* **72,** 1335 (1997).
[10] A. P. Wolffe and K. Ura, *Methods* **12,** 10 (1997).
[11] G. Schnitzler, *in* "Current Protocols in Molecular Biology" (F. A. Ausubel *et al.,* eds.), **3,** p. 21.5.7. Wiley, New York, 2001.
[12] K. Luger, T. J. Rechsteiner, and T. J. Richmond, *Methods Enzymol.* **304,** 3 (1999).

involving minor histone variant proteins, critical structural mutants, and the introduction of cross-linking moieties.[13]

DNA Labeling and Attachment Methods

Selection of a DNA template for chromatin assembly and subsequent single-molecule analysis involves two major criteria: template length and sequence. With the experimental configuration previously described by Brower-Toland *et al.*,[8] a DNA template of around 4000 bp is optimal. A shorter DNA template will lead to greater uncertainty in the determination of the DNA extension and the tension in the DNA.[9] This is especially a concern for a saturated nucleosomal DNA due to its shortened DNA tether. A longer DNA template will introduce more Brownian motions of the trapped microsphere. DNA sequence choice is governed entirely by the goal of the experiment. We have previously utilized a DNA molecule composed of 17 repetitions of the sea urchin 5S nucleosomal positioning element in order to produce nucleosomal arrays containing, as far as possible, identical nucleosomal units.[8] However, nucleosomes assembled by salt dialysis on a nonrepetitive sequence with no bias for nucleosome positioning produce single-molecule data of sufficient quality for analysis.[14] As expected, nucleosomes on such arrays exhibit a broader range of binding energies than those assembled on naturally occurring positioning elements such as the 5S sequence.

Labeling and purification of these DNA molecules have been accomplished by standard enzymatic manipulation and chemical fractionation methods. Nonrepetitive sequences can be asymmetrically end-labeled using the polymerase chain reaction (PCR) with differentially end-labeled primers. Repetitive sequences not amenable to PCR amplification have been labeled by Klenow fill-in reaction with the appropriate labeled NTPs. Experiments performed in our lab have utilized biotin and digoxigenin-labeled nucleic acids successfully. These two labels are especially convenient because of the wide commerical availability of avidin-coated microspheres, and the existence of sufficiently high-affinity anti-digoxigenin antibodies (Roche Applied Science, Indianapolis, IN). Removal of residual unincorporated label is critical to the success of these experiments.

[13] A. Flaus, K. Luger, S. Tan, and T. J. Richmond, *Proc. Natl. Acad. Sci. USA* **93,** 1370 (1996).
[14] B. Brower-Toland and M. D. Wang, unpublished data.

Chromatin Assembly

Assembly of chromatin arrays for analysis from highly purified DNA and histone components can be achieved by chemical (gradient salt dialysis)[15] or by enzymatic means.[16] The salt dialysis method of assembly has the advantage of preserving sample purity and minimizing the amount of post-assembly purification required before experimentation.

Enzymatic assembly of chromatin has the pitfall of introducing a large number of protein impurities if an assembly extract is utilized. The recent development by the Kadonaga lab of a completely recombinant assembly system minimizes this complication.[17] Chromatin assembly by this method is more rapid than by salt dialysis. Moreover, it has the advantage of producing extremely regular arrays of nucleosomes in a sequence-independent fashion, without introducing artifactual structures such as dinucleosomes. With either technique, optimization of assembly conditions by post-assembly electrophoretic analysis is necessary prior to single-molecule experimentation, both to avoid artifactual structures and to ascertain the quality of array formation.

Preparation of Experimental Samples

For single-molecule studies, samples are prepared by sequential infusion of solutions into sample chambers for microscopic observation. Chamber volume is approximately 10 μl. Fluid flow through the chamber is by capillary action produced by placing an absorbent wick at one end of the chamber while delivering solutions by micropipette at the other end. All procedures are carried out at room temperature, and incubations are performed in a humid chamber to avoid evaporation. Samples prepared in this way are exquisitely sensitive to changes in physical and chemical conditions, so that consistency in all aspects of sample preparation, especially temperature, is critical. Once prepared, chromatin samples have a useful experimental lifetime of about 2 h at room temperature.

Buffer solutions:

1. PBS: 140 m*M* NaCl, 2.7 m*M* KCl, 10 m*M* Na_2HPO_4, 1.8 m*M* NaH_2PO_4, pH 7.3.
2. Blocking buffer (BB): PBS + 0.2% purified non-fat milk protein.
3. Chromatin dilution buffer (CDB): 10 m*M* Tris-HCl (pH 8.0), 1 m*M* Na_2EDTA, 150 m*M* NaCl.

[15] K.-M. Lee and G. Narlikar, *in* "Current Protocols in Molecular Biology" (F. A. Ausubel *et al*., eds.), **3,** p. 21.6.3. Wiley, New York, 2001.
[16] M. Bulger and J. T. Kadonaga, *Methods Mol. Genet.* **5,** 241 (1994).
[17] M. E. Levenstein and J. T. Kadonaga, *J. Biol. Chem.* **277,** 8749 (2002).

4. Experimental sample buffer (ESB): 10 m*M* Tris-HCl (pH 8.0), 1 m*M* Na_2EDTA, 100 m*M* NaCl, 1.5 m*M* $MgCl_2$, 0.02% (v/v) Tween-20, and 0.01% (w/v) milk protein.

The blocking agent that has worked best in our hands is a purified milk protein powder (Biorad Laboratories, Hercules, CA). For attachment of digoxigenin-labeled DNA samples to sample chamber surfaces we have used polyclonal sheep anti-digoxigenin (Roche Applied Science, Indianapolis, IN) with relatively uniform results. Chromatin assembled by the salt dialysis method referred to earlier are stored at concentrations $\geq$100 ng/μl at 4°, and diluted to 0.2–1 ng/μl in CDB immediately before application to sample chamber for experimentation.

1. Rinse sample chamber with 5 chamber volumes of PBS.
2. Immediately infuse 1 volume of anti-digoxigenin solution (25 ng/μl in PBS). Incubate 10 min.
3. Rinse with 5 volumes of BB. Incubate with residual blocker, 5 min.
4. Rinse with 5 volumes of PBS.
5. Immediately infuse 1 volume of diluted chromatin sample in CDB. Incubate 7.5 min.
6. Rinse with 5 volumes of CDB.
7. Infuse 1 volume of avidin-coated beads (10 pM in CDB + 0.01% (w/v) purified non-fat milk protein). Incubate 5 min.
8. Rinse with 5 volumes of ESB. Seal chamber with nail polish if no additional solutions will be infused.

Instrumentation

Mechanical measurements of a single nucleosomal array can be obtained by using a single-beam optical trapping microscope.[5,8] Here, we provide a brief overview of the instrument. The reader should refer to Wang *et al.*[9] and Koch *et al.*[5] for more detailed descriptions of the design, construction, and calibration of the optical trapping setup.

Optical Trapping System

A schematic of our optical setup is shown in Fig. 1A. The trapping laser has a wavelength of 1064 nm (Spectra-Physics Lasers, Mountain View, CA). The laser beam passes through a single-mode optical fiber (Oz Optics, Carp, ON) and an acousto-optic deflector (NEOS Technologies, Melbourne, FL), and is focused onto the sample plane by a 100×, 1.4-NA, oil immersion objective on an Eclipse TE200 DIC microscope (Nikon USA, Melville, NY). The focus of the laser serves as the center of the trap for a micron-sized microsphere. The laser light is collected by a 1.4-NA oil

immersion condenser and projected onto a quadrant photodiode (Hamamatsu, Bridgewater, NJ). The photocurrents from each quadrant of the photodiode are amplified and converted to voltage signals using a position detection amplifier (On-Trak Photonics, Lake Forest, CA). The position of the optical trap relative to the sample can be adjusted with a servo-controlled 1-D piezoelectric stage (Physik Instrumente GmbH & Co., Waldbronn, Germany). Analog voltage signals from the position detector and stage position sensor are anti-alias filtered at 5 kHz (Krohn-Hite, Avon, MA) and digitized at 7–13 kHz for each channel using a multiplexed analog to digital conversion PCI board (National Instruments Corporation, Austin, TX).

Calibration of the Optical Trapping System

The instrument calibration methods may follow those of Wang *et al*.[9] In brief, the first step of the calibration determines the position of the trap center relative to the beam waist and the height of the trap center relative to the coverslip. The second step of the calibration determines the position detector sensitivity and trap stiffness. The third step of the calibration locates the anchor position of the DNA tether on the coverslip, and is performed prior to each measurement by stretching the DNA at low load (<5 pN). These calibrations are subsequently used to convert data into force and extension.

Experimental Control and Data Acquisition

To disrupt nucleosomes as shown in Fig. 1B, the coverslip is moved relative to the trapped microsphere with a piezoelectric stage to stretch the DNA. Once a surface-tethered microsphere is optically trapped, the coverslip is then moved with a piezoelectric stage to stretch the nucleosomal DNA with either a velocity clamp or a force clamp.[5,8] Both of these clamps may be implemented with digital feedback algorithms, with an average rate for a complete feedback cycle of 7–13 kHz. In the velocity clamp mode, the coverslip is moved at a constant velocity relative to the microsphere, whose position is kept constant by modulating the light intensity (trap stiffness) of the trapping laser. A disruption event, during which DNA is released from a histone octamer, is observed as a sudden reduction in the tension of the DNA. In the force-clamp mode, the position of the coverslip is modulated so that the trapping force on the microsphere is held constant by keeping its position fixed in a trap of constant stiffness. In this mode, a disruption event is observed as a step in the coverslip position.

Determination of the DNA Elasticity

Determination of the force-extension relation of a naked DNA is essential for the conversion of force and extension to number of base pairs of naked DNA (see section on Data Analysis). Marko and Siggia proposed the Worm-Like-Chain (WLC) model, which accounts for the entropic elasticity and well describes the force-extension relation in the low-force region (<5 pN).[18] Wang *et al*. extended this model to also include the enthalpic elasticity in the high-force region (>5 pN),[9] and referred to this modified form as the Marko-Siggia (MMS) model. This MMS model incorporates both enthalpic and entropic contributions to stiffness and fits the experimental results extremely well for forces up to 50 pN. In the MMS model, the elastic stiffness of DNA is parameterized by its contour length under zero tension, L_0, its persistence length, L_P, and its elastic modulus, K_0. The force (F) and extension (ξ) are simply related:

$$F = \left(\frac{k_B T}{L_p}\right)\left[\frac{1}{4(1-\xi/L_0+F/K_0)^2} - \frac{1}{4} + \frac{\xi}{L_0} - \frac{F}{K_0}\right].$$

The elastic parameters of dsDNA can be obtained following Wang *et al*.[9] Under our experimental conditions, L_0 per base is 0.338 nm, L_P is 43.1 nm, and K_0 is 1205 pN. Therefore, if both the force and extension are known, this relation can be used to obtain L_0, which can be readily converted to the number of base pairs.

Data Analysis

Nucleosomes can be disrupted in various ways. The two ways presented here are velocity-clamp stretching and force-clamp stretching. These two methods are roughly equivalent, but with some subtle differences. Velocity clamp allows disruption of all nucleosomes at a specified stretching velocity regardless of the strength of protein-DNA interactions within nucleosome. However, nucleosomes in an array are disrupted under slightly different force conditions, which depend on the number of nucleosomes remaining in the array at a specific disruption. Force clamp allows disruption of all nucleosomes under identical force conditions (i.e., the same force). However, more experimentation is required to determine a workable range of force: If the force is too small or too large, the time to disrupt the nucleosomes will be too long or too short to be experimentally accessible.

[18] J. F. Marko and E. D. Siggia, *Macromolecules* **28,** 8759 (1995).

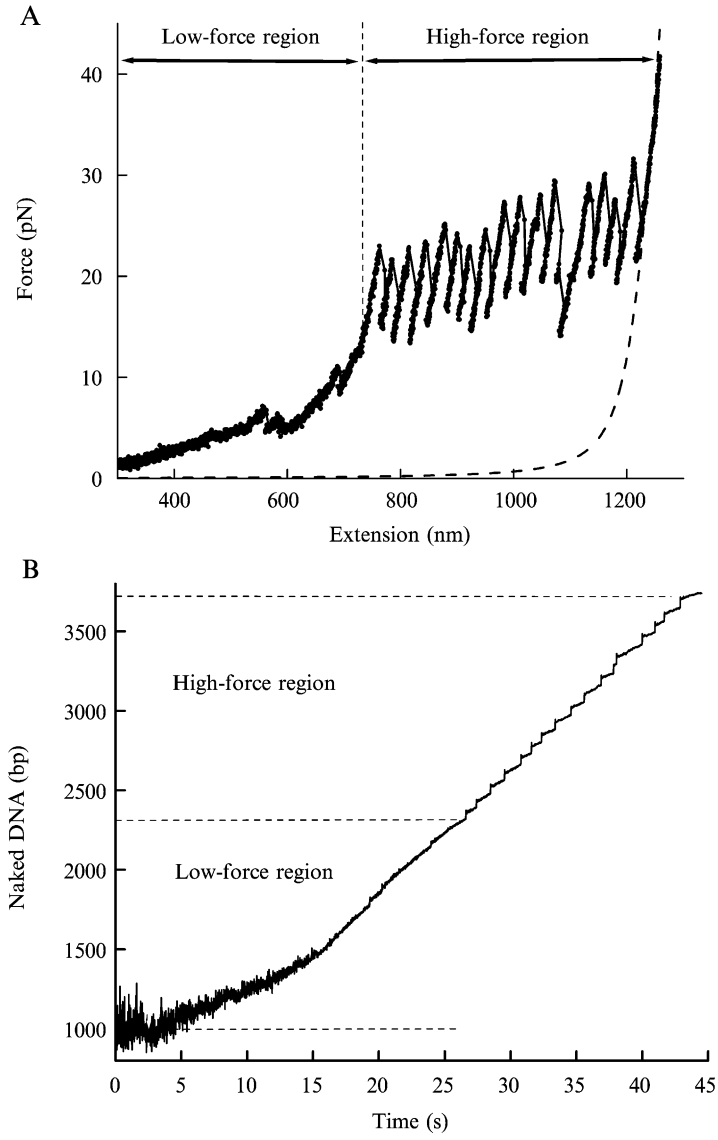

FIG. 2. Stretching a nucleosomal array with a velocity clamp at 28 nm/s. Nucleosomal arrays were prepared with avian core histones and a 3684-bp DNA fragment containing 17 direct tandem repeats of the sea urchin 5S positioning element. The biotin and digoxigenin linkers at the two ends of the DNA effectively contribute ~50 bp of DNA. (A) Force-extension curve of a fully saturated nucleosomal array. At higher force (>15 pN), a sawtooth pattern containing 17 disruption peaks was observed. Force-extension characteristics of a

Velocity Clamp

An example of data taken with a velocity clamp is shown in Fig. 2. At low force (<15 pN), the force-extension curve starts to deviate from that of the corresponding naked DNA; at higher force (>15 pN), a distinctive sawtooth pattern starts to appear; at even higher force (>40 pN), the force-extension resembles that of a naked DNA (dotted curve). Previously, Brower-Toland *et al.*[8] demonstrated that the high-force sawtooth pattern is indicative of a nucleosomal array, with each peak corresponding to a single nucleosome. Under the conditions used in our experiments, the spacing between adjacent peaks is ~27 nm. The observed sawtooth pattern suggests separate disruption of strong DNA-histone interactions in individual nucleosomes.

To determine the amount of DNA released from a nucleosome, the MMS model can be applied. This conversion attributes extension only to naked DNA, that is, linker DNA and DNA peeled from nucleosome core particles (NCP). This method of conversion from force-extension curve to number of base pairs of naked DNA is similar to that previously used for single-molecule studies of transcription.[3] The MMS model is only an approximation for a nucleosomal array. To achieve better precision for the conversion, a more refined model will be necessary. Conversion of the data in Fig. 2A is shown in Fig. 2B, where the amount of naked DNA is plotted as a function of time during stretching. At the beginning of stretching (0–2 s), the average amount of naked DNA is constant, indicating no DNA release from NCPs. This should correspond to the amount of linker DNA for a relaxed nucleosomal array. As force rises in the low-force region, DNA release is gradual, indicating a simultaneous release of DNA from all nucleosomes, with ~76 bp of DNA release per nucleosome. At high force, the sawtooth peaks in Fig. 2A, are converted to steps. DNA release is sudden, indicating a separate release of DNA from each nucleosome of ~80 bp.

Force Clamp

An example of data taken with a force clamp at 20.2 pN is shown in Fig. 3. Unlike the velocity clamp measurements, all the nucleosomes experienced the same force before disruption. Here, sudden disruptions of nucleosomes resulted in stepwise increases in the DNA extension, with

full-length naked DNA (dotted line) are shown for comparison. (B) Amount of naked DNA as a function of time derived from data shown in Fig. 2A. The top dotted line is a comparison with a full-length naked DNA. At higher force, the curves show 17 steps, which correspond to the 17 disruption peaks in Fig. 2A.

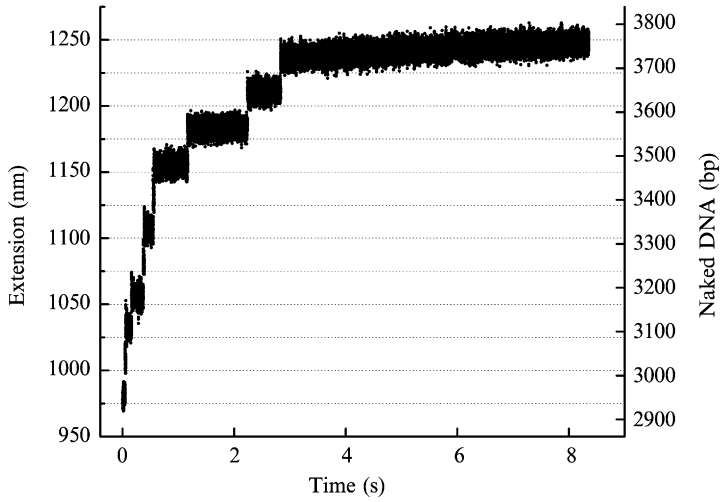

FIG. 3. Stretching a nucleosomal array with a force clamp. The graphs are plots of DNA extension (left axis) and the amount of naked DNA (right axis) versus time under constant force of 20.2 pN.

each step corresponding to one nucleosome disruption. The extension (left axis) is readily converted to number of base pairs of naked DNA (right axis). These steps (~80 bp) provide a measure of DNA release per nucleosome at high force.

Conclusion

Renewed interest in chromatin as a mediator of the structure, maintenance, and regulation of eukaryotic genomes has inspired the development of a variety of novel chromatin techniques. Optical trapping technology provides a useful addition to this repertoire of techniques. We anticipate that single-molecule optical trapping experiments on chromatin structure will complement more traditional technologies, and aid in the elucidation of the structural role in chromatin of histone and nonhistone proteins and their post-translational modifications. Likewise, optical trapping methods will be adaptable to the study of enzymatic activities such as RNA polymerases and ATP-dependent chromatin remodelers operating on chromatin structure.

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