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Micromechanical Studies of Mitotic Chromosomes

M. G. Poirier[*] *and John F. Marko*[*†]
Departments of *Physics and †Bioengineering
University of Illinois at Chicago, Chicago, Illinois 60607

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We review micromechanical experiments studying mechanoelastic properties of mitotic chromosomes. We discuss the history of this field, starting from the classic *in vivo* experiments of Nicklas (1983). We then focus on experiments where chromosomes were extracted from prometaphase cells and then studied by micromanipulation and microfluidic biochemical techniques. These experiments reveal that chromosomes have a well-behaved elastic response over a fivefold range of stretching, with an elastic modulus similar to that of a loosely tethered polymer network. Perturbation by microfluidic "spraying" of various ions reveals that the mitotic chromosome can be rapidly and reversibly decondensed or overcondensed, i.e., that the native state is not maximally compacted. We compare our results for chromosomes from cells to results of experiments by Houchmandzadeh and Dimitrov (1999) on chromatids reconstituted using *Xenopus* egg extracts. Remarkably, while the stretching elastic response of reconstituted chromosomes is similar to that observed for chromosomes from cells, reconstituted chromosomes are far more easily bent. This result suggests that reconstituted chromatids have a large-scale structure that is quite different from chromosomes in somatic cells. Finally, we discuss microspraying experiments of DNA-cutting enzymes, which reveal that the element that gives mitotic chromosomes their mechanical integrity is DNA itself. These experiments indicate that chromatin-condensing proteins are not organized into a mechanically contiguous "scaffold," but instead that the mitotic chromosome is best thought of as a cross-linked network of chromatin. Preliminary results from restriction enzyme digestion experiments indicate a spacing between chromatin "cross-links" of roughly 15 kb, a size similar to that inferred from classical chromatin loop isolation studies. These results suggest a general strategy for the use of micromanipulation methods for the study of chromosome structure.

I. Introduction

The question of how double-stranded DNAs (dsDNA) that encode the genomes of cells are physically organized, or "folded," is a fundamental yet unresolved problem of cell biology. This is remarkable given the large amount of effort that has been devoted to the traditional microscopy of higher order chromatin structures. The fact that new models for large-scale

chromosome structure (Kimura *et al.*, 1999; Machado and Andrew, 2000a; Dietzel and Belmont, 2001; Losada and Hirano, 2001, Stack and Anderson, 2001) continue to be proposed indicates that this question remains open.

During interphase, gene expression is closely related to chromatin organization. It is inevitable that the physical layout of genes in the nucleus affects their expression, e.g., by affecting the transport of regulatory factors to and mRNAs from transcription loci. However, very little is known about the structures that organize interphase chromosomes. During mitosis, gene expression stops and chromosomes undergo a gross reorganization, or "condensation," into segregated, cigar-shaped mitotic chromatids. Again, very little is known about how the chromatin is folded up at this stage of the cell cycle.

There are many reasons why determination of the chromosome structure in any cell is challenging. However, one of the main problems is certainly *that chromosomes have a dynamic structure*, which changes drastically during the cell cycle (Fig. 1). Studies of chromosome structure make sense only in the context of particular points of the cell cycle in defined cell types. This chapter focuses on the folding of the chromosome in amphibian cells during mitosis, specifically at the stage between prophase and metaphase when chromosomes are completely condensed and the nuclear envelope has been disassembled, but where the chromosomes are not yet attached to the mitotic spindle. We will mainly discuss the structure of prometaphase chromosomes, specifically in epithelial cells from newt (*Notophthalmus viridescens*) and frog (*Xenopus laevis*). These are model organisms for the study of mitotic

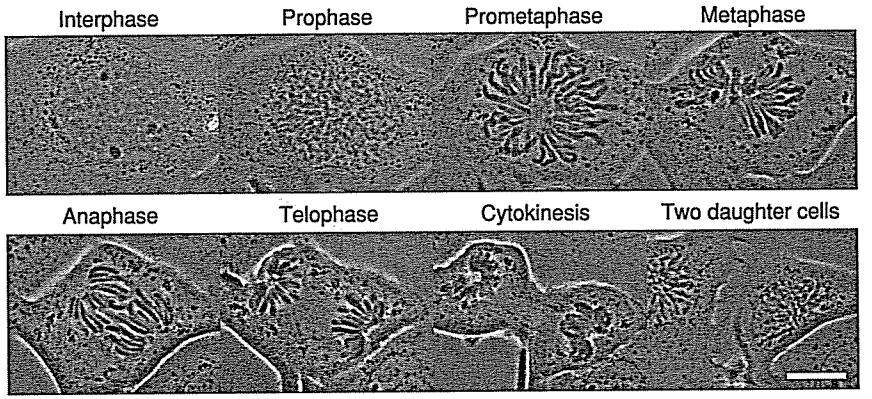


Figure 1 Cell cycle in a newt cell. During mitosis, chromosomes condense inside the nucleus; during prophase, the nuclear envelope disassembles and chromosomes float loose in the cytoplasm; and during prometaphase, they are captured and aligned by the spindle at metaphase. The two duplicate chromatids of each chromosome are pulled apart at anaphase. Bar: 10 μm. Image is a phase-contrast, 60× oil objective.

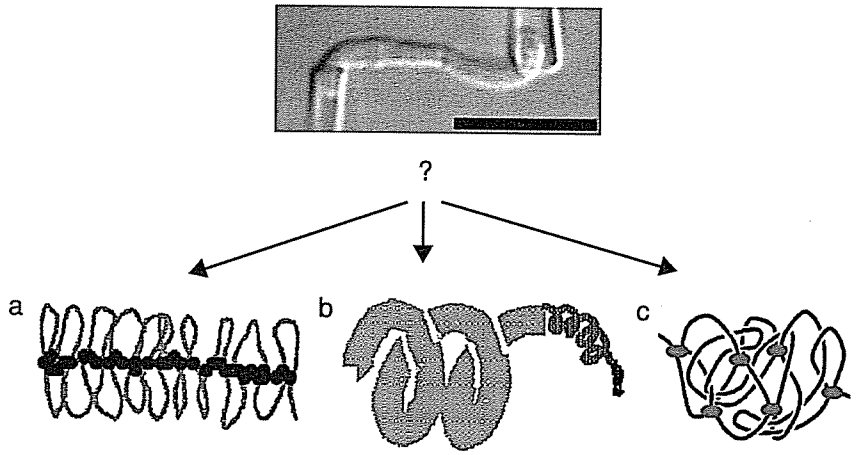


Figure 2 (Top) Prometaphase chromosome attached at its ends to pipettes outside a cell. Bar: 10 μm. Image is a DIC, 60× oil objective. (Bottom) Three possible models of how chromatin is arranged within a mitotic chromosome.

chromosome structure for the simple reason that their chromosomes are large (Fig. 2).

A second problem that chromosome researchers must confront is that *chromosomes are soft physical objects*, with elastic stiffness far less than that of DNAs and proteins from which they are composed. This means that the structures of chromosomes can be destroyed—or changed—by preparations that leave protein and DNA secondary structures intact. This chapter is concerned with reviewing recent studies of mechanical properties of mitotic chromosomes that quantify their softness. Emphasis will be placed on the idea that mechanical measurements can be used to assay structural changes introduced biochemically. We will show how such studies can provide information about higher order chromosome structure.

Section II provides a brief review of previous biophysical studies of chromosome structure and the force response of single DNA molecules and chromatin fibers, the basic constituents of chromosomes. Section II concludes with a summary that seeks to convince the reader that micromanipulation experiments are a useful tool for answering the many questions about the mitotic chromosome structure that are either contentious or unanswered. Section III reviews experiments studying the stretching elastic response of whole mitotic chromosomes. Section IV discusses the bending elasticity of mitotic chromosomes, emphasizing the connection expected between bending and stretching elasticity. The dynamics of stress relaxation in chromosomes are then discussed briefly in Section V.

Section VI then discusses experiments that modify chromosome structure chemically and biochemically, while monitoring the changes in chromosome mechanical properties. This includes discussion of the effects of shifts in salt concentration and DNA-cutting enzymes. The experiments discussed in this section have clear implications for mitotic chromosome structure, and in particular rule out the "contiguous protein scaffold" model, which posits that chromatin fibers are organized as loop domains tethered to an internal and physically connected protein skeleton. Finally, Section VII presents a preliminary model of mitotic chromosome structure based on these results and then discusses some of the many open questions, including the topic of DNA connections between mitotic chromosomes.

Work of Poirier *et al.* (2000, 2001, 2002; Poirier and Marko, 2002a,b) is described in more detail in Poirier (2001). Web materials, including images and movies of experiments, are available at http://www.uic.edu/~jmarko.

II. Architecture and Components of Eukaryote Chromosomes

This section reviews current understanding of the components of chromosomes and overall chromosome structure. It also discusses physical properties of the components of chromosomes, essentially DNA and chromatin fiber, with emphasis on recent micromanipulation experiments. This section is not a complete review of the large literature on the chromosome structure (see Koshland and Strunnikov, 1996; Hirano, 2000), but is meant to brief the reader on some basic structural and biophysical facts about eukaryote chromosomes important to understanding the later sections. The plan of this section is to start from what is best known—the structure of the nucleosome—and then work up to gradually larger chromatin structures, which are less well understood.

A. Eukaryote Chromosomes Are Made of Chromatin Fiber

Eukaryote chromosomes contain similar amounts of genomic dsDNA and protein. Chromosomes of animals contain on the order of 100 Mb of dsDNA (note the useful dsDNA relation 1 Gb = 1 pg). Paradoxically, size and complexity of genomes are not obviously related (Gall, 1981): the largest human chromosomes contain about 300 Mb, whereas some amphibian chromosomes contain more than 1 Gb.

At all stages of the cell cycle, this large amount of DNA is organized into nucleosomes (Kornberg, 1974), octamers of histone proteins around which dsDNA is wrapped. Each nucleosome is about 10 nm in diameter and involves about 200 bp of dsDNA (146 bp wrapped, with the balance as internucleosomal "linker" DNA). The structure of the nucleosome has

been determined precisely using X-ray crystallography (Klug, 1984; Arents *et al.*, 1991; Luger *et al.*, 1997). Remarkable progress has been made in the understanding of the remodeling of nucleosome structure and chemical modification of histones themselves during gene expression (Wolffe and Guschin, 2000). It is clear that there are many structural states of chromatin to understand.

The molecular mass of 200 bp of dsDNA is about 120 kDa, and the molecular mass of the histone octamer plus one "linker" histone (which sits on the linker DNA) is about 125 kDa. Thus the relative weight of dsDNA and histones in chromosomes is roughly equal; histones are a major protein component of chromosomes.

It is known that DNA bound to nucleosomes is able to unbind transiently. Quantitative experiments (Widom, 1997; Polach and Widom, 1995; Anderson and Widom, 2000) show that restriction enzyme access to DNA is attenuated exponentially as one moves into nucleosome-bound DNA. This raises the interesting question of on what time scale, and for what factors, transient access to DNA may occur via conformational fluctuation of the nucleosome itself.

The clarity of understanding of nucleosome structure contrasts with the confusion about how the ~10-nm-diameter nucleosomes are organized into larger scale ("higher order") chromatin structures. Electron microscope (Thoma *et al.*, 1979) and X-ray diffraction (Widom and Klug, 1985) studies suggest that the nucleosomes fold into a ~30-nm-diameter chromatin fiber, possibly with a helical structure. However, little else about supranucleosomal organization ("higher order chromatin structure") is solidly understood. This is a result of the relative softness of chromatin fiber, which leads to the apparent flexible polymer properties of chromatin (Cui and Bustamante, 2000; Marko and Siggia, 1997a; Sec.II.C), plus the inhomogeneity inherent to chromatin. Polymer-like flexibility may also account for observations of nonhelical chromatin fiber structures (Horowitz *et al.*, 1994; Woodcock and Horowitz, 1995).

Chromatin fiber structure is sensitive to ionic conditions. When chromatin fibers are extracted into solution at subphysiological 10 m*M* univalent ionic strength, they are observed in the electron microscope as 10-nm-thick "beads on a string." At the more physiological ionic strength of 100 to 150 m*M* univalent ions, nucleosomes stack into a more condensed, and thicker, 30-nm-thick fiber (Fig. 3). At physiological ionic strength, lateral internucleosomal attractions tend to lead to aggregation of isolated fibers (Van Holde, 1989).

The sensitivity of chromatin fiber to ionic strength is connected to two key concepts. First, *nucleosome–nucleosome interactions have a strong electrostatic component*. The effect of altering univalent ionic strength is to change the strength and range of electrostatic interactions. At low ionic strength,

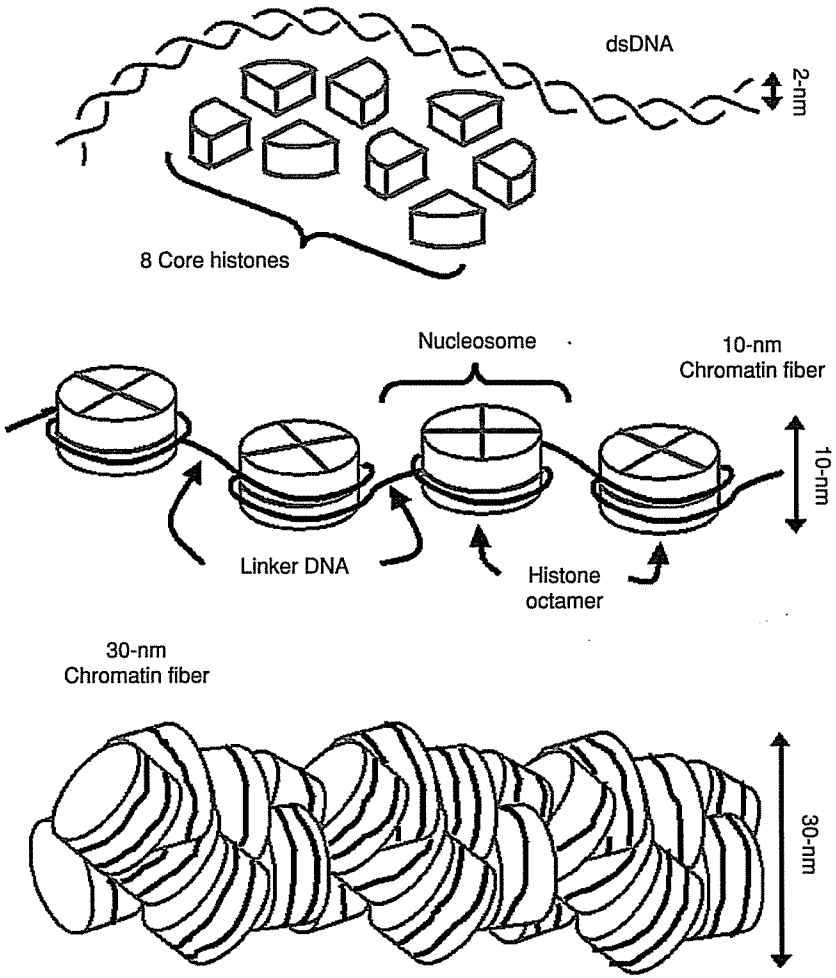


Figure 3 dsDNA, histones, nucleosome, 10-nm chromatin fiber, and 30-nm chromatin fiber. Structural–biological studies of chromatin have focused on the ultrastructure of isolated nucleosomes and on studying the conformation of nucleosomes in the 10- and 30-nm fiber.

electrostatic interactions are strong and have a long range, causing the like-charged nucleosomes (chromatin fiber has a net negative charge, similar to dsDNA) to repel sufficiently to open chromatin fiber up. At higher ionic strength, the reduced strength and range of electrostatic repulsion are overcome by attractive nucleosome–nucleosome interactions mediated by histone tails and histone H1, and the fiber folds up.

The second key concept, which is perhaps less familiar, is that *chromatin fiber is relatively soft*, or equivalently that internucleosomal interactions

are relatively weak. The change in ionic strength from 10 to 100 mM has a drastic effect on chromatin fiber, yet the nucleosomes themselves do not undergo major conformational changes. The strong electrostatic histone–dsDNA interactions are relatively unperturbed until much higher ionic strengths (~0.8 M Na^+) are reached. Similarly, the dsDNA structure is essentially insensitive to this change in ionic strength; over the range 0.01 to 0.1 M Na^+ the main effect on the double helix is an increase in the melting temperature of about 10°. The softness of chromatin fiber, relative to the relative "stiffness" of dsDNA and nucleosome structure, is important in understanding chromosome physical properties.

The physiological 30-nm chromatin fiber is thought to be anywhere from 10- to 50-fold shorter in contour length than the underlying dsDNA. A widely used estimate results from the compaction of the 1200-bp associated with six nucleosomes into one 10-nm-thick turn of helical chromatin fiber: the resulting 120 bp/nm for chromatin is about 40 times less than the 3 bp/nm for dsDNA. In fact, this 40-fold compaction factor has not been convincingly given by experiments. Given that it is known that some nucleosomes are positioned, some are mobile, and that there are a wide range of histone modifications and variants, it seems unlikely that there is a universal chromatin fiber structure or length compaction factor.

B. Micromechanics of Double-Stranded DNA

A new approach to biophysical characterization of DNA is mechanical manipulation of single molecules, with molecular tension as an experimentally controllable and measurable quantity. The general idea is to quantify intermolecular interactions by direct force measurement and to observe self-organization processes of single or small numbers of molecules. Methods used to study single dsDNAs are all based on attaching the ends of the molecule to large objects, which act as "handles" (Bustamante *et al.*, 2000). The handles are used to apply controllable forces and to provide an optical marker for the molecule ends and therefore end-to-end extension. Although these techniques usually are restricted in application to molecules of at least a few kilobases in length, ingenious techniques (Bustamante *et al.*, 2000) have been developed to measure conformational changes of just a few nanometers (Liphardt *et al.*, 2001). This section focuses mainly on what has been learned about dsDNA mechanical properties using these techniques in preparation for discussing similar force–distance experiments on whole chromosomes. We also use the example of dsDNA to introduce some of the basic ideas of polymer elasticity used to discuss chromosome extensibility.

dsDNA itself has mechanical properties that are well characterized and understood. dsDNA has a *persistence length* of about A=50 nm (150 bp in

B form) (Hagerman, 1988). The persistence length is the contour length over which thermal (Brownian) fluctuations can dynamically bend the double helix appreciably (e.g., through a 60° bend). Thus, over dsDNA lengths of less than 150 bp, the contour is of fixed shape (the double helix is in general roughly straight, but some sequences are intrinsically rather severly bent). Over distances longer than 150 bp, a dsDNA undergoes appreciable dynamic bending.

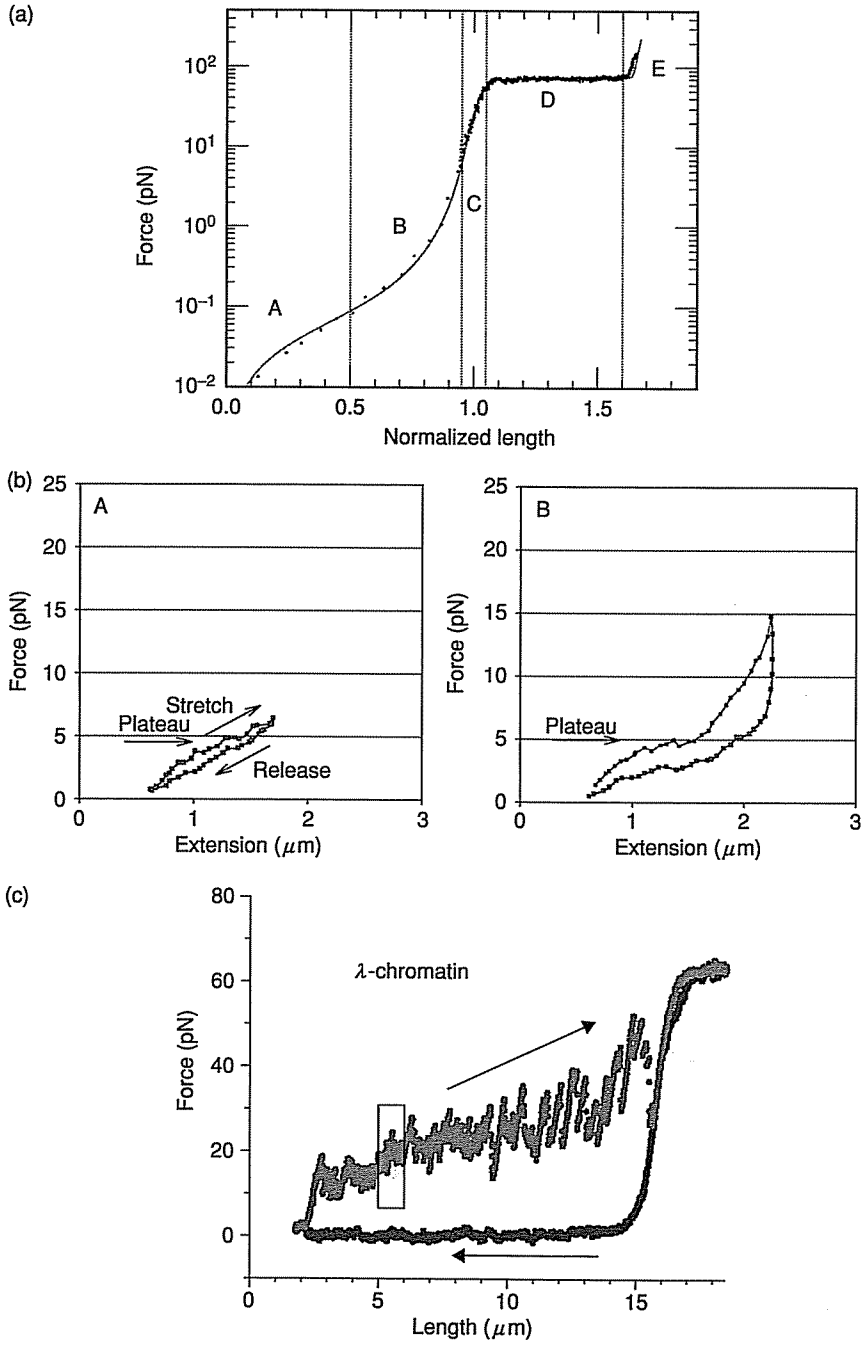
If one stretches a long dsDNA out, thermally excited bends will require a certain tension to be straightened (Fig. 4a). This tension is about kT/A, where $kT = 4.1 \times 10^{-21}$ J is the energy associated with a single thermal fluctuation at room temperature $T \sim 300$ K (note $kT = RT/N_A$, where R is the familiar gas constant and N_A is Avogadro's number; RT is simply the thermal energy of 1 mol of thermal fluctuation, i.e., about the heat inside 1 mol of a simple gas or liquid). This characteristic tension is about 0.1×10^{-12} Newtons (N; note 1 J/m = 1 N) or about 0.1 piconewtons (pN). Below 0.1 pN, one can think of a dsDNA as being a spring, with extension proportional to applied tension; at 0.1 pN a dsDNA is extended to slightly greater than half its total contour length. At higher forces (0.1 to 10 pN) dsDNA elasticity is highly nonlinear, with tension increasing quickly as the length approaches that of the B form (3 bp/nm) (Smith *et al.*, 1992; Bustamante *et al.*, 1994).

The characteristic tension to begin to extend a dsDNA (0.1 pN) is a small force, even by single molecule standards. Cellular motor proteins generate forces ranging from a few piconewtons (myosin: 5 pN, kinesin: 8 pN) to tens of pN (RNA polymerase: 40 pN; Yin *et al.*, 1995), roughly because they convert chemical energy at the rate of a few kT per nanometer of motion (note that 1 kT/nm = 4 pN). Another source of tension on dsDNA *in vivo* is DNA–protein interaction; e.g., it has been demonstrated that polymerization of RecA onto dsDNA generates forces in excess of 50 pN (Leger *et al.*, 1998). In the cell, dsDNA thus can be stretched out and modified structurally by forces generated by the machinery that transcribes (Yin *et al.*, 1995), replicates (Wuite *et al.*, 2000), and repairs it.

From forces of 0.1 to 10 pN, the dsDNA elastic response is well expressed by the empirical force law (Bustamante *et al.*, 1994):

$$f = \frac{kT}{A}\left[\frac{x}{L} + \frac{1}{4(1-\frac{x}{L})^2} - \frac{1}{4}\right] \tag{1}$$

where A is the persistence length of 50 nm and where x is the molecule end-to-end extension and L its total B-form contour length. Equation (1) captures the weak initial elastic response where force increases from 0 to about kT/A as x/L increases from 0 to about 0.5, and the strong nonlinear force increase as x approaches L. These two features are generic for all flexible



polymers that undergo random walk-like bending fluctuations when unstretched.

For even larger forces (10 to 100 pN), the dsDNA secondary structure starts first to stretch (10–50 pN) and then the double helix is disrupted and stretches to an extended form at ~65pN (Cluzel *et al.*, 1996; Smith *et al.*, 1996). This disruption has a strong DNA twisting dependence (Allemand *et al.*, 1998; Leger *et al.*, 1999). Measurements of the stretching of the double helix structure have shown that dsDNA can be thought of as an elastic rod, of elastic *Young modulus* $Y \sim 300$ MPa. The meaning of Y comes from the force needed to stretch an elastic rod of uniform and circular cross section and equilibrium (unstretched) length L so as to increase its length by ΔL:

$$f = \pi r^2 Y \frac{\Delta L}{L} \tag{2}$$

Here, r is the cross-sectional radius of the rod (for dsDNA, $r = 1$ nm; note that for the general cross-section shape, πr^2 can be replaced by the rod cross-sectional area).

The Young modulus is thus the stress (force per cross-sectional area) at which an elastic rod would be doubled in length if its initial linear elasticity could be extrapolated: Y characterizes the stretching elasticity of a material in a shape-independent way. Similarly, $f_0 = \pi r^2 Y$ is the force at which a rod would double in length, based on extrapolation of its linear elasticity. For dsDNA, $f_0 \sim 1000$ pN, and like most solid materials, Eq. (2) applies only for $\Delta L/L$ much less than unity [for dsDNA, the regime where Eq. (2) applies is from $\Delta L/L = 0.0$ to about 0.05, where 0.0 refers to the B-DNA length).

The bending flexibility of an elastic rod is also related to Y. The bending modulus of an elastic rod, again assuming linear elasticity and circular uniform cross section, is

$$B = \frac{\pi}{4} r^4 Y \tag{3}$$

This quantity has dimensions of energy times length. If our elastic rod is bent into a circular arc of bending radius R, the torque that must be applied is B/R, and the force that must be applied is B/R^2. For dsDNA, $Y = 300$ MPa gives $B = 2\times10^{-28}$ Jm.

Figure 4 Comparison of elastic response of (a) single dsDNAs and (b and c) chromatin fibers. dsDNA and the chromatin fiber both display an initial low-force (sub-pN) elastic regime, followed by a higher force (few pN) regime. However, dsDNA shows a very stiff and nonlinear response (A), whereas the chromatin fiber shows a more gradual elastic response (B) (Cui and Bustamante, 2000) as it is extended. This is believed to be due to driving the chromatin fiber opening transition (10- to 30-nm fiber transition of Fig. 3) by force. (C) Low-force elasticity is also seen (Bennink *et al.*, 2001); at higher forces, force jumps corresponding to nucleosome removal events are observed. (See Color Insert.)

For rods that are thin enough to be bent by thermal fluctuation (e.g., the double helix), it is useful to relate B to the bending persistence length A:

$$A = \frac{B}{kT} = \frac{\pi r^4 Y}{4\, kT} \tag{4}$$

For dsDNA, we therefore see that Y=300 MPa gives an estimate of A = 50 nm, essentially the observed value. The connection between the value of Y obtained from stretching the double helix with that obtained from separate measurement of the persistence length A shows that elementary concepts of elasticity at least roughly apply at the nanometer scale of the interior of the double helix.

Micromechanical studies of DNA have allowed detailed studies of DNA stretching (Smith *et al.*, 1992; Bustamante *et al.*, 1994), DNA twisting and supercoiling (Strick *et al.*, 1996), stress-driven DNA structural transitions (Cluzel *et al.*, 1996; Smith *et al.*, 1996; Allemand *et al.*, 1999; Leger *et al.*, 1999), and DNA strand separation by force (Essevaz-Roulet *et al.*, 1997). Application of these techniques to the study of nucleic acid processing and reorganization by proteins is a direction of intensive current research. Protein–DNA interactions, which have been studied micromechanically, include force generation during transcription (Yin *et al.*, 1995), force generated by DNA polymerase (Wuite *et al.*, 2000), direct study of a single DNA loop formed by lac repressors (Finzi *et al.*, 1995), dynamics of stretching of DNA by RecA (Leger *et al.*, 1998), and observation of DNA strand exchange by topoisomerase II (Strick *et al.*, 2000).

C. Micromechanics of Chromatin Fibers

The force–extension properties of chromatin fiber extracted from chicken erythrocytes (Cui and Bustamante, 2000) have been measured. Because chromatin fibers are far more complex than single dsDNAs, their mechanical response is complicated. Three different force regimes have been reported. First, a very low-force "entropic elasticity" regime is observed, similar to that seen for dsDNA. This initial low-force (below 0.1 pN) force response is thought to be due to the polymer flexibility of chromatin and allows an estimate of chromatin persistence length of about 30 nm, slightly shorter than dsDNA itself. This low persistence length is possible due to the zig-zag path of the linker DNA: a spring (a "Slinky" toy is a good example) can be bent more easily than the wire from which it is formed. However, quantitatively useful data for chromatin low-force (<0.1 pN) "polymer" elasticity under physiological conditions have not yet been published.

At higher forces (0.1 to 5 pN), what is observed depends strongly on ionic conditions, as one would expect based on the 10- to 30-nm fiber transition

observed with increasing ionic strength. At relatively low (10 mM Na^+) ionic strength, a strongly nonlinear elastic response similar to that of dsDNA is observed. However, at closer to physiological ionic strength (40 mM Na^+), a more gradual, nearly linear elastic response is observed for forces between 0.1 and 5 pN (visible at the left of Fig. 4b, data from Bennink *et al.*, 2001). This can be explained in terms of the unstacking of adjacent nucleosomes, i.e., by the idea that force can be used to drive the 30- to 10-nm fiber transition. This transition is observed to be reversible and is characterized by a force constant $f_0 \sim 5$ pN and a high degree of smooth extensibility (compare with "bare" dsDNA, which has a stretching force constant of 1000 pN and can be stretched by only about 5% before transforming to a new stretched form). The doubling in length of the chromatin fiber over a 5 pN increase in force observed by Cui and Bustamante (2000) can be combined with the native fiber 30-nm diameter to estimate an effective Young modulus, $Y \sim 100$ kPa, far below the effective modulus of straight DNA ~300 MPa. As DNA is folded up into chromatin, its effective modulus is reduced.

At higher forces (20 pN), irreversible extension of chromatin fiber occurs (Cui and Bustamante, 2000). This has been observed to be in the form of a series of jumps of quantized length (Fig. 4b). These jumps are thought to be associated with the removal of single nucleosomes. Brower-Toland *et al.* (2002) showed that half-nucleosome (~80 bp) winds of DNA can also be released using similar tensions. It is likely that this threshold for nucleosome removal is highly extension rate dependent, as the known binding free energy ~20 to 30 kT/nucleosome indicates that one should expect equilibrium between bound and free nucleosomes for forces near 2 to 3 pN (Marko and Siggia, 1997b).

Observation of this equilibrium for pure chromatin fiber would require long experimental time scales, as the barrier associated with nucleosome removal or rebinding is likely close to the 20-kT binding energy. However, use of nucleosome assembly factors such as NAP-1, which act in thermal equilibrium, may make it practical to observe chemical equilibrium between octamer on- vs off-states (S. Leuba, private communication).

The experiments of Cui and Bustamante (2000) used chromatin fibers isolated from cells, but more recent experiments have assembled chromatin fibers *in vitro* onto initially bare molecules of dsDNA. One way to proceed is to use salt dialysis assembly (Brower-Toland *et al.*, 2002), which titrates histone–DNA interaction strength allowing nucleosomes to form along dsDNA. Another strategy is to use cell extract-derived chromatin assembly systems (Ladoux *et al.*, 2000; Bennink *et al.*, 2001), which have allowed measurement of the ~10-pN forces applied during chromatin assembly. Purified chromatin assembly enzymes such as NAP-1 promise to provide a biochemically defined assembly system. These strategies promise to allow

assembly of nucleosome arrays where the underlying DNA is known and which fold into 30-nm-like fibers.

A key result of the chromatin fiber studies to date is that the elastic response of chromatin fiber is very different from that of the underlying dsDNA. The presence of nucleosomes masks the divergent force response of dsDNA elasticity. Weak initial entropic elasticity is followed by reversible unfolding at the few piconewton force scale, whereas at larger forces ~10 pN, nucleosomes are irreversibly popped off. Many questions remain, just as one example the degree of variation of chromatin physical properties as a function of histone modifications that occur *in vivo*.

D. Chromosome Structure at Scales Larger Than the Chromatin Fiber

Beyond the chromatin fiber, it is thought that nonnucleosome proteins act to define chromosome structure. During interphase, this includes the machinery of gene regulation and expression, centers of DNA replication (Cook, 1991), and the nuclear matrix (Wolffe, 1995, Section II,D,2), all of which are beyond the scope of this chapter. This section focuses on what is known about the large-scale chromosome structure, with emphasis on the mitotic chromosome structure. We describe conclusions of classical microscopy-based studies, plus newer insights obtained from three-dimensional studies of chromosome structure and dynamics. Finally, we discuss recent work on biochemical characterization of proteins from cell-free chromosome assembly systems.

1. Structural–Biological Studies of Mitotic Chromosome Structure

Much of our understanding of the mitotic chromosome structure at larger scales is mainly based on relatively invasive electron microscopy (EM) studies and on optical microscopy. Based on EM visualization of DNA loops extending from an apparent protein-rich chromosome body after histone depletion (Paulson and Laemmli, 1977; Paulson, 1988), and to some extent on direct visualization of these chromatin loops in fixed cells, one commonly discussed model for mitotic chromosome structure is based on labile chromatin loops interconnected by a protein-rich "scaffold" (Marsden and Laemmli, 1979; Fig. 2). Other studies suggest that the scaffolding is coiled (Boy de la Tour and Laemmli, 1988).

These experiments are often taken to imply the existence of a connected protein "skeleton" inside the mitotic chromosome (see Lewin, 2000; Lodish *et al.*, 1995; Wolffe, 1995). Paulson and Lamelli (1977) concluded that the scaffold was a fibrous network of nonhistone proteins and was responsible for the basic shape of metaphase chromosomes, and Lamelli

et al. (1978) emphasized that the scaffold could be isolated as a structurally independent stable entity. However, slightly later discussions (Marsden and Lamelli, 1979) suggested that the question of whether the native scaffold is stabilized through protein–protein interactions is unresolved. Intriguingly, an old literature of whole chromosome DNaase digestion experiments (Cole, 1967) suggests that if DNA is cut sufficiently often, the chromosome disintegrates. Laemmli (2002) has emphasized to one of us that the conclusion that the internal protein skeleton is mechanically contiguous does not follow from his results. Therefore, the question of connectivity and mechanical integrity of the DNA and non-DNA components of the mitotic chromosome remains open; this is a primary focus of Section VI.

Other microscopy studies suggest a hierarchical structure formed from a succession of coils at larger length scales (Belmont *et al.*, 1987, 1989; Fig. 2). Proposals have since been made for a mitotic chromosome structure that combines loop and helix-folding motifs (Saitoh and Laemmli, 1993). Existing microscopy studies do not give a clear and consistent idea of chromatin structure in mitotic chromosomes, in part because of the invasive preparations necessary for EM visualization and the inability of light microscopy to observe structures smaller than ~200 nm *in vivo*.

The folding scheme of interphase chromatin inside the nucleus pre-1990 was highly unsettled. With no techniques to differentiate different chromosomes or chromosomal regions, light microscopy by itself reveals little, and electron microscopy again leads to conflicting views of chromatin structure at length scales from 10 to 100 nm. Biochemical analysis of chromatin domains (Jackson *et al.*, 1990) suggests that the interphase chromatin is organized into ~50-kb domains.

2. Three-Dimensional Microscopy Study of Chromosome Structure and Dynamics

An increasing use of fluorescent labeling and optical sectioning microscopy techniques in the 1990s allowed many features of chromosome structure to be determined by mapping the physical position of specific DNA sequences with ~300 nm precision. Fluorescent *in situ* hybridization (FISH) and other techniques applied to whole chromosomes show that different chromosomes occupy different regions or "territories" of the interphase nucleus (Cremer *et al.*, 1993; Zink *et al.*, 1998) and has also shown the existence of interchromosomal regions.

Similar studies where specific chromosome loci were tagged have been used to measure the real-space distance between genetic markers as a function of the chromatin length between the markers. Remarkably, these studies show interphase chromosomes to have a random walk-like organization at

<1-Mb scales and a "loop" organization at 1- to 100-Mb scales (Yokota *et al.*, 1995). Similar studies have been used to study attachments of chromosomes to the nuclear envelope (Marshall *et al.*, 1996). The structure of the bulk of the interphase nucleus remains uncertain, with the role of a nucleoskeleton ("nuclear matrix") in chromosome organization still unclear (Pederson, 2000).

A FISH study of loci along metaphase chromosomes has also been done to verify that genes are in linear order at >1-Mb scales. However, markers spaced by less than 1 Mb are often seen in random order, indicating that at the corresponding <1-μm scale, metaphase chromatin is not rigidly ordered (Trask *et al.*, 1993). This lack of determined structure is consistent with the flexible loop domain picture of the metaphase chromosome structure (Fig. 2), although one might argue that the fixation used somehow distorted structures at these scales.

Structural studies have also been done *in vivo* by the use of live cell dyes for specific structures, by the incorporation of fluorescent nucleotides (Manders *et al.*, 1999), and by the expression of fusions of chromosome-specific proteins with green fluorescent protein (GFP) (Tsukamoto *et al.*, 2000; Belmont, 2001). One study used both techniques to show that there are ~1-μm position fluctuations of interphase chromosome loci from a range of species (Marshall *et al.*, 1997). These fluctuations persisted even in poisoned cells, suggesting that ~Mb chromosome segments are free to undergo thermal fluctuation, in the manner of flexible polymers. This result is at odds with the idea of a dense, rigid nucleoskeleton and suggests instead that chromosomes have intermittent attachments, with ~Mb regions of chromatin free to move on micrometer-length scales.

A study of the yeast (*Saccharomyces cerevisiae*) interphase chromosome structure by Dekker *et al.* (2002) is unique in its methodology and results. This study used cross-linking of isolated nuclei, followed by restriction enzyme digestion. The fragments were self-ligated, and the resulting fragments were polymerase chain reaction amplified and analyzed. The result was a statistical "map" of *in vivo* chromatin contacts, giving a statistical three-dimensional chromosome model. This technique may provide a way to map chromosome structure and dynamics in unprecedented detail.

3. Chromosome-Folding Proteins Identified Using Cell-Free Chromosome Assembly Systems

An alternative to the deconstruction of chromosomes from live cells is to study chromosomes assembled *in vitro* using cell-derived factors. *Xenopus* egg extracts provide an excellent system for doing this, allowing the conversion of *Xenopus* sperm chromatin into either interphase nuclei or metaphase-like chromatids (Smythe and Newport, 1991). This system has

permitted the identification of proteins thought to be critical to organizing mitotic chromosomes, most notably the SMC protein family (Hirano and Mitchison, 1994; Strunnikov *et al.*, 1993, 1995; Strunnikov, 1998).

Hirano and Mitchison (1994) showed that if the XCAP-C/E proteins (two of the SMC proteins in *Xenopus*) were removed from the *in vitro* mitotic chromosome system, then only a cloud of tangled chromatin fibers would result instead of mitotic chromatids. Furthermore, anti-XCAPs were found to destabilize assembled mitotic chromatids, indicating that XCAP-C/Es were needed both for assembly and for maintenance of the mitotic chromosome structure. Hirano and Mitchison (1994) also found that the XCAPs were localized inside the mitotic chromatids, possibly on a helical or lattice structure. Further work of Hirano and co-workers established that XCAPs in "condensin" complexes (Hirano, 1997) show an ATP-dependent DNA supercoiling capability that was interpreted in terms of a DNA coiling function (Kimura *et al.*, 1997, 1999). Other SMC-type proteins have other roles in modulating the 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) suggested that the balance between condensin and cohesin SMCs determines large-scale metaphase chromosome morphology.

Many questions remain about the SMC proteins, which have a remarkable structure of ~100-nm coiled-coils with a central hinge (Melby *et al.*, 1998) and ATP-binding and -hydrolyzing end domains (Fig. 5). Their distribution inside mitotic chromatids, clear revelation of their function in chromosome condensation, and whether they are the major proteins of the "mitotic protein scaffold" all remain unknown. Thanks to the biochemical characterizations described earlier, these questions may be answered through gradual "biochemical dissection" (Hirano, 1995, 1998, 1999).

Transcriptionally functional interphase nuclei can also be assembled readily from *Xenopus* egg extracts (Smythe and Newport, 1991). This system has been used to analyze nuclear assembly, transcription, and nuclear import. To date, there has been only limited progress in identifying chromosome-organizing nuclear proteins based on *in vitro*-assembled nuclei, presumably due to the increased complexity of the interphase nucleus relative to the metaphase case.

4. Topoisomerase II

One of the most common proteins found in mitotic chromosomes is topo II (Gasser *et al.*, 1986), the enzyme that passes dsDNA through dsDNA and which is assumed to be the enzyme primarily responsible for removing entanglements of chromatin fiber during chromosome condensation and segregation. This idea is strongly supported by experiments using

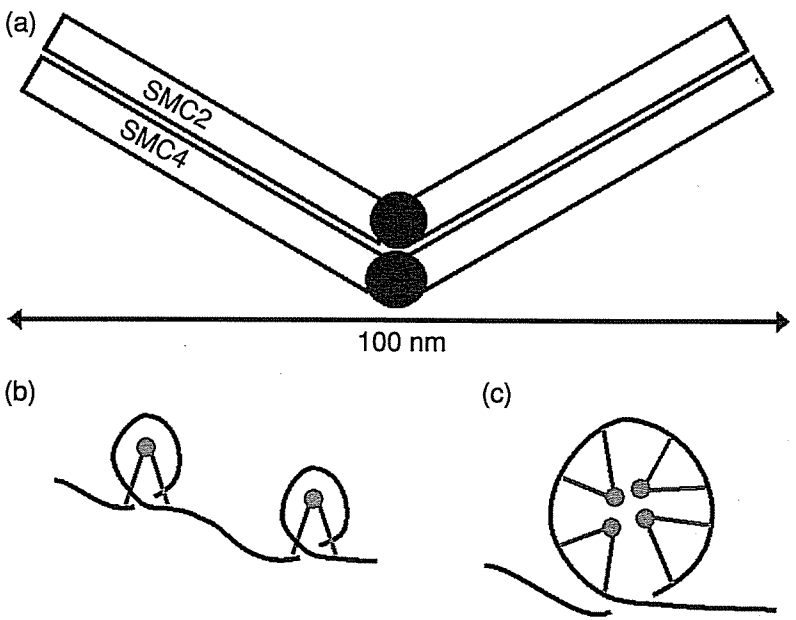


Figure 5 SMC proteins play a role in the higher order mitotic chromosome structure. Condensin SMC complexes (a) include a ~100-nm-long hinged dimer with ATP- and DNA-binding domains at each end. These complexes are thought to bind together (b), or perhaps coil (c), chromatin fibers.

mitotic *Xenopus* egg extracts: when topo II is depleted, sperm chromatin just forms a cloud of apparently entangled chromatin fibers, which never form condensed and segregated chromatids (Adachi *et al.*, 1991).

However, a second hypothesis that topo II also plays a structural role in mitotic chromosomes is contentious (Warburton and Earnshaw, 1997). Immunofluorescence studies show that topo II is localized into helical tracks inside chromatids (Boy de la Tour and Lamelli, 1988; Sumner, 1996). Combined with the fact that topo II interacts with two strands of dsDNA, this result suggests that topo II might be part of an internal protein structure in the mitotic chromosome. However, other experiments use salt treatment to deplete topo II from mitotic chromosomes, with no apparent deleterious effect on their structure (Hirano and Mitchison, 1993). It has been reported that the axial distribution of topo II may be triggered by cell lysis while *in vivo* it is mobile (Christensen *et al.*, 2002). These experiments can be reconciled by supposing that topo II is critical for establishment of the mitotic chromosome structure by allowing dsDNA disentanglement, that it is present in a high copy number on the assembled mitotic chromosome, but

that it does not play a crucial role in holding the mitotic chromosome together.

5. Chromosomal Titin

Titin is a huge protein of filamentous structure and is the elastic restoring element of sarcomeres (Trinick, 1996). The mechanical response of isolated titin molecules has been measured precisely using single molecule manipulation (Kellermayer *et al.*, 1997; Reif *et al.*, 1997; Tskhovrebova *et al.*, 1997). Because of its structure, a long series of independently folded globular domains, titin displays initial linear elasticity followed by a series of irreversible force jumps associated with successive domain unfolding events. Remarkably, it was found that muscle titin antibodies localize onto mitotic chromosomes (Machado *et al.*, 1998; Machado and Andrew, 2000a,b). It has been therefore speculated that a putative chromosomal titin might play a role in chromosome condensation and might be a contributor to the chromosome elastic response (Houchmandzdeh and Dimitrov, 1999).

E. Why Study Mitotic Chromosomes Micromechanically?

The structure of chromosomes beyond the nucleosomal scale is poorly understood, partly because chromosomes are dynamic, having quite different structures at different points of the cell cycle, and partly because chromatin is inhomogeneous and soft. In particular, mitotic chromosomes are soft, without a regular structure that can be studied by X-ray crystallography. The mitotic chromosome is a logical starting point for the study of chromosome structure, as in this stage of the cell cycle the chromosome is packaged (condensed), the chromosomes are segregated from one another, and gene expression is halted, all of which appear to be simplifying factors. In addition, study of the mitotic chromosome structure will presumably shed light into the mechanism of chromosome disentanglement and condensation (Hirano, 2000), and lessons learned from study of the mitotic chromosome may be applicable to the presumably more difficult problem of understanding the interphase nucleus.

Basic questions about the mitotic chromosome of interest to us include the following: What is the physical arrangement of chromatin fiber (randomly or regularly coiled or folded?)? What are the molecules (proteins?) that accomplish this folding? What molecules are necessary to keep the mitotic chromosome folded up? How are the processes of chromosome condensation and disentanglement coordinated? All of these questions have a mechanistic as well as a biochemical character and might be attacked using a combination of biochemical and *micromechanical* experimental methods.

In addition to studying chromosome structure, biophysical chromosome experiments provide information relevant to understanding a range of *in vivo* chromosome biology questions. For example, stresses applied to chromosomes are known to play a role in chromosome alignment and segregation during mitosis (Alut and Nicklas, 1989; Li and Nicklas, 1995, 1997; Nicklas, 1997, 1998; Nicklas *et al.*, 1994, 1995, 2001; King *et al.*, 2000). Kinetochore chromatin elasticity is central to a recent model for the capture of mitotic chromosomes on the mitotic spindle (Joglekar and Hunt, 2002), and chromosome stretching has been used to study the roles of specific proteins in chromatin compaction (Thrower and Bloom, 2001). Chromosome stiffness has also been proposed to play a role in the mechanism of meiotic synapsis (Kleckner, 1996; Zickler and Kleckner, 1999).

The next sections focus on our experiments that seek to study mitotic chromosome structure using their elastic response. Elegant and pioneering experiments of Nicklas (1983) showed that meiotic metaphase I chromosomes have well-defined elastic properties. We have carried out studies reaching the same general conclusion for mitotic chromosomes removed from amphibian cells. Combining micromanipulation and the *in situ* reaction techniques of Maniotis *et al.* (1997), we are able to monitor the elastic response of whole chromosomes while biochemical reactions are being carried out on the underlying chromatin. The goal of these studies is to diagnose changes in chromosome structure made biochemically via observation of changes in chromosome elasticity.

III. Stretching Elasticity of Chromosomes

The extensibility of chromosomes has been studied by a number of researchers and for many years (Callan, 1954; Bak *et al.*, 1977, 1979; Nicklas, 1983; Claussen, 1994). Mitotic chromosomes can be observed to occasionally be stretched out by spindle forces (Fig. 6), and their extensibility is possibly a result of the fact that they contain up to meter-long DNA molecules. However, when studied in more detail, mitotic chromosomes display remarkable elasticity, with stretching properties reminiscent of a network of highly elastic filaments. This section presents some history of chromosome extensibility studies and then describes experiments studying chromosome-stretching elasticity.

A. Lampbrush Chromosomes

One of the earliest discussions of extensibility of chromosomes was by Callan (1954), who carried out manipulation experiments on amphibian lampbrush chromosomes using glass microneedles. The lampbrush phase

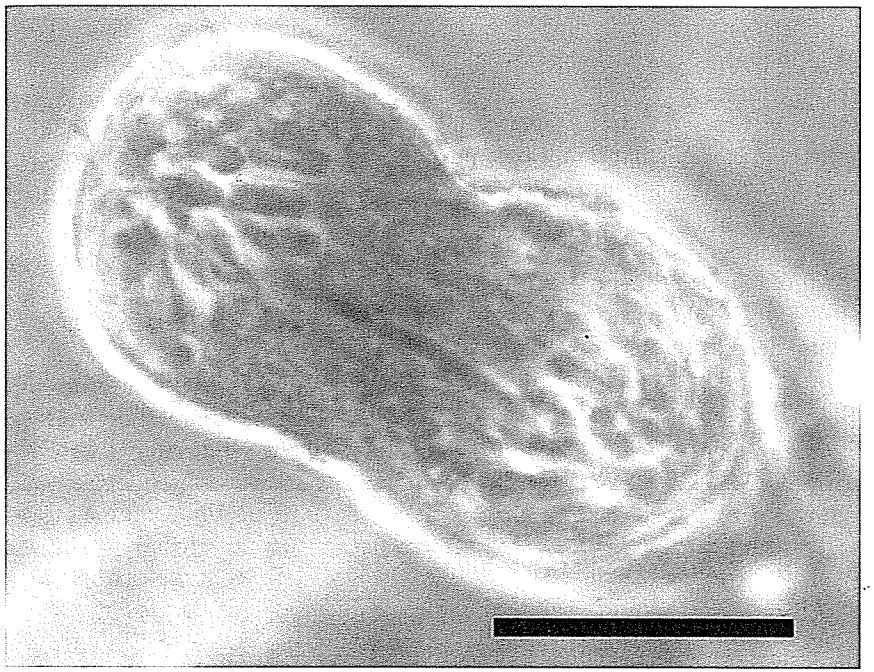


Figure 6 A newt (*N. viridescens*) tissure culture cell showing a chromosome being stretched to about twice its native length by the mitotic spindle during anaphase. Spindle forces are known to be on the order of 1 nN, indicating that the force constant of a whole chromosome is on a similar scale. Bar: 20 μm. Photo courtesy of Professor J. Tang.

occurs during female meiotic prophase in birds and amphibians and has played a special role in cell biology for three reasons. First, lampbrush chromosomes are huge, even by amphibian standards, up to ~1 mm long. Second, they display large flexible loop domains tethered to a central axis (Gall, 1956). The basic idea of chromatin loops tethered to a central chromosome axis, clearly the case for lampbrushes, has been used as a basic model for chromosome structure at other cell stages, notably mitosis. Third, the large lampbrush loops are "puffed up" by RNA transcripts coming off tandem polymerases. Electron microscope observation of the tandem transcription units along lampbrush loops provided early and convincing evidence of the processive nature of transcription (Miller and Beatty, 1969; Miller and Hamkalo, 1972). Particularly this aspect of lampbrush structure continues to be an area of active research (Morgan, 2002).

Callan (1954) carried out lampbrush chromosome-stretching experiments using glass microneedles and observed that they could be stretched to centimeter lengths. Observations of DNAase breakage of lampbrush

chromosomes by Callan and Macgregor (1958) and Gall (1963) were used to support the hypothesis that each chromatid contains a single linear DNA. This hypothesis, which has been proven to be true for small eukaryote chromosomes (e.g., for yeast, by isolation and analysis of whole genomic DNAs), is assumed to be true for all eukaryotes. In addition, these experiments made clear that lampbrush chromosomes are held together by nucleic acid and not by an internal non-DNA structure. The highly quantitative work of Gall (1963) further established that the large lampbrush loops are extended regions of individual chromatids.

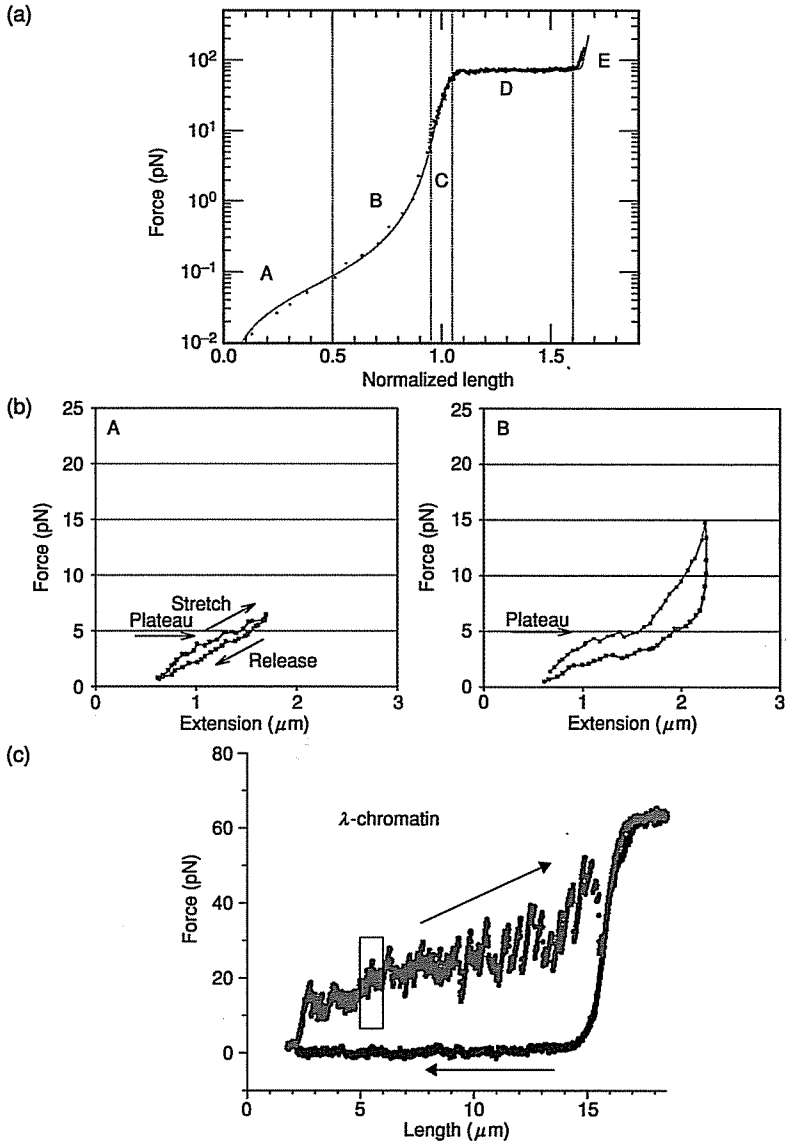
Marvelous pictures of lampbrush chromosomes can be found in Callan (1986); it should be noted that the large loops are apparently not in sharp focus, despite the use of flash photography. This is because the loops are in *motion*, i.e., undergoing thermal conformational fluctuation (Callan, 1986). This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

B. Mitotic Chromosome Extensibility and Elasticity

As mentioned earlier, direct observation of stretching of chromatids by the mitotic spindle, plus the huge length of DNA per chromatid, leads naturally to the notion that mitotic chromosomes should be extensible. This expectation was verified in work by Nicklas and Staehly (1967), who used microneedles to hook chromosomes inside grasshopper spermatocytes and demonstrated that meitoic chromosomes (metaphase I through anaphase I) were extensible and elastic, i.e., would return to native length after being stretched by up to eight times.

1. Nicklas' Study of Chromosome Elasticity in Grasshopper Spermatocytes

The first experiment to quantify the elastic response of a chromosome *in vivo* was carried out by Nicklas (1983) using microneedles to carry out experiments inside living cells. The cells used were grasshopper (*Melanoplus sanguinipes*) spermatocyte cells, which have a soft cell cortex that allows needles to grab chromosomes without breaking the cell membrane (a few additional experiments on cricket spermatocytes are also reported). Then, forces were measured by observing the bending of the microneedle and then calibrating the force needed to cause such bending. Microneedles were used that required between 0.076 and 0.25 nN/μm of deflection (1 nN = 10^{-9} Newton; recall 1 Newton = 1 kg m/s^2). Using a film analysis technique, the minimum resolvable deflection of about 0.25 μm gave a force resolution of roughly 0.05 nN.



Chapter 2, Figure 4 Comparison of elastic response of (a) single dsDNAs and (b and c) chromatin fibers. dsDNA and the chromatin fiber both display an initial low-force (sub-pN) elastic regime, followed by a higher force (few pN) regime. However, dsDNA shows a very stiff and nonlinear response (A), whereas the chromatin fiber shows a more gradual elastic response (B) (Cui and Bustamante, 2000) as it is extended. This is believed to be due to driving the chromatin fiber opening transition (10- to 30-nm fiber transition of Fig. 3) by force. (c) Low-force elasticity is also seen (Bennink *et al.*, 2001); at higher forces, force jumps corresponding to nucleosome removal events are observed.

The spermatocytes go through two divisions, which reduce the original four sets of homologous chromatids to the single chromatids of sperm. Nicklas studied the first meitoic division, focusing on anaphase I. Although the main focus of this remarkable paper is on spindle force generation (the maximum force that could be applied to a chromosome by the spindle was found to be nearly 1 nN), Nicklas also reported a complete series of measurements of chromosome elasticity.

Nicklas (1983) noted that during anaphase I it was possible to measure the elastic response of one and two chromatids independently by carrying out experiments on chromosomes either before or after their chromatid separation (during anaphase I, the chromatids "unpeel" except for the kinetochore). Using a statistical analysis of data on a number of chromosomes, he showed that attached pairs of chromatids required twice as much force to be doubled in length as did single chromatids. The elasticity observed was linear [force proportional to change in length and to cross-sectional area, see Eq. (2)]. The force needed to double a grasshopper meiotic anaphase I chromosome (two chromatids) was determined to be $f_0 = 0.75$ nN; single chromatids were found to have $f_0 = 0.32$ nN (when reading Nicklas' paper, keep in mind 1 nN $= 10^{-4}$ dyne). This result was used to infer that the (average) Young-stretching modulus of an anaphase I chromosome was 430 Pa (again, note 1 Pa $= 1$ N/m$^2 = 10$ dyne/cm^2). The range of linear elastic response was reported to be at least up to $\Delta L/L = 2$ (threefold extension).

The experiments of Nicklas (1983) are superb in being *in vivo* measurements, which are sufficiently quantitative that it is completely convincing that the elastic response of the chromosomes, and not some aspect of the cell membrane or cytoskeleton, is being measured. However, this depended on the very fluid cell surface of insect spermatocytes (Nicklas, 1983; Zhang and Nicklas, 1995, 1999), a feature not shared by mammalian somatic cells. This is emphasized by Skibben and Salmon (1997), who were able to do elegant chromosome manipulations inside cultured newt lung cells during mitosis only using very stiff microneedles, with consequently no possibility to use their bending to measure force. Nevertheless, micromanipulation techniques based on microneedles have been used to carry out remarkable studies of spindle mechanics and regulation in somatic vertebrate cells.

2. Stretching Mitotic Chromosomes after Their Removal from Cells

Given that stretching chromosomes inside mitotic vertebrate cells is not possible, the next best approach to study chromosome stretching is to remove chromosomes from cells into the cell buffer. This approach will always be subject to the criticism that chemical conditions outside the cell will alter chromosome structure, but using comparisons with available

in vivo information, the relationship between *in vivo* and *ex vivo* chromosome structures can be understood. As described later; our own experiments, combined with those of others, convinced us that there is little or no change in chromosome structure, at least initially after removal from a mitotic cell.

Classen and co-workers (1994) noted that chromosomes prepared for metaphase spread karyotyping could be highly extended. That group has used chromosome stretching to develop high-resolution chromosome-banding techniques (Hliscs *et al.*, 1997a,b). However, the first quantitative measure of the elastic response of a mitotic chromosome extracted from a cell was carried out by Houchmandzadeh *et al.* (1997) using a technique reminiscent of that of Nicklas (1983). The experiments of Houchmandzadeh *et al.* (1997) were done on mitotic cells in primary cultures of newt lung epithelia (*Notophthalmus viridescens*). This organism is attractive for chromosome research because it is a vertebrate with relatively few (haploid $n = 11$), large (haploid genome ~ 35 pg of dsDNA) chromosomes (Gregory, 2001). Each *N. viridescens* chromatid therefore contains about 3 pg = 3 Gbp, or about 1 m, of dsDNA. At metaphase, the chromosomes are between 10 and 20 μm long and have a diameter of about 2 μm. Newt epithelia cells are cultured easily as a monolayer on dishes built on cover glasses, which are open to room atmosphere, making them excellent for micromanipulation experiments (Reider and Hard, 1990).

Houchmandzadeh *et al.* (1997) used glass micropipettes (inside diameter ~ 2 μm; Brown and Flaming, 1986) to puncture mitotic cells and then to grab onto the chromosomes. The micropipettes were introduced into the open culture dish from above using an inverted microscope. Chromosomes were grabbed by aspirating the chromosome end into the pipette opening, with the other chromosome end anchored in the cell. The main method used by Houchmandzadeh *et al.* (1997) to apply controlled stretching forces to chromosomes was to use aspiration into a pipette that had been treated with bovine serum albumin so that the chromosome could slide freely while in contact with the bore of the pipette. The chromosome acted as a piston, and by controlling the aspiration pressure, it could be stretched. This technique allows sensitive measurements, but has the defect that the chromosome–pipette seal is not perfect, and the "piston" will be leaky. This results in an overestimation of the modulus, as part of the pressure applied to the pipette drives flow.

The results were essentially that mitotic chromosomes are elastic, with a Young modulus estimated to be approximately 1000 Pa at prometaphase (i.e., chromosomes condensed, but not yet attached to spindle), compatible with the results of Nicklas after taking into account the flow effect mentioned earlier: Over a range of twofold extension, the elasticity was remarkably linear (see Fig. 8; Houchmandzadeh *et al.*, 1997). Experiments

were also carried out just after nuclear envelope breakdown (end of prophase), and it was found that chromosomes had a higher elastic modulus $Y = 5000$ Pa.

In addition, Houchmandzadeh *et al.* (1997) discussed the result of severe deformation of chromosomes using untreated pipettes to which chromosomes adhere permanently. It was found that prometaphase chromosomes could be extended to as large as 100 times their native length without breaking. For extensions beyond 10 times length, the chromosomes did not return to their native length, plus a number of effects due to "plastic" deformation of chromosomes were observed. When rapid extensions were made, the native chromosome could be converted to a thin fiber that was much stiffer than the native chromosome. Using calibrated pipette bending, the thin fiber was found to have $Y \sim 10{,}000$ Pa. Finally, the thinned chromosome was observed to coil helically after stress was released.

Following the study of Houchmandzadeh *et al.* (1997), we further developed the micropipette-based manipulation technique in order to more quantitatively measure newt chromosome mechanical properties (Poirier *et al.*, 2000). Primary cultures of newt lung epithelia were used using the pipettes to tear holes in the cells. Calibrated micropipette bending was used as the force measurement scheme for chromosomes removed from cells and suspended between two pipettes. This allows both ends of the chromosome to be monitored, and therefore chromosome extension can be controlled precisely. Digital image acquisition and analysis were used to measure pipette bending. Measurement of the correlation between pipette images allows pipette shifts (and therefore deflections) to be determined to about 10-nm accuracy. Typically pipettes were used with bending moments ~ 1 nN/μm of deflection, setting a limit on our force resolution of 0.01 nN = 10 pN. In practice, force resolution is usually limited by slow mechanical drifts of the pipettes. Figure 7 shows the experimental setup as viewed in the microscope.

Measurements of Poirier *et al.* (2000) of the force–extension response of single mitotic (prometaphase) chromosomes are shown in Fig. 8. A completely reversible elastic force response was observed for extensions up to about five times native extension, with a force constant $f_0 \sim 1$ nN. Given the 1.6 μm diameter of the chromosomes, this corresponds to a Young modulus near 500 Pa, near to the value obtained by Nicklas (1983). [The 300 Pa quoted in Poirier *et al.* (2000) is based on a slight overestimate of the chromosome thickness; our current best estimate is a prometaphase chromosome diameter of about 1.6 μm.] Although on the same order of magnitude as the modulus measured by Houchmandzadeh *et al.* (1997), the lower modulus observed by Poirier *et al.* (2000) indicates that the aspiration technique overestimates chromosome elasticity.

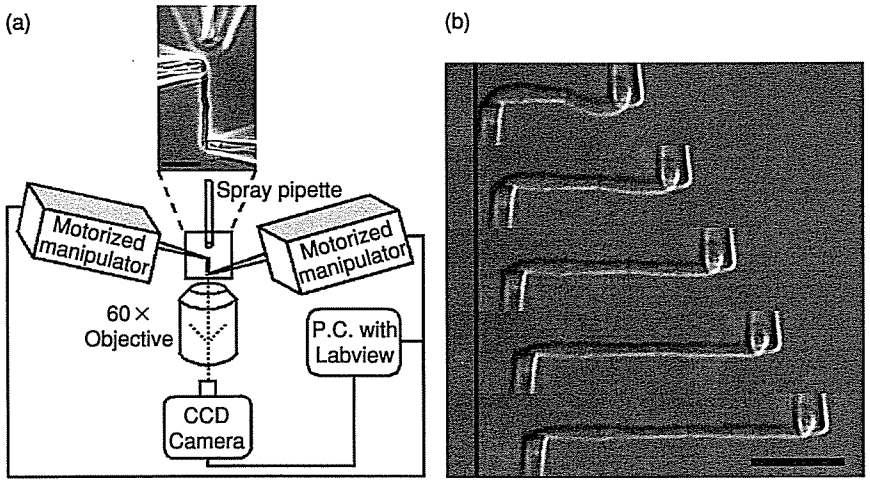


Figure 7 A two-pipette chromosome experiment as carried out in Poirier *et al.* (2000). (a) Schematic diagram of the experimental setup. Two pipettes are used to hold a mitotic chromosome, with one pipette fabricated with a deflection force constant ~1 nN/μm to allow chromosome tension to be measured. A third pipette can be moved near to chromosome to microspray reagents for combined chemical–micromechanical experiments (see Section VI). (b) Example images collected during the 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 an accuracy of about 10 nm. Bar: 10 μm.

To date we have carried out about 100 chromosome stretching experiments on newt mitotic chromosomes, and in accord with Nicklas (1983), we find appreciable variation in the force constant, roughly from $f_0 = 0.5$ to 2 nN (see histograms of Fig. 8). Unfortunately, there are no apparent cytological markers on newt chromosomes (for a karyotype, see Hutchison and Pardue, 1975) so we are unable at present to determine whether particular newt chromosomes have consistently higher or lower force constants. It might be possible to correlate the chromosome elastic response with the chromosome number by *in situ* karyotyping, e.g., using sequence-sensitive DNA dyes, following force measurement.

A feature of chromosome stretching that is quite obvious in all the aforementioned studies is that mitotic chromosomes do not become thin as they are stretched in the reversible elastic regime. Our measurements (Poirier *et al.*, 2000) indicate that the fractional decrease in chromosome width is less than 0.1 times the fractional chromosome length increase. For a solidly bonded elastic medium, this ratio (called the Poisson ratio, see Fig. 8a, inset) is usually close to 0.5, corresponding to volume conservation. In contrast, the volume of a mitotic chromosome actually increases as it is being

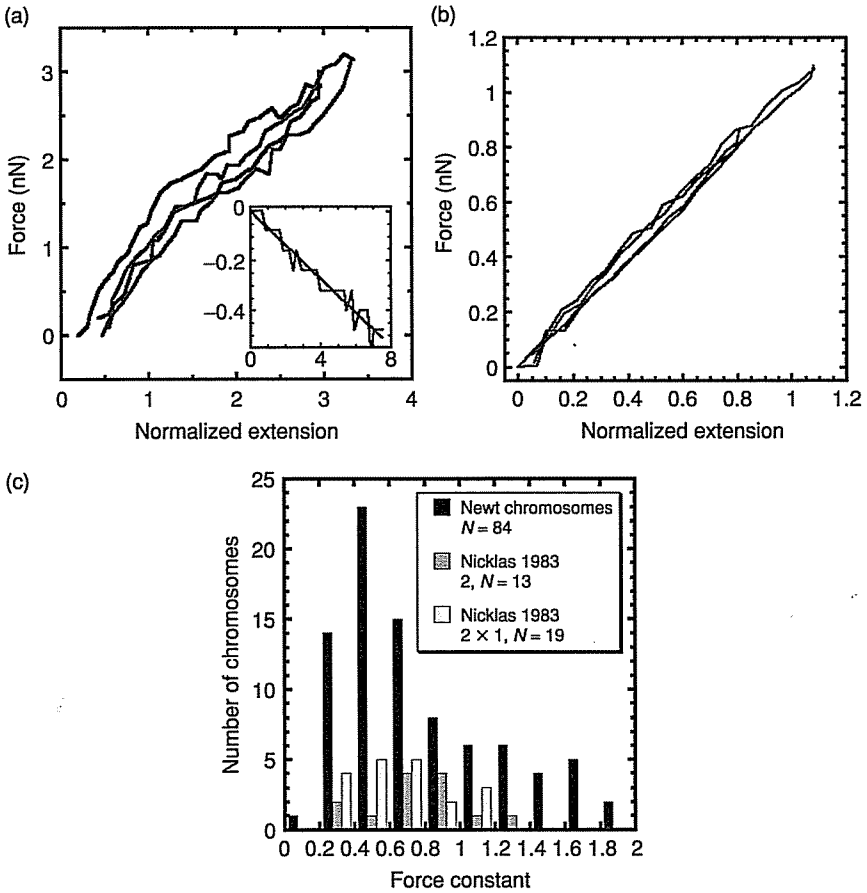


Figure 8 Force–extension data for newt chromosomes. (a) Data from Poirier *et al.* (2000) for primary cultures of newt lung cells. The different curves show successive extension–retraction cycles; their coincidence indicates that the chromosome has reversible elasticity over the fourfold range of extension shown. The elastic response is nearly linear, and the initial force increase shows that the chromosome force constant is about 1 nN. (Inset) The fractional change in chromosome width as a function of extension, and the chromosome Poisson ratio is less than 0.1. (b) Data for the newt TVI cell line for small extensions (up to two times native length) after chromosome extraction using dilute Triton X-100 (see text). In this range, the chromosome elastic response is strikingly linear, again with a force constant near 1 nN. (c) Histogram of force constants of 84 extracted newt prometaphase chromosomes, plus histograms of *in vivo* force constants of grasshopper spermatocyte metaphase I chromosomes (two chromatids) and single chromatids (Nicklas, 1983). Single chromatid grasshopper data had forces doubled for direct comparison with the two chromatid data sets. Distributions of force constants are similar in the newt and grasshopper systems.

stretched. This can only occur if the fluid medium surrounding the chromosome flows into it as it is stretched, which in turn indicates that the chromatin fibers inside a mitotic chromosome do not adhere to one another.

In Poirier *et al.* (2000), the behavior of chromosomes under high extensions (5 to 50 times native length) is also reported. In contrast to Houchmandzadeh *et al.* (1997), high extensions were studied using very slow rates of strain, typically 0.01/s (i.e., chromosome length is doubled in 100s). At these slow strain rates, it is found that permanent plastic deformation of chromosomes occurs beyond about fivefold extensions. Remarkably, after extensions beyond about 30 times native length, when stress is released, the chromosome relaxes to an elongated and swollen structure with a very low elastic modulus. This "ghost" chromosome appears to have the same histone content as the native chromosome, as assayed using fluorescent-labeled antihistone introduced *in situ*. This irreversible swelling behavior is in accord with the Poisson ratio result discussed earlier, again suggesting that the chromatin fibers inside the mitotic chromosome are not adhering to each other strongly. In the interpretation of Poirier *et al.* (2000), the "ghost" chromosome results from mechanical rupturing of the cross-linking elements that hold the chromosome in its condensed mitotic form.

Following the work of Poirier *et al.* (2000), we further developed a number of aspects of the newt chromosome experiment. First, we obtained a newt eye lens epithelial tissue culture line (TVI line; Reese, 1976), which provides many more metaphase cells per experiment dish and avoids all the troubles of working with animals and primary cell cultures. Next, we developed a technique of using a micropipette loaded with a 0.05% solution of Triton X-100 in 60% phosphate-buffered saline, (PBS), which we spray onto the surface of a mitotic cell to produce a hole through which the mitotic chromosomes are disgorged. Finally, we now generally anchor the force-measuring pipette to the sample slide rather than placing it on a micromanipulation to reduce its mechanical drift. None of these changes in system or technique resulted in any changes to the results reported in Poirier (2000). The result of a recent experiment is shown in Fig. 8b and indicates that the initial elastic response of a mitotic chromosome is essentially linear, with a force constant near to 1 nN.

We have also carried out experiments on *Xenopus* A6 tissue culture cells. These amphibian cells are very similar to newt cells, but have smaller chromosomes ($n=18$, haploid DNA content ~ 3 pg $= 3$ Gb, or about 150 Mb/chromosome). These chromosomes can be isolated and manipulated at prometaphase; they show the same general elastic properties as newt chromosomes, with a force constant of about 1 nN. This force constant indicates a Young modulus Y$\sim$1000 Pa (Poirier *et al.*, 2002b).

C. *In Vitro*-Assembled Chromosomes

Dimitrov and Houchmandzadeh (1999) carried out an important study of the mechanical properties of mitotic chromatids assembled *in vitro* using *Xenopus* egg extracts. It is important to note that the system studied is assembled from sperm DNA and, as a result, isolated chromatids are assembled. Also, the chromosomes assembled in the usual egg extract "mitotic" reaction may have a structure unique to the first division of a fertilized egg, i.e., not precisely the structure of a somatic mitotic chromosome.

Micropipettes were used to grab, manipulate, and stretch the chromatids; a force measurement was done via observation and calibration of micropipette bending using the same general scheme as shown in Fig. 7. Micropipette bending stiffnesses were roughly 1 nN/μm. The stretching experiments were carried out in buffer, following chromosome assembly.

The *in vitro*-assembled chromatids display stretching elasticity similar to that of chromosomes isolated from cells. For small extensions, linear elasticity was observed, with a force constant $\sim$1 nN, and Young modulus Y$\sim$1000 Pa. However, for extensions beyond about two times native length, the force observed during retraction is significantly less than the force observed during extension, indicating that irreversible changes have occurred. Finally, for extensions about 10 times native length, the *in vitro*-assembled chromatids show a force "plateau" and fail mechanically. Houchmandzadeh and Dimitrov (1999) also presented an explanation for the mitotic chromatid elastic response in terms of a titin-like elastic "core."

Roughly, the *in vitro* chromatids have stretching elasticity rather similar to chromosomes from cells, but are somewhat more fragile at higher extensions. It would be of great interest to have stretching data on *replicated in vitro*-assembled chromosomes, which would have two duplicate chromatids; replicated chromosomes can be assembled using "cycling" egg extracts (Smythe and Newport, 1991).

D. Summary

All the available experimental data on the low-extension stretching elasticity of mitotic chromosomes are in excellent agreement; grasshopper, newt, and frog chromosomes from cells, plus egg extract chromatids, all require roughly 1 nN of force to be doubled in length. This level of force constant corresponds to Young moduli of roughly 500 Pa. Chromosomes from cells display a reversible force–extension response when stretched five times their native length; irreversible stretching occurs for larger extensions, with chromosome breakage occurring for large extensions >20 times native

length. *In vitro*-assembled chromatids are mechanically less robust than *in vivo* chromosomes.

An intriguing feature of the mitotic chromosome elastic response is that chromosome volume increases with initial extension. This indicates that mitotic chromosomes are not bonded solidly together and instead behave rather like a polymer network containing an appreciable amount of cytoplasm ("solvent").

The initial elastic response of mitotic chromosomes is not due to gross alteration of the chromatin fiber structure, as can be seen from comparisons of chromosome and chromatin (Cui and Bustamante, 2000) elastic responses. Chromatin fibers extracted from chicken eurythrocytes display reversible elasticity with a force constant of roughly 10 pN ($pN = 10^{-12}$ N). Because there will be on the order of a few thousand chromatin fibers in a chromosome cross section (the chromosomes discussed earlier are roughly a micrometer in cross section, and each fiber is roughly 30 nm thick), the 1 nN force at which chromosome length is doubled corresponds to a maximum force per chromatin fiber of a fraction of a piconewton. Therefore, the chromatin fiber structure is not being altered appreciably when chromosomes are being stretched by a factor of two; the initial elastic response of chromosomes must be due to modification of a larger scale condensed chromatin structure. Furthermore, the relatively low modulus of the chromosome indicates that the large-scale chromatin structure is remarkably soft, yet elastic.

IV. Bending Elasticity of Chromosomes

During mitosis, chromosomes are observed to be bent by spindle-generated forces; knowing the bending stiffness of a mitotic chromosome therefore gives additional information about forces generated by the mitotic apparatus. However, an additional motivation for measurement of the bending stiffness of a mitotic chromosome is to study the homogeneity of the elastic response, and therefore of structure. The previous section, following the approach of Nicklas (1983), implicitly assumed that chromosomes are uniform elastic media; definition of a Young modulus really makes sense only for homogeneous materials. This assumption can be checked easily: because bending of the rod is just stretching that varies across the rod cross section (the inside of the bend is compressed, while the outside is elongated), if a chromosome has uniform elongational properties, its bending and elongational stiffnesses will be related by Eq. (3).

The main result of experiments that compare the elongational and bending stiffness of chromosomes is that *in vivo* and for chromosomes extracted from cells, bending and stretching properties are related in the way that we

expect for uniform elastic media (Poirier *et al.*, 2002b). This indicates that chromosome elasticity is due to the bulk of the cross section of the chromosome and is not mainly due to a thin, stiff, central structure. In contrast, the *in vitro*-assembled *Xenopus* chromatids studied by Houchmandzadeh and Dimitrov (1999) are far more flexible than one would expect from their Young modulus of about 1000 Pa. This is a strong indication that *in vitro*-assembled mitotic chromatids have an internal structure distinct from that of *in vivo* mitotic chromosomes.

The method that has been used to measure bending moduli of chromosomes is to measure spontaneous thermal-bending fluctuations. The idea is that a small rod in solution will be hit continually by the molecules of the solvent, and as a result its shape will undergo conformational fluctuations. This is the mechanism by which flexible polymers undergo conformational diffusion. Chromosomes turn out to be flexible enough that their bending fluctuations can be observed. Essentially, the bending modulus B is inversely proportional to the amplitude squared of bending fluctuations, and so by measurement of fluctuation amplitude, bending stiffness can be inferred. This technique has been used to measure the bending elasticity of a number of filamentous cell structures. Elegant experiments of this type by Gittes *et al.* (1993) were used to measure the bending rigidity of actin filaments and microtubules.

Perhaps the simplest experiment to envision is to anchor one end of the filament being studied to a solid object (e.g., a very stiff micropipette) and then observe the fluctuations along the rod (Fig. 9a). As one moves down the rod from the anchor point, the amplitude of fluctuation perpendicular

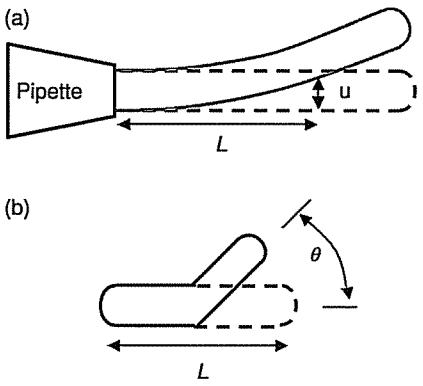


Figure 9 Experimental arrangements for the observation of thermally excited rod bending. (a) Rod clamped at one end, and the transverse fluctuation amplitude is measured as a function of distance from the pipette along the rod. (b) Short rod measured at three nearby points equidistant along a segment of length L so as to measure the fluctuation angle.

to the rod will increase. The precise relation expected for a straight, uniform rod is

$$\overline{u^2} = \frac{32kTL^3}{\pi^4 B} \tag{5}$$

where the bar indicates the average of the fluctuation–amplitude squared. As one would expect, a higher temperature gives larger fluctuation, a higher bending stiffness B gives lower fluctuation, and fluctuation amplitude increases with distance from the anchor point.

Equation (5) applies only near enough to the anchor point so that the typical value of the amplitude u is small compared to the distance to the anchor L. Chromosomes from amphibian cells will turn out to be stiff enough that this is always true. However, *in vitro*-assembled chromatids are so flexible that they undergo random walk fluctuations along their length, and to analyze their fluctuations, the generalization of Eq. (5) is needed (see Houchmandzadeh and Dimitrov, 1999).

A second type of experiment can be done by the observation of bending angles along a short segment of an untethered elastic rod (Fig. 9b). If the positions of three points at the ends and midpoint of a segment of the rod of length L are measured, then a bending angle can be determined. Fluctuations of the bending angle are related to the bending modulus by a relation similar to Eq. (5):

$$\overline{\Delta\theta^2} = \frac{kTL^3}{B} \tag{6}$$

The probability distribution of the angle fluctuations is simply Gaussian:

$$p(\Delta\theta)\exp\left[-\frac{(\Delta\theta)^2}{2\overline{\Delta\theta^2}}\right] \tag{7}$$

and, if it can be measured, provides a check that the fluctuations are thermal and not mechanical noise. This type of measurement can be done in situations where a small rod cannot be manipulated, e.g., for a chromosome inside a cell.

A. Expected Bending Flexibility and Fluctuations of Mitotic Newt Chromosomes

The previous section showed that the Young modulus of a mitotic newt chromosome was roughly Y = 500 Pa. Using Eq. (3), a chromosome cross-section radius of $r = 0.8$ μm, we obtain an expected $B = 1.6 \times 10^{-22}$ N m^2, based on the assumption that the chromosome behaves as a uniform elastic

medium. Plugging this value of B into Eq. (5), and the maximum chromosome length $L = 20$ μm, we find the root mean square fluctuation $\sqrt{\overline{u^2}} = 0.3$ μm. Thus, the tip of a 20-μm-long newt chromosome should thermally wobble over by roughly half a micrometer, an easily observable fluctuation amplitude.

B. Bending Fluctuations of Chromosomes Extracted from Cells

We have measured bending fluctuations for chromosomes extracted from newt cells and immobilized at one end in a pipette (Fig. 10). The main

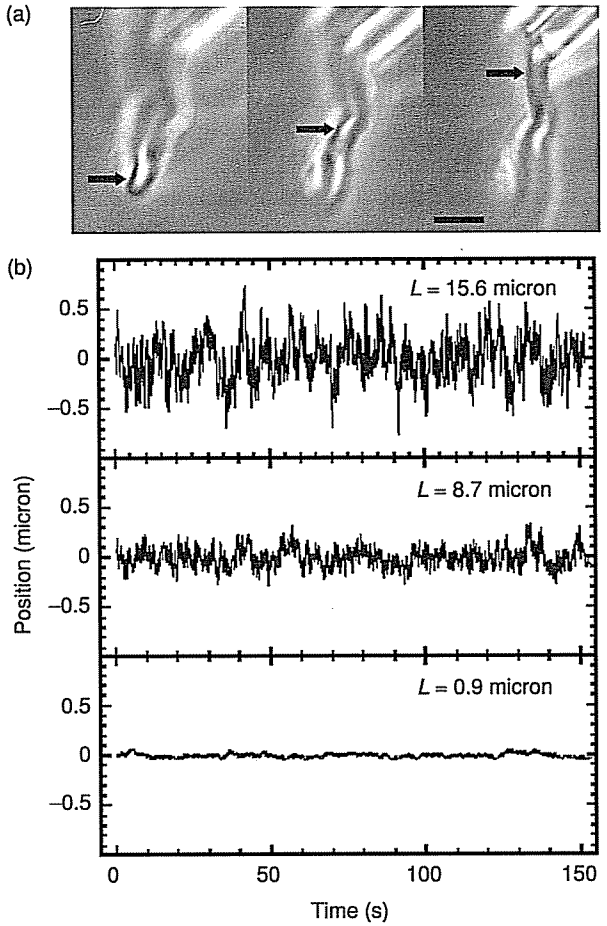


Figure 10 *(continues)*

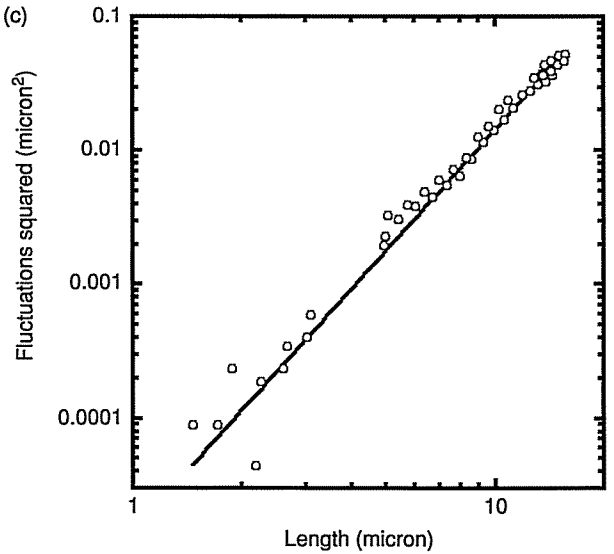


Figure 10 Measurement of bending fluctuations for newt (*N. viridescens*) prometaphase chromosomes. (a) A chromosome is anchored at one end in a pipette, and bending fluctuations are observed. Bar: 4 μm. (b) Amplitude time series as a function of time for the three positions indicated by arrows in part a; the distance from the anchored end is indicated in each segment. With increasing distance from the anchor, the fluctuations increase. (c) Mean square fluctuations versus distance from the anchor point; on a log–log plot data fall on the cubic power law given by Eq. (5). The fit shown allows the bending modulus to be extracted from data.

difficulty in this experiment was finding chromosomes that had no attachments to any cell structures so that a completely free end was obtained. The fluctuations have amplitudes satisfying Eq. (5) and lead to bending moduli between 1 and 3×10^{-22} N m^2 (Poirier and Marko, 2002a), in good accord with the aforementioned estimate. Newt chromosomes show no sign of "hinges" or other easily bent regions along their length. In particular, there is no sign of hinging behavior at the kinetochore. Each newt chromosome that had its bending modulus measured subsequently had its Young modulus measured by attachment of a force-measuring pipette to the free end. The actual Young modulus agreed well will the Young modulus inferred from the bending fluctuations.

In the same study (Poirier and Marko, 2002a), chromosomes extracted from *Xenopus* A6 cells were found to be somewhat more flexible, with bending moduli between 5 and 20 $\times$ 10^{-24} N m^2. This flexibility is due to the smaller cross section of the frog chromosomes ($r \sim 0.5$ μm). The bending

modulus value is consistent with measured A6 Young moduli (200 to 800 Pa in our experiments) via Eq. (3).

C. Bending Fluctuations of Chromosomes *in Vivo*

To check the relationship between bending moduli of newt chromosomes extracted from cells and *in vivo*, it would be useful to have data for mitotic chromosomes in live newt cells. Marshall *et al.* (2001) first did this using observations of bending fluctuations to measure the bending rigidity of mitotic chromosomes in *Drosophila* embryo cells. During mitosis, the mitotic spindle induces large bending fluctuations. Marshall *et al.* (2001) therefore compared native cells (large nonthermal bending fluctuations) with colchicine-treated cells (no microtubules, and therefore much smaller bending fluctuations).

The small fluctuations of the *Drosophila* chromosomes in the colchicine-treated cells led to an estimate of B = 6×10^{-24} N m^2 and a Young modulus estimate of 40 Pa. The much larger fluctuations in the native cells were then used to quantify the forces being applied to the chromosomes by the mitotic apparatus. Unfortunately, no stretching data are available for *Drosophila* embryo mitotic chromosomes; the small size of the chromosomes makes their micromanipulation extremely challenging.

We used the basic method of Marshall *et al.* (2001), colchicine treating newt cells, to obtain *in vivo* thermal bending data for mitotic chromosomes (Poirier and Marko, 2002a; note that cochicine treatment leads to essentially metaphase chromosomes). Angle fluctuations were measured *in vivo* over short distances along a number of chromosomes, and the probability distribution was the expected Gaussian form [Eq. (7)]. Chromosome-bending stifnesses were from 0.2 to 0.5 $\times$ 10^{-22} N m^2, about a factor of four smaller than obtained for isolated chromosomes.

The somewhat smaller values of B obtained *in vivo* may reflect a change in physical properties due to the chemical differences between the cytoplasm and the extracellular medium. Alternately, there may be sources of nonthermal fluctuation that are weak and not disrupted by colchicine treatment. Just as one example, SMC "condensin" proteins have a possible motor function and could result in forces on top of thermal forces, which tend to move chromosomes around. Also, the live cells continue to crawl on their substrate, and it may be that cytoplasmic flows driven by cell crawling cause some nonthermal fluctuations. Because nonthermal forces will generally increase bending fluctuations, we can expect the *in vivo* measurements to provide lower bounds on B. Thus we conclude that newt chromosomes have B *in vivo* comparable to that measured in the extracellular medium, roughly 10^{-22} N m^2.

D. Bending Fluctuations of *in Vitro*-Assembled *Xenopus* Chromatids

Houchmandzadeh and Dimitrov (1999) measured the bending stiffness of mitotic chromatids assembled in *Xenopus* egg extracts. They observed that the roughly 20μm-long chromatids were very flexible, finding $B = 1.2 \times 10^{-26}$ N m^2. This is about 1000 times smaller than the value of B that we have obtained for chromosomes from *Xenopus* A6 cells. Thus, *in vitro*-assembled chromosomes are far more flexible to bend than somatic cell chromosomes from the same species.

The bending modulus of *in vitro*-assembled chromatids is so low that they undergo flexible polymer-like bending fluctuations. The thermal persistence length of *in vitro*-assembled chromatids is $A = B/(kT) = 2.5$ μm, meaning that they should have many thermally fluctuating bends along their length. Indeed, movies of *in vitro*-assembled chromatids display observable dynamical bending on a few-micrometer length scale (S. Dimitrov, private communication). In collaboration with Professor R. Heald (U. C. Berkeley), we have observed *in vitro*-assembled chromatids and have verified that they are extremely flexible. To the eye, they behave entirely differently from mitotic chromosomes isolated from cells.

Paradoxically, *in vitro*-assembled chromosomes are extremely flexible to bend, yet have a Young (stretching) modulus Y$\sim$1000 Pa similar to that of mitotic chromosomes from cells. In starker terms, Eq. (3) fails for *in vitro*-assembled chromosomes by a factor of 1000. This fact led Houchmandzadeh and Dimitrov (1997) to suggest that the *in vitro*-assembled chromosomes should be organized around a thin core, which would provide stretching elasticity, but with very little bending rigidity. Those authors present a quantitative analysis indicating that one or a few titin molecules, suspected to be a chromosomal component (Machado *et al.*, 1998; Machado and Andrew, 2000a,b), could produce the observed elasticity while being bent easily.

In comparison with the 1000-fold larger value of B obtained for chromosomes from cells (recall that for both newt and frog chromosomes from cells, the bending behavior was consistent with the Young modulus), it seems inescapable that *in vitro*-assembled chromosomes have a different internal structure from chromosomes in somatic cells. Given that *in vitro*-assembled chromatids are a widely used model system for studying mitosis, this observation may be fairly important. A further interesting question is whether *in vitro*-assembled chromosomes, which are run through a round of DNA replication so that they are chromatid pairs, have a larger bending rigidity consistent with their Young modulus. One possibility is that cycle 1 of the fertilized frog egg may simply assemble a distinct chromosome structure; another possibility is that the reaction on unreplicated DNA may not be able to fully condense the chromatids.

E. Bending of Chromosomes during Mitosis

If one observes cells in culture going through mitosis, chromosomes can be observed to be bent during prometaphase as they are being aligned, and then during anaphase as the chromatids are being pulled toward the spindle poles. During anaphase, the chromosomes can be bent quite severely, and to the eye it appears that the chromosome arms are being pulled back by some retarding force.

Roughly, the retarding force needed to bend a chromosome into an anaphase "U" shape is the bending modulus divided by the square of the width of the "U" (Houchmandzadeh *et al.*, 1997). For a newt chromosome with $B = 10^{-22}$ N m^2 and a U width of a few micrometers, this retarding force is roughly 10^{-11} N. A basic question is whether this force is possibly due to viscous drag. The drag force on the chromosome will be roughly its length times viscosity times its velocity; for newt chromosomes ($L = 10$ μm, cytoplasm viscosity = 0.01 Pa s, velocity = 0.01 μm/s), we obtain a drag force of about 10^{-15} N. Drag cannot generate the relatively large force needed to bend an anaphase chromosome (Nicklas, 1983).

Based on this estimate, it is clear that the force that bends chromosomes and chromatids during mitosis is not simple viscous drag due to the cytoplasm. One explanation suggested by Houchmandzadeh *et al.* (1997) is that there is a larger amount of friction due to motion of the chromosomes through the cell than estimated on the basis of estimates of cytoplasm viscosity, perhaps because of cytosketetal filaments that oppose the motion of large objects. However, it is worth considering the alternative possibility that opposing forces are applied to the kinetochore and chromosome arms (or telomeres), which are large compared to viscous drag forces.

F. Summary

Mitotic chromosomes have well-defined bending properties, and mitotic amphibian cell chromosomes have a bending modulus consistent with their stretching moduli via Eq. (3), implying that their cross sections are relatively homogeneous. Measurements of bending fluctuations inside and outside cells indicate that there is not a large difference in bending elasticity caused by isolation of a chromosome. Remarkably, *in vitro*-assembled chromatids are bent far more easily than would be expected on the basis of their stretching modulus. This suggests that *in vitro*-assembled chromatids have an internal structure qualitatively different from that of metaphase chromosomes in somatic cells.

V. Viscoelasticity of Chromosomes

The previous three sections emphasized two main points: that chromosomes contain a huge length of dsDNA, organized into chromatin fiber, which itself is a bulky but flexible polymer that is highly elastic, and that mitotic chromosomes have a well-defined stretching elasticity (Y~500 Pa), allowing them to recoil into their native form after being stretched up to five times. This stretching elasticity is also responsible for the well-defined bending elasticity of chromosomes *in vivo*, with a bending modulus in accord with the Young modulus via Eq. (3). Our main focus has been on the *reversible, equilibrium* stress response, i.e., elastic-restoring forces in the regime where extensions are small enough not to damage chromosome internal structure and where sufficient time has elapsed that the internal stress in the chromosome is in equilibrium with the force applied by the measurement apparatus (e.g., a microneedle or micropipette).

If one stretches a chromosome rapidly enough, a stretching force in excess of the equilibrium force will be required, as the stress in the chromosome will be partly due to the intrinsic elasticity, plus additional *viscous* stress associated with the fact that the chromosome internal structure is not able to reach its equilibrium at each moment in time. The viscous stress can also be thought of arising from the sliding friction of adjacent chromatin domains. This section is concerned with quantifying this effect, and its conclusion will be that a mitotic chromosome has an immense "internal viscosity" in quantitative terms $\sim 10^5$ times the viscosity of water.

A. Observations of Slow Stress Relaxation

During mitosis, chromosomes are often stretched out at anaphase due to interchromatid attachments (Fig. 6), and sometimes one can observe what happens when the chromatids release while a large amount of stress (e.g., a total force near to 1 nN) is acting on them. Following stress release, one observes the chromatid end to retract back until it returns to near-native length over a time of a few seconds. Nicklas and Staehly (1967) carried out micromanipulation experiments using microneedles and intact grasshopper spermatocytes and noted that if they stretched a metaphase I chromosome to about eight times its native length and then released it, it would recoil to its native length in about 4 s. This measurement amounts to a determination of the internal viscosity of a chromosome given the subsequent measurement of the grasshopper spermatocyte metaphase I force constant of about 1 nN (Nicklas, 1983).

In linear approximation, the situations described earlier are described by the force balance equation:

$$Yx = \eta' \frac{dx}{dt} \tag{8}$$

where $x = \Delta L/L$, the chromosome extension as a fraction of its native length L (Poirier *et al.*, 2001a). The left side of Eq. (8) is equilibrium elastic stress, just the Young modulus times the strain (note that Y has dimensions of stress, or force per area), and has the linear form observed for whole chromosome elasticity (Fig. 8). The right side, which is zero when the extension is stationary, is the simplest model of viscous stress inside the chromosome and the starting point for the description of *viscoelasticity* (Landau and Lifshitz, 1986). The constant η' has dimensions of force per area times time, or viscosity. When applying Eq. (8) to free relaxation of a chromosome, we should keep in mind that the external fluid will contribute to η' (recall water has viscosity of 10^{-3} Pa s); however, this will be a tiny correction to the mainly "internal" viscosity.

If at time $t = 0$ our chromosome is released with extension x_0, its subsequent extension [the solution to Eq. (8)] is just a simple exponential decay:

$$x(t) = x_0 \exp[-t/t_0] \tag{9}$$

where the time constant for the decay is $t_0 = \eta'/Y$. The observation by Nicklas and Staehly (1967) of $t_0 \sim 4$ s and the measurement of $Y \sim 500$ Pa (Nicklas 1983) therefore give an estimate of that $\eta' \sim 2000$ Pa s, more than 10^6 times the viscosity of water. This is a very rough estimate and involves extrapolation of Eq. (8) into the high-extension regime where nonlinear corrections are important. However, it is clear that the internal relaxation rate of a mitotic chromosome is extremely slow.

In our first experiments measuring newt chromosome elasticity we found that in order to obtain reversible elastic equilibrium force responses using micropipettes, very slow extension and retraction rates were necessary (Poirier *et al.*, 2000). If one elongates a newt chromosome by a factor of two over a time less than about 10 s, the extension curve is above the equilibrium force, while the retraction curve lags below it. The nicely reversible extension–retraction curves of Fig. 8 therefore take >5 min per extension–retraction cycle. This effect is in accord with the observations of Nicklas and Staehly (1967) and with Eq. (8), which can be amended to include the effect of the external stress applied by a pipette.

We subsequently measured the internal viscosity of the prometaphase newt chromosome more carefully (Poirier *et al.*, 2001). We did this using chromosomes attached to two pipettes, simply by moving one pipette rapidly while acquiring visual data for the deflection of the other, force-measuring pipette. The results of this experiment are shown in Fig. 11. Generally, one

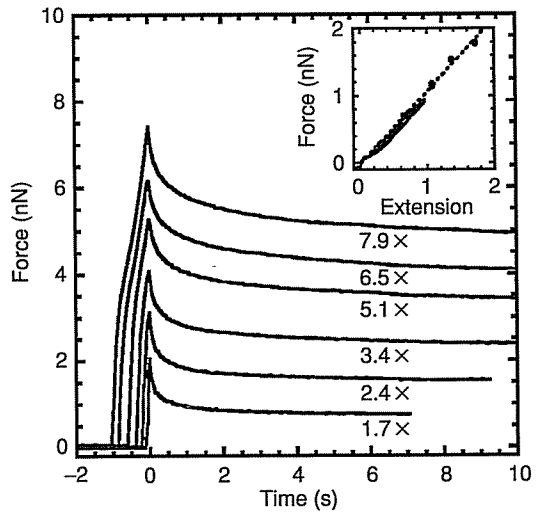


Figure 11 Measurement of the dynamics of stress relaxation in a single mitotic newt (*N. viridescens*) chromosome. A chromosome was attached between two pipettes, and then one pipette was moved rapidly while the other pipette deflection was observed, from which the force in the chromosome was inferred. The force jumps up to a peak and decays down to its final equilibrium value. For short extensions the end of decay is observed to be exponential, with a displacement-independent decay time of about 1s. (Inset) The equilibrium chromosome elastic response (line) and final equilibrium forces reached in the step experiments (points) are in accord for small extensions where Eq. (8) is valid.

observes an initial force pulse just shortly after the pipette is moved, followed by a decay to the final equilibrium force. For small step extensions to a final chromosome extension less than about three times native length, the final force decays are nearly all the same exponential shape, in accord with Eq. (8). For small extensions, the final equilibrium force is in agreement with the slow extension–retraction chromosome elastic response, which was measured at the beginning of the experiment (inset, Fig. 11). This indicates that the chromosome is reaching its elastic equilibrium without being damaged appreciably by the rapid pipette motion.

Most simply, if we use Eq. (9), we can combine the linear regime exponential relaxation time $t_{\text{relax}} = 1$ s with the chromosome elastic modulus $Y = 500$ Pa to obtain an internal viscosity $\eta' = 500$ Pa s, similar to the aforementioned estimate based on the observations of Nicklas and Staehly. A more complete analysis takes into account the bending stiffness of the pipette, which turns out to be the dominant elastic element in the experiment (Poirier *et al.*, 2001a); the relaxation time turns out to be $\eta' L/k_p$ (pipette force constant $k_p \sim 1$ nN/μm, larger than the typical chromosome force constant of $\sim$0.1 nN/μm for a $\sim$10-μm chromosome segment). This gives an internal

viscosity $\eta' = 100$ Pa s, about 10^5 times the viscosity of water. The only reasonable way to explain this large internal viscosity is to suppose that when the chromosome is stretched rapidly, the chromatin inside it must rearrange, and the time needed for this rearrangement is on the order of 1 s.

One might imagine that since a chromosome must increase in volume as it is stretched (recall the Poisson ratio of a newt chromosome is less than 0.1, see Fig. 8a), perhaps slow relaxation is due to the flow of buffer into the chromosome (Tanaka and Fillmore, 1979). However, if this were the case, one would expect to see the chromosome width undergo a sharp contraction in the step experiments, followed by a gradual decay back up to its equilibrium width. This is not observed (Poirier *et al.*, 2001); the chromosome width jumps to its final equilibrium value much faster than the stress inside it decays. We therefore conclude that the internal viscosity is due to chromatin rearrangements, which we infer to be taking place on $\sim$1-s time scales.

Our conclusion that chromatin domains take $\sim$1-s time scales to relax when the chromosome is stretched implies that there are internal fluctuations of chromatin conformation on the $\sim$1-s time scale inside a quiescent mitotic chromosome. Internal motions of chromatin domains can possibly be on this long time scale as the chromosome is one long filament. The "loop" domains that must result from loose condensation of a long filament can be expected to have to undergo slow sliding motions past one another (de Gennes, 1979; Poirier *et al.*, 2001), with slow dynamics.

B. Dynamics of Bending of Mitotic Newt Chromosomes

The previous section showed that mitotic chromosomes had well-defined bending elasticity that could be measured via observation of their spontaneous thermal fluctuations, i.e., the mean square fluctuation was proportional to the cube of the length from the anchor point [Eq. (5)]. Furthermore, the bending stiffness obtained was that expected from the Young modulus [Eq. (3)]. Our conclusion was that spontaneous thermal bending fluctuations of mitotic chromosomes have the amplitudes expected for a thin elastic filament, much as observed for actin filaments and microtubules (Gittes, 1993).

However, the time dependence of the fluctuations (time series of Fig. 10a) turns out to be quite distinct from the fluctuations for actin and microtubules, where the dominant friction is the external hydrodynamic drag on the filament. In that case, the characteristic time of fluctuations at the free tip grows with the fourth power of the overall length of the filament (discussed, for example, in Harnau and Reineker, 1999):

$$t_{hydro} \approx \frac{\eta L^4}{B} \tag{10}$$

This formula indicates that if one compares the tip fluctuations of two chromosomes that are a factor of two different in length, the tip motion of the shorter one should be about $2^4 = 16$ times faster than the long one. This is not the case (Poirier and Marko, 2002b); in experiments on mitotic newt chromosomes of different lengths, the *same* tip fluctuation lifetime was observed, even while the time-averaged fluctuation *amplitudes* obey Eq. (5). The characteristic lifetime of the fluctuations was in each case about 0.7 s.

An explanation follows from the fact that the conventional theory of bending fluctuations [Eq. (10)] ignores internal viscosity, which for actin and microtubules is negligible in experimental measurements done to date. When internal viscosity is taken into account, the tip fluctuation time turns out to be the larger of Eq. (10) and the internal viscous relaxation time $t_0 = \eta'/Y$. For newt mitotic chromosomes, $t_0 > t_{\text{fluct}}$ for chromosome lengths satisfying $L/r < (\eta'/\eta)^{1/4}$, where the relation between Y and B [Eq. (3)] has been used. Since we know $\eta'/\eta \sim 10^5$ and $r \sim 1$ μm, the tip fluctuations will not show any length dependence for $L < 20$ μm, as we have observed (Poirier and Marko, 2002c).

C. Summary

Newt mitotic chromosomes, although well-defined elastic solid media, have an exceedingly slow stress response. Measurements of free relaxation of stretched chromosomes and dynamical stretching experiments on newt chromosomes reveal a characteristic time $t_0 = \eta'/Y$, where $t_0 \sim 1$ s, and where $\eta' \sim 500$ Pa s (about 10^5 times the viscosity of water). This characteristic relaxation time corresponds to the time of spontaneous internal chromatin rearrangements, which are so sluggish that external hydrodynamic friction is irrelevant. These spontaneous internal rearrangements limit the rate at which bending fluctuations occur to a degree that again external hydrodynamic damping, the usual friction relevant to flexible polymers, plays no role. Mitotic chromosomes thus behave like micrometer-thick filaments composed of cross-linked flexible polymers, with slow internal dynamics on the 1-s time scale.

VI. Combined Biochemical–Micromechanical Study of Mitotic Chromosomes

The previous sections discussed the physical properties of mitotic chromosomes, focusing on elastic properties *in vivo*, and for chromosomes extracted from cells. By themselves, they indirectly reveal information about mitotic

chromosome structure, such as the flexibility and conformational freedom of chromatin inside the chromosome. However, a more direct and powerful method to analyze mitotic chromosome structure is to use changes in chromosome elasticity as an indicator of changes in the chromosome structure introduced chemically.

This section focuses on two sets of experiments: reversible changes in chromosome structure driven by shifting ionic conditions and irreversible changes driven by DNA-cutting enzymes. For these experiments, work of the previous sections provides a baseline elastic response. The ion experiments will provide additional information about the flexibility of chromatin in mitotic chromosomes, while the enzyme experiments will reveal that mitotic chromosomes can be disassembled completely by cutting DNA alone. These experiments suggest that the chromosome elastic response is due to chromatin itself and is not due to some internal non-DNA structure, which is to some degree counter to conventional wisdom concerning mitotic chromosome structure.

The strategy of carrying out real-time observation of chemical reactions on whole chromosomes is not new. For example, the drug actinomycin-D was used to release RNA transcripts from the large "puffed up" loops on amphibian lampbrush chromosomes; the subsequent collapse of the loops showed that their open morphology was due to active transcription (Izawa, 1963; Callan, 1982, 1986). For mitotic chromosomes, observations of morphological changes caused by salts, proteases, and DNAase on chromosomes were carried out in the early 1960s (Cole, 1967). More recently, Maniotis *et al.* (1997) carried out a series of experiments on clusters of metaphase chromosomes extracted from cells using microneedles. In that work the effects of a wide range of chemicals, including salts and nucleases, were studied by observing morphological changes in the light microscope.

A. Whole Genome Extraction Experiments

Maniotis *et al.* (1997) developed a technique for extracting whole genomes from human and bovine tissue culture cells in both interphase (i.e., from the nucleus) and during mitosis. They used microneedles to "harpoon" either nucleoli during metaphase or mitotic chromosomes. Chemical experiments were then done on the chromosomes while observing on the inverted microscope, using drops of enzyme introduced into the slide on which the cells are cultured and manipulated.

Maniotis *et al.* (1997) emphasized that when these mechanical extractions are done, the whole genome (i.e., essentially all the chromatin) is obtained due to mechanical connections between the chromosomes. These interchromosome connections are invisible fibers (evidenced by their mechanical

effects), which are RNa ase and protease insensitive, but which are cut by DNa ase and micrococcal nuclease. They therefore concluded that the chromosomes of mammalian cells are *connected together* at the chromatin level, i.e., that the molecule that holds the genome together is DNA.

A very wide range of interesting experiments have been done by Maniotis *et al.* (1997), with emphasis on interphase chromosome organization and chromosome–cytoskeleton interactions, topics that are slightly outside of the focus of this review. However, two other experiments are done of particular relevance here. One class of experiments involves shifts in ionic conditions. It was observed that mitotic chromosomes can be decondensed rapidly by the introduction of drops of high concentrations of ions (500 m*M* $MgCl_2$, 500 m*M* $CaCl_2$, 1 *M* $CuCl_2$, 1 *M* NaCl) and that this decondensation was reversible, unless very high concentrations of ions were used. These experiments indicate that mitotic chromatin is compacted by interactions of primarily ionic character and suggest that the condensation of the mitotic chromosome is not a precise folding, as it can be cycled chemically on a short time scale.

Second, Maniotis *et al.* (1997) used drops of proteases (5 mg/ml trypsin and 50 mg/ml proteinase K) to examine the role of proteins in mitotic chromosome organization. It was found that these enzymes cause rapid decondensation of chromosomes into "swollen clouds," a result consistent with results of experiments discussed by Cole (1967). Remarkably, Maniotis *et al.* (1997) found that the decondensed chromosomes could be recondensed by adding linker histone H1 at 1 mg/ml. Core histones and other nonhistone proteins could not produce this effect. Apparently the main effect of protein digestion is to disrupt nucleosome-stacking interactions, as mitotic chromosome morphology can be "rescued" using H1.

It is striking that H1 is sufficient for this rescue, as one might imagine that other, rarer proteins that define a higher order chromatin structure (i.e., above the level of the 30-nm fiber) would be cut by the proteases and that this would limit the degree of recondensation that H1 could affect. Perhaps the large concentration of H1 and its accessibility (H1 is chemically exchanging on short time scales; Lever *et al.*, 2000; Misteli *et al.*, 2000) make it a main target in this experiment, whereas the rare and perhaps other well-buried proteins that stabilize the higher level chromosome structure remain undamaged.

B. Combined Micromechanical–Chemical Experiments

Our experiments focus on combining the *in situ* biochemical reaction approach of Maniotis *et al.* (1997) with single chromosome elasticity measurements, the aim being to do real-time quantitative monitoring of chromosome

structure changes. Our focus is on the study of mitotic chromosome structure, with less emphasis on the interchromosome connections studied by Maniotis *et al.* (1997). The basic method is to extract mitotic chromosomes to set up two-pipette micromanipulation and then to measure the initial, native stretching elastic response. Then, we bring in a third pipette of inside ~4 μm, larger than the chromosome-grabbing pipettes, which has been loaded with some reagent in suitable solution (typically 60% PBS or Tris buffer, pH 7.6; see Fig. 7).

This third pipette is brought within 10 μm of the chromosome and then pressure is used to spray the reagent at the chromosome. Calibration experiments using fluorescent dyes show that this results in a jet of reagent exiting the pipette, with concentrations near to those in the pipette up to 20 μm away. Beyond this distance, the reagent diffuses rapidly into the large (~1 ml) volume of the sample dish. In a typical experiment, volumes of a few thousand cubic micrometers are typically sprayed (1000 cubic μm is 10^{-12} liter = 1 picoliter). Using this technique, arbitrary reagents can be introduced onto a mitotic chromosome, and the kinetics of reactions can be observed micromechanically to some extent via the force-measuring pipette. Then, when reagent flow is stopped, the chromosome is returned rapidly (<1 s) to the initial (extracellular) buffer condition, in which the effect of the reaction on elastic properties can be measured quantitatively.

C. Shifts in Ionic Conditions Can Decondense or Hypercondense Mitotic Chromosomes

Because chromatin is electrically charged, one can expect its solvency, packing, and elasticity properties to be modified by changes in ionic conditions. In single fiber experiments, Cui and Bustamante (2000) showed that unfolding of single chromatin fibers could be modulated with an univalent salt concentration, and Maniotis *et al.* (1997) showed that an increased ionic strength could strongly decondense isolated mitotic chromosomes. Using our microspraying techniques, we quantified the effect of shifts in salt concentration, and we have reproduced the abrupt decondensation effects reported by Maniotis *et al.* (1997) with >200 m*M* univalent and divalent salt concentrations (Poirier *et al.*, 2002). In experiments where force was monitored, we found that applied tension was reduced entirely by high concentrations of Na^+ and Mg^{2+}.

However, we have also found that in the 20 to 100 m*M* Mg^{2+} and Ca^{2+} concentration range, mitotic chromosomes go through a range of rather strong condensation, generating contractile forces of up to 0.2 nN. As the divalent cation concentration is ramped up from zero, we observe condensation near 20 m*M*, followed by an abrupt return to the native degree of

compaction near 50 mM and then finally the strong decondensation observed by Maniotis *et al.* (1997) at >200 mM. Our results are in excellent accord with old observations of the morphological change of mitotic chromosomes during ionic condition shifts (Cole, 1967). We emphasize that the use of force measurement, in addition to morphological observation, provides a quantitative measure of the degree to which chromosome structure is changed reversibly, and irreversibly, in these types of experiments (Fig. 12).

A similar condensation–decondenation effect is observed with increasing concentrations of trivalent ions. No compaction occurs with any concentration of Na^+ or K^+. All these decondensation and condensation effects occur *istropically*; under zero tension, the fractional length and fractional width changes are nearly equal. This behavior is reconciled easily with a chromosome model, which is an isotropic network of chromatin fibers and is difficult to square with an aniostropic chromatin loop attached to scaffold model.

The effects of divalent ions are in line with similar reentrant bundling (i.e., bundling followed by dissolution as the divalent ion concentration is raised) of stiff polyelectolytes observed in DNA (Pelta *et al.*, 1996; Saminathan *et al.*, 1999) and actin solutions (Tang *et al.*, 1996). These phenomena again make clear that native mitotic chromatids are not near their maximum possible compaction [we observe up to a 30% volume decrease using trivalent ions (Poirier *et al.*, 2002)] and also that charge interactions are important in determining the precise degree of chromatin compaction. Divalent ions at 20–100 mM concentrations overcondense chromatin, whereas ionic concentrations >300 mM lead to strong but apparently reversible decondensation.

The rapid decondensation and hypercondensation discussed earlier occurred during experiments where the ionic conditions were shifted for less than 1 min. In these experiments, force curves measured after the ionic exposures showed that no appreciable changes in mitotic chromosome

elasticity, and from our point of view, mitotic chromosome structure, occurred. However, sufficiently long exposures (>10 min) to high ionic strength conditions result in an irreversible change in chromosome structure and elasticity (Poirier *et al.*, 2002); high salt exposures are well known to eventually remove even core histones from chromatin. However, appreciable protein removal does not appear to be occuring during the ~10-s experiments discussed previously. This result applied to ~10 mM concentrations

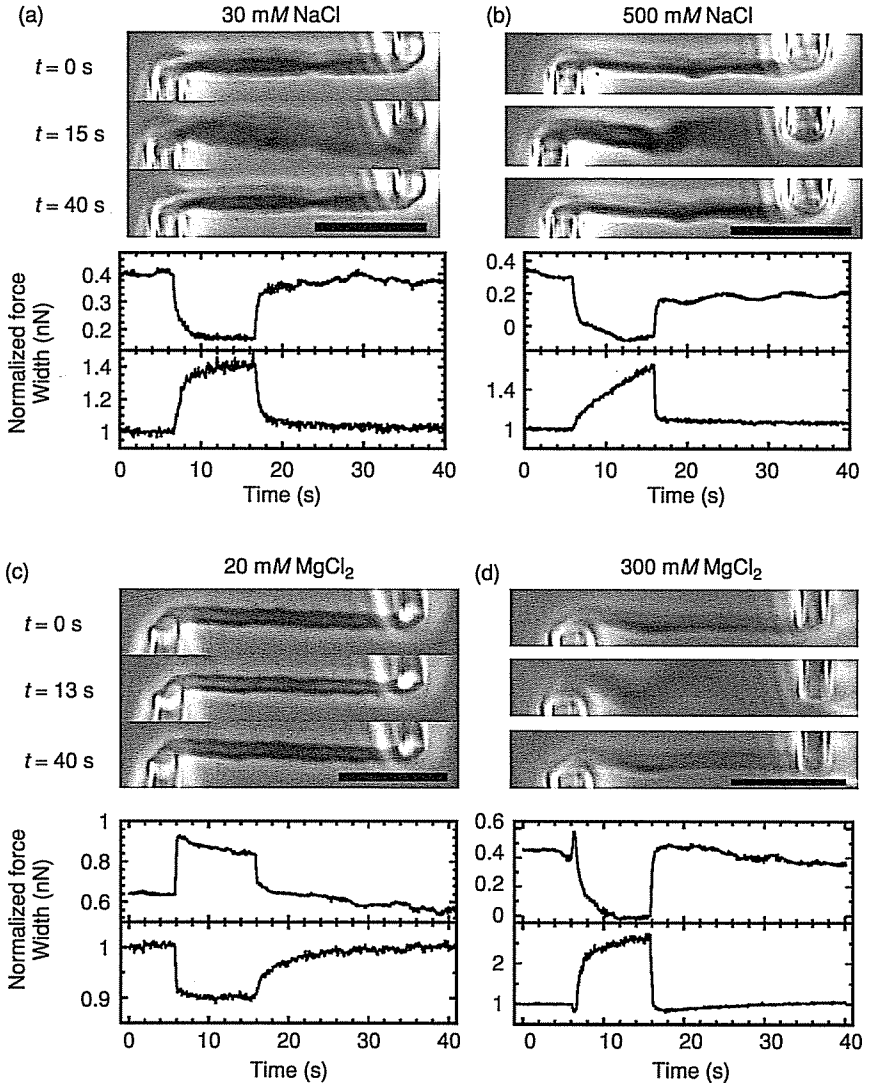


Figure 12 Effects of shifts in ionic conditions on the newt chromosome structure. Images of combined chemical–micromechanical experiments with 30 mM NaCl (a), 500 mM NaCl (b), 20 mM $MgCl_2$ (c), and 300 mM $MgCl_2$ (d). Images show the chromosome before, during, and after an exposure to the different ionic conditions. Plots show the time series of the force the chromosome supports and width of the chromosome. For 30 and 500 mM NaCl, the force decreases and the width increases. However, 20 mM $MgCl_2$ induces an increase in the force and a decrease in width, whereas 300 mM $MgCl_2$ causes a decrease in force and an increase in width. Note that the force signal for the 300 mM spray of $MgCl_2$ shows a brief force "spike" due to transient chromatin condensation induced by the ion concentration sweeping through ~10 mM concentrations. The time scale of the response of the chromosome to the ionic shifts occurs on the second time scale and shows that the internal structure of a mitotic chromosome can be changed rapidly. Bars: 10 μm. From Poirier *et al.* (2002).

of Mg^{2+} is an important control for enzyme experiments where divalent ions are often present in about this concentration (see later).

D. Micrococcal Nuclease Completely Distintegrates Mitotic Chromosomes

Micrococcal nuclease (MNase) nonspecifically cuts dsDNA and is widely used to cut chromatin up into nucleosomes. This enzyme was an obvious choice to use to examine the effect of cutting away the chromatin itself, and we were originally motivated to determine whether we could reveal whether the often-discussed internal protein "scaffold" (Paulson and Laemmli, 1977; Marsden and Laemmli, 1979; Earnshaw and Laemmli, 1983; Boy de la Tour and Laemmli, 1988; Saitoh and Laemmli, 1994) was mechanically contiguous. A second aim of the experiments was to determine just how much of the chromosome elastic response was due to chromatin (i.e., dsDNA) itself. Finally, we were motivated by curiosity about how to reconcile the old literature suggesting that DNase could disintegrate mitotic chromosomes (Cole, 1967) with the observation that the protein-rich chromosome scaffold could survive the biochemical removal of histones (Paulson and Laemmli, 1977).

We therefore sprayed isolated newt TVI mitotic chromosomes with 1 n*M* MNase in suitable reaction buffer (60% PBS plus 1 m*M* $CaCl_2$) with a small tension (0.1 nN) applied initially (Poirier and Marko 2002c). Tension is

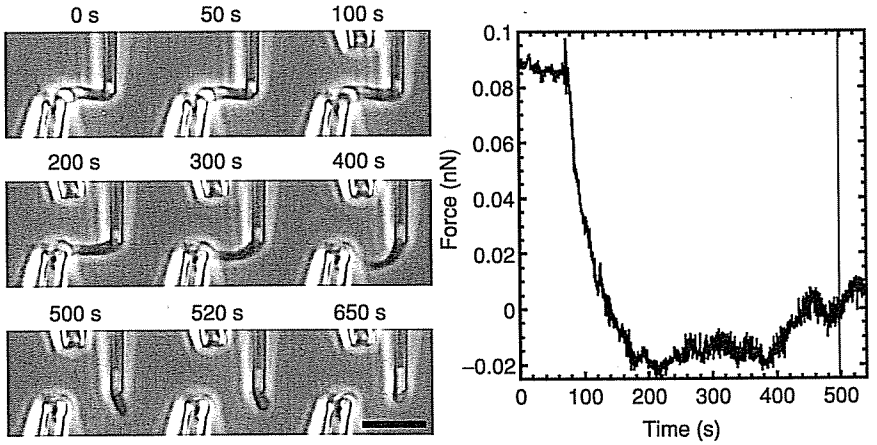


Figure 13 Time course of tension in a chromosome, and chromosome morphology, during digestion by 1 n*M* MNase with an initial tension of 0.1 nN. Spraying starts at 80 s; force decays after 30 s; and the chromosome is cut after 450 s. The spray pipette can be seen in the upper center of the $t > 120$-s frames. Bar: 10 μm.

monitored during the spray experiment (Fig. 13). When the spray starts, the tension jumps briefly due to the slight compaction induced by the divalent Ca^{2+}, but then the tension drops below our force resolution (~ 0.01 nN = 10 pN) after 30 s. During this initial period, the morphology of the chromosome is unaffected. However, between the period 100 of 200 s, the chromosome disintegrates and is eventually severed (Fig. 13). This experiment indicates that the force-bearing and structural element of the mitotic chromosome is DNA based, i.e., chromatin itself, and indicates that the chromosome is not held together by a mechanically contiguous internal protein "scaffold."

A second type of experiment was also done where digestion was done with zero applied force and was then stopped before the chromosome was altered morphologically (at 30 s of 1 n*M* exposure). The chromosome could then be extended into a string of blobs, connected by thin chromatin strands. These strands could then be severed by a brief spray of MNase, where the peak tension applied was <100 pN. This latter experiment makes clear that the disassembly effect observed using MNase is not tension dependent. The forces applied in this experiment are below those required to break single protein or nucleic acid chains.

These experiments, which are in accord with the old literature of disintegration of mitotic chromosomes by DNAase (Cole 1967), indicate that the mitotic chromosome is essentially a cross-linked network of chromatin, i.e., that the higher order chromosome structure is stabilized by non-DNA molecules (most likely proteins), which are isolated from one another. It is difficult to reconcile our MNase results with the "textbook" model (Lewin, 2000; Lodish *et al.*, 1995; Wolffe, 1995) of chromatin loops hanging from an internal mechanically contiguous protein scaffold.

E. Restriction Enzymes with Four-Base Specificity Can Disintegrate Mitotic Chromosomes

Following the MNase experiments we carried out experiments with blunt-cutting restriction enzymes, which cut dsDNA, leaving no overhangs, at specific base pair sequences (Poirier and Marko 2002c). These enzymes are powerful tools for analyzing the network connectivity of mitotic chromosomes. We selected enzymes that were active in physiological-like buffers (i.e., pH near 7, ionic conditions near 100 m*M* univalent plus ~10 m*M* divalents).

Two enzymes with four-base recognition sequences, *Alu*I (AG^CT) and *Hae*III (GG^CC), which occur every 256 bases on random-sequence DNA, were used, and they cut up mitotic chromosomes in the fashion of MNase. Figure 14 shows the result for *Alu*I, which severs the chromosome

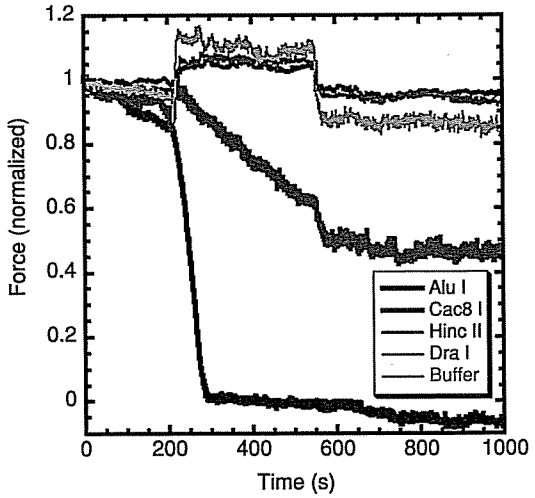


Figure 14 Time course of tension in a chromosome during digestion by blunt-cutting restriction enzymes. Initial force in all experiments was 0.6 nN; each force curve is normalized to this initial value. Enzyme exposure is from 200 to 550 s. (Bottom curves) *Alu*I completely reducing force to zero (cutting chromosome completely). (Middle curves) Cac 8I only partially relaxing applied tension (partially cutting chromosome). (Top curves) Only small effects of *Hinc*II, *Dra*I, and restriction enzyme activity buffer (no enzyme). The "step" increases in force for the top curves reflect the slight condensation of the chromosome driven reversibly by the divalent ions of the activity buffer.

completely after <100 s (again the force increase seen at the onset of spraying is the reversible condensing effect of the ~10 m*M* Mg^{2+} in the enzyme buffer, easily understood in the light of our previous salt experiments). After factoring in the 10-fold reduction in sequence accessibility in chromatin vs bare DNA, this experiment shows that mitotic chromosomes are not cross-linked more often than once every few kilobases.

Experiments with six-base recognition sequence enzymes, *Stu*I (AGG^CCT) and *Dra*I (TTT^AAA), show essentially a zero force effect (Fig. 14 shows *Dra*I), indicating that the accessible six-base sites are rarer than chromatin cross-links.

To test the accessibility of six-base-wide sites further, Fig. 14 also shows results for Cac81 where four bases are recognized out of a six-base region (GCN^NGC). This enzyme shows an intermediate effect, partially reducing applied force, but not totally severing the chromosome. Thus, the six-base site size is partially available to the restriction enzymes. Taken together, these results are all consistent with a chromatin network model with a cross-link every few tens of kilobases and are inconsistent with an internal protein scaffold model (unless the "scaffold" has the form of many small

localized protein structures, which is of course again a cross-linked network of chromatin).

F. Summary

We have described how micromechanical measurements can be combined with microspraying of reactants to carry out experiments that give unique information about the mitotic chromosome structure. Experiments with varying ionic conditions show that the mitotic chromosome can be decondensed rapidly (~1 s) and reversibly or, in the case of ~10 m*M* concentrations of divalent and trivalent cations, hypercondensed. This shows that the mitotic chromosome structure is stabilized at a less than maximal level of condensation by interactions of strong electrostatic character.

Experiments with DNA-cutting enzymes show that the contiguous element that defines mitotic chromosome structure is DNA itself. Chromosomes can be dissolved completely by MNase and four-site blunt-cutting restriction enzymes, a result that is hard to reconcile with a chromatin loop scaffold model unless the underlying non-DNA scaffold disassembles spontaneously as a result of cutting of DNA.

VII. Conclusion

A. Summary of Physical Properties of Mitotic Chromosomes

The previous sections presented data for elastomechanical properties of mitotic chromosomes. To generalize, we have found that mitotic chromosomes stretch and bend as if they are classical elastic media, but with an enormous range of extensibility. We find that mitotic newt chromosomes can be reversibly stretched fivefold and that over this range their elastic response is nearly linear, with a Young (stretch) modulus of about 500 Pa. The mitotic chromosomes of newt and *Xenopus* are therefore doubled in length by forces ~1 nN, similar to the elastic response of grasshopper spermatocyte metaphase I chromosomes (Nicklas, 1983), and also similar to the maximum forces applied by the mitotic spindle to chromosomes during anaphase.

The large range of the linear elastic response of mitotic chromosomes is distinct from well-bonded materials such as metals (which fracture when stretched by less than a percent in length) and even most polymer gels. This basic elastic response is in accord with the classic *in vivo* results of Nicklas (1983) for grasshopper spermatocyte (metaphase I) chromosomes and with *Xenopus* mitotic chromosomes. Houchmandzadeh and Dimitrov (1999)

found similar elastic behavior in their study of *in vitro*-assembled *Xenopus* chromatids.

Mitotic chromosomes taken out of cells have bending elasticity consistent with their Young modulus via Eq. (3), indicating that they may be considered to be roughly uniform (at least regarding their mechanical properties). In our experiments on newt and *Xenopus* chromosomes (Poirier and Marko, 2002a) removed from cells, we observed remarkably uniform bending properties, with no sign of "hinged" regions. In cochicine-treated cells we were able to observe bending fluctuations, which indicated that *in vivo* the bending elasticity of mitotic newt and *Xenopus* chromosomes is similar to those observed for extracted chromosomes.

Our observation of bending stiffness in accord with the stretching elasticity is distinct from results for *in vitro*-assembled *Xenopus* chromatids (Houchmandzadeh and Dimitrov, 1999), who found bending elasticity roughly 1/1000 of what was expected on the basis of their ~1-nN stretching constant. The tremendous flexibility of the *in vitro*-assembled chromatids relative to chromosomes in and extracted from cells in striking and suggests to us that *in vitro*-assembled chromatids have an internal structure quite distinct from metaphase chromosomes *in vivo*. An important experiment is therefore measurement of the bending flexibility of *in vitro chromosomes* assembled in egg extracts and cycled through one round of DNA replication.

Nicklas and Staehly (1967) noted that after being stretched and released, grasshopper chromosomes slowly recoiled to their native length. We have found that this slow relaxation is an intrinsic physical property of mitotic chromosomes and that the effective internal viscosity inside a mitotic chromosome is roughly 100,000 times that of water. The same slow relaxation is responsible for making the bending fluctuation time of a mitotic chromosome independent of the chromosome length, which from the point of conventional polymer and colloid physics is peculiar. This slow stress relaxation indicates that chromatin fiber domains inside the folded mitotic chromosome are undergoing continual slow thermal rearrangements with a time scale on the order of 1 s. These slow rearrangements are a natural outcome of the slow motion of entangled domains of chromatin fiber.

B. Elasticity of Mitotic Chromosomes versus Elasticity of Chromatin Fiber

Individual chromatin fibers extend by about a factor of two relative to their native length, under tensions of about 5 pN (Cui and Bustamante, 2000; Bennink *et al.*, 2001; Brower-Toland, 2002). There are ~2000 chromatin fibers crossing a cross-section of a newt mitotic chromosome (the cross-sectional area ~3 μm^2, divided by the ~800-nm^2 area per

chromatin fiber, divided by two since part of the internal volume of the chromosome is cytoplasm "solvent"). This indicates that we can expect a force of roughly 10 nN (2000 times 5 pN) to be associated with opening up a mitotic chromosome to the end of its elastic response. The paradox here is that at this point a mitotic chromosome is actually extended about 10× its native length. This indicates that the initial elastic response of a whole mitotic chromosome is due to opening of a higher order chromatin structure.

Regarding mechanical properties, to date it is not clear what the relation is between extracted chromatin fibers (Cui and Bustamante, 2000) or *in vitro*-assembled chromatin (Bennink *et al.*, 2001; Brower-Toland *et al.*, 2002) and mitotic chromosomes. One possibility is that the large range of elastic response is due to the opening of SMC-compacted chromatin fiber. Kimura *et al* (1997, 1999) suggested that condensins coil DNA, and perhaps when applied to chromatin, a spring-like coil results. This could be tested by single molecule experiments on chromatin fiber compacted by active condensins.

C. Ionic Condition Shift Experiments

Maniotis *et al.* (1997) showed that mitotic chromosomes may be decondensed easily and, to some degree, reversibly recondensed, in addition to their observation that chromosomes are interconnected throughout the cell cycle. Our study of effects of shifts in ionic conditions on extracted mitotic newt chromosomes verifies this result and extends it. First, we find that either lowering or raising the univalent ionic strength relative to the ~100 n*M* Na^+ of the amphibian cell culture medium decondenses mitotic newt chromosomes. For lowered ionic strength, decondensation most likely results from opening of a 30-nm fiber into 10-nm "beads on a string." For a high salt concentration, decondensation is most likely due to a reduction in the range of electrostatic interactions, with consequent opening of folded chromatin (Poirier *et al.*, 2002).

For multivalent ions added to cell buffer, condensation (even by Mg^{2+}) occurs at low (~10 m*M*), concentrations; at higher concentrations (>100 m*M*), decondensation occurs. The low-concentration condensation may be due to "bridging" interactions, i.e., net attractive interactions induced by localization of the multivalent ions (Ha and Liu, 1997). An alternative explanation is that condensation occurs when the charge neutral point is reached, eliminating coulomb repulsion and allowing other attractive interactions to dominate (Nguyen *et al.*, 2000; Nguyen and Shklovskii, 2001).

To us, the most significant result of ionic condition shift experiments is that the mitotic chromosome structure can be cycled through condensation

or decondensation chemically on short (1 s) time scales. Moreover, when flow is stopped to return to the original buffer conditions, the chromosome immediately returns to its native state, as assayed by morphology and mechanical properties. Irreversible lengthening and softening of the chromosome, presumably due to protein loss, occur only at high (>1 *M*) ionic strengths after >10-min exposures (Poirier *et al.*, 2002).

D. DNA-Cutting Experiments

Cutting dsDNA inside the mitotic newt chromosome with sufficient frequency disconnects the chromosome completely (Poirier and Marko, 2002c). MNase and four-base blunt-cutting restriction enzymes dissolve the chromosome into optically invisible fragments. By far the simplest interpretation of this experiment is that the elastic response and mechanical continuity of the mitotic chromosome are due to chromatin fiber, i.e., DNA itself. A rough estimate of the genomic distance between cuts required to disconnect the chromosome is 15 kb, based on the reduction in effect of more rarely cutting restriction enzymes (Poirier and Marko, 2002c). Six-base blunt-cutting restriction enzymes have no effect on the mechanical properties of whole mitotic newt chromosomes.

E. Implications for Structure of the Mitotic Chromosome

The experiments reviewed earlier, taken together, consistently suggest that the mitotic chromosome has a *network* structure, i.e., is organized by isolated chromatin–chromatin attachments (Fig. 15). The purely mechanical measurements (stretching and bending) indicate that chromosome stretching is supported by stress spread across its whole cross section, and therefore that the mitotic chromosome structure appears to be, at the scale of a

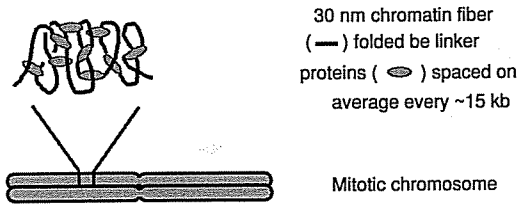


Figure 15 Network model of a mitotic chromatid. Black curve indicates the single linear chromatin fiber, and black blobs show isolated non-DNA cross-linking elements. If the chromatin is cut sufficiently often, the chromosome will be severed; the non-DNA cross-linkers are not mechanically contiguous through the chromosome.

whole chromatid, homogeneous. This hypothesis is also supported by the homogeneous way that whole chromosomes elongate.

Dynamic stretching and bending experiments both show that the interior of a mitotic chromosome relaxes extremely slowly, on a roughly 1-s time. We hypothesize that this long time scale is due to chromatin conformational fluctuation and that the long time scale has its origin in entanglements. This implies that mitotic chromatin is not heavily constrained by chromatin-folding proteins, i.e., that there are long stretches of chromatin between "cross-links." These stretches of chromatin are apparently free to undergo slow conformational motions.

Shifts in ionic conditions can decondense and overcondense a mitotic chromosome rapidly. These morphological changes are reversible for short (10 s) salt treatments, and at zero stress are isotropic, again suggesting a homogeneous and not terribly highly ordered mitotic chromatin organization. At least one-third of the chromosome volume is mobile cytoplasm or buffer based on condensation experiments. Finally, our DNA-cutting experiments make clear that the mechanically contiguous structural element of the mitotic chromosome is DNA (i.e., chromatin) itself. The nonchromatin fiber content of the mitotic chromosome must be disconnected.

Taken together, these results indicate that the mitotic chromosome must be a network of chromatin fiber, with isolated nonchromatin cross-links (Fig. 15). We must rule out models for mitotic chromosome structure based on mechanically well-defined non-DNA skeletons or scaffolds. It must be emphasized that the identity of these putative chromatin cross-linkers is unknown; at present the most likely suspects are the condensin-type SMC protein complexes.

F. Future Experiments

The combined chemical–micromechanical method for the study of a large-scale chromosome structure explored here provides information complementary to usual biochemical assay and microscopy approaches. Traditional biochemical approaches give information about local interactions and the products of chemical reactions. Traditional microscopy gives information about morphology and structure in a given cell state or in a given preparation of molecules. Our approach allows direct study of elastomechanical properties of chromosomes and to observe how those properties are modified dynamically by chemical reactions. Our conclusions about the flexibility and connectivity of chromatin fiber in the mitotic chromosome are difficult to support by traditional biochemical and microscopy approaches, but are rather obvious results of a combined chemical–micromechanical approach. The question of mitotic chromosome organization therefore can be attacked

Table I Symbols, Units and Relationships

Symbol	Name	Unit	Value	Comment
A	Persistence length	m	0.1	Mitotic newt chromosome
B	Bending modulus	$N{\cdot}m^2$	10^{-22}	Mitotic newt chromosome
f_0	Force constant	N	10^{-9}	Mitotic newt chromosome
θ	Angle	rad	10^{-2}	Chromosome segment bend
η	Fluid viscosity	Pa·sec	10^{-3}	Water and cell culture media
η'	Internal viscosity	Pa·sec	100	Mitotic newt chromosome
k	Boltzmann constant	J/K	1.381×10^{-23}	Equal to R/N_A
kT	Unit of thermal energy	J	4.1×10^{-21}	At 300°K = 27°C
k_p	Pipette stiffness	N/m	10^{-3}	Typical force-measuring pipette
L	Length	m	20×10^{-6}	Mitotic newt chromosome
N_A	Avagadro's number		6.022×10^{23}	
r	Cross-section radius	m	1×10^{-6}	Mitotic newt chromosome
R	Gas constant	J/K	8.316	Equal to $N_A k$
u	Transverse fluctuation	m	1×10^{-7}	Mitotic newt chromosome
Y	Young modulus	Pa	500	Mitotic newt chromosome
Distance:	1 m = 10^6 μm = 10^9 nm = 10^{10} Å			
Force:	1 Newton (N) = 1 $kg{\cdot}m/s^2$ = 10^4 dyne			
	1 nN = 10^{-9} N			
	1 pN = 10^{-12} N			
	1 kT/nm = 4.1 pN (at 300°K = 27°C)			
Energy:	1 Joule (J) = 1 $kg{\cdot}m^2/s^2$ = 10^7 erg = 0.239 cal			
	1 kT = 0.59 kcal/mol (at 300°K = 27°C)			
Pressure:	1 Pascal (Pa) = 1 N/m^2			
Bending modulus:	1 $N{\cdot}m^2$ = 1 J·m			
dsDNA:	1 Gbp (10^9 bp) of dsDNA = 1.013 p (pg = 10^{-12} g) = 0.34 m contour length			

profitably by integrating information from all these approaches. Many interesting questions remain to be answered.

A very basic variation on the stretching experiments would be the study of relative elasticity of different regions of the mitotic chromosome. Use of labels for centromere, telomere, and euchromatin regions of the chromosome would allow the elasticity of different types of chromatin to be studied. For example, elasticity of the kinetochore is relevant to the modeling of chromosome capture by the mitotic spindle (Joglekar and Hunt, 2002).

To back up the network model of the mitotic chromosome, it is be extremely important to analyze the sizes of chromatin fragments produced. This could be done via aspiration of the fragments followed by fluorescence quantification of them after dispersal onto a slide. Also, further digestion experiments using other DNA cutters, RNases, and proteases need to be done. Effects of other chemical modifications of chromatin (e.g., acetylation,

Table II Physical Properties of Mitotic Chromosomes[a]

Chromosome type	Experimental condition	Young's modulus, Y (Pa)	Bending Rigidity, B (J·m)	Internal viscosity (Pa·s)	Reference
Drosophila metaphase chromosome	*In vivo*	N.D.[b]	$\sim 6 \times 10^{-24}$	N.D.	Marshall *et al.* (2001)
Grasshopper metaphase I and anaphase I chromosome	*In vivo*	200 to 1000 (avg = 430)	N.D.	~100	Nicklas and Staehly (1967), Nicklas (1983)
Newt (*N. viridescens*) prometaphase chromosome	Cell culture medium	100 to 1000	$1\text{–}3 \times 10^{-22}$	100	Houchmandzadeh *et al.* (1997); Poirier *et al.* (2000, 2001, 2002a)
Newt prometaphase chromosome	*In vivo*	N.D.	$2\text{–}5 \times 10^{-23}$	N.D.	Poirier *et al.* (2002a)
Xenopus prometaphase chromosome	Cell culture medium	200 to 800	$0.5\text{–}2 \times 10^{-23}$	N.D.	Poirier *et al.* (2002a)
Xenopus prometaphase chromatid	Cell culture medium	~300	$\sim 5 \times 10^{-24}$	N.D.	Poirier *et al.* (2002a)
Xenopus reconstituted chromatid	*Xenopus* egg extract	1000	1.2×10^{-26}	N.D.	Houchmandzadeh and Dimitrov (1999)

[a] Ranges for values indicate the width of distribution of measured values, not measurement errors. [b] Indicates quantity not measured directly.

phosphorylation) on mitotic chromosome condensation, monitored precisely via elasticity, would also be interesting. These kinds of experiments in general give information on the poorly understood question of enzyme access in dense chromatin.

Development of our techniques to study the structural roles of specific proteins might be possible. We have already demonstrated antibody labeling using microspraying for antihistone (Poirier *et al.*, 2000) and for anti-XCAPs (unpublished); our method used directly fluorescent labeling and thus observation of primary antibody reactions *in situ*. The simplest types of experiments would be visualization of targeting in our experiment as a function of chromosome stretching. This general technique might be useful for chromosome mapping (Clausen, 1994; Hliscs, 1997a,b), especially if different parts of a chromosome could be exposed to different reagents using microchannel arrays.

More ambitious experiments would use fluorophores, which can generate large amounts of hydroxyls, lysing the antibody targets (Beerman and Jay, 1994). This technique has been used successfully to study the disruption of cytoskeletal proteins and might be used in conjunction with chromosome elasticity measurement to study the effect of condensin or cohesin disruption on the mitotic chromosome structure. This type of experiment could directly test models of SMC function such as that of Losada and Hirano (2002).

Study of the orientational ordering of chromatin using polarization microscopy could be informative. Purified and concentrated nucleosomes have been demonstrated to form chiral liquid crystal phases (Leforestier *et al.*, 1999; Livolant and Leforestier, 2000); optical activity has also been observed for certain chromosomes (Livolant, 1978; Livolant and Maestre, 1988). A major question is whether animal chromosomes have similar liquid crystal organization, either in native or in stretched forms. Some very preliminary experiments on newt chromosomes in our laboratory using the CRI Polscope showed undetectable birefringence for chromosomes stretched up to four times native length. This suggests that ordered domains of mitotic chromatin are smaller than the wavelength of light, i.e., <100 nm, and that appreciable stretching of chromosomes does not induce strong orientational ordering of chromatin. However, further experiments are necessary.

Our results suggest that chromatin in mitotic chromosomes is to some degree flexible and so it might be possible to pull chromatin fibers out of them. This could be done using pipettes to pull on small particles coated with antihistone or other chromatin-binding factors. Comparison of mitotic chromatin fiber physical properties obtained in such an experiment, with results of single chromatin fiber mechanical experiments (Cui and Bustamante, 2000; Bennink, 2001), would be interesting.

We have repeatedly observed interchromosome fibers between mitotic chromosomes as discussed by Maniotis *et al.* (1997), and these objects require further study. Initial experiments have verified the result of Maniotis *et al.* (1997) that these fibers are cut by MNase and therefore contain nucleic acid (most likely DNA). Rough stretching experiments show that these fibers are highly and reversibly extensible, with an estimated force constant in the nanonewton range. These are therefore a more folded structure than the 30-nm fiber, but because they are barely visible in the light microscope, we estimate their thickness to be less than 200 nm. DNA staining and quantification are an objective of our current studies. We also hypothesize that these fibers are telomeric structures (the interchromosome fibers at metaphase almost always come from chromosome ends), and therefore probes for telomere DNA should be tested. An interesting question is whether these fibers are intrinsic to transformed cells (most of our work is in tumor cell lines), and therefore parallel studies in primary cell cultures are of strong interest.

Other chromosome structures could be studied by combined chemical–micromechanical techniques. We are interested in comparing mitotic chromosomes to meiotic chromosomes. The range of physical structures occurring during meiosis provides a motivation for micromechanical experiments. Mechanical properties of meiotic chromosomes may play a crucial role in general recombination (Kleckner, 1996; Zickler and Kleckner, 1999) and may be related to polymer physics of the chromatin loops (Marko and Siggia, 1997a). Interphase chromosomes would be extremely interesting to study as isolated objects. Maniotis *et al.* (1997) used purely mechanical techniques to extract whole interphase genomes, an important first step. We are searching for a biochemical method to open the nuclear envelope to allow more gentle interphase genome extractions.

Finally, we note that Hinnebusch and Bendich (1997) have demonstrated that bacterial chromosomes can be extracted and studied physically Cunha *et al.* (2000a, 2001b) have succeeded in isolating and chemically manipulating *Escherichia coli* nucleoids, which might also be studied using micromechanical techniques. The wide range of genetic and biochemical tools developed for *E. coli*, plus the many very basic and open questions regarding the bacterial chromosome structure, make it a highly attractive system for micromanipulation study.

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