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Micromechanical studies of mitotic chromosomes

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Abstract

We review micromechanical experiments on mitotic chromosomes. We focus on work where chromosomes were extracted from prometaphase amphibian cells, and then studied by micromanipulation and microfluidic biochemical techniques. These experiments reveal that chromosomes have 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. Finally, we discuss microspraying experiments of DNA-cutting enzymes which reveal that the element which 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. We compare our results to similar experiments done by Houchmandzadeh and Dimitrov (J Cell Biol **145:** 215–213 (1999)) on chromatids reconstituted using *Xenopus* egg extracts. Remarkably, while the stretching elastic response of the reconstituted chromosomes is similar to that observed for chromosomes from cells, the reconstituted chromosomes are far more easily bent. This result suggests that reconstituted chromatids have a large-scale structure which is quite different from chromosomes in somatic cells. More generally our results suggest a strategy for the use of micromanipulation methods for the study of chromosome structure.

Nomenclature

Symbol	*Name*	*Value*
A	persistence length (mitotic newt chromosome)	0.1 m
B	bending modulus (mitotic newt chromosome)	10^{-22} N m^2
f_0	force constant (mitotic newt chromosome)	10^{-9} N
η	fluid viscosity (water and cell culture media)	10^{-3} Pa s
η'	internal viscosity (mitotic newt chromosome)	100 Pa s
k	Boltzmann constant (equal to R/N_A)	1.381×10^{-23} J/K
kT	unit of thermal energy (at 300 K = 27°C)	4.1×10^{-21} J
k_p	pipette stiffness (typical force-measuring pipette)	10^{-3} N/m
ℓ	distance to pipette (for bending experiment)	10^{-6} m
L	length (mitotic newt chromosome)	20×10^{-6} m
N_A	Avagadro's number	6.022×10^{23}
r	cross-section radius (mitotic newt chromosome)	1×10^{-6} m
R	gas constant (equal to $N_A k$)	8.316 J/K
u	transverse fluctuation (mitotic newt chromosome)	1×10^{-7} m
Y	Young modulus	500 Pa

Distance: 1 m = 10^6 μm = 10^9 nm = 10^{10} Å
Force: 1 newton (N) = 1 kg m/sec^2 = 10^5 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 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 m^2 = 1 J m
dsDNA: 1 Gbp (10^9 bp) of dsDNA = 1.013 picograms (pg = 10^{-12} g) = 0.34 m contour length

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Introduction

The question of how the millimeter to centimeter-long double-stranded DNAs 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 traditional microscopy of higher-order chromatin structure. The fact that new models for large-scale chromosome structure (Kimura *et al*., 1999; Machado and Andrew, 2000; Dietzel and Belmont, 2001; Losada and Hirano, 2001; Stack and Anderson, 2001) continue to be proposed in the literature indicates that this question remains open.

There are many reasons why determination of 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 (Figure 1). In this article, the focus is on the folding of the chromosome in vertebrate 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 be mainly considering chromosomes from newt (*Notophthalmus viridescens*) and frog (*Xenopus laevis*). These are model organisms for study of mitotic chromosome structure in part simply because their chromosomes are large (Figure 2).

A second problem that chromosome researchers must confront is that *chromosomes are soft physical objects*, with elastic stiffness far less than that of the DNAs and proteins from which they are composed. This means that the structures of chromosomes can be destroyed – or changed – by preps which leave protein and DNA secondary structures intact. This paper 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 for structural changes introduced biochemically.

'Architecture and components of Eukaryote chromosomes' provides a brief review of previous biophysical studies of chromosome structure, and DNA and chromatin physical properties. 'Elasticity of mitotic chromosomes' reviews experiments studying stretching, bending, and dynamic elastic responses of mitotic chromosomes (Table 1). 'Combined biochemical-micromechanical study of mitotic chromosomes' 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. 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

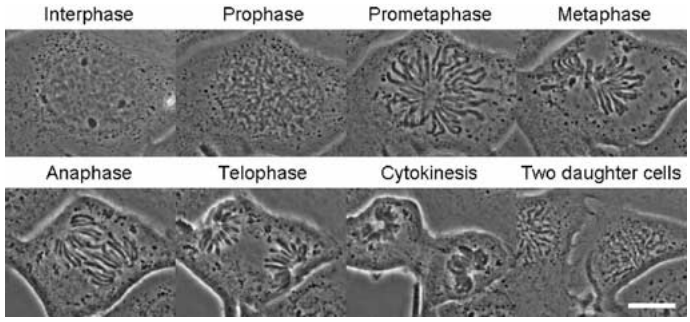

Fig. 1. Cell cycle in a newt cell. During mitosis, chromosomes condense inside the nucleus during prophase, the nuclear envelope disassembles and chromosomes float loose in the cytoplasm during prometaphase, they are captured and aligned by the spindle at metaphase, and the two duplicate chromatids of each chromosome are pulled apart at anaphase. Bar is 20 μm, image is phase contrast, 60x oil objective.

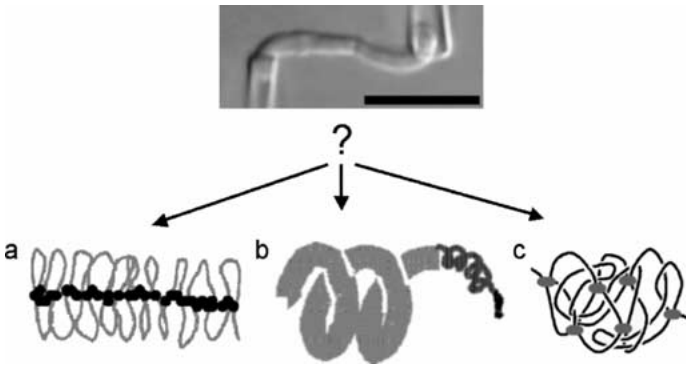

Fig. 2. Prometaphase chromosome, attached at its ends to pipettes outside a cell. Bar is 10 μm, image is DIC, 60x oil objective. Below are three possible models of how chromatin is arranged within a mitotic chromosome.

Table 1. Physical properties of mitotic chromosomes

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

ND indicates quantity not directly measured.
Ranges for values indicate the width of distribution of measured values, and not measurement errors.

physically connected protein skeleton. Finally, 'Conclusion' presents a preliminary model of mitotic chromosome structure based on these results, and then discusses some of the many open questions, including the controversial topic of DNA connections between mitotic chromosomes.

Work of Poirier *et al.* (2000, 2001a, 2002a, b, c) is described in more detail in the PhD thesis of Poirier (2001b). These documents plus images and movies of experiments are available at http://www.uic.edu/~jmarko.

Architecture and components of eukaryote chromosomes

In this section, we review current understanding of the components of chromosomes and overall chromosome structure (see Koshland and Strunnikov, 1996; Hirano, 2000 for more detail on this topic). We also discuss physical properties of DNA and chromatin fiber, with emphasis on recent micromanipulation experiments.

Eukaryote chromosomes are made of chromatin fiber

Chromosomes of animals contain on the order of 10 Mb to 1 Gb of dsDNA (for dsDNA 1 Gbp = 1 pg). At all stages of the cell cycle, the 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 precisely determined using X-ray crystallography (Richmond *et al.*, 1984; Arents *et al.*, 1991; Luger *et al.*, 1997). Discovery of the remodeling of nucleosome structure and chemical modification of histones themselves during gene expression (Wolffe and Guschin, 2000) indicates that there are most likely many chemical-structural states of chromatin to understand.

The molecular weight of 200 bp of dsDNA is about 120 kD, and the molecular weight of the histone octamer plus one 'linker' histone (which sits on the linker DNA) is about 125 kD. 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 the DNA bound to nucleosomes is able to transiently unbind. Precise experiments (Polach and Widom, 1995; Widom, 1997; Anderson and Widom, 2000) show that restriction-enzyme access to DNA is exponentially attenuated as one moves into nucleosome-bound DNA. This raises the interesting question of on what timescale, and for what factors, transient access to DNA may occur, via conformational fluctuation of the nucleosome itself.

Electron microscope (Thoma *et al.*, 1979) and X-ray diffraction (Widom and Klug, 1985) studies suggest that the nucleosomes fold into a chromatin fiber of ~30 nm diameter, 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 chromatin's apparent flexible-polymer properties (Cui and Bustamante, 2000; Marko and Siggia, 1997a, see below), plus the inhomogeneity inherent to chromatin. Polymer-like flexibility may account for observations of non-helical chromatin fiber structures (Horowitz *et al.*, 1994; Woodcock and Horowitz, 1995).

Chromatin is sensitive to ionic conditions. When chromatin fibers are extracted into solution at sub-physiological 10 mM univalent ionic strength, they are observed in the electron microscope as an 10 nm-thick 'beads-on-a-string'. At the more physiological ionic strength of 100–150 mM univalent ions, nucleosomes stack into a more condensed, and thicker, 30 nm-thick fiber (Figure 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 indicates that nucleosome–nucleosome interactions have

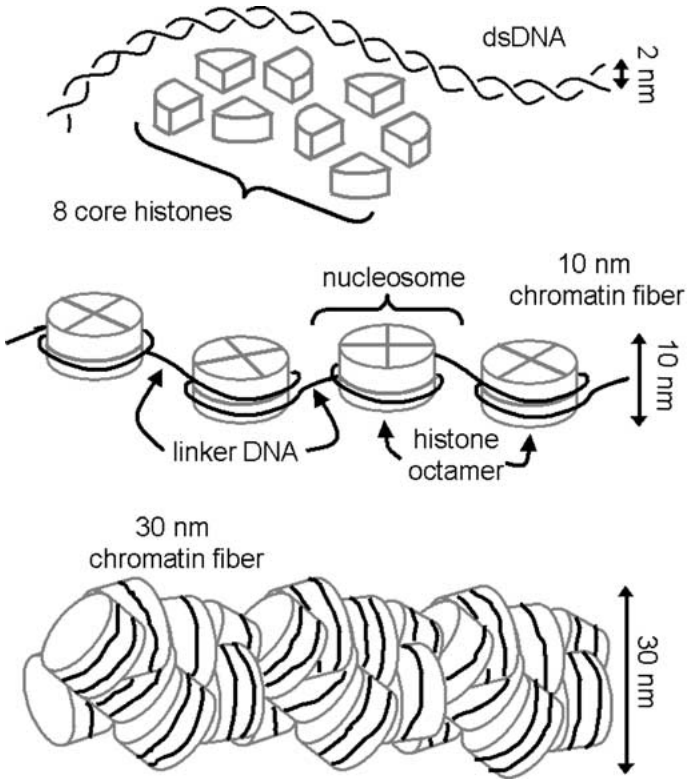

Fig. 3. dsDNA, histones, nucleosome, 10 nm chromatin fiber, 30 nm chromatin fiber. Structural-biological studies of chromatin have focused on the ultrastructure of isolated nucleosomes, and on studying the conformation of nucleosomes in the 10 and 30 nm fiber.

a strong electrostatic component. At low ionic strength, electrostatic interactions have a long range, and the like-charged nucleosomes (chromatin fiber has a net negative charge, similar to dsDNA) repel sufficiently to open chromatin fiber up. At higher ionic strength, this repulsion is overcome by attractive nucleosome–nucleosome interactions, and the fiber folds up.

The drastic structural effect of the change in ionic strength from 10 to 100 mM shows that chromatin fiber is relatively soft, or equivalently that internucleosomal interactions are relatively weak. During the unfolding of chromatin fiber by changing ionic conditions, the nucleosomes themselves do not undergo major conformational changes; the strong electrostatic histone-dsDNA interactions are relatively unperturbed until much higher ionic strengths ($\sim$0.8 M Na^+) are reached. Similarly, dsDNA structure is essentially insensitive to this change in ionic strength.

The 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 shown to apply *in vivo*. 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.

Micromechanics of dsDNA

A new approach to biophysical characterization of DNA is mechanical manipulation of single molecules, with molecular tension as an experimentally controllable and measurable quantity. 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 relative positions to as little as a few nanometers (Liphardt *et al.*, 2001). We use the example of dsDNA to introduce some basic ideas of polymer elasticity that will later be used to discuss chromosome extensibility.

The double helix has a persistence length of about $A = 50$ nm (150 bp for B-DNA, Hagerman, 1988). The persistence length is the contour length over which thermal (Brownian) fluctuations typically bend the double helix through a 60° bend. Over dsDNA lengths of less than 150 bp, the contour is of fixed shape (the double helix is nominally roughly straight, but some sequences are intrinsically rather severly bent). Over

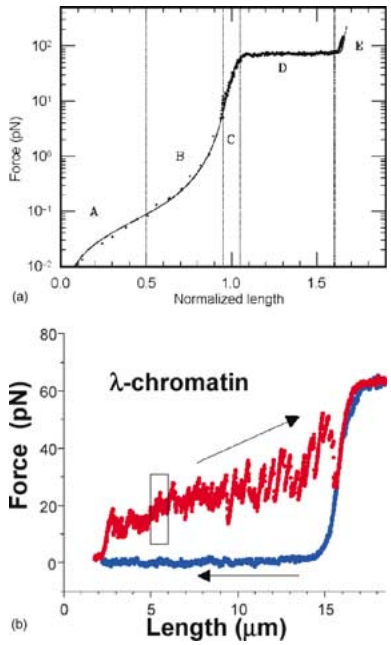

Fig. 4. Comparison of elastic response of (a) single dsDNAs, and (b) chromatin fiber. dsDNA and chromatin fiber both display an initial low-force (sub-pN) elastic regime, followed by a higher-force (few pN) regime. However, dsDNA (a) shows a very stiff and non-linear response, while chromatin fiber (b) shows a more gradual elastic response (Bennink *et al.*, 2001, figure reproduced with permission) as it is extended. This is believed to be due to driving the chromatin fiber opening transition (10–30 nm fiber transition of Figure 3) by force. At ~20 pN, force jumps corresponding to nucleosome removal events are observed.

distances longer than 150 bp, a dsDNA undergoes appreciable dynamic bending.

Thermally excited bends along a long DNA are straightened out by tensions (Figure 4a) of greater than kT/A ($kT = 4.1 \times 10^{-21}$ J, the energy of a single thermal fluctuation at room temperature $T \sim 300$ K; recall $k = R/N_A$ where R is the familiar gas constant and N_A is Avogadro's number), or about 0.1 piconewtons (pN). At 0.1 pN a dsDNA is extended to slightly greater than half its total contour length. At higher forces (0.1–10 pN) dsDNA elasticity is highly non-linear, with tension increasing quickly as the length approaches that of 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 pN (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* are DNA-protein interactions; for example 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 structurally modified by forces generated by the machinery which transcribes (Yin *et al.*, 1995), replicates (Wuite *et al.*, 2000), and repairs it.

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

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

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 non-linear force increase as x approaches L. These two features are generic for all flexible polymers which undergo random-walk-like bending fluctuations when unstretched.

For even larger forces (10–100 pN), dsDNA secondary structure starts first to stretch (10–50 pN), and then the double helix is disrupted, and stretches to an extended form at ~65 pN (Cluzel *et al.*, 1996; Smith *et al.*, 1996); this disruption has a strong DNA twisting-dependence (Allemand *et al.*, 1998; Leger *et al.*, 1999). For forces of 10–50 pN, dsDNA can be thought of as an elastic rod, with 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 (Landau and Lifshitz, 1986):

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

Here, r is the cross-sectional radius of the rod (for dsDNA, $r = 1$ nm; note that for 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, Equation (2) applies only for $\Delta L/L$ much less than unity (for dsDNA, the regime where (2) applies is from $\Delta L/L = 1.0$–1.05, where 1.0 refers to the B-DNA length).

The bending flexibility of an elastic rod is also related to Y. An elastic rod's bending modulus, 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 $B = 2 \times 10^{-28}$ Jm.

For rods which 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:

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

For dsDNA, we therefore see that $Y = 300$ MPa gives rise to 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, show that elementary concepts of elasticity apply at the nanometer scale of the interior of the double helix.

Micromechanics of chromatin fibers

Recently, the force-extension properties of chromatin fiber extracted from chicken erythrocytes (Cui and Bustamante, 2000) were measured. Three different force regimes were observed. First, 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 far more easily bent than the wire from which it is formed. However, to date completely convincing data for chromatin low-force (< 0.1 pN) 'polymer' elasticity under physiological conditions have not yet been published.

At higher forces (0.1–5 pN), what is observed depends strongly on ionic conditions, as one would expect based on the 10–30 nm fiber transition observed with increasing ionic strength. At relatively low (10 mM Na^+) ionic strength, a strongly non-linear 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 (barely visible in Figure 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 30–10 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, $Y \sim 10^5$ Pa, far below the effective modulus of straight DNA ~300 MPa. As DNA is folded up, its effective modulus is reduced.

At higher forces (20 pN), irreversible extension of chromatin fiber occurs (Cui and Bustamante, 2000). Recent experiments observe this to be in the form of a series of jumps of quantized length (Figure 4b). These jumps are thought to be associated with removal of single nucleosomes, and possibly individual ~80 bp winds of DNA (Brower-Toland *et al*., 2002). It is likely that this threshold for nucleosome removal is highly extension-rate dependent, since the known binding free energy ~20–30 kT/nucleosome indicates that one should expect equilibrium between bound and free nucleosomes for forces near 2–3 pN (Marko and Siggia, 1997b).

Observation of this equilibrium for pure chromatin fiber would require long experimental time scales since 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). Recent experiments have moved in this direction, assembling chromatin fibers *in vitro*, onto initially bare molecules of dsDNA, using cell-extract-derived chromatin-assembly systems (Ladoux *et al*., 2000; Bennink *et al*., 2001), which have allowed measurement of the ~10 pN forces applied by chromatin-assembly enzymes.

Chromosome structure at scales larger than the chromatin fiber

Beyond the chromatin fiber, it is thought that many species of protein 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, Sec. 2.4.2). We focus on large-scale chromosome structure as observed by traditional microscopy, in recent three-dimensional studies of chromosome structure and dynamics.

Structural-biological studies of mitotic chromosome structure

Much of our understanding of mitotic chromosome structure at larger scales is 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 (Figure 2) interconnected by a protein-rich 'scaffold' (Marsden and Laemmli, 1979). Other studies suggest that the scaffolding is coiled (Boy de la Tour and Laemmli, 1988).

Although these experiments are often taken to imply the existence of a connected protein 'skeleton' inside the mitotic chromosome (see the textbooks Lewin, 2000,

pp. 551–552, Lodish *et al*., 1995, pp. 349–353, Wolffe, 1995, pp. 52–55), Laemmli has recently emphasized to one of us that the conclusion that the internal protein skeleton is mechanically contiguous does not follow from his group's experiments and is not meant to be implied by any of his publications (see discussion and figures in Laemmli, 2002). The issue of the connectivity and mechanical integrity of the DNA and non-DNA components of the mitotic chromosome will be a primary focus of the section 'Combined biochemical-microchemical study of mitotic chromosomes'.

Other microscopy studies suggest a hierarchical structure (Figure 2) formed from a succession of coils at larger length scales (Belmont *et al*., 1987, 1989). Proposals have since been made for mitotic chromosome structure which combine loop and helix folding motifs (Saitoh and Laemmli, 1993). Existing microscopy studies do not give a consistent picture of mitotic chromosome structure, in part because of the invasive preparations necessary for EM visualization, and the inability of light microscopy to resolve detail smaller than ~200 nm.

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 EM 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) suggest that interphase chromatin is organized into ~50 kb domains.

Three-dimensional microscopy study of chromosome structure and dynamics

Increasing use of fluorescent labeling and optical sectioning microscopy techniques in the 1990s has allowed many features of chromosome structure to be determined by mapping physical position of specific DNA sequences with ~300 nm precision. Fluorescent *in situ* hybridization and other techniques applied to whole chromosomes shows 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 1–100 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).

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 metaphase chromosome structure (Figure 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 incorporation of fluorescent nucleotides (Manders *et al*., 1999), and by 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 micron length-scales.

A recent study of yeast (*S. 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 PCR-amplified and analyzed. The result was a statistical 'map' of *in vivo* chromatin contacts, giving a statistical three-dimensional chromosome model.

Chromosome-folding proteins identified using cell-free chromosome assembly systems

It is possible to study chromosomes assembled *in vitro*. *Xenopus* egg extracts provide an excellent system for doing this, converting *Xenopus* sperm chromatin into either interphase nuclei, or metaphase-like chromatids (Smythe and Newport, 1991). This system has permitted identification of proteins thought to be critical to organizing mitotic chromosomes, most notably the SMC protein family (Hirano and Mitchinson, 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 *Xenopus* egg extracts, 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 mitotic chromosome structure. Hirano and Mitchison also found that the XCAPs were localized inside the mitotic chromatids. Further work established that XCAPs in

condensin complexes (Hirano, 1997) show an ATP-dependent DNA-coiling capability (Kimura *et al*., 1997, 1999). Other SMC-type proteins have other roles in modulating chromosome structure (Strunnikov and Jessberger, 1999), notably holding sister mitotic chromatids together during prophase ('cohesins', see Michaelis *et al*., 1997; Guacci *et al*., 1997; Losada *et al*., 1998). Losada and Hirano (2001) have suggested that 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 hydrolysing end domains. Their distribution inside mitotic chromatids, clear revelation of their function in chromosome condensation, and the question of whether or not they are the major proteins of the 'mitotic protein scaffold' remain unanswered. Thanks to the biochemical characterizations described above, these questions may be answerable through gradual 'biochemical dissection' (Hirano, 1995, 1998, 1999).

Topoisomerase II

One of the most common proteins found in mitotic chromosomes is topo II (Gasser *et al*., 1986), the enzyme which 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 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). Recently it was reported that the axial distribution of topo II may be triggered by cell lysis (Christensen *et al*., 2002). At present, these experiments can be reconciled by supposing that topo II is critical for establishment of mitotic chromosome structure by allowing dsDNA disentanglement, that it is present in high copy number on the assembled mitotic chromosome, but that it does not play a crucial role in holding the mitotic chromosome together.

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 precisely measured 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 chromosomal titin might play a role in chromosome condensation, and might be a contributor to chromosome elastic response (Houchmandzdeh and Dimitrov, 1999).

Why study mitotic chromosomes micromechanically?

The structure of chromosomes, beyond the nucleosomal scale, is poorly understood, in spite of a huge focus of effort. This is because chromosome structure is dynamic, and because chromatin is inhomogeneous and soft. Mitotic chromosomes are a logical starting point for study of chromosome structure, since they are packaged (condensed), segregated from one another, and gene expression is halted, all of which are simplifying factors. Study of mitotic chromosome structure will presumably shed light into the mechanism of chromosome disentanglement and condensation (Hirano, 2000).

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?) which 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 structural 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; Nicklas and Ward, 1994; Li and Nicklas, 1995, 1997; Nicklas *et al*., 1995, 1998, 2001; Nicklas, 1997; King *et al*., 2000). Kinetochore chromatin elasticity is central to in a recent model for 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).

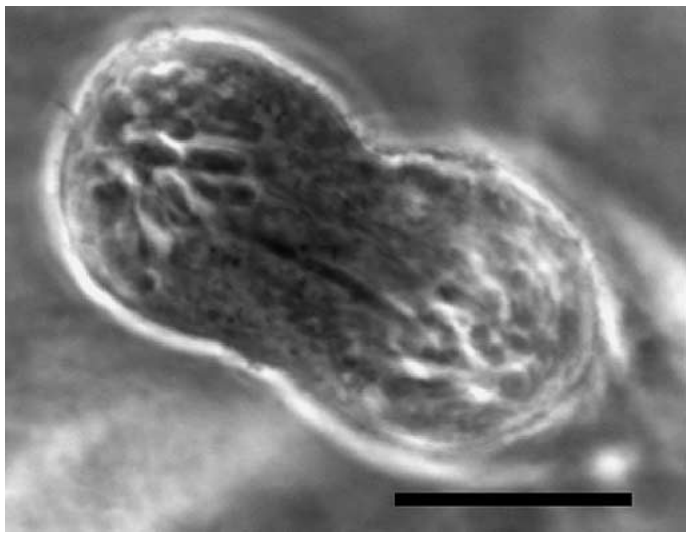

Fig. 5. Newt (*N. viridescens*) tissue culture cell, showing a chromosome being stretched to about twice its native length, by the mitotic spindle during anaphase. The spindle forces are known to be on the order of 1 nN, indicating that the force constant of a whole chromosome is on a similar scale. Bar is 20 μm. Photo courtesy of Prof J. Tang.

Elasticity of mitotic chromosomes

Mitotic chromosomes can be observed to occasionally be stretched out by the ~nN (nanonewton) spindle forces *in vivo* (Figure 5). This has led to a number of studies of chromosome stretching (Callan, 1955; Bak *et al*., 1977, 1979; Nicklas, 1983, 1988; Claussen, 1994).

Lampbrush chromosomes

One of the earliest discussions of chromosome extensibility was by Callan (1955) who carried out manipulation experiments on amphibian lampbrush chromosomes using glass microneedles. These observations plus DNA-ase experiments of Gall (1963) were used to support the hypothesis that each chromatid contains a single linear DNA.

The lampbrush phase 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, clearly 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 huge numbers of 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; Morgan, 2002).

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

Mitotic chromosome extensibility and elasticity

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 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.

Nicklas' fundamental 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*) spermatocytes which have a soft cell cortex which allows needles to grab chromosomes without breaking the cell membrane. Forces were measured by observing microneedle bending. Microneedles were used which required between 0.076 and 0.25 nN/μm per micron of deflection (1 nN = 10^{-9} N; recall 1 N = 1 kg m/s^2). Using a film analysis technique, the resolvable deflection of about 0.25 μm gave force resolution of roughly 0.05 nN.

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 Equation 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 aspects of the cell membrane or cytoskeleton, are 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 Skibbens 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 forces.

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 of 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 relation between *in vivo* and *ex vivo* chromosome structure can be understood. As we will describe below, our own experiments combined with those of others convince us that there is little or no change in chromosome structure at least initially after removal from a mitotic cell.

Classen *et al.* (1994) noted that metaphase chromosomes could be highly extended, and have used chromosome stretching to develop high-resolution chromosome banding techniques (Hliscs *et al.*, 1997a, b). The first measurement of the elastic response of a mitotic chromosome extracted from a cell was carried out by Houchmandzadeh *et al.* (1997), using mitotic cells in primary cultures of newt lung epithelia (*Notophthalmus viridescens*). This organism is attractive for chromosome research since it is a vertebrate with relatively few (haploid $n = 11$), large (haploid genome ~35 pg of dsDNA) chromosomes (Gregory, 2001). Each *N. viri* chromatid therefore contains about 3 pg = 3 Gbp, or about one meter, of DNA. At metaphase, the chromosomes are between 10 and 20 μm long, and have a diameter of about 2 μm. Newt epitheilia cells are easily cultured as a monolayer on dishes built on cover glass which are open to room atmosphere, making them well-suited for micromanipulation experiments (Reider and Hard, 1990).

Houchmandzadeh *et al.*, 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.*, to apply controlled stretching forces to chromosomes was to use aspiration into a pipette that had been treated with BSA 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 will result in an overestimation of the modulus, since 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 account of the flow effect mentioned above. Over a range of two-fold extension, the elasticity was remarkably linear (see Figure 8 of Houchmandzadeh *et al.*, 1997). Experiments were also carried out just after nuclear envelope breakdown (end of prophase), and it was found that chromosome had a higher elastic modulus $Y = 5000$ Pa. In addition, Houchmandzadeh *et al.* (1997) discuss 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.

Poirier *et al.* (2000) then used the micropipette-based manipulation technique to more quantitatively measure newt chromosome mechanical properties. Calibrated micropipette bending was used as the force-measurement scheme, for chromosomes removed from cells and suspended between two pipettes (Figure 6). This allows both ends of the chromosome to be monitored, and therefore chromosome extension can be precisely controlled. Digital image acquisition and analysis were used to measure pipette bending. Measurement of the corre-

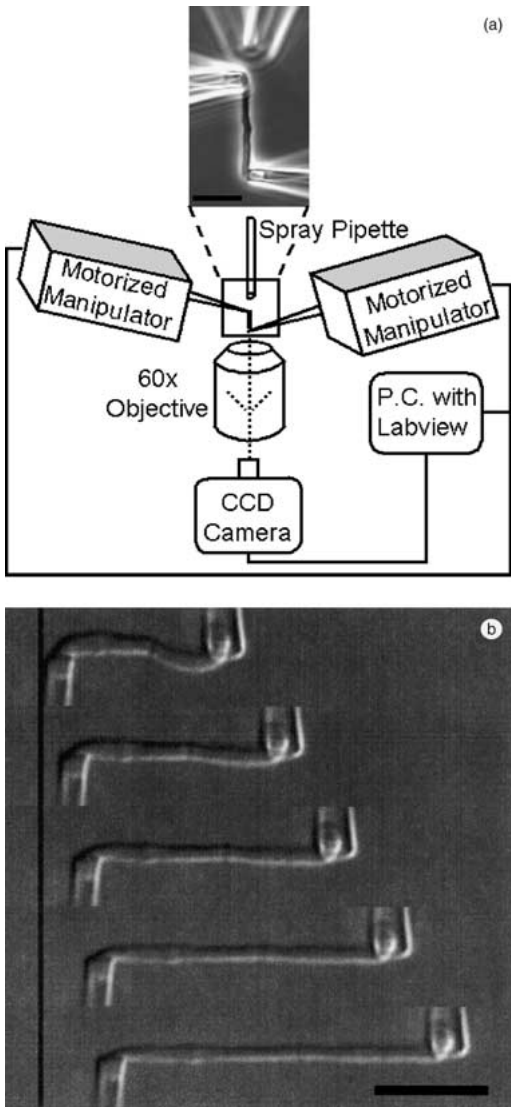

Fig. 6. Two-pipette chromosome experiment, as carried out in Poirier (2000). (a) Schematic diagram of experimental setup. Two pipettes are used to hold a mitotic chromosome, with one pipette fabricated with a deflection force constant ~1 nN/μm to allow chromosome tension to be measured. A third pipette can be moved near to the chromosome to microspray reagents for combined chemical-micromechanical experiments. (b) Example images collected during force-extension experiment. As the left pipette is moved, the right pipette is observed to deflect. Digital image analysis allows pipette deflections to be measured to about 10 nm accuracy. Bar is 10 μm.

lation between pipette images allows pipette shifts (and therefore deflections) to be determined to about 10 nm accuracy. Pipettes were used with bending moments ~1 nN/μm micron of deflection, thus setting a theoretical limit on force resolution of 0.01 nN = 10 pN. In practice, force resolution is usually limited by slow mechanical drifts of the pipettes.

The force-extension response of single mitotic (prometaphase) chromosomes are shown in Figure 7. 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. 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 $f_0 = 0.5$–2 nN (see histograms of Figure 7c). Unfortunately there are no obvious 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.

A feature of chromosome stretching which is quite obvious in all the above studies is that mitotic chromosomes do not narrow as they are stretched, in the reversible elastic regime. Our measurements (Figure 7a inset) 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 is usually close to 0.5, corresponding to volume conservation. By contrast, the volume of a mitotic chromosome actually increases as it is being stretched. This can only occur if the fluid medium surrounding the chromosome flows into it as it is stretched, and in turn this indicates that the chromatin fibers inside a mitotic chromosome do not adhere to one another.

We have recently improved a number of aspects of this 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. Second, we developed a technique of using a micropipette loaded with a 0.05% solution of Triton X-100 in 60% 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 to reduce its mechanical drift. The updated method leads to results consistent with those of Poirier (2000). A chromosome force measurement using a chromosome Triton-extracted from a TVI cell is shown in Figure 7b; its initial elastic response is reversible and 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 160 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, and a Young modulus $Y \sim 1000$ Pa (Poirier *et al.*,

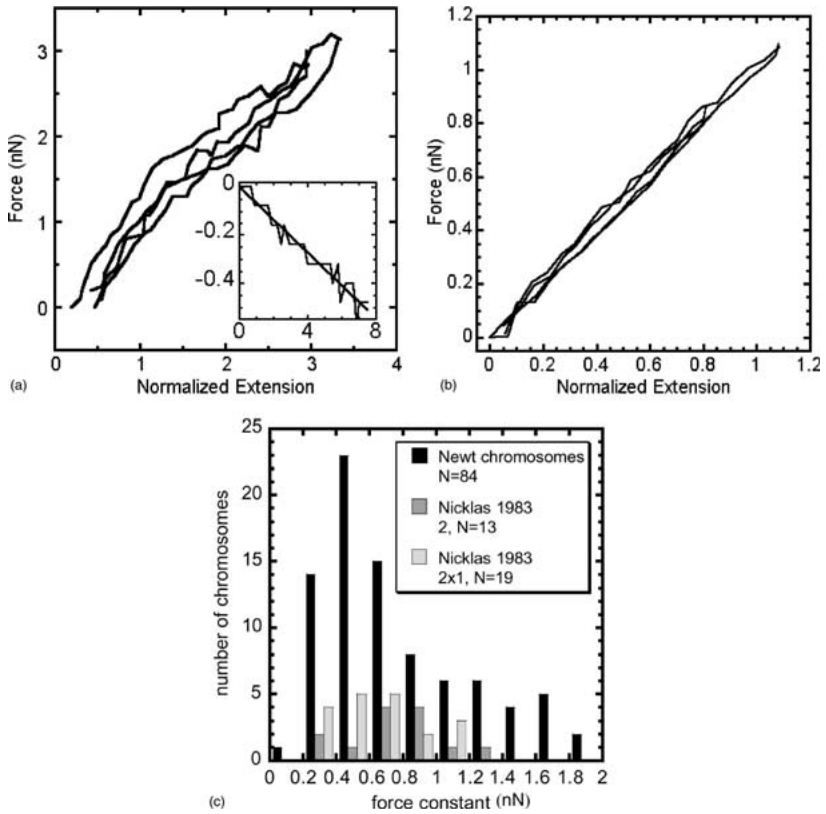

Fig. 7. Force-extension data for newt chromosomes. (a) Data from Poirier *et al.* (2000) for primary cultures of newt lung cells. The different curves show successive extension-retraction cycles; their coincidence indicates that the chromosome has reversible elasticity over the fourfold range of extension shown. The elastic response is nearly linear, and the initial force increase shows that the chromosome force constant is about 1 nN. Inset of (a) shows the fractional change in chromosome width as a function of extension, and indicates that the chromosome Poisson ratio is less than 0.1. (b) Data for 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 (2 chromatids), and single chromatids (Nicklas, 1983). The single-chromatid grasshopper data has had forces doubled for direct comparison with the two-chromatid data sets. The distributions of force constants are essentially identical in the newt and grasshopper systems.

2002b). Thus, grasshopper, newt and frog mitotic chromosomes 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.

In vitro *assembled chromosomes*

Houchmandzadeh and Dimitrov (1999) carried out an important study of the mechanical properties of mito tic 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. Micropipettes were used to grab, manipulate, and stretch the chromatids; force measurement was done via observation and calibration of micropipette bending (stiffnesses $\sim$1 nN/μm) using the same general scheme as shown in Figure 6. 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'. Houchmandzadeh and Dimitrov (1999) also present an explanation for 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).

Relation to chromatin fiber elasticity

The initial elastic response of mitotic chromosomes is not due to gross alteration of chromatin fiber structure, as can be seen from comparison of chromosome (Figure 7) and chromatin (Figure 4b) elastic responses. Chromatin fibers display reversible elasticity with a force constant of roughly 5 pN (pN $= 10^{-12}$ N). Since there will be on the order of a few thousand chromatin

fibers in a chromosome cross-section (the chromosomes discussed above are roughly a micron 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 pN. Therefore chromatin fiber structure is not being appreciably altered when chromosomes are being stretched by a factor of two; the initial elastic response of chromosomes must be due to modification of larger-scale condensed chromatin structure. The relatively low modulus of the chromosome indicates that large-scale chromatin structure is remarkably soft, yet elastic.

Bending elasticity of chromosomes

The Young modulus definition (2) strictly applies only to homogeneous materials. The degree to which this assumption is correct can be checked by measuring chromosome bending, and then comparing the bending stiffness with (3), the result expected for a rod made of a homogeneous elastic material.

The main result of experiments that compare 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. Remarkably, 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 which is distinct from that of *in vivo* mitotic chromosomes.

Bending moduli of chromosomes have been measured using observation of spontaneous thermal bending fluctuations. This approach 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. The bending modulus B is inversely proportional to the amplitude-squared of bending fluctuations. The simplest experiment to envision is one where one end of the filament being studied is anchored to a solid object (e.g. a very stiff micropipette, Figure 8a). As one moves down the rod from the anchor point, the amplitude of fluctuation perpendicular to the rod will increase. In the case where the fluctuations are small (rod length small compared to rod persistence length), we expect:

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

where the bar indicates the average of the fluctuation-amplitude-squared (Poirier *et al*., 2002b).

Bending flexibility of extracted mitotic chromosomes

In the previous section we saw that the Young modulus of a mitotic newt chromosome inferred from stretching was

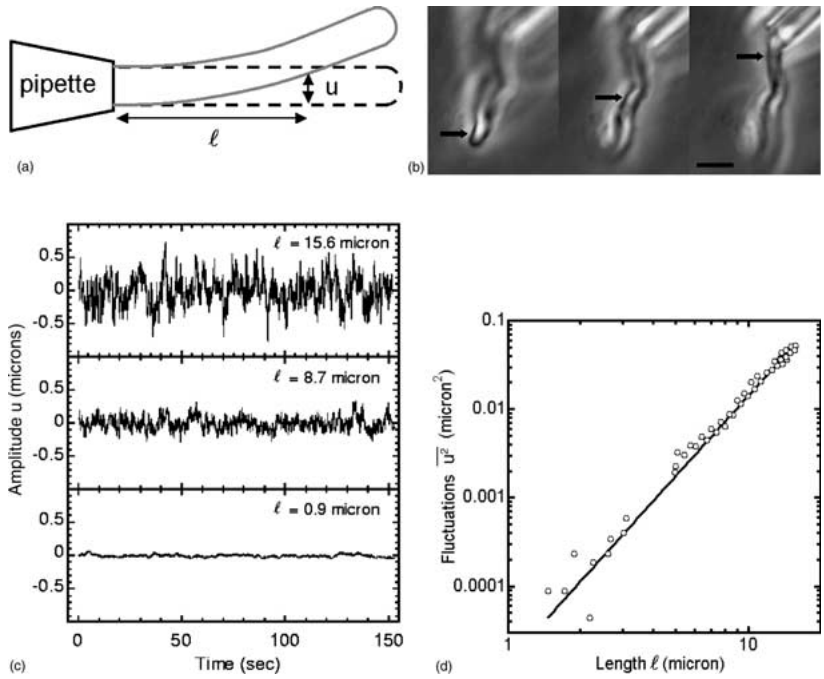

Fig. 8. Measurement of bending fluctuations for newt (*N. viridescens*) prometaphase chromosomes. (a) Rod geometry showing transverse fluctuation-amplitude u as a function of distance ℓ from the pipette along the rod. (b) Chromosome anchored at one end in a pipette. Bar is 4 μm. (c) Amplitude time series as a function of time for the three positions indicated by arrows in part b; the distance ℓ from the anchored end is indicated in each panel. With increasing distance from the anchor, the fluctuations increase. (d) Mean square fluctuations vs. distance from anchor point; on a log–log plot the data fall on the cubic power law given by Equation (3). The fit shown allows the bending modulus to be extracted from the data.

roughly $Y = 500$ Pa. Using Equation 3, a chromosome cross-section radius of $r = 0.8$ μm, we obtain an expected $B = 1.6 \times 10^{-22}$ N m^2. Plugging this expected value of B into Equation 5, and the maximum chromosome length $L \cong 20$ μm, we find the root-mean-square fluctuation $\sqrt{\overline{u^2}} = 0.3$ μm. Observed fluctuations of newt chromosomes (Figure 8c) have about this amplitude, and lead to an estimate of B between 1 and 3×10^{-22} N (Poirier *et al.*, 2002b). The newt chromosomes have bending stiffness consistent with their stretching elasticity via (3). Furthermore, they show no sign of hinges or other easily bent regions along their length.

Xenopus A6 chromosomes were found to be somewhat more flexible, with bending moduli between 5 and 20×10^{-24} N m^2. This flexibility is due to the smaller cross-section of the frog chromosomes ($r \sim 0.5$ μm) and is consistent with measured A6 Young moduli (200–800 Pa, Poirier *et al.*, 2002b).

Bending fluctuations of chromosomes in vivo

To check the relation 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.* therefore compared native cells (large non-thermal 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 $B = 6 \times 10^{-24}$ 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. No stretching data are available for *Drosophila* embryo mitotic chromosomes.

We used the basic method of Marshall *et al.* colchicine-treating newt cells, to obtain *in vivo* thermal bending data for mitotic chromosomes (Poirier *et al.*, 2002b). Using a variation on the approach described above an *in vivo* estimate of B of $0.2–0.5 \times 10^{-22}$ N m^2 was obtained, 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 cytoplasm and the extracellular medium. Alternately, there may be sources of non-thermal fluctuation which are weak and which are not disrupted by colchicine treatment. 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 non-thermal fluctuations. Since non-thermal forces will generally increase bending fluctuations, we can expect the *in vivo* measurements to provide lower bounds on B. We conclude that newt chromosomes have B *in vivo* comparable to that measured in the extracellular medium, roughly 10^{-22} N m^2.

Bending flexibility 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 20 μm-long chromatids were very flexible, and in precise experiments measured $B = 1.2 \times 10^{-26}$ N m^2. This is about 1000 times smaller than the value of B that we have obtained for chromosomes from *Xenopus* A6 cells. The *in vitro*-assembled chromatids are so flexible that they undergo polymer-like bending fluctuations. The thermal persistence length of the *in vitro*-assembled chromatids is $A = B/(kT) = 2.5$ μm, and movies of *in vitro*-assembled chromatids display observable dynamical bending on a few-micron length scale (S. Dimitrov, private communication).

This extreme flexibility 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. They propose that a few molecules of titin, suspected to be a chromosomal component (Machado *et al.*, 1998; Machado and Andrew, 2000a, b) could produce the observed elastic response.

The 1000-fold difference in B indicates that *in vitro*-assembled chromosomes must have a different internal structure from chromosomes in somatic cells. An 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.

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 towards the spindle poles. During anaphase, the chromosomes can be quite severely bent, 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 microns this retarding force is roughly 10^{-11} N. A basic question is whether this force is plausibly 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).

Viscoelasticity of mitotic chromosomes

If one stretches a chromosome rapidly enough, a stretching force in excess of the equilibrium force will be required, since 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. This effect was observed (Poirier *et al*., 2000). The internal viscosity of a chromosome will also limit the rate at which it retracts following release of stress, as observed by Nicklas and Staehly (1967). This relaxation time can be related to a viscosity using the Young modulus via $t_0\ Y = \eta'$ (Poirier *et al*., 2001a). Plugging in $t_0 = 1$ s and $Y = 500$ Pa estimates an internal viscosity of η' of 500 Pa s, more than 10^5 times that of water.

Experiments specifically aimed at determining internal viscosity of a prometaphase newt chromosome (Poirier *et al*., 2001a) were done using chromosomes attached to pipettes, by rapidly moving one pipette while acquiring visual data for the deflection of the other, force-measuring pipette. Generally, one observes an initial force pulse just shortly after the pipette is moved, followed by a decay to some final equilibrium force. In the linear regime (stretching to less than three times native length) gives $\eta' = 100$ Pa s, about 10^5 times the viscosity of water. The relaxation of observed width is faster than that of force, ruling out the possibility that the slow dynamics are due to gel-filling hydrodynamics (Tanaka and Fillmore, 1979).

A remaining possibility to explain the large η' is that when the chromosome is rapidly stretched, the chromatin inside it must rearrange, and the time needed for this rearrangement is on the order of 1 s. Entanglement dynamics of the long, tethered chromatin domains can easily be on this time scale (de Gennes, 1979, pp. 230–233, Poirier *et al*., 2001a). A second measurement of chromosome internal viscosity is obtained from analysis of bending fluctuation dynamics (Poirier and Marko, 2002c). The characteristic lifetime of the fluctuations ($t_0 \sim 0.7$ s, see Figure 8c) again indicates $\eta' \sim 100$ Pa s, providing further evidence of chromatin domains undergoing conformational fluctuations on the second timescale.

Combined biochemical-micromechanical study of mitotic chromosomes

A direct method to analyze mitotic chromosome structure is to use changes in chromosome elasticity as an indicator of *changes* in chromosome structure introduced chemically. The strategy of real-time observation of chemical reactions on whole chromosomes is rather old. For example, actinomycin-D was used to release the 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, p. 109). The new feature discussed below is mechanical measurement during such experiments.

Whole-genome-extraction experiments

Maniotis *et al*. (1997) developed a technique for extracting whole genomes from human and bovine tissue culture cells, during interphase (i.e. from the nucleus) and during mitosis. Microneedles were used to 'harpoon' either interphase nucleoli, or mitotic chromosomes. Chemical experiments were then done on the extracted genomes while observing on the inverted microscope. Remarkably, when these 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 RNAase and protease sensitive, but which are cut by DNAase and micrococcal nuclease. They conclude that the chromosomes of mammalian cells are *connected together* at the chromatin level, i.e. that the molecule which holds the genome together is DNA.

Two experiments of Maniotis *et al*. (1997) on metaphase chromosomes are highly relevant here. First, it was observed that mitotic chromosomes can be rapidly decondensed by introduction of drops of high concentrations of ions (500 mM $MgCl_2$, 500 mM $CaCl_2$, 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, since it can be cycled chemically on a short timescale.

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

It is striking that H1 is sufficient for this rescue, since one might imagine that other, rarer proteins which define 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 effect. Perhaps the large concentration of H1 and its accessibility (H1 is chemically exchanging on short timescales, Lever *et al*. (2000), Misteli *et al*. (2000)) make it a main target in this experiment, while the rare and perhaps other well-buried proteins which stabilize higher-level chromosome structure remain undamaged.

Combined micromechanical-chemical experiments

Our recent experiments focus on combining the *in situ* biochemical treatments with single-chromosome elasticity measurements, the aim being to do real-time quantitative monitoring of chromosome structure changes. Our focus is on study of mitotic chromosome structure. Mitotic chromosomes are extracted and set up for two-pipette micromanipulation, and their initial, native stretching elastic response is measured. Then, a third pipette of ID ~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 Figure 6) is brought within 10 μm of the chromosome. Pressure is then 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 rapidly diffuses into the large (~1 ml) volume of the sample dish. In a typical experiment, volumes of a few thousand cubic microns are typically sprayed (1000 cubic microns is 10^{-12} l = 1 picolitre). Any reagent can be used, with reaction kinetics micromechanically observed via the force-measuring pipette. When reagent flow is stopped, the chromosome is rapidly (<1 s) returned to the initial (extracellular) buffer condition, in which the reaction's effect on elastic properties can be measured.

Shifts in ionic conditions can decondense or hypercondense 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 mM univalent and divalent salt concentrations (Poirier *et al.*, 2002a). In experiments where force was monitored, we found that applied tension could be entirely eliminated using high concentrations of Na^+ and Mg^{2+}; however, after the ~10 s sprays end, the chromosome folds back up into a native-like structure. Following sufficiently long (>10 min) exposures to high salt concentrations, mitotic chromosomes do not fully recondense, presumably as a result of protein loss.

We have also found that in the range 20–100 mM Mg^{2+} and Ca^{2+} concentration, mitotic chromosomes go through a range of rather strong *condensation*, generating contractile forces of up to 0.2 nN. As divalent cation concentration is ramped up from zero, we observe condensation near 20 mM, followed by an abrupt return to the native degree of compaction near 50 mM, and then finally strong decondensation at >200 mM. A similar condensation–decondenation effect is observed with increasing concentration of trivalent ions. No compaction occurs with any concentration of Na^+ or K^+. All these decondensation and condensation effects occur *isotropically*; under zero tension, the fractional length and fractional width changes are nearly equal. This behavior is easily reconciled with a chromosome model, which is an isotropic network of chromatin fibers, and is difficult to square with an anisotropic chromatin-loop-attached-to-scaffold model. The condensation effects show that mitotic chromatids are not near their maximum possible compaction (we observe up to a 30% volume decrease using trivalent ions, Poirier *et al.* (2002a)), and that charge interactions are important to controlling chromatin compaction.

Effects of divalent ions are in line with similar reentrant bundling (i.e. a bundling followed by an dissolution as divalent ion concentration is raised) of stiff polyelectrolytes recently observed, in DNA (Pelta *et al.*, 1996; Saminathan *et al.*, 1999) and actin solutions (Tang *et al.*, 1996). The 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 the 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).

Micrococcal nuclease completely disintegrates mitotic chromosomes

Micrococcal nuclease (MNase) non-specifically cuts dsDNA, and is widely used to cut chromatin between nucleosomes. We were motivated to use MNase to determine whether or not the internal protein 'scaffold' (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.

We sprayed isolated newt TVI mitotic chromosomes with 1 nM MNase in suitable reaction buffer (60% PBS plus 1 mM $CaCl_2$), with a small tension (0.1 nN) initially applied. When the spray starts, chromosome tension (Figure 9) briefly jumps 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. Then, over the time interval 100–200 s, the chromosome disintegrates, and is eventually severed. 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' (Poirier and Marko, 2002d).

A second type of experiment where digestion was done with zero applied force was stopped before the chromosome was morphologically altered (at 30 s of 1 nM exposure). The chromosome then could be extended into a string of blobs, connected by thin chromatin strands. The strands could be severed by a

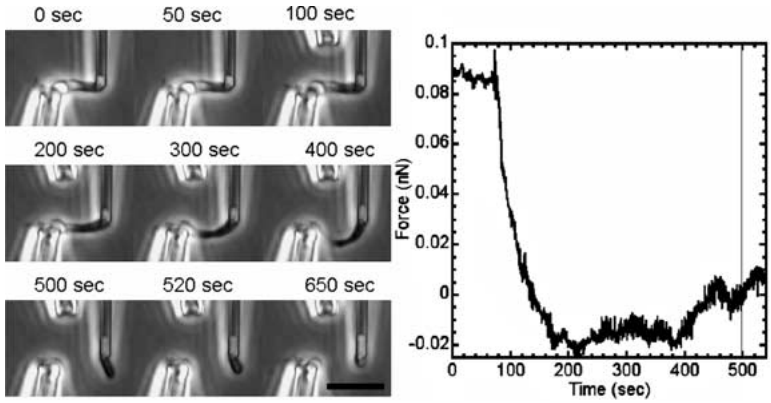

Fig. 9. Time course of tension in a chromosome, and chromosome morphology, during digestion by 1 nM MNase, with initial tension 0.1 nN. Spraying starts at 80 s; force decays after 30 s; chromosome is cut after 450 s. The spray pipette can be seen in the upper center of the $t > 120$ s frames. Bar is 10 μm.

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 imply that the mitotic chromosome is essentially a cross-linked network of chromatin, i.e. that higher-order chromosome structure is stabilized by non-DNA molecules (most likely proteins), which are isolated from one another. It is difficult to reconcile the MNase results with chromatin loops hanging from an internal mechanically contiguous protein scaffold.

Restriction enzymes with 4-base specificity can disintegrate mitotic chromosomes

We also carried out experiments with blunt-cutting restriction enzymes, which cut dsDNA at specific base-pair sequences (Poirier and Marko, 2002d). Enzymes were selected that were active in physiological-like buffers (i.e. pH near 7, ionic conditions near 100 mM univalent plus ~10 Mm divalents). Two enzymes with 4-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. Alu I severs the chromosome completely after < 100 s (Figure 10; force increase at spraying onset is condensing effect of the ~10 mM Mg^{2+} in the enzyme buffer, easily understood given our 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 kb.

Experiments with 6-base-recognition sequence enzymes Stu I (AGG^CCT) and Dra I (TTT^AAA) show essentially zero force effect (Figure 10), indicating that the accessible 6-base sites are rarer than chromatin cross-links. To test further the accessibility of 6-base-wide sites, we also used Cac8I where 4 bases are

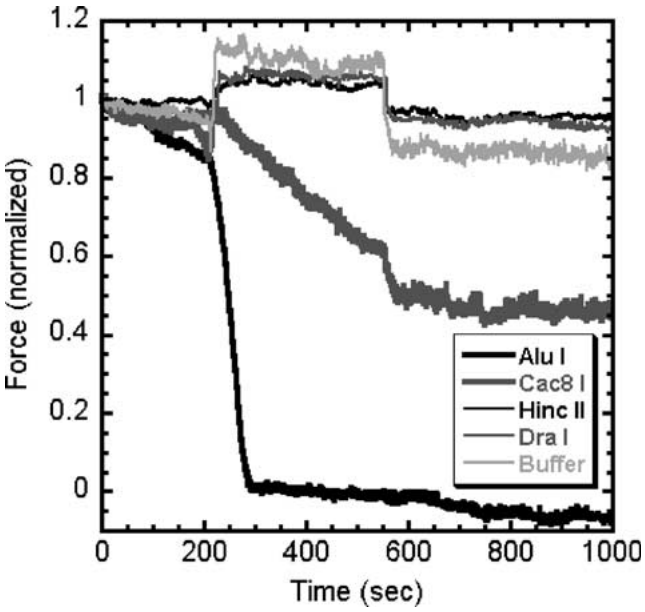

Fig. 10. Time course of tension in a chromosome during digestion by blunt-cutting restriction enzymes. Initial force in all experiments was 0.6 nN; each force curve is normalized to this initial value. Enzyme exposure is from 200 to 550 s. Bottom curve shows Alu I completely reducing force to zero (cutting chromosome completely). Middle curve shows Cac 8I only partially relaxing applied tension (partially cutting chromosome) Top curves show 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 reversibly driven by the divalent ions of the activity buffer.

recognized out of a 6-base region (GCN^NGC). This enzyme shows an intermediate effect (Figure 10), partially reducing applied force, but not totally severing the chromosome. Thus, the 6-base site size is partially available to the restriction enzymes. As for MNase, these results are consistent with a chromatin network model with a cross-link every few tens of kb, 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.

Conclusion

Mechanical properties of mitotic chromosomes

Mitotic chromosomes stretch and bend as if they are classical elastic media, but with an enormous range of extensibility. Mitotic newt chromosomes can be reversibly stretched fivefold, and 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. Houchmandzadeh and Dimitrov (1999) found similar stretching behavior in their study of *in vitro* assembled *Xenopus* chromatids.

Our observation of newt and *Xenopus* chromosome bending stiffnesses in accord with their stretching properties is distinct from the extreme bending flexibility observed for *in vitro* assembled *Xenopus* chromatids (Houchmandzadeh and Dimitrov, 1999). *In vitro* assembled chromatids must 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.

DNA-cutting experiments

Cutting dsDNA inside the mitotic newt chromosome with sufficient frequency completely disconnects the chromosome. MNase and 4-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 is 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 gradual reduction in effect of more rarely cutting restriction enzymes. 6-base blunt-cutting restriction enzymes have no effect on the mechanical properties of whole mitotic newt chromosomes.

Implications for structure of the mitotic chromosome

We suggest that the mitotic chromosome has a *network* structure, i.e. is organized by isolated chromatin-chromatin attachments (Figure 11). The purely mechanical measurements (stretching and bending) indicate that chromosome stretching is supported by stress spread across its whole cross-section, and therefore that mitotic chromosome structure appears to be, at the scale of a whole chromatid, homogeneous. This hypothesis is also supported by the homogeneous way whole chromosomes elongate.

Dynamic stretching and bending experiments both show that mitotic chromatin relaxes extremely slowly, on a roughly 1 s time. We hypothesize that this long timescale is due to chromatin conformational fluctuation, and that the long timescale 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 rapidly decondense and overcondense a mitotic chromosome. These morphological changes are reversible for short (10 s) salt treatments, and at zero stress are isotropic, again suggesting a homogeneous and disordered mitotic chromatin organization. At least 1/3 of chromosome volume is mobile cytoplasm or buffer based on condensation experiments. Finally, the DNA-cutting experiments indicate that the mechanically contiguous structural element of the mitotic chromosome is DNA (i.e. chromatin) itself. The non-chromatin fiber content of the mitotic chromosome must be disconnected. We rule out models for mitotic chromosome structure based on mechanically contiguous non-DNA skeletons or scaffolds, in favor of a network model. At present, the identity of the chromatin cross-linkers (the network 'nodes') is unknown; at present the most likely suspects are the condensin-type SMC protein complexes.

Future experiments

Combined chemical-micromechanical experiments provide complementary information to usual biochemical

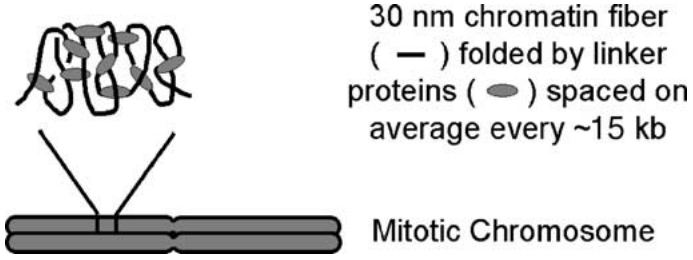

Fig. 11. Network model of a mitotic chromatid. Black curve indicates the single linear chromatin fiber, gray blobs show isolated non-DNA cross-linking elements. If the chromatin is cut sufficiently often, the chromosome will be severed; the non-DNA cross-linkers are not mechanically contiguous through the chromosome.

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 the direct study of elasto-mechanical properties of chromosomes, and to observe how those properties are modified dynamically by chemical reactions. Flexibility and connectivity of chromatin fiber in the mitotic chromosome are difficult to study 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 profitably attacked by integrating information from all these approaches.

A basic variation on the stretching experiments would be 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 modeling of chromosome capture by the mitotic spindle (Joglekar and Hunt, 2002).

To further analyze our network model of the mitotic chromosome it is important to analyze the sizes of chromatin fragments produced by enzyme microdigestion. 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, RNAases, and proteases need to be done. Effects of other chemical modifications of chromatin (e.g. acetylation, 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 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). The simplest types of experiments would be visualization of antibody binding patterns as a function of chromosome stretching. This general technique might also 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 disruption of cytoskeletal proteins, and might be used in conjunction with chromosome elasticity measurement to study the effect of condensin or cohesin disruption on mitotic chromosome structure. This type of experiment could directly test models of SMC function such as that of Losada and Hirano (2001).

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 stretched forms. Preliminary experiments on newt chromosomes in our showed no detectable birefringence for chromosomes stretched up to four times native length, suggesting 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.

We have repeatedly observed interchromosome fibers between mitotic chromosomes as discussed by Maniotis *et al*. (1997), and these objects require further study. In initial experiments we 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) have 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 gentler interphase genome extractions.

Finally, we note that Hinnebusch and Bendich (1997) have demonstrated that bacterial chromosomes can be extracted and physically studied. Cunha *et al*. (2001a, b) have succeeded in isolating and chemically manipulating *E. 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 bacterial chromosome structure, make it a highly attractive system for micromanipulation study.

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