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Micromechanics of chromatin and chromosomes

John F. Marko and Michael G. Poirier

Abstract: The enzymes that transcribe, recombine, package, and duplicate the eukaryotic genome all are highly processive and capable of generating large forces. Understanding chromosome function therefore will require analysis of mechanics as well as biochemistry. Here we review development of new biophysical–biochemical techniques for studying the mechanical properties of isolated chromatin fibers and chromosomes. We also discuss microscopy-based experiments on cells that visualize chromosome structure and dynamics. Experiments on chromatin tell us about its flexibility and fluctuation, as well as quantifying the forces generated during chromatin assembly. Experiments on whole chromosomes provide insight into the higher-order organization of chromatin; for example, recent experiments have shown that the mitotic chromosome is held together by isolated chromatin–chromatin links and not a large, mechanically contiguous non-DNA "scaffold".

Key words: DNA struture, chromatin, chromosomes, mitosis.

Résumé : Toutes les enzymes qui transcrivent, recombinent, enroulent et copient le génome eucaryote sont très processives et peuvent engendrer de grandes forces. Donc, la compréhension du rôle du chromosome nécessitera une analyse non seulement de la biochimie, mais également des mécanismes. Dans cet article, nous faisons une revue de la mise au point de nouvelles techniques biophysiques et biochimiques pour étudier les caractéristiques mécaniques de chromosomes et de fibres chromatiniennes isolés. Nous discutons également d'expériences sur des cellules utilisant la microscopie et permettant de visualiser la structure et la dynamique des chromosomes. Des expériences sur la chromatine nous renseignent sur sa flexibilité et son oscillation; de plus, elles nous permettent de quantifier les forces engendrées lors de l'assemblage de la chromatine. Des expériences sur des chromosomes entiers nous renseignent sur l'organisation d'ordre supérieur de la chromatine; par exemple, des expériences récentes ont montré que le chromosome mitotique est maintenu ensemble par des liaisons isolées entre des fibres chromatiniennes et non pas grâce à un gros échafaudage mécaniquement contigu, de nature autre que l'ADN.

Mots clés : stucture de l'ADN, chromatine, chromosomes, mitose.

[Traduit par la Rédaction]

Introduction

Although we now possess the sequences of many genomes, the physical organization of the eukaryote chromosome remains a mystery (Dietzel and Belmont 2001; Belmont 2002). Fully understanding chromosome structure and dynamics would shed light on many fundamental questions — how regulatory factors find their target sequences, how distant chromosomal sequences meet and interact, how chromatin is folded at various stages of the cell cycle, how chromosomes are disentangled from one another after their replication, and how homologous chromosome regions find one another during meiosis. All of these questions are related, and are all biophysical as much as they are biochemical issues. To understand them we will need to understand the physical properties of chromatin and chromosomes.

This paper will review our understanding of chromatin organization, from a biophysical point of view. In the next section (Chromatin fiber is soft and dynamic) we will discuss experiments that study the polymer dynamics and elasticity of individual chromatin fibers, and we will review experiments that reveal that many familiar DNA-processing enzymes are also powerful motors. A main point of that section will be that chromatin structure is subject to both continual thermal agitation, and the effects of powerful mechanoenzymes. This suggests that chromatin structure will have to be described at least partly in statistical terms.

The third section (Large-scale organization) will discuss current ideas about how chromatin is folded up into chromosomes. This will include discussion of the current "chromosome territory" picture of the interphase nucleus, a review of experiments that examine in vivo motions of interphase chromatin, and a discussion of our own work on physical

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properties of mitotic chromosomes. The final section (Open questions) will discuss some of the major problems that could be profitably studied by a combination of biological and biophysical approaches.

Chromatin fiber is soft and dynamic

X-ray studies give us a crystal-clear picture of nucleosome structure (Richmond et al. 1984; Arents et al. 1991; Luger et al. 1997), providing a solid starting point for biochemists and biophysicists thinking about eukaryote chromosome structure at the <10 nm scale. Unfortunately, we know essentially nothing at this level of detail about chromatin structure at larger than 10 nm, with continuing controversy concerning basic issues such as to what degree the 30 nm fiber has a regular structure (Woodcock and Horowitz 1995). The reason for this disconnect between our structural understanding of the nucleosome and the chromatin fiber stems from the fact that the nucleosome is held together into a compact structure by a large number of strong DNA–protein interactions, while the chromatin fiber is folded by far weaker interactions.

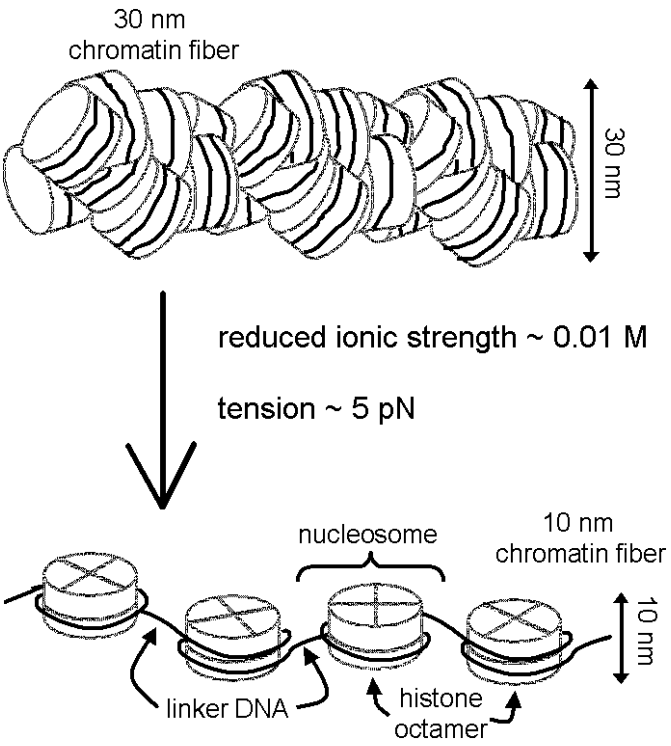
A simple way to see this is to review the classical topic of chromatin structure as a function of univalent salt concentration (van Holde 1989). As NaCl concentration is ramped up from 10 mM to 150 mM, isolated chromatin fibers change from their extended, unfolded "10 nm beads on a string" structure at low salt, to a condensed "30 nm fiber", and finally to precipitated chromatin sludge. By contrast, over this salt range there is relatively little change of histone or DNA secondary structure. Thus, at the single-fiber level, chromatin is folded by relatively weak interactions the strength of which can be modulated by changing the range of electrostatic nucleosome–nucleosome repulsions (Fig. 1; note that the range of electrostatic interactions in univalent salt solution is 0.3 nm divided by the square root of the salt molarity, thus about 1 nm at the physiological level of 0.1 M (Israelachvilli 1985)). This softness suggests that in vivo we may have to think of chromatin as a flexible polymer that will wander randomly in response to thermal forces unless it is organized by directed forces of processive mechanoenzymes or by the binding forces of other architectural proteins.

Nucleosome dynamics

Photos of structures inferred from X-ray data can easily mislead us into thinking about the nucleosome as a monolithic and static object. Elegant experiments have been carried out that demonstrate that isolated core particles undergo huge conformational fluctuations in the form of transient "unpeeling" of DNA (Widom 1997; Polach and Widom 1995; Anderson and Widom 2000). These experiments showed that restriction enzyme access was suppressed at sites progressively further towards the middle of the ~150 bp nucleosomal DNA.

Analysis of this suppression of enzyme activity suggests that the free energy cost of unpeeling DNA is roughly 1 kcal/mol per 10 bp (Marko and Siggia 1997*a*). Using this number we estimate the free energy holding all the DNA onto the core particle at 15 kcal/mol, very roughly consistent with thermodynamic measurements (Cotton and Hamkalo 1981; Ausio et al. 1984*a*, 1984*b*).

Fig. 1. Conversion of chromatin fiber from native "30 nm fiber" to extended "10 nm beads on a string" can be driven chemically by reducing univalent salt concentration, or alternately by applying about 5 pN tension. Linker histones are not shown in this sketch.



The idea that DNA may transiently release from the nucleosome is important for problems of nucleosome transfer along a DNA segment. It has been suggested that this kind of transient DNA release may allow slow "diffusion" of nucleosomes along a long DNA template (Schiessel et al. 2001).

Single chromatin fiber experiments

Over the past decade, experimental techniques for doing ultra-low-force micromechanical experiments on single DNA molecules of 10 kb to 100 kb (for bare DNA recall that 1 bp has a 0.34 nm rise, thus 3 kb is 1 μm in contour length; Bustamante et al. 2000) have been developed to the point where it is practical to study the micromechanics of DNA–protein composites, including single chromatin fibers. The first experiment of this sort (Cui and Bustamante 2000) used purified chicken erythrocyte chromatin segments containing a few hundred kilobases of DNA, which were chemically attached to 3 μm diameter microspheres. The microspheres allowed manipulation of the chromatin segments, via their trapping in a focused laser spot ("laser tweezer") and by suction on the end of a glass micropipette.

Cui and Bustamante (2000) found that single chromatin fibers showed first a very-low-force regime where forces <1 pN (1 pN = 10^{-12} N, recall that the newton is the SI unit of force, and is roughly the weight of a small apple) were

needed to stretch the initially random-coiled fibers out to their "natural" contour length. This general behavior is similar to that displayed by long, flexible polymers such as dsDNA (Smith et al. 1992; Bustamante et al. 1994; Bustamante et al. 2000). The fibers could then be gradually stretched to double their natural length by application of about 5 pN force. This unfolding was attributed to the opening up of the fiber by force (Fig. 1), and led Cui and Bustamante to estimate that the binding free energy between adjacent nucleosomes was on the order of 2 kcal/mol (compare to the estimated 15 kcal/mol binding energy of DNA to nucleosome). Finally, for large forces, the fibers could be irreversibly lengthened, presumably by force-driven removal of histones (Marko and Siggia 1997*a*).

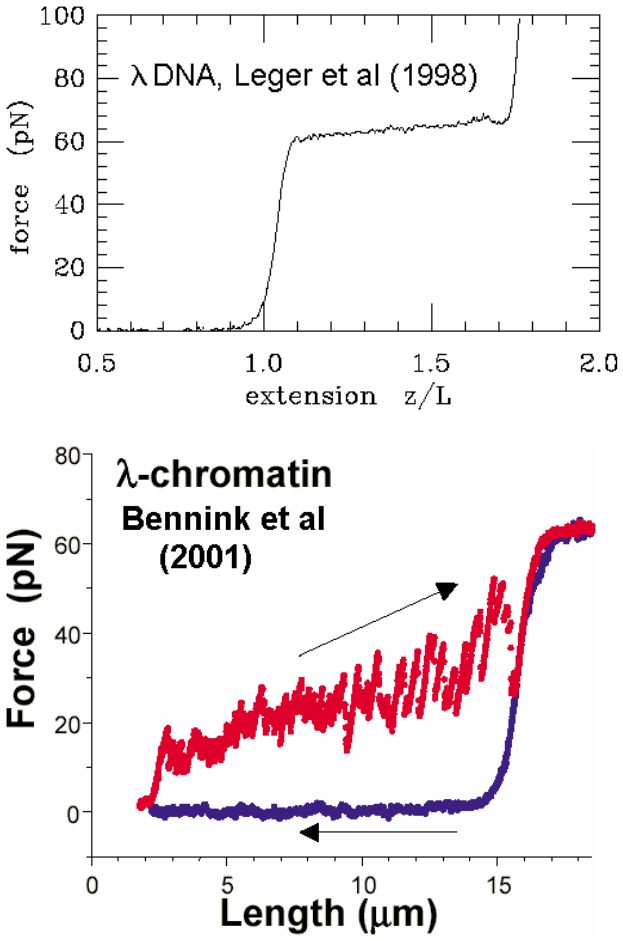
A main defect of the Cui–Bustamante experiment is that the isolated chromatin fibers studied contained unknown DNA and had an unknown protein content. An alternative approach of assembling chromatin in situ onto a tethered λ-DNA of known 48.5 kb length using diluted *Xenopus* egg extracts was carried out by Bennink et al. (2001). The DNA was end-grafted to 3 μm diameter microspheres, and again manipulated using a combination of laser tweezers and micropipettes. The initially 16 μm long DNA was compacted by chromatin assembly, down to a natural length of a little more than 1 μm.

The basic mechanical properties for assembled "λ-chromatin" observed by Bennink et al. (2001) (Fig. 2) were in accord with those found by Cui and Bustamante. The initially collapsed coils could be stretched to their natural length by low forces (<1 pN), and the fibers could be roughly doubled in length by about 5 pN. The major new result of Bennink et al. (2001) was the observation of force peaks spaced by roughly 60 nm distance increases that could be interpreted as single-nucleosome-removal events. These nucleosome "pop-offs" were observed for forces of about 20 to 30 pN.

Subsequent experiments of Brower-Toland et al. (2002) explored a third chromatin system using single-molecule manipulation. Brower-Toland et al. reconstituted nucleosomes using the classical salt dialysis technique (van Holde 1989) onto an engineered DNA containing a tandem array of nucleosome positioning sequences. This artificial chromatin was then end-attached to microspheres and manipulated using laser tweezers. In this experiment, force signals of again roughly 20 pN were observed with roughly 30 nm spacing, suggesting that nucleosomes could be removed one DNA turn at a time (recall that the roughly 150 bp or 50 nm of DNA in a nucleosome is wrapped in 1.75 turns around it). This experiment suggests that a single turn of DNA (about 80 bp) wrapped around core histones may be quite stable.

All these experiments observed nucleosomes to start to pop off at forces near 20 pN. Given that about 50 nm of extension occurs during each pop-off event (essentially the contour length of the 150 bp of DNA released), the work done to remove a nucleosome is the force–distance product (20 pN times 50 nm) of about 1000 pN·nm. A piconewton nanometre is an energy of 0.15 kcal/mol, so the work done in the micromechanical experiments to remove a nucleosome is therefore roughly 150 kcal/mol. This is far in excess of any existing measurements of the free energy of

Fig. 2. Force-extension behavior of bare DNA (data from Leger et al. 1998, top) and λ-chromatin (bottom, from Bennink et al. 2001, reproduced with permission by Nature's Publishing Group). Both DNA and chromatin show an initial low-force (<1 pN) regime where the initially randomly coiled state extends to its natural contour length. DNA shows some stiff elastic behavior (rapid upturn in force curve, top) followed by a sharp transition to an extended form about 1.7-times the B-DNA natural length (force "plateau" near 60 pN, top). Chromatin shows a more gradual stretching behavior as it is extended to about 2-times its natural length by forces of about 5 pN (left end of red curve, bottom). Nucleosome removal occurs for forces of 20 to 40 pN, and finally when all nucleosomes are removed, bare DNA elastic response (16 micrometre length, 60 pN plateau, and blue retraction curve) is recovered. Roughly 60 nm spacing of removal events (red zig-zags, bottom) are consistent with the expected ~200 bp liberated per nucleosome removed. Other experiments of Brower-Toland et al. (2002) also show 30 nm spacings, suggesting that tension-driven nucleosome removal can occur one DNA turn at a time.



the DNA–nucleosome interactions, which are at most 20 kcal/mol (Cotton and Hamkalo 1981). The most likely explanation for this disparity is that the pop-off experiments are done on timescales far shorter than those required to observe thermal equilibrium. Assembly and removal of whole nucleosomes likely involves passage over a large energy barrier, which might be traversed by thermal fluctuations only on very long time scales. Experiments on nucleosome

pop-off need to be done as a function of pulling rate, and under conditions where barriers to nucleosome assembly and (or) removal are reduced.

DNA-acting mechanoenzymes

Single-molecule experiments have been used to assay forces generated by a number of enzymes that processively interact with DNA, with the result that we now know that these enzymes are typically powerful motors. The first DNA-processing enzyme to be mechanically characterized was RNA polymerase, which turns out to be able to generate forces of up to 40 pN (*Escherichia coli* RNApol, Yin et al. 1995; Wang et al. 1998). By comparison, kinesin and myosin type motors generate less than 10 pN; RNA polymerase is thought to generate a larger force because of its short step length and thus "low gear" (1 nm; note the steps taken by kinesin are thought to be 4 nm, Schnitzer et al. 2000). These forces are sufficient to drive nucleosome pop off as observed in single-molecule experiments (Bennink et al. 2001), and are therefore consistent with the results of experiments where transcription is observed to displace nucleosomes on DNA plasmids (Felsenfeld et al. 1996).

T7 DNA polymerase has also been observed to generate large forces (>30 pN, Wuite et al. 2000; Maier et al. 2000). DNA helicases have not yet had their force-generating properties directly measured in single-DNA experiments (Dohoney and Gelles 2001; Bianco et al. 2001), but they must be able to generate at least the roughly 20 pN needed to mechanically "unzip" the double helix (Essavez-Roulet et al. 1997; Bockelmann et al. 2002). Since progress of these enzymes is critical to cell survival and propagation, and must occur in a crowded nucleus (or bacterial nucleoid), it is no surprise that they are mechanically powerful.

A related phenomenon is force generation by proteins that complex with DNA. A simple example is RecA, which in the test tube can polymerize onto the double helix, and in the process stretch it by about 1.6 times. Single-DNA experiments have shown that RecA polymerization alone (with no ATP hydrolysis) is able to overcome a 100 pN retarding force (Leger et al. 1998). This is consistent with the 60 pN force known to be needed to generate DNA "overstretching" (Cluzel et al. 1996; Smith et al. 1996). These large forces are not surprising if protein binding generates a DNA conformational change (bending, stretching, looping, etc.). The force generated during binding is roughly the ratio of the binding energy to the length change: for example, a 0.5 nm length change as a result of a 5 kcal/mol protein–DNA interaction can be expected to generate a force of 5/0.5 = 10 $kcal \cdot mol^{-1} \cdot nm^{-1}$ = 60 pN (note the relation 1 $kcal \cdot mol^{-1} \cdot nm^{-1}$ = 6 pN). Experiments on the DNA-folding proteins IHF (Ali et al. 2001) and HU (B. Schnurr, personal communication) are consistent with this general idea that tension on a DNA–protein complex can shift its dissociation point (Marko and Siggia 1997*a*).

Although more complex than the protein–DNA interactions discussed just above, chromatin assembly reactions fall into the same general category of force-generating protein–DNA systems. Experiments of Ladoux et al. (2000) have observed that diluted *Xenopus* egg extracts, a standard chromatin-assembly system, compact DNA with roughly 10 pN forces. A similar level of force generation has been reported to occur during experiments using only the purified chromatin assembly factor NAP-1 (Leuba et al. 2003). Finally, we note that chromatin-remodeling enzymes, which consume ATP and physically reorganize nucleosomes (Flaus and Owen-Hughes 2001), are likely to also be powerful mechanoenzymes.

These in vitro experiments indicate that chromatin in the cell is likely to be subjected to appreciable forces, both from processive enzymes, and from binding of proteins which change DNA conformation. Forces of up to 40 pN are generated by transcription, enough to move nucleosomes around. Binding of proteins to DNA can generate even larger forces on the 100 pN level, enough to tension-denature the DNA double helix. These large forces are far above the few pN needed to extend a chromatin fiber from its zero-tension coiled conformation.

Large-scale organization of chromosomes

Chromosome layout in the nucleus

Development of methods for fluorescence labeling of chromosomes, and high-resolution optical microscopy, has led to a tremendous improvement in our understanding of how chromosomes are arranged inside the interphase nucleus. A very basic result is that nuclear DNA can be released from the nucleus only when DNA cuts are made at sufficient frequency; this result suggests that chromatin is organized into "loops". Experiments of Jackson et al. (1990) found a loop size in HeLa cells of roughly 50 kb. However, there is not yet a clear answer to the next logical question: What are the molecules that organize those loops? The appealing idea of a nucleoskeleton ("nuclear matrix") that would provide a structure from which chromosomes could be hung remains controversial (Pederson 2000).

There has been progress made in understanding the organization of chromosomes themselves, thanks to the use of techniques for observation of the positions of specific DNA sequences both in fixed and live cells. One of the most important developments has been establishment of the idea that each chromosome occupies a spatially localized "territory" of the nucleus (Cremer et al. 1993; Cremer and Cremer 2001). This idea is based on chromosome visualization experiments where chromosomes in fixed cells were labeled with fluorescent labeled probe oligos (FISH). By introducing probes for sequences on specific chromosomes, it was found that the territories of different chromosomes were spatially separated. Other experiments indicate that chromosomes occupy specific positions inside the interphase nucleus (Croft et al. 1999).

Inside of individual chromosomes, FISH has also been used to map the relative positions of specific loci. The pioneering study of Yokota et al. (1995) measured the relative positions of pairs of specific sites on the same chromosome for separations from 150 kb to 200 Mb apart, collecting sufficient data to determine how the average separation of sites depended on their genomic separation. It was found that for genomic separations <1 Mb, the mean square distance between sites was linear in the sequence distance, with a proportionality constant of about 2 μm^2/Mb = 60 nm^2/kb. This scaling behavior suggests that interphase chromosome has a "random walk" organization (random walks also have the

property that their mean square displacement increases linearly with the number of steps taken). At genomic separations beyond roughly 1 Mb, Yokota et al. found a reduction of the rate of increase of mean squared displacement. To explain their data, they suggested a "giant loop" structure of the interphase chromosome, with a loop size of roughly 3 Mb.

A recent study of yeast (*Saccharomyces cerevisiae*) interphase chromosome structure by Dekker et al. (2002) is unique in its methodology and results. This study used crosslinking of isolated nuclei, followed by restriction enzyme digestion. The fragments were self-ligated, and the resulting fragments were PCR-amplified and analyzed. The result was a statistical "map" of in vivo chromatin contacts, giving a statistical three-dimensional chromosome model.

The results of Yokota et al. (1995) and Dekker et al. (2002) can be interpreted in a unified way. In terms of a random-walk picture (Yokota et al. 1995), they suggest a "persistence length" (length over which appreciable bending typically occurs) on the order of 30 nm (see Marko and Siggia 1997*b*; Dekker et al. 2002), a value roughly consistent with chromatin persistence length inferred from single-chromatin fiber mechanical experiments (Cui et al. 2000). A persistence length of 30 nm corresponds to roughly 15 nucleosomes containing 3 kb in a tightly packed-solenoid model.

Dynamics of chromatin fiber observed during interphase

The in vitro and "single-molecule" experiments described above indicate that isolated chromatin fibers behave to some extent like flexible biopolymers. This is far from saying that chromatin has polymer-like dynamics in vivo; one possibility is that chromatin is in a dynamically frozen state during most of the cell cycle, e.g., as a result of its being tethered to a dense and rigid nucleoskeleton. However, a few experiments have looked at the physical dynamics of chromatin inside living cells and provide data at odds with such a static picture.

Marshall et al. (1997) carried out a number of experiments on cells from different species examining relative motion of labeled chromatin loci. They observed that the loci pairs underwent random fluctuations with a correlation time on the order of 1 min. These fluctuations continued with reduced amplitude and on a similar time scale in azide-poisoned cells, suggesting that thermal, flexible-polymer-like motion plays a role in moving chromatin around inside the cell nucleus. However, observation of suppression of this motion by azide poisoning suggested that active transport processes contribute appreciably to chromatin motion.

One of the techniques used by Marshall et al. (1997) involved observation of positions of tandem arrays of lac-repressor binding sites, using gfp-lac-repressor expressed in transgenic cell lines (Robinett et al. 1996; Belmont 2001). This promises to be a powerful method for studying chromosome structure and dynamics in vivo, or in fixed cells with high spatial resolution (Dietzel and Belmont 2001).

Two recent physical measurements characterized the diffusive motion of histone H1 through the cell nucleus. Gfp-H1 fusion protein was expressed in a cell line, which gave a fluorescent signal across the nucleus (Lever et al. 2000; Misteli et al. 2000). When photobleaching of part of the nucleus was done, the bleached region recovered its fluorescence on a few minute timescale. This experiment indicates that in vivo histone H1 is in chemical kinetic equilibrium on this timescale, and is apparently free to move diffusively through the whole nucleus.

These experiments suggest that the interior of the nucleus is a dynamic place, with apparently unfettered diffusion of proteins, and large-scale conformational changes of chromosomes on the few-minute timescale. Note that they do not rule out the possibility that there is a dense nucleoskeleton in vivo, which would not necessarily greatly inhibit free diffusion, or chromatin motion. However, two other biophysical experiments examining global structure of the nucleus are difficult to reconcile with a nucleoskeleton. Maniotis et al. (1997) have developed a technique for removing the whole genome from an interphase cell, using small glass needles to hook and then pull all the chromosomes from a small hole punched in the nucleus. A string of interphase chromosomes can be obtained, appearing rather like a string of sausages. Maniotis et al. went on to study how extracted genomes could be chemically manipulated using salts, nucleases, proteases, and other agents. There is certainly massive damage to the chromosomes in such an experiment. However, this experimental result appears incompatible with chromosomes sutured at many places to an internal nucleoskeleton.

Another experiment was carried out by our group, as part of a study of chromosome condensation dynamics (Sarkar et al. 2002). We noticed that when a chromosome condensing salt (cobalt hexamine trichloride, a trivalent cation, well known to condense negatively charged biopolymers) was injected into the nucleus, the interphase chromosomes could be essentially instantaneously condensed into distinct thick fibers extending across the nucleus. Micrometre-diameter particles could then be seen to be undergoing apparently free diffusion in the apparent void where the chromosomes had just been. Then, when the flow of cations was stopped, after a few minutes, the chromosomes swelled back up to fill the nucleus. Again, this is a brutal and damaging experiment, but again, it is hard to imagine these results occuring for interphase chromosomes intimately attached to a dense nucleoskeleton. The results of the experiments discussed in this section are all consistent with an alternative picture of chromosomes being arranged in the nucleus in distinct territories, and with attachments to the nuclear envelope (Marshall et al. 1996), but without other constraint by nonchromosomal structures.

Mitotic chromosome

The mitotic chromosome is an attractive object for study for chromatin structure enthusiasts for several reasons. First, the presumably complicating factors of nuclear structure and dynamics, such as gene expression, are out of the picture. Second, mitotic chromosomes are distinguishable objects with strikingly well-defined structures: the mitotic chromosomes of a given species can be described quantitatively by their metaphase lengths and thicknesses, and identified easily by their banding patterns. What is the origin of this structural regularity?

Historically, there have been two main models for mitotic chromosome structure. The first posits that loops of chro-

matin are folded onto a "scaffold" in the interior region, often presumed to be composed of protein (Paulson and Laemmli 1977; Laemmli et al. 1978; Marsden and Laemmli 1979; Gasser et al. 1986; Boy de la Tour and Laemmli 1988; Saitoh and Laemmli 1993, 1994). This model is supported mainly by experiments where the chromatin is histone depleted, leading to generation of a halo of DNA around a protein-rich, roughly chromosome-shaped residue, which can be visualized on the electron microscope grid (Paulson and Laemmli 1977). Isolation of these protein-rich objects showed them to contain topo II and proteins later identified as condensins as major components (Laemlli et al. 1978; Gasser et al. 1986). The scaffold model is attractive in part because it offers a simple answer to the question of why mitotic chromosomes have their well-defined cylindrical shapes. The main alternative model is one where the mitotic chromosome is assembled by being coiled or folded into successively thicker fiber forms, by a heirarchy of folding processes (Belmont et al. 1987, 1989).

A major step forward in understanding large-scale mitotic chromosome structure came in the mid-1990s with the identification of the "SMC" class of proteins. The first such proteins to be identified are elements of complexes now known as "condensins", and were first found in yeast (Strunnikov et al. 1993, 1995) and in *Xenopus* (Hirano and Mitchison 1994). Elegant experiments of Hirano and Mitchison using the *Xenopus* egg extract in vitro chromosome assembly system demonstrated that they were necessary for establishing and for maintaining mitotic chromatid structure.

The condensin complexes are built around a pair of gigantic filamentous coiled-coils about 100 nm in length, with a hinge region in the middle, and DNA-binding and ATP-hydrolysing domains at the end. Their structure suggests a function as active, DNA-folding "machines", but there has not yet been a convincing in vitro demonstration of a DNA-compacting function. Remarkably, condensin-like proteins have been found in eubacteria and characterized (Melby et al. 1998; Hirano 1999); mutants show a nucleoid-condensation defect. Other SMC proteins have separate roles in modulating chromosome structure (Strunnikov and Jessberger 1999), notably holding sister mitotic chromatids together during prophase ("cohesins", see Michaelis et al. 1997; Guacci et al. 1997; Losada et al. 1998). Losada and Hirano (2001) have suggested that a balance of activities of condensin and cohesin complexes determines large-scale mitotic chromosome morphology.

Micromechanical experiments on mitotic chromosomes

Our own research has focused on the mechanical properties of mitotic chromosomes, which we have combined with enzyme digestions to develop methods to study chromosome structure. Mitotic chromosomes have very well-defined elastic properties, showing reversible stretching even after five-fold extensions (Nicklas 1983; Houchmandzadeh et al. 1997; Poirier et al. 2000). During mitosis, chromosomes are often stretched, by spindle-generated forces on the order of 1 nN (1 nN = 1000 pN) in large animal cells (Nicklas 1983). The extensibility of mitotic chromosomes is sometimes used to increase the resolution of banding (Claussen et al. 1994).

Nicklas (1983) made the first measurements of the elasticity of mitotic chromosomes (to be precise, Nicklas studied meitoic metaphase I and II chromosomes, i.e., the mitotic-like stages of meiosis). He used microneedles to push and hook chromosomes inside grasshopper cells, by pushing on the cell membrane. Bending of the microneedle provided a way to measure forces; it is possible to pull needles with a long taper, which need a force in the 1 nN range to be deflected by a micrometre.

A rather similar experimental approach that we have followed is to use glass micropipettes, similar in size and stiffness to the microneedles of Nicklaus, but which can be used to apply suction. We typically open up a cell using brief exposure to 0.05% Triton X-100, and then we "grab" a chromosome using a bit of suction. The chromosome then "corks" the pipette and becomes permanently attached to it. By attaching each end to a different pipette, we are able to manipulate chromosomes, and by measuring pipette deflections, measure forces (Poirier et al. 2000) (Fig. 3). Our experiments are done outside cells, in the surrounding cell culture medium.

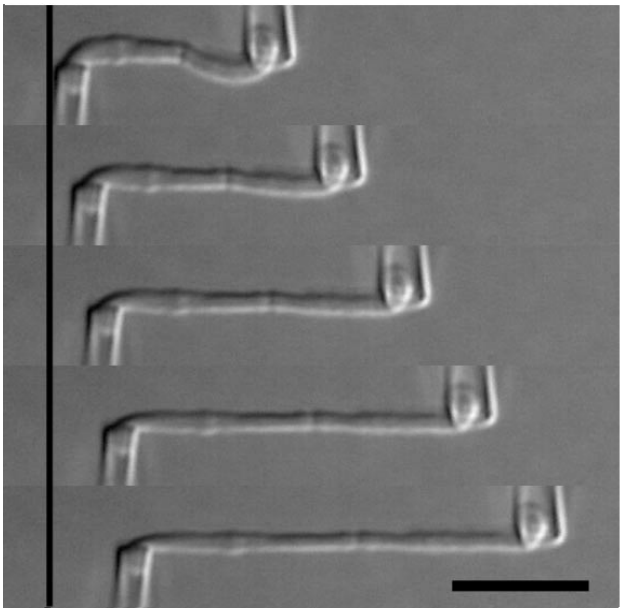
To describe the elastic properties of a material, one often quotes its elastic modulus. This expresses what stress (force per area) must be put on an object to double its length, and for a mitotic chromosome, this stress is about 500 Pa (1 Pa = 1 N/mr^2, the SI unit of pressure and stress). A 500 Pa modulus is low, even for a very loose high-polymer gel. Agarose gels as used in the biochemistry lab have a modulus of about 10 kPa (10 000 Pa), plexiglass and folded biomolecules (B DNA and globular protein domains) have a modulus near 1 GPa (1 000 000 000 Pa), and covalently bonded materials (metals, glasses) have moduli of about 10 GPa. The modulus is useful since it expresses the strength of the interactions holding a material together, in a way that is independent of size or shape.

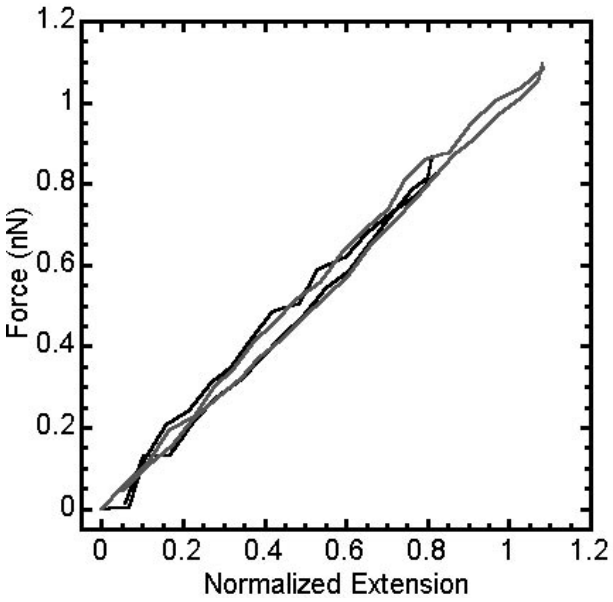
Thus, mitotic chromosomes have a low modulus, indicating that they are relatively loosely linked internally. Also, their extreme extensibility of up to five times without apparent damage (Nicklaus 1983; Poirier et al. 2000) indicates that their internal structure must involve loosely compacted domains of chromatin that can easily unfold under force. We also note that the basic elastic properties of mitotic chromosomes were observed to be similar for experiments done inside cells (Nicklaus 1983), and for experiments done on chromosomes that had been removed from mitotic cells (Poirier et al. 2000).

For extensions beyond five times, mitotic chromosomes are permanently lengthened by stretching, suggesting that internal "links" holding chromatin in its compacted form are being broken (Poirier et al. 2000). After extensions beyond about 30 times native length followed by relaxation, the mitotic chromosome ends up not only longer than native, but also appreciably thicker as well (Poirier et al. 2000). This suggests that if sufficient numbers of chromatin interconnects are broken up, the remaining chromatin fiber then "swells up". Even in these high-extension experiments, the force per chromatin fiber is likely only about 1 pN, not enough to remove nucleosomes by force (Bennink et al. 2001). Using fluorescent antibodies, histone content was observed not to change appreciably during this type of experiment (Poirier et al. 2000).

These experiments suggest that chromatin inside the mitotic chromosome is compacted by force, i.e., that chromatin

Fig. 3. Two-pipette chromosome experiment (reprinted from Poirier and Marko 2002*a*, *Molecular Biology of the Cell*, 2002, **13**: 2170–2179 with permission from the American Society for Cell Biology). Pipettes are used to hold a mitotic chromosome, with left pipette fabricated with a deflection force constant ~1 nN/μm to allow chromosome tension to be measured. Left: images collected during force-extension experiment. As the right pipette is moved, the left pipette is observed to deflect. Digital image analysis allows pipette deflections to be measured to about 10 nm accuracy, translating to about 10 pN force resolution. Bar is 10 μm. Right: Force-extension data for a newt chromosome, in linear elastic regime. Extension of 1 corresponds to a doubling of length of a chromosome, and requires about 1 nN force, similar to forces exerted by the spindle during mitosis.





itself does not assemble into mitotic chromatids by virtue of direct chromatin–chromatin attractive interactions. The corollary is that the mitotic state is not necessarily a maximally compacted state of chromatin. Additional experiments show a slow response to applied forces consistent with the elastic response of a polymer network (Poirier et al. 2001; Poirier and Marko 2002*c*). Measurements of Daban (2000) show that the net DNA concentration in chromosomes is well below that in the chromatin fiber, indicating that chromosomes do not consist of very tightly packed chromatin.

Mitotic chromosome bending and chromatids reconstituted from Xenopus egg extracts

A rod made of a material with a well-defined elastic stretching modulus has a bending stiffness that is simply related to that modulus. For mitotic chromosomes extracted from cells into the surrounding culture buffer, this relationship appears to hold (Poirier et al. 2002; also see Marshall et al. 2001). For the biologist, this would be a bit of biophysical trivia except for the following fact. Similar stretching and bending experiments have also been done for *Xenopus* chromatids assembled in vitro using egg extracts (Houchmandzadeh and Dimitrov 1999), with the result that the bending stiffness is roughly 1000-times smaller than one would expect from their stretching properties.

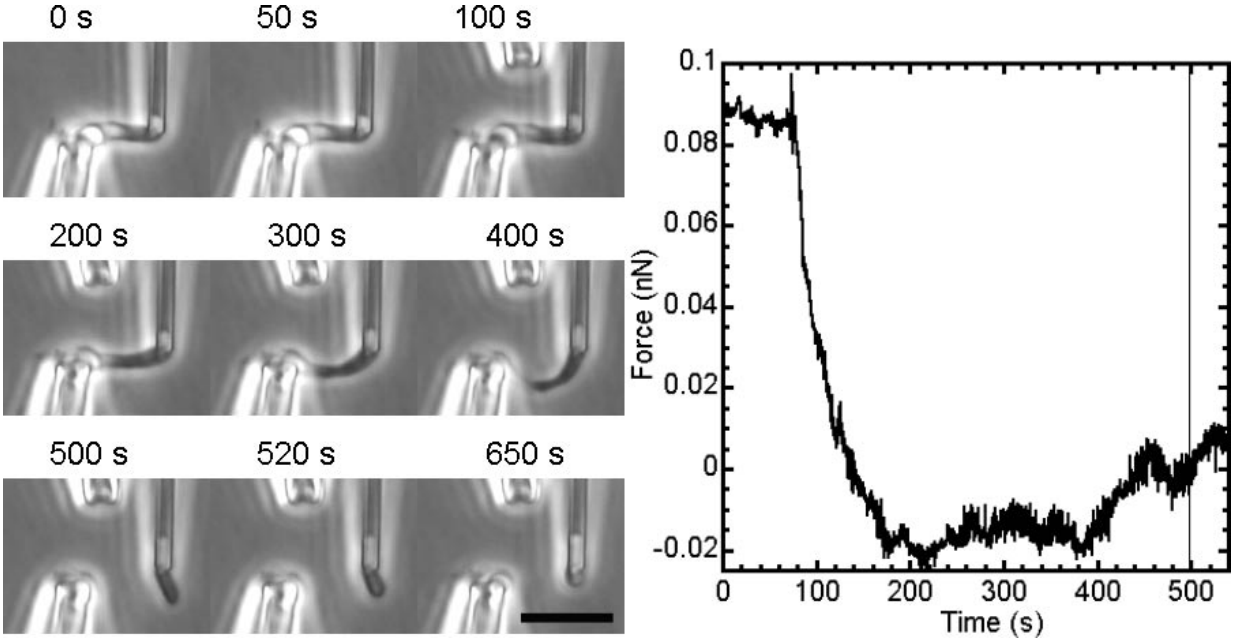
Thus, the egg-extract chromatids must have a quite different internal structure from somatic mitotic chromosomes. Our favored speculation is that the egg-extract system is missing some chromatin-linking elements of the in vivo system, presumably proteins. If some cross-linkers that play a role in the largest-scale folding of the prophase chromatin were absent or inactive in the egg-extract system, it is possible that the chromatids could be relatively easily bent, while having stretching elasticity similar to that of native chromosome. Given that the egg-extract system is widely used as a model for in vivo chromosome condensation, one should keep in mind that it is pretty clear that it generates chromatids that lack some levels of the in vivo organization. This example also illustrates how quantitative material property measurements can allow one to classify different structural states in biological systems.

Combined micromechanical–biochemical experiments

Maniotis et al. (1997) developed methods for taking groups of chromosomes out of cells using microneedles, and then exposing them to changes in salt concentration and digestion by enzymes. We were surprised by their finding that mitotic chromosomes could be abruptly decondensed and recondensed merely by shifting salt concentration. However, there is an old literature concerning this general type of experiment (Cole 1967), indicating that mitotic chromosomes can be hypercondensed, or greatly decondensed by salts. We have developed techniques for carrying out experiments of this type, but where reagents are introduced onto chromosomes from a micropipette of micrometre diameter (Poirier et al. 2002). This allows rapid turning on and off of reagent flow (when flow is stopped, the tiny volume of chemicals diffuses rapidly away) while observing changes in mechanical properties of an individual chromosome.

We found that not only could mitotic chromosomes be hypercondensed or decondensed on <1 s timescales, but also chromosome elastic response after such treatments matched the pre-treatment response (Poirier et al. 2002). This indicates that far from being tightly bound together, chromatin

Fig. 4. Time course of tension in a chromosome, and chromosome morphology, during digestion by 1 nM MNase, with initial tension 0.1 nN. Spraying starts at 80 s, force decays after 30 s, chromosome is cut after 450 s. The spray pipette can be seen in the upper center of the t >120 s frames. Bar is 10 μm. From Poirier and Marko (2002*b*).



in mitotic chromosomes is folded up largely by relatively weak, nonspecific electrostatic interactions that are easily disrupted. Furthermore the native state can be easily recovered. Interestingly, by use of trivalent cations, the volume of a chromosome can be reduced by about a third. Thus, the native state is quite far from its maximum density; much of visible mitotic chromatid volume is mobile small molecule species.

Understanding the effect of ionic condition shifts allowed us to move on to nuclease experiments to study what contribution the connectivity of DNA makes to chromosome mechanical integrity. We originally expected to be able to use nucleases to cut the "loops" of chromatin away from the "protein scaffold", thus allowing us to study the scaffold by itself. However, we found that nonspecific micrococcal nuclease (Fig. 4) and a number of blunt, 4-base-target restriction enzymes quickly eliminated all elastic modulus, and then completely dissolved the chromosome (Poirier and Marko 2002*b*). We were forced to conclude that the mechanical integrity of the mitotic chromosome comes from DNA (chromatin) itself, and not from some other non-DNA structure.

Our current model of the mitotic chromatid is that it is a "chromatin network", held together with isolated "cross-link" elements. These elements depend on the chromatin itself to hold the chromosome together; the most simple picture is that they are actually spatially and mechanically separated. Although we do not yet know the identity of these "cross-linkers", it is a plausible speculation that they are the condensin protein complexes.

Open questions

Physical properties of chromatin fiber

Techniques for carrying out single-chromatin-fiber experiments are now available, and many questions about chromatin can be answered with them. One extremely basic question is under what (if any) circumstances, chromatin fibers undergo large-scale "random-walk" conformational fluctuations. Recent single-fiber experiments have focused heavily on tension-driven nucleosome removal (Cui and Bustamante 2000; Bennink et al. 2001; Brower-Toland et al. 2001), but have not yet revealed the polymer properties of chromatin in the same clear way that single-DNA experiments have done (Bustamante et al. 2000). It may well be that the fibers in single-chromatin-fiber experiments fold up because of the same nonspecific nucleosome–nucleosome interactions that lead to chromatin aggregation at physiological ionic strength (van Holde 1989). If this is the case, there is a basic question: What leads to in vivo chromatin random walk behavior, both static (Yokota et al. 1995) and dynamic (Marshall et al. 1997)? This question may be related to the question of how chromatin physical properties vary with histone modification, which is so far a topic that has not been touched using single-chromatin-fiber methods.

There is another question following from tension-driven nucleosome removal experiments (Bennink et al. 2001; Brower-Toland et al. 2002), which show nucleosome removal to require forces of about 20 pN. This is far above the minimum force of about 2 pN that one would expect based on thermodynamic arguments (Marko and Siggia 1997*a*), suggesting that experiments done on longer timescales — or perhaps in the presence of non-ATP-hydrolysing nucleosome-assembly factors — may be able to remove nucleosomes with far less tension.

Another feature of chromatin fiber that may be revealed using single-fiber micromanipulation experiments is the question of the rate at which nucleosomes are able to move ("diffuse") along DNA (Schiessel et al. 2001). This might be studied using fluorescent labeled histones on single fibers, using high-sensitivity fluorescence detection techniques.

Nuclear organization and dynamics

Methods for studying chromatin conformation in the nucleus are also developing rapidly. The prevailing picture is one where at large length scales, chromosomes are rather well organized, with different chromosomes occupying different regions ("territories") of the nucleus (Cremer et al. 1993; Cremer and Cremer 2001), while at the few micrometre length scale there is random-walk-like conformation (Yokota et al. 1995) and dynamics (Marshall et al. 1997). This suggests questions about the domains of different dynamical organization. How small a scale does one have to look at before the motion of chromatin domains is dominated by thermal, truly random motion? Is there an intermediate range of lengths where chromatin is moved around mainly by nonrandom forces generated by active processes (e.g., transcription)? These kinds of questions may be answered in the next few years using in vivo visualization techniques, such as the gfp-lac-repressor in vivo labeling technique (Belmont 2001).

In conclusion, experiments suggest that nuclear function is strongly affected by random motion, with thermal conformational fluctuation of chromatin and diffusive motion of proteins playing transport functions. Active transport mechanisms must continually operate against this background of random forces to keep the nucleus ordered. This opposition of random disordering and directed forces means that at some level the interphase chromosome has a structure that is best described statistically. The random-walk–giant loop model of Yokota et al. (1995) and the contact-correlation picture of Dekker et al. (2002) are important steps towards developing such a statistical description.

Mitotic chromosomes

The folding scheme of the mitotic chromosome remains to be understood (Belmont 2002). Our single-chromosome digestion experiments indicate that the internal structure of the mitotic chromosome must involve chromatin domains which are loosely "crosslinked" together (Poirier and Marko 2002*b*). We believe our experiments rule out a large-scale protein "scaffold". If we accept this result, we are then faced with the problem of determining the identity of the crosslinking elements.

Condensins are certainly a good candidate for the mitotic crosslinkers, given their essential role in condensing chromosomes (Hirano and Mitchison 1994). Over the next few years it is likely that condensin function may be clearly assayed using single-molecule techniques. However, the result that in vitro chromosomes are much floppier than in vivo chromosomes suggests that there may be other molecules that help to tighten up the structure of mitotic chromosomes. Belmont (2002) has commented that it is likely that mitotic chromosomes may be organized by a hierarchy of folding schemes.

Another aspect of mitotic chromosomes that is open to biophysical study is the question of how their compaction during prophase is compatible with the removal of entanglements between replicated chromatids. Hirano (1998) has suggested that chromosome condensation may be coupled to resolution of entanglements, and this general picture can be supported by visualization of chromatid separation (Sumner 1991). A theoretical argument can be made in favor of this, where one views chromosome condensation as a process of gradually increasing the density of chromatin crosslinking (Marko and Siggia 1997*b*), but where the crosslinks have a short lifetime, and where topoisomerase II is assumed to be around to pass chromatin through chromatin. The two chromatids will tend to separate as the crosslinks are cycled on and off, because of the lower free energy of the chromatid-segregated (less crowded) state.

Meiosis

Some of the most spectacular chromosome dynamics occur during meiosis, where first there is pairing of homologous chromosomes, recombination, and finally two mitosis-like divisions. In addition, during meiotic prophase, there is in many species the formation of "lampbrush" chromosome structures, where massive tandem transcription generates large (tens of micrometres) visible loops to extend laterally from the chromosome axis. At this stage, the chromosomes may become on the order of a millimetre in length (Callan 1986). The lampbrush structure played an important role in early chromosome structure research, allowing Callan and MacGregor (1958) to show via DNase digestion experiments that each chromosome was held together by DNA. Gall (1963) then carried out quantitative experiments on lampbrushes that established that each chromatid contained just one DNA molecule.

Marvelous pictures of lampbrush chromosomes can be found in the monograph of Callan (1986); it should be noted that the large loops are apparently not in sharp focus, despite the use of flash photography. This is because the loops are in motion, i.e., undergoing thermal conformational fluctuation (Callan 1986, pp. 28–29) This feature of lampbrush chromosomes is an example of flexible polymer behavior of chromatin, on a huge and directly observable scale (Marko and Siggia 1997*b*).

A fundamental question about meiosis concerns the nature of the mechanism of homology search leading to chromatid pairing. Kleckner and co-workers have shown that yeast chromatids start to pair before meiosis, with initial pairing occuring between sites roughly 65 kb apart (Weiner and Kleckner 1994; Kleckner 1996). Does this initial pairing proceed via random collision of chromatin domains, or is there a more active search mechanism? Kleckner has also emphasized the possible role of meiotic chromosome physical properties in chiasma formation and crossover interference (Kleckner 1996; Zickler and Kleckner 1999; Blat et al. 2002). It is of interest to directly study the physical properties of meiotic prophase chromosomes, with particular attention to the role of chromatin loops in controlling chromosome mechanical properties (Marko and Siggia 1997*b*).

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