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Micromechanical studies of mitotic chromosomes

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Abstract

Mitotic chromosomes respond elastically to forces in the nanonewton range, a property important to transduction of stresses used as mechanical regulatory signals during cell division. In addition to being important biologically, chromosome elasticity can be used as a tool for investigating the folding of chromatin. This paper reviews experiments studying stretching and bending stiffness of mitotic chromosomes, plus experiments where changes in chromosome elasticity resulting from chemical and enzyme treatments were used to analyse connectivity of chromatin inside chromosomes. Experiments with nucleases indicate that non-DNA elements constraining mitotic chromatin must be isolated from one another, leading to the conclusion that mitotic chromosomes have a chromatin 'network' or 'gel' organization, with stretches of chromatin strung between 'crosslinking' points. The as-yet unresolved questions of the identities of the putative chromatin crosslinkers and their organization inside mitotic chromosomes are discussed.

Abbreviations

BAF	barrier to autointegration factor
CAP	chromosome-associated protein
Muk	Mukaku, Japanese for anucleate
MukBEF	*E. coli* condensin complex
NEB	nuclear envelope breakdown
SMC	structural maintenance of chromosomes complex
topo II	topoisomerase II

Introduction

How DNA in eukaryote cells is physically organized into chromosomes is largely a mystery. During interphase, different chromosomes occupy different nuclear regions or 'territories' (Cremer *et al*. 1993, 1996, 2000, Cremer & Cremer 2001, Bolzer *et al*. 2005). Interphase chromosomes are highly dynamic, with active chromatin in an open conformation and undergoing continual exchange of protein components (Misteli *et al*. 2000, Phair & Misteli 2000). Chromosomal loci are known to be mobile (Marshall *et al*. 1997, Bystricky *et al*. 2004, Levi *et al*. 2005), and recently it has been shown that gene positioning affects gene expression (Brickner & Walter 2004, Ahmed & Brickner 2007, Akhtar & Gasser 2007, Lanctot *et al*. 2007).

During mitosis, gene expression stops, and chromosomes are folded into their segregated mitotic forms (Figure 1). Although condensin structural maintenance of chromosome (SMC) protein complexes are essential to mitotic condensation (Strunnikov *et al*. 1993, Hirano & Mitchison 1994, Strunnikov *et al*. 1995, Hirano 2006), exactly how these proteins act on chromatin is unknown. Given the many different models for mitotic chromosome structure (Saitoh & Laemmli 1994, Houchmandzadeh & Dimitrov 1999, Kimura *et al*. 1999, Machado & Andrew

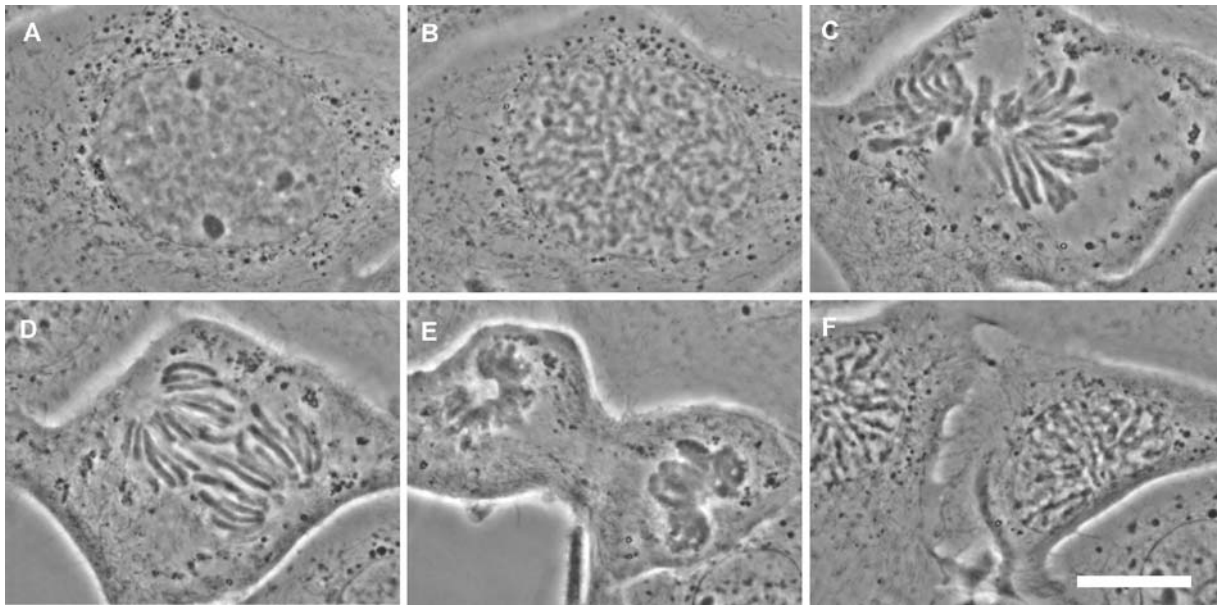

Figure 1. Cell division in newt epithelial cell, phase-contrast imaging. **(A)** Early prophase; **(B)** late prophase showing long and thin prophase chromosomes; **(C)** spindle-aligned metaphase chromosomes; **(D)** separation of chromatids at anaphase; **(E)** telophase chromosomes beginning to decondense; **(F)** interphase nuclei in daughter cells. Bar=20 μm. (Images courtesy of M.G. Poirier).

2000, Maeshima & Laemmli 2003, Dietzel *et al*. 2004, Kireeva *et al*. 2004, Hirano 2006, Sheval and Polyakov 2006, Fukui & Uchiyama 2007), it is clear that we do not yet understand large-scale chromosome folding (Belmont 2006).

Our lack of understanding of chromosome organization is rooted in physical properties of chromatin. The dynamic nature of chromosomes resulting from gene positioning, cell-cycle reorganization, and continual thermal (Brownian) motions indicates that chromatin structure must be described statistically, rather than in terms of precise folds. Furthermore, chromatin and chromosomes are soft materials, with rigidities far less than those of the molecules from which they are composed, leading to the pitfall that large-scale structure of chromosomes can be altered by preparations which leave protein and DNA molecular structures intact.

This paper reviews studies of structural and mechanical properties of mitotic chromosomes, focusing on micropipette-based micromanipulation experiments which permit measurement of changes in mechanical properties to assay structural changes driven biochemically. Most of these types of experiments have studied prometaphase or metaphase chromosomes of animals; many experiments that will be discussed below involve amphibian chromosomes from the newt *Notophthalmus viridescens* (Hutchison & Pardue 1975) and the frog *Xenopus laevis*. These are model organisms for study of mitosis simply because their chromosomes are large. In the case of *Xenopus*, there is the additional feature that egg extracts may be used to assemble chromosomes, mitotic apparatus, and nuclei *in vitro* (Almouzni & Wolffe 1993, Maresca *et al*. 2005).

The next section of this paper (Mitotic Chromosome Architecture) summarizes molecular-biological understanding of mitotic chromosome organization. The third section (Micromanipulation and Mechanics of Mitotic Chromosomes) discusses experimental methods for measuring chromosome elasticity, and reviews experimental results for stretching and bending elasticity, emphasizing the surprisingly low bending stiffness of chromatids reconstituted using *Xenopus* egg extracts relative to the bending stiffness of chromosomes extracted from cells. That section also discusses the ability of mitotic chromosomes to be rapidly unfolded and reversibly refolded by shifts in ionic concentration.

The fourth section of the paper (Molecular Connectivity of Mitotic Chromosomes) focuses on the question of how mitotic chromosomes are held together, and on experiments with enzymes that illuminate this question. A ‘chromatin network’ or ‘gel’ model is suggested by these results, where

chromatin is intermittently crosslinked to itself. A short review of the fascinating phenomenon of interchromosome linkers is included. The final section (Implications for Chromosome Structure) discusses the consequences of biochemical and biophysical data for models of mitotic chromosome folding.

Mitotic chromosome architecture

Proteomic experiments are moving towards providing a comprehensive catalogue of mitotic chromosome proteins (Uchiyama *et al*. 2004, 2005, Fukui & Uchiyama 2007). Here only a subset of the proteins in the mitotic chromosome will be discussed (Figure 2), from a coarse-grained structural and biophysical point of view.

Chromatin fibre

Chromosomes are composed of chromatin fibre, which consists of DNA complexed with histones into repeated nucleosome units. The structure of the entire nucleosome (147 bp of DNA plus core histones) has been determined using X-ray crystallography (Luger *et al*. 1997). Each ~10 nm-diameter nucleosome contains 147 bp of DNA wrapped around eight core histone proteins (two each of histones H2A, H2B, H3 and H4, for a total octamer mass of roughly 95 kDa). Given one nucleosome every ~180 bp of DNA (of mass 110 kDa), in a chromosome the total mass of core histones is about the same as that of DNA.

Formation of a nucleosome reduces the total 60 nm contour length of 180 bp of DNA to roughly 10 nm.

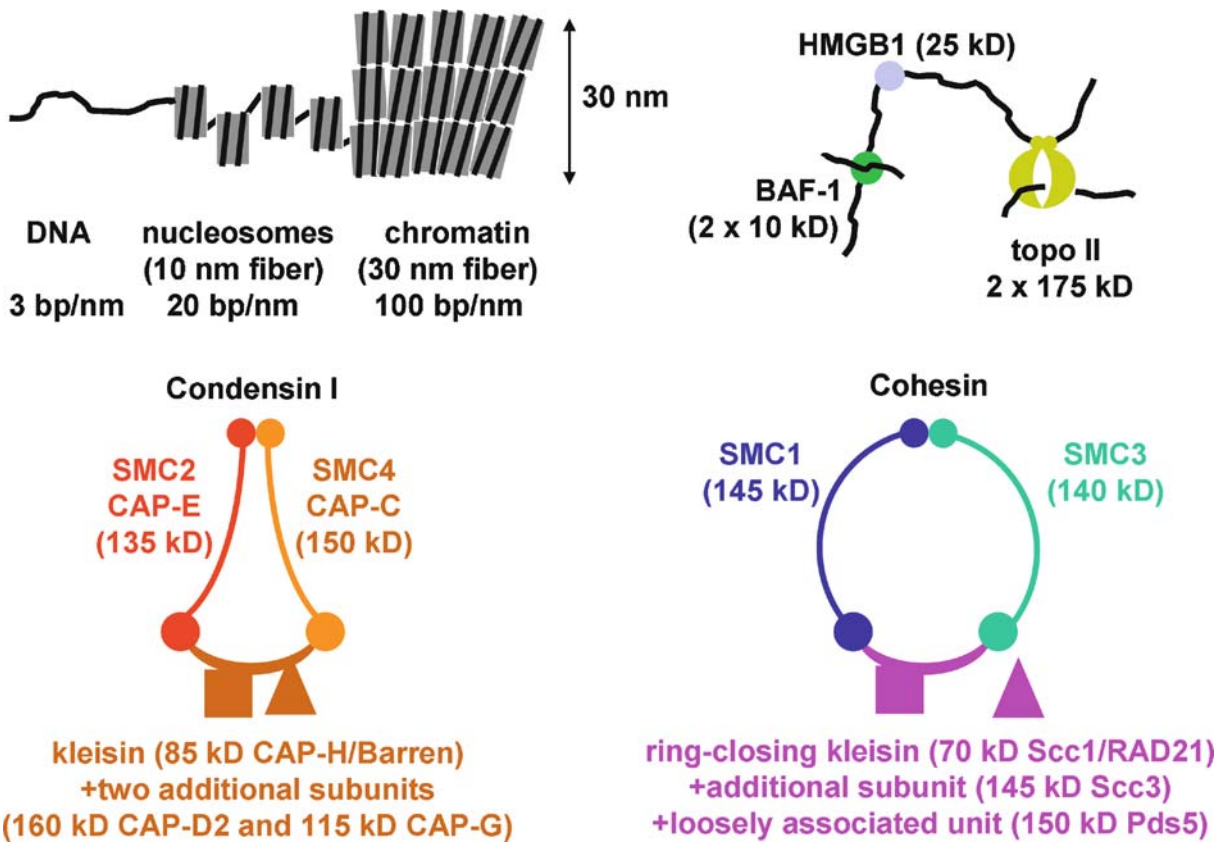

Figure 2. Cartoons of elements of eukaryote mitotic chromosomes, sketched roughly to the same scale. *Upper left:* DNA is complexed with histones to form nucleosomes, which then fold into chromatin fibre containing roughly 100 bp/nm; linker histones are not shown. *Upper right:* small HMGB1 and BAF-1 proteins bend and crosslink DNA; a larger (10 nm) topo II is shown bound to one DNA, while passing a second DNA through it. *Lower left:* much larger (50 nm) condensin I complex composed of two long SMCs plus a bridging kleisin unit and two additional accessory proteins. Condensin II is not shown; its structure is similar to that of I (see text). *Lower right:* a cohesin complex composed of long SMCs plus kleisin and accessory units has a large open structure with a hole large enough to pass 30 nm chromatin fibre.

Thus naked DNA, with 3 bp/nm, can be compacted into a string of nucleosomes with roughly 20 bp/nm, a linear compaction of about 6-fold.

Nucleosomes have associated with them linker histones (H1 or H5, ~20 kDa). Linker histones have long been thought to be to be involved in compaction of chromatin fibre to a folded 30 nm-thick form (Thoma *et al*. 1979), but the details of how this occurs remain poorly understood. The questions of linker histone to nucleosome stoichiometry (Woodcock *et al*. 2006), and exactly how linker histone binds to chromatin are not settled (Brown *et al*. 2006).

An often-used estimate is that when compacted into 30 nm form, there are about 6 nucleosomes per 10 nm of chromatin fiber length, or 100 bp/nm, about 30-fold shorter than the original DNA. Recent X-ray studies of crystallized nucleosome arrays (Dorigo *et al*. 2004) and tetranucleosomes (Schalch *et al*. 2005) support this estimate, and suggest that the 30 nm fiber has a two-start helix organization, with linker DNA in the interior of the fiber. This is in accord with neutron scattering experiments on chromatin containing histone H1 which indicate that linker histone is concentrated in the fiber center (Graziano *et al*. 1994). Daban (2000) has emphasized that the known very high DNA concentration in chromosomes may provide useful constraints on models of chromatin fibre folding.

Chromatin fibre structure is sensitive to ionic conditions. When chromatin fibres are extracted into solution at sub-physiological 10 mM univalent ion concentration, they are observed in the electron microscope as 10 nm-thick 'beads-on-a-string'. As one approaches the more physiological level of 100–150 mM univalent ions, nucleosomes stack into the 30 nm fibre. At physiological ionic strength, lateral internucleosomal attractions also tend to lead to aggregation of isolated fibres (van Holde 1988). This sensitivity indicates that nucleosome–nucleosome interactions have a strong electrostatic component, and also indicates that chromatin is soft and easily deformed. This softness and consequent variable structure of chromatin has made it difficult to arrive at consensus regarding the folded structure of the 30 nm fibre.

Many small DNA-binding proteins are found in chromatin, e.g., transcription factors. Two small proteins that are likely to be important to chromatin folding are high-mobility-group (HMG) proteins, and the barrier-to-integration-factor (BAF-1) protein (Margalit *et al*. 2007), which are present in mitotic chromosomes in numbers comparable to those of histones (Uchiyama *et al*. 2005). HMG proteins are divided into three families with different functions (Hock *et al*. 2007): HMGA proteins bind AT-rich DNA, HMGB proteins bend DNA (Thomas & Travers 2001), and HMGN proteins reorganize nucleosomes.

Intriguingly, BAF-1 molecules (10 kDa) organize into dimers, with two DNA-binding domains capable of interacting with two different DNA helices. BAF-1 has been demonstrated to be able to link DNA helices (Lee & Craigie 1998, Bradley *et al*. 2005). BAF-1 is an abundant chromosomal protein capable of noncovalently crosslinking chromatin.

Topoisomerase II

Topo II is a large dimeric protein (each polypeptide chain is ~175 kDa) responsible for passing DNA through DNA in an ATP-dependent manner, so as to resolve DNA entanglements such as those between sister chromatids resulting from DNA replication. Remarkably, topo II has been demonstrated to be able to drive entanglements below the number expected for random strand passages, indicating that it can use energy liberated during ATP hydrolysis to selectively remove DNA entanglements (Rybenkov *et al*. 1997).

Topo II's activity is essential to condensation and segregation of mitotic chromosomes (Hirano & Mitchison 1993). Metazoan cells contain topo IIα and β isoforms; during mitosis topo IIα is mainly resident on chromosomes, while topo IIβ is mainly cytoplasmic (Meyer *et al*. 1997). Observations of GFP-topo IIα fusion proteins *in vivo* show it to rapidly exchange on and off chromosomes (Christensen *et al*. 2002, Tavormina *et al*. 2002). Estimates for amounts on mitotic chromosomes vary over the range of one topo IIα for every 20–50 kb of DNA (Gasser *et al*. 1986, Fukui & Uchiyama 2007).

Topo II has been suggested not only to efficiently disentangle DNA but also to play a structural role in mitotic chromosomes. Analysis of non-histone proteins in mitotic chromosomes found 'scaffold protein I' (Lewis & Laemmli 1982), later identified as topo II (Earnshaw *et al*. 1985, Gasser *et al*. 1986). Electron-microscopic studies have indicated that topo II can bind a crossover of two DNAs (Zechiedrich & Osheroff 1990), and topo II has been observed to be able to recondense protease-decondensed chromosomes (Bojanowski *et al*. 1998). Immunofluores-

cence experiments have observed topo II localized in chromatid-axial patterns in mitotic chromosomes (Boy de la Tour & Laemmli 1988, Saitoh & Laemmli 1994, Maeshima & Laemmli 2003, Kireeva *et al*. 2004, Maeshima *et al*. 2005) (Figure 3).

However, although topo II is required for assembly of mitotic chromatids using *Xenopus* egg extracts, 500 mM univalent salt treatment extracts topo II after assembly, without causing noticeable changes in chromatid structure (Hirano & Mitchison 1993). Topo II does not appear to be essential for maintenance of mitotic chromosome structure.

Condensin structural maintenance of chromosome (SMC) complexes

SMC proteins are large (~150 kDa) ATPases folded into long (50 nm) coiled-coils terminated by globular domains (Figure 2). Together with additional 'kleisin' and accessory proteins, SMCs form large complexes that play major roles in chromosome condensation and segregation, as well as in other aspects of chromosome dynamics. The SMC-kleisin complexes of interest here are the condensin and cohesin SMC complexes (Nasmyth & Haering 2005, Hirano 2006).

Condensins consist of two SMCs (a heterodimer of a 135 kDa SMC2 and a 150 kDa SMC4) complexed with a lighter kleisin unit (70 kDa) and at least two additional accessory units (Figure 2). Condensins were first characterized in yeast (Strunnikov *et al*. 1993, 1995) and *Xenopus* (Hirano & Mitchison 1994). It was soon realized that one of the SMCs had been identified as non-histone 'scaffold protein II' from mitotic chromosomes (Lewis & Laemmli 1982). Electron microscopy (EM) indicates that the two SMCs bind together to form a hinged structure nearly 100 nm in length if extended, suggesting a function as a chromatin–chromatin linker. Experiments with *Xenopus* egg extracts established that the

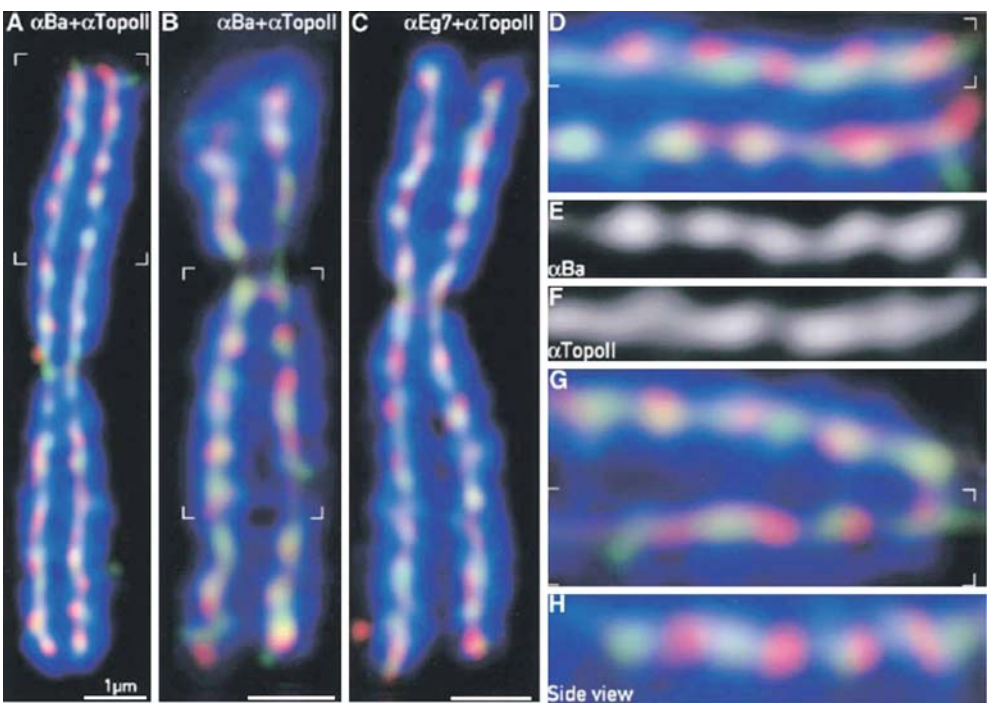

Figure 3. Condensin and topo II distributions on HeLa metaphase chromosomes. Chromosomes were stained with DAPI (blue), anti-topo IIα (green), anti-condensin I (aBa, red in panels **A**, **B**), and anti-condensin II (Eg7, red in panel **C**). Antibody signals occur along the chromatid axis, with condensin and topo II in alternating or coiled regions. **(D)** A higher magnification image of the box of panel **A**; **(E)** and **(F)** show individual antibody signals of **(D)**. **(G)** A higher magnification image of the boxed region of **(B)**. **(H)** A side of the boxed region of **(B)** obtained from a series of images taken along the focusing axis. Bars=1 μm. Reprinted from *Developmental Cell* **4**, Maeshima K and Laemmli UK, A two-step scaffolding model for mitotic chromosome assembly, Pages 467–80, Copyright (2003), with permission from Elsevier and Cell Press.

SMC units were essential for establishment and maintenance of mitotic chromatid structure (Hirano & Mitchison 1994).

Further experiments with *Xenopus* egg extracts and human cells revealed that two distinct condensins (I and II) are involved in mitotic chromosome condensation (Ono *et al*. 2003). These two complexes are built on the same SMC2-SMC4 heterodimer, but have different kleisin and accessory units (condensin II, not shown in Figure 2, contains distinct CAP-H2, CAP-D3 and CAP-G2 units). Condensin I and II appear to have distinct architectural functions; in human cells, depletion of the condensin-I-specific G subunit led to poorly condensed, fat and fuzzy metaphase chromosomes, while depletion of the condensin-II-specific G2 subunit led to 'curly' chromosomes (Ono *et al*. 2003).

The dynamics of the two condensin units are quite different. Vertebrate condensin II loads onto chromosomes in the nucleus, participating in prophase chromosome condensation, while condensin I is cytoplasmic, and loads onto chromosomes only after nuclear envelope breakdown (NEB) (Maeshima & Laemmli 2003, Hirota *et al*. 2004, Ono *et al*. 2004). Photobleaching experiments find condensin II to be immobile on human chromosomes even during prophase, while condensin I is highly mobile, exchanging on a roughly 4-minute timescale throughout mitosis (Gerlich *et al*. 2006).

The roles and dynamics of different condensin complexes may be different in different organisms. Yeast contains only condensin II. In *Drosophila*, condensin I has been reported to dominate mitotic chromosome condensation and to be necessary for stability of chromosomes during mitosis (Oliveira *et al*. 2005). It is loaded onto chromosomes in early prophase, and remains highly dynamic throughout mitosis, undergoing binding–unbinding turnover on a timescale of a few minutes (Oliveira *et al*. 2007).

Estimates of the number of condensin complexes on mitotic chromosomes are in the range of 1 per 10–30 kb of DNA (Takemoto *et al*. 2004, Fukui & Uchiyama 2007). In chromosomes assembled using *Xenopus* extracts, it has been estimated that there is one condensin per 5–10 kb (Kimura & Hirano 1997); 10 kb contains about 60 nucleosomes, or about 100 nm of 30 nm chromatin fibre.

Condensin activity on individual tethered DNA molecules has been observed. Strick *et al*. (2004) carried out experiments with purified *Xenopus* condensin I, finding that the complex was able to condense single DNAs by roughly 30 nm steps, in an ATP-dependent fashion. This result establishes that condensin has an ATP-dependent DNA-condensing function in a biochemically defined system.

A second and important single-DNA experiment of (Strick *et al*. 2004) started by introducing condensin without ATP; no condensation occurred. Then, all condensin in solution was washed away. Finally, ATP alone was introduced, triggering stepwise condensation of DNA. Thus, condensin is able to associate with DNA in the absence of ATP, and then after ATP becomes available, to reorganize *along* DNA so as to condense it. A second *in cis* capability of condensin is generation of chiral knots and supercoiling along DNA (Kimura & Hirano 1997, Kimura *et al*. 1999, Petrushenko *et al*. 2006).

In vivo experiments suggest that condensin subunits may to some extent be dispensable. In a conditional knockout system (Hudson *et al*. 2003) observed that in the absence of one of the condensin SMCs, mitotic chromosome condensation was delayed but eventually proceeded. When isolated, the mitotic chromosomes appeared more easily damaged, and less mechanically robust. (Gerlich *et al*. 2006) found that depletion of non-SMC condensin I or II subunits led to problems with chromosome segregation, and made it more likely that chromosomes would be damaged by spindle forces during mitosis; however, chromosome condensation did occur.

Cohesin SMC complexes

Eukaryote cells also contain cohesin complexes, which like condensins are based on a heterodimer of ~50 nm-long coiled-coil SMC proteins and a kleisin unit, plus additional subunits (Figure 2) (Nasmyth & Haering 2005). Cohesins have a more open, ring-like form, appearing in EM studies as asymmetric polygons (Nasmyth & Haering 2005) large enough to encircle chromatin fibres (Haering *et al*. 2002, Gruber *et al*. 2003).

Cohesins associate with DNA before S-phase (Ivanov & Nasmyth 2005, Lengronne *et al*. 2006). After DNA replication, cohesins link the sister DNAs together, holding them together until anaphase, when a regulated protease cuts the cohesion, allowing sister separation (Gruber *et al*. 2003, Wirth *et al*.

2006). Remarkably, in yeast it has been established the spatial distribution of cohesion units changes after their initial loading, eventually becoming concentrated at regions of convergent transcription spaced by roughly 10–15 kb (Glynn *et al*. 2004, Lengronne *et al*. 2004). Experiments of Haering *et al*. (2002), Gruber *et al*. (2003) and Ivanov & Nasmyth (2005) support a model whereby cohesins topologically link sister chromatids together, and are able to slide during their redistribution. Other authors have presented evidence suggesting that cohesin binds to individual chromatids (Milutinovich *et al*. 2007), and that cohesins are reorganized by transcription-driven dissociation (Bausch *et al*. 2007).

In metazoan cells, much of the cohesin initially loaded is removed following S-phase, during prophase and prometaphase. However, an appreciable amount of cohesin remains near centromeres (Waizenegger *et al*. 2000), and at least some cohesin stays bound along arms of vertebrate mitotic chromatids up to the point when anaphase segregation occurs (Gimenez-Abian *et al*. 2004).

Universality of SMC complexes

SMC-containing complexes are also found in prokaryotes, the prime example being the MukBEF complex in *E. coli*, which is based on a homodimer of the MukB SMC. MukB was identified genetically via a chromosome segregation defect (Niki *et al*. 1991). Overexpression of MukB has been observed to cause chromosome overcondensation *in vivo* (Wang *et al*. 2006). Estimates of 1000 bsSMC condensins in *B. subtilis* (Lindow *et al*. 2002) suggest that there is roughly one bacterial condensin per 10 kb of (replicated) DNA, not terribly different from the eukaryote ratio. SMCs are also found in archaeal species (Hirano 2005), making them a chromosomal protein that can be found in all three domains of life.

Large-scale mitotic chromosome organization

Mitotic animal chromosomes have a 'noodle' shape at metaphase (Figure 1), with two parallel chromatids (Figures 3 and 4) held together by cohesins. The longest human metaphase chromosome is roughly 10 μm long, and slightly less than 2 μm in width, with 250 Mb of DNA in each linear chromatid. The longest metaphase newt (*Notophthalmus viridescens*) chromosome is about 20 μm long (Hutchison & Pardue 1975) and slightly more than 2 μm in width. Here the focus is on chromatin packing along the arms of the chromosome without discussion of the specialized chromatin folding at centromeres (Carroll & Straight 2006).

Our understanding of chromatin folding in mitotic chromosomes at sub-optical scales (<200 nm) is largely based on EM studies. EM visualization of DNA loops extending from a protein-rich chromosome body after histone depletion (Paulson & Laemmli 1977), plus visualization of structures consistent with a loop organization in serial-sectioned fixed cells (Marsden & Laemmli 1979, Adolph 1980, 1981, Adolph & Phelps 1982, Adolph *et al*. 1986, Maeshima *et al*. 2005), support a model for mitotic chromosome structure based on chromatin loops interconnected by a non-histone-protein-rich 'scaffold' (Marsden & Laemmli 1979). In human cells, mitotic loops observed in EM experiments are 50–100 kb in size.

Other EM studies suggest a hierarchical folding formed from a succession of coils or folds at progressively larger length scales (Belmont *et al*. 1987, 1989, Strukov *et al*. 2003). Proposals have also been made for mitotic chromosome structure which combine loop and helix folding motifs (Rattner & Lin 1985, Saitoh & Laemmli 1993, 1994), and which include an axial 'glue' acting on a hierarchically folded chromosome (Kireeva *et al*. 2004).

The general idea that folded domains of chromatin are attached to a chromatid-axial structure is supported by many studies which have observed axial distribution of non-histone chromosome structural proteins (Figures 3 and 4). As mentioned above, topo II has been observed to be axially or helically organized in mitotic chromosomes (Boy de la Tour & Laemmli 1988, Sumner 1996, Tavormina *et al*. 2002, Cuvier & Hirano 2003, Maeshima & Laemmli 2003, Kireeva *et al*. 2004, Maeshima *et al*. 2005), although the degree to which an axial distribution is observed appears sensitive to experimental details (Hirano & Mitchison 1993).

Immunofluorescence studies of expanded chromosomes by (Earnshaw & Heck 1985, Maeshima & Laemmli 2003, Gassmann *et al*. 2004) revealed a punctuate, discontinous distribution of topo II. Live-cell experiments with dyed topo II (Swedlow *et al*. 1993) and GFP fusion proteins (Christensen

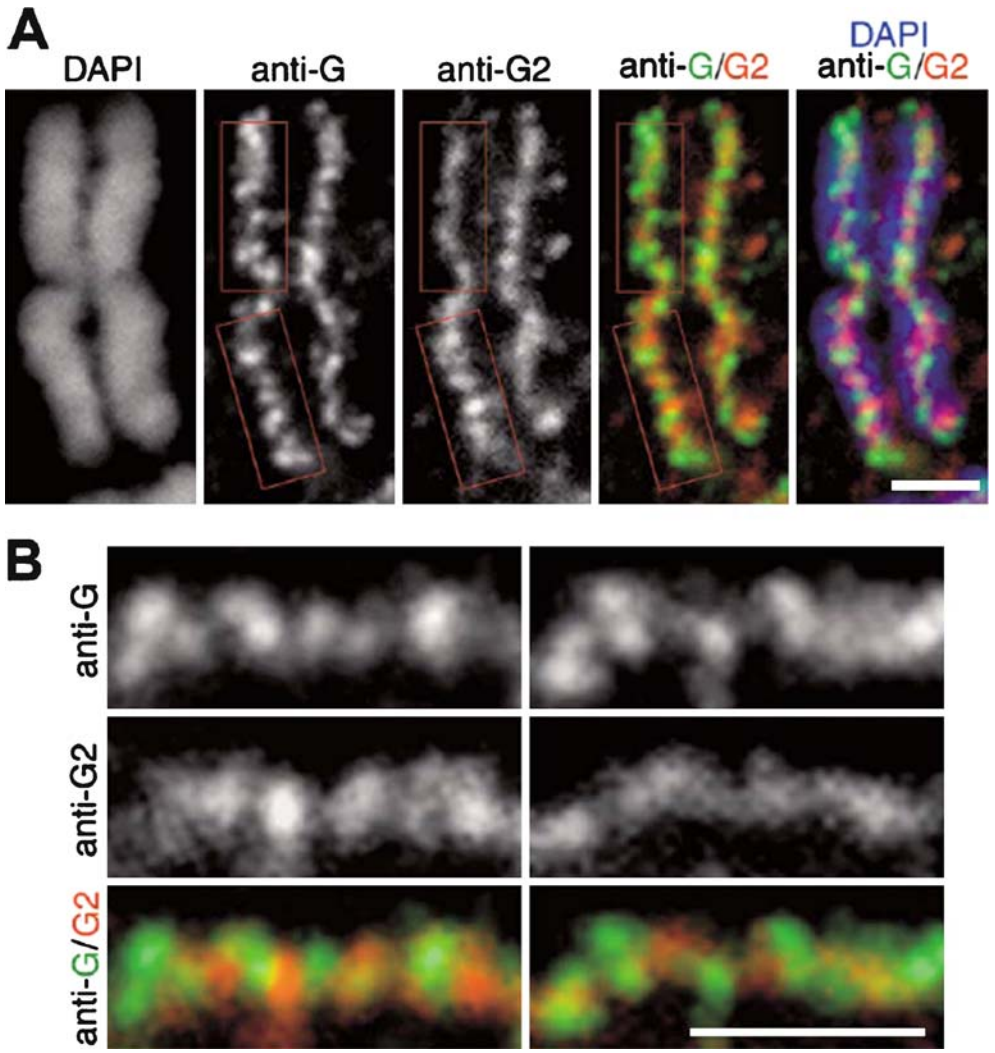

Figure 4. Condensin I and condensin II distribution on HeLa chromosomes. **(A)** Metaphase HeLa chromosome stained with DAPI (blue) and biotinylated anti-hCAP-G (condensin I) and anti-hCAP-G2 (condensin II). Condensins occupy chromatid-axial distributions, with condensin I and II in separate alternating or coiled regions. Right panels show merged images. Bar=2 μm. **(B)** Higher-magnification images of boxed regions of **(A)** show alternating condensin I and II domains, with condensin I (green in merge) possibly exterior to condensin II (red). Bar= 2 μm. Reprinted from *Cell* **115**, Ono T, Fang Y, Spector DL and Hirano T, Spatial and temporal regulation of Condensins I and II in mitotic chromosome assembly in human cells, Pages 109–21, Copyright (2003), with permission from Elsevier and Cell Press.

et al. 2002, Tavormina *et al*. 2002) disagree as to the degree of its axial localization. Swedlow *et al*. (1993), Sumner (1996) have suggested that topo II will be found where DNA interlocks occur, which in conjunction with topo II's dynamic exchange on and off chromosomes might be responsible for the variability in axial localization observed experimentally. The data might be unified if a portion of topo IIα is rather stably bound along chromatid axes, with less stably bound topo IIα populating the chromatid exteriors.

Condensin units have been observed to be axially organized in mitotic chromatids (Figures 3 and 4) (Hirano & Mitchison 1994, Maeshima & Laemmli 2003, Ono *et al*. 2003, 2004, Kireeva *et al*. 2004, Maeshima *et al*. 2005). Immunofluorescence studies indicate that in animal cells, condensin II may be localized nearer to the chromatid axis than condensin I (Ono *et al*. 2003), reflecting the loading of condensin II before condensin I. The same study suggests that condensin I and II may have alternating or helically interwound axial distributions (Figure 4)

(Ono *et al*. 2003). A similar alternating distribution along chromatid axes was observed for condensins and axial topo II (Figure 3) (Maeshima & Laemmli 2003).

Experiments with *Xenopus* egg extracts have shown that varying the amount of linker histone dramatically affects large-scale mitotic chromatid structure assembled *in vitro*. Comparison of experiments with native extracts, linker-histone-depleted extracts, and mock-depleted extracts revealed that absence of linker histone resulted in an approximately 2-fold-longer chromatid (Maresca *et al*. 2005, Maresca & Heald 2006). When linker histone was added to the depleted extracts, a shorter chromatid was recovered. This important result shows that local changes to chromatin fibre folding strongly affect global mitotic chromatid folding, in an anisotropic way.

Micromanipulation and mechanics of mitotic chromosomes

Chromosome-stretching experiments

Micromanipulation of chromosomes can be useful in studying chromosome structure, through observation of how modification or removal of specific molecules impacts chromosome mechanics. Several of the experiments discussed in the previous section used observations of qualitative changes in chromosome mechanical stability following interference with or depletion of condensin (Hirano & Mitchison 1994, Hudson *et al*. 2003, Ono *et al*. 2003, Gerlich *et al*. 2006) to infer their chromosome-folding functions. Measurement of chromosome mechanical properties is also important to understanding the mitotic apparatus, which is in part regulated via roughly nanonewton forces (1 nN=10^{-9} newtons; a newton approximately is the gravitational force exerted by a 100 gram mass on the earth) generated in chromosomes during mitosis (Nicklas 1983, Skibbens *et al*. 1995, Skibbens & Salmon 1997, Nicklas *et al*. 2001, Gardner *et al*. 2005). Chromosome mechanics has been suggested to play a variety of roles in mitotic and meiotic chromosome dynamics (Kleckner 1995, 1996, Marko & Siggia 1997, Kleckner *et al*. 2004).

Methods have been developed to isolate and to carry out stretching experiments on single prometaphase chromosomes, using glass micropipettes (Houchmandzadeh *et al*. 1997, Poirier *et al*. 2000, Poirier & Marko 2002b, 2003, Marko & Poirier 2003, Pope *et al*. 2006). The method is broadly similar to microneedle-based manipulation of meiotic metaphase chromosomes inside grasshopper spermatocytes (Nicklas 1963, 1983), as well as to classic studies of lampbrush chromosome mechanics (Callan 1954). Manipulation experiments without force measurement have been done by Maniotis *et al*. (1997, 2005) and Bojanowski *et al*. (1998) who have developed methods for taking whole genomes out of cells using microneedles, and exposing them to changes in buffer conditions and enzymes.

For single-chromosome experiments we culture cells in open dishes, prepared on thin, ethanol-cleaned, but otherwise untreated, glass microscope slides, onto which rubber O-rings (~25 mm diameter) are affixed by wax. These slides are kept in an incubator until use; 80% confluent samples are typically used for experiments. Experiments are done using an inverted microscope (Olympus IX-70, 60× 1.4 NA objective) with a motorized stage (Prior Scientific, Rockland, MA, USA). The microscope has a video camera connected to a PC computer with a video frame grabber (National Scientific) which allows real-time acquisition and analysis of images (Figure 5).

Micropipettes used for manipulation and force measurement are fabricated from borosilicate glass capillaries (1 mm OD, 0.75 mm ID, World Precision Instruments) using a puller (Sutter P-97) and home-built forge. Micropipette taper can be adjusted over a wide range to produce either very stiff or very easily bent pipettes. We generally use stiff pipettes with ~5 mm taper and a 2 μm end opening, and floppy pipettes with ~12 mm taper. Floppy pipettes require roughly 0.1 nN applied to their ends to be deflected (bent) by about 1 μm, and are used for force measurements. Stiff pipettes are not detectably bent by the nanonewton forces used in our experiments.

The two pipettes for holding the ends of the chromosome are attached to motorized three-axis micromanipulators (Sutter Instruments MP-285). These can be controlled manually or automatically with a Labview (National Instruments) program. The pipettes are mounted in holders connected to flexible tubing which terminates in reservoirs of 10 ml volume that have manually adjustable position; manual adjustment of the height of the reservoirs by a few centimetres allows either suction or flow to be generated.

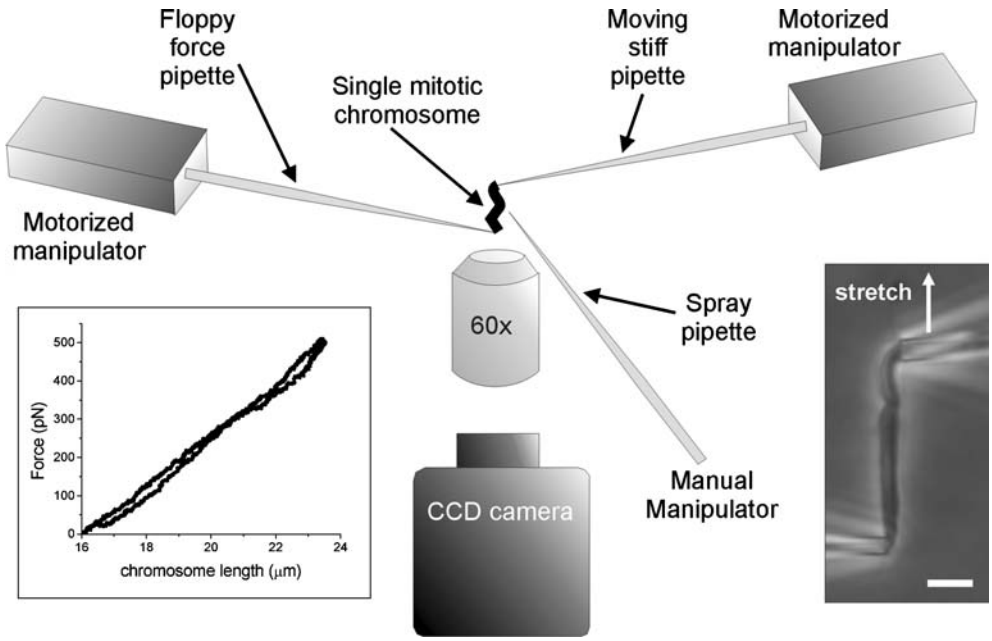

Figure 5. Experimental setup for chromosome micromanipulation. Cells and extracted chromosomes are imaged through a 60× contact objective. Manipulation is done from above using micropipettes. A single chromosome is extracted from a cell and suspended between a stiff moving pipette and a floppy fixed pipette (Bar=5 μm). As the stiff pipette moves (arrow), the deflection of the fixed floppy pipette is used as a force sensor. Force versus chromosome length data are extracted from images; stretch data for a native chromosome are shown. A third pipette is introduced into the sample dish to spray the chromosomes with reagents. (Adapted from Pope *et al*. 2006).

A third pipette with a wider aperture (10 μm) for spraying reagents onto chromosomes is attached to a mechanical micromanipulator (Taurus, World Precision Instruments). This allows rapid introduction of any reagent by flow, while observing changes in mechanical properties of an individual chromosome. When flow is stopped, the tiny volume of chemicals diffuses rapidly away, rapidly turning off the reaction.

The procedure for isolating an individual chromosome is straightforward (Figure 6). A stiff micropipette is filled with a 0.05% solution of Triton X-100 (Fisher) in 60% phosphate-buffered saline (PBS, Cambrex; for mammalian cells undiluted, PBS is used). This pipette is positioned close to the edge of a cell identified to be in prometaphase. A few seconds of spraying produces a hole in the cell membrane, through which the chromosomes flow out into the extracellular medium. At this point, a second, floppy pipette filled with 60% PBS is used to catch one end of one of the chromosomes: the chromosomes adhere tightly to the untreated glass surface. The other end of the chromosome is next detached from the other chromosomes, and sucked into a stiff moveable pipette.

The result is that a single isolated chromosome is suspended between the two micropipettes in the extracellular medium, ready for mechanical study. Stretching elasticity measurements proceed by moving the stiff pipette, and by observation of deflection of the stationary floppy pipette (Figure 7). Positions of pipettes are computed in real time using Labview (National Instruments) programs which determine the amount the images of the pipettes shift with time.

Experiments begin with measurement of the force (deflection) generated on the initial, 'native' chromosome by its stretching, then spraying of one or more reagents onto the chromosome, followed by measurement of the possibly modified force-versus-extension response of the chromosome. At the end of the experiment, the force-measuring floppy pipette is removed and cleaned, and has its force constant calibrated. This calibration is done by pushing the floppy pipette against a stiffer pipette with known force constant; this calibration pipette has itself been calibrated in the same way. Through a sequence of three or four calibrations against progressively stiffer pipettes, one obtains a pipette that can have its stiffness measured by pushing it against an analytical

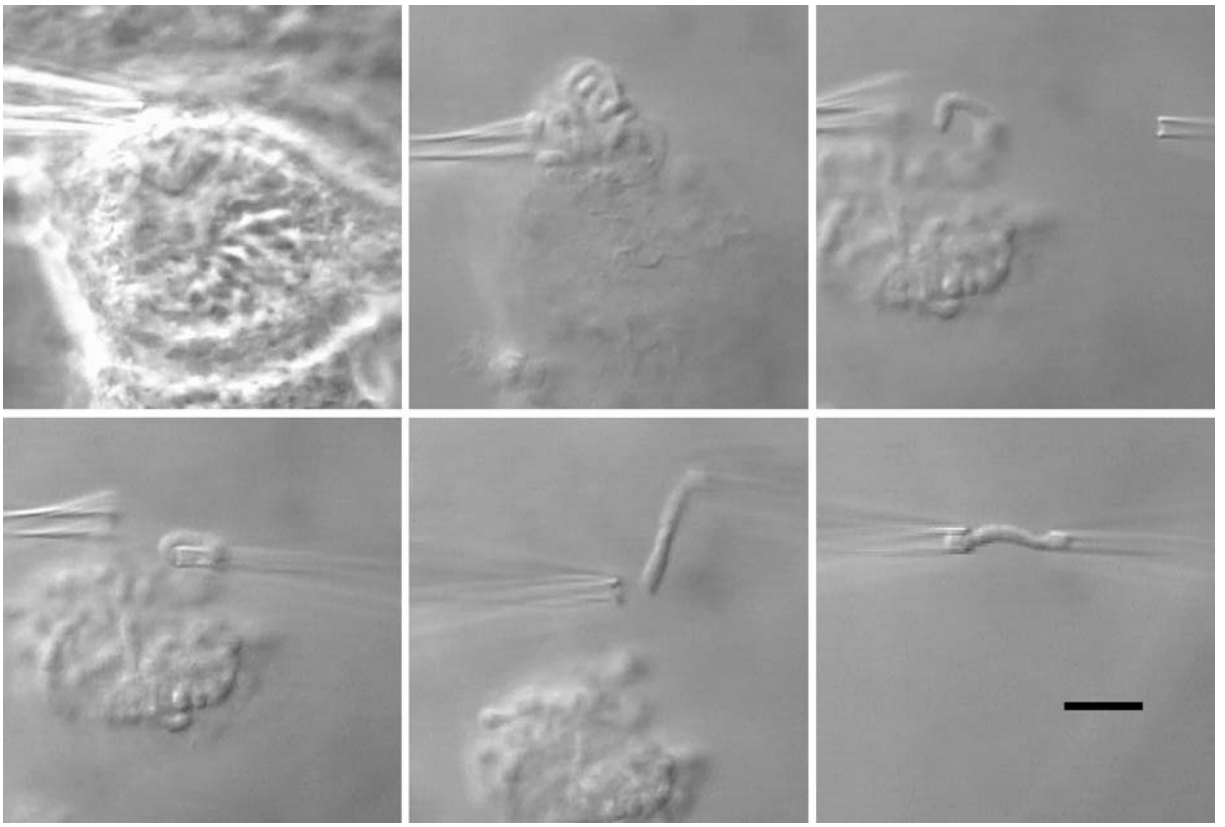

Figure 6. Isolation of metaphase chromosome from dividing newt cell. *Upper left:* cell is sprayed with dilute solution of Triton surfactant which destabilizes cell membrane. *Upper centre:* chromosomes flow out of cell. *Upper right and lower left:* one chromosome is captured in a pipette using suction. *Lower centre:* chromosome is moved away from cell by right pipette, note stretching of isolated chromosome caused by attachment to other chromosomes. *Lower right:* chromosome is attached to second pipette using suction. A video version of this procedure is available at http://markolab.bmbcb.northwestern.edu/ Bar=10 μm. (Images courtesy of M.G. Poirier).

balance with a micrometer (Poirier *et al*. 2000). Calibration of the force-measuring pipette stiffness allows the deflection–extension data to be converted to force–extension. Typical native newt chromosome data are shown in Figure 5, lower left.

Our group has carried out experiments of this type on chromosomes extracted from cells. Houchmandzadeh & Dimitrov (1999) and Almagro *et al*. (2004) have used similar techniques to study unreplicated chromatids assembled using *Xenopus* egg extracts.

Mitotic chromosome stretching elasticity

Mitotic chromosomes have robust elasticity, returning to native length even after 5-fold extensions (Nicklas 1983, Houchmandzadeh *et al*. 1997, Poirier *et al*. 2000). During mitosis, chromosomes are often doubled in length by spindle-generated forces on the order of 1 nN in large animal or insect cells (Nicklas 1983). The extensibility of mitotic chromosomes has been used to increase the resolution of chromosome banding (Claussen *et al*. 1994).

Nicklas made the first measurements of the elasticity of mitotic chromosomes (actually meiotic metaphase I and II chromosomes), using microneedles to push and hook chromosomes *inside* grasshopper cells, by pushing on the cell membrane (Nicklas 1963, 1983). Bending of the microneedle provided a way to measure forces, and Nicklas found that roughly nanonewton forces caused chromosomes to be stretched to double their native length *in vivo*.

Our experiments on newt and *Xenopus* mitotic chromosomes removed from cells and manipulated with micropipettes showed that mitotic newt

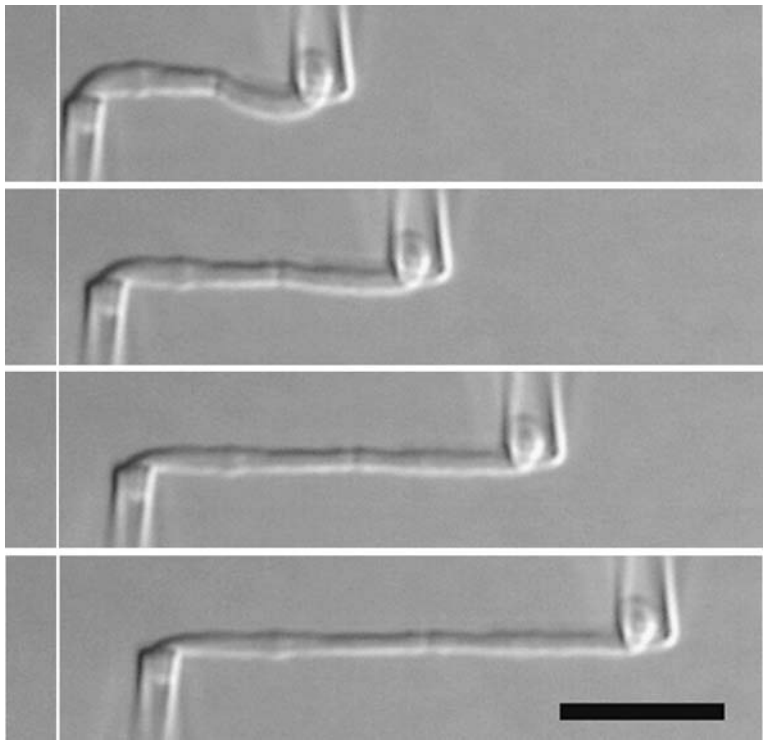

Figure 7. Chromosome stretching experiment. Pipettes are used to hold a mitotic chromosome, with the left pipette fabricated with a deflection force constant ~1 nN/μm to allow chromosome tension to be measured. Top image shows relaxed chromosome. As the right pipette is moved, the left pipette is observed to deflect from its zero-force position (thin white line). Digital image analysis allows pipette deflections to be measured to about 10 nm accuracy, translating to about 10 pN force resolution. Bar=10 μm. (Adapted from Poirier *et al*. 2002a).

chromosomes could be doubled in length by roughly 1 nN forces (Houchmandzadeh *et al*. 1997, Poirier *et al*. 2000, Poirier & Marko 2003), in good accord with Nicklas (1963). We also found stretching force to vary nearly linearly with extension for elongations up to four times the native length, allowing us to summarize the elastic response with a number, the 'force constant' or slope of the force-versus-elongation curve. We emphasize that for newt chromosomes, if one makes sufficiently slow extension–relaxation cycles, the same forces are measured during retraction as during extension; i.e., no hysteresis or irreversibility is observed. Similar results were obtained for chromatids reconstituted using *Xenopus* egg extracts (Houchmandzadeh & Dimitrov 1999): forces of about 1 nN were required to double chromatid length, with well-defined linear reversible elasticity. Interestingly, a broad distribution of chromosome force constants is obtained from single-chromosome stretching experiments (Nicklas 1983, Poirier & Marko 2003); it is not clear whether this variation is due to mitotic stage or is chromosome-specific.

This level of force (1 nN) on a whole newt chromosome is insufficient to remove histones from DNA. Stretching experiments on assembled chromatin fibers in buffer (typically 10 to 100 mM NaCl, pH 7.5) (Cui & Bustamante 2000, Bennink *et al*. 2001, Brower-Toland *et al*. 2002) show that nucleosome removal occurs only when tension exceeds roughly 10 piconewtons (pN) (note 1 pN=10^{-3} nN; individual protein motors such as kinesin generate pN forces). By contrast, in a whole newt chromosome of ~1 μm radius and therefore ~3 μm^2 cross-sectional area, several thousand 30 nm chromatin fibres pass through each chromosome cross-section. Therefore, forces of a few nanonewtons on a whole chromosome reduce to roughly 1 pN forces per chromatin fibre, insufficient force to dislodge histones. However, this level of force is sufficient to stretch out chromatin fibre from a folded (30 nm) to an extended (10 nm or "beads-on-a-string") conformation; in this force range chromatin fibres have been observed to display a nearly linear force-versus-extension response (Cui & Bustamante 2000,

Bennink *et al*. 2001). More detailed estimates indicate that the linear reversible elastic range of stretching of whole chromosomes can be attributed to this folding elasticity of the 30 nm chromatin fibre (Cocco *et al*. 2003). However, one should keep in mind that the possibility that at least part of whole-chromosome elasticity may arise from the opening of large-scale chromatin folding.

To describe the elastic properties of a material, one often quotes its elastic modulus. This expresses what stress (force per area) would be required to double an object's length, if the initial linear elasticity were extrapolated. For a mitotic chromosome, this stress is about 500 pascals (Nicklas & Staehly 1967, Poirier *et al*. 2000, Poirier & Marko 2002b) (1 pascal (Pa)=1 newton/meter^2 is the SI unit of pressure and stress). A 500 Pa modulus is low, even for a very loose high-polymer gel: 1% agarose gels have moduli of about 10 kPa (10 000 Pa), plexiglass and folded biomolecules (B-DNA and globular protein domains) have moduli near 1 GPa (10^9 Pa), and covalently bonded materials (metals, glasses) have moduli of about 10 GPa. The modulus is useful because it expresses the strength of the interactions holding a material together, in a way which is independent of size or shape. Table 1 lists moduli of the mitotic chromosomes studied to date.

Mitotic chromosomes have a modulus roughly a millionth of the modulus of the molecules from which they are composed, indicating that they are relatively loosely internally linked. The extreme extensibility of up to five times without apparent damage indicates that the internal structure must involve loosely compacted domains of chromatin that can readily unfold under force. Further evidence for unfolding of polymer-like folded domains is given by dynamic experiments that show a slow, viscous response to applied forces consistent with the elastic response of a flexible polymer network (Poirier *et al*. 2000, 2001, Poirier & Marko 2002a). All of our elastic experiments require very slow (100 s) extension–relaxation cycles to stay in mechanical equilibrium; sufficiently fast stretching can cause irreversible changes to chromosomes (Houchmandzadeh *et al*. 1997).

By contrast, following very slow extension to 5-fold or greater extensions and forces in the 10–20 nN range, mitotic chromosomes are permanently lengthened, suggesting that internal 'links' holding chromatin in its compacted form are being broken (Poirier *et al*. 2000); similar irreversible elasticity is

Table 1. Physical properties of mitotic chromosomes. Ranges for values indicate the width of distribution of measured values, and not measurement errors

Chromosome type	Experiment conditions	Stretching (Young) modulus(Pa)	Bending rigidity(J m)	References
Drosophila metaphase chromosome	*In vivo*	ND	$\sim 6\times10^{-24}$	Marshall *et al*. (2001)
Grasshopper metaphase I and anaphase I chromosome	*In vivo*	200–1000 (avg=430)	ND	Nicklas (1967, 1983)
Newt (*N. viridescens*) prometaphase chromosome	Cell culture medium	100–1000	$1–3\times10^{-22}$	Houchmandzadeh *et al*. (1997), Poirier *et al*. (2000, 2002a,b), Pope *et al*. (2006)
Newt prometaphase chromosome	*In vivo*	ND	$2–5\times10^{-23}$	Poirier *et al*. (2002a)
Xenopus prometaphase chromosome	Cell culture medium	200–800	$0.5–2\times10^{-23}$	Poirier *et al*. (2002a)
Xenopus anaphase chromatid	Cell culture medium	~300	$\sim 5\times10^{-24}$	Poirier *et al*. (2002a)
Xenopus reconstituted chromatid	Buffer EB	1000	1.2×10^{-26}	Houchmandzadeh & Dimitrov (1999), Almagro *et al*. (2004)

ND indicates quantity not directly measured.

seen for unreplicated mitotic chromatids following sufficient extension (Houchmandzadeh & Dimitrov 1999). After slow extensions beyond about 30 times native length followed by relaxation, mitotic chromosomes end up not only longer than native but also wider (Figure 8) (Poirier *et al*. 2000). This suggests that if sufficient numbers of chromatin interconnects are broken up, the now less constrained chromatin swells up. Using fluorescent antibodies, histone content was observed not to change appreciably during this experiment (Poirier *et al*. 2000).

Note that experiments in the irreversible stretching regime involved forces of at most 20 nN, corresponding to forces of several piconewtons per chromatin fibre by the cross-section argument mentioned above. This is insufficient force to quickly break chemical bonds (Grandbois *et al*. 1999), but it is sufficient to break protein–DNA and protein–protein interactions. The irreversible stretching behaviour of chromosomes is most likely due to disruption of chromatin-crosslinking elements.

Mitotic chromosome bending stiffness

The utility of the bending stiffness of a chromosome is that it can be measured without applying external stresses. Any small flexible rod will undergo random bending fluctuations at room temperature by thermal forces. The approach of measuring thermal bending fluctuations has been widely used to study mechanical properties of biopolymers and biopolymer complexes (e.g. Gittes *et al*. 1993). One usually measures the length over which thermally excited bends occur, or the "persistence length" (Gittes *et al*. 1993, Houchmandzadeh & Dimitrov 1999). The bending stiffness is just the persistence length times a thermal energy factor ($k_BT=4\times10^{-21}$ joules; here T is absolute temperature, essentially the same for all laboratory temperatures). Thus, the bending constant is measured in joule-metres (J m) (Table 1).

This approach has been applied to mitotic chromosomes. When prometaphase chromosomes are isolated from either newt or *Xenopus* cells, very small bending fluctuations are observed: the 'persistence length' obtained from quantitative analysis is found to be many times the length of the chromosome (Poirier *et al*. 2002a). However, when *Xenopus* chromatids assembled using egg extracts are observed (after dilution into suitable buffer to avoid non-thermal fluctuations generated by condensins and other ATPases), one sees drastic thermal bending fluctuations by large angles, and one measures a persistence length of roughly 2 μm, much shorter than the 20 μm-long chromatids (Houchmandzadeh & Dimitrov 1999). Reconstituted *Xenopus* chromatids have a bending stiffness about 500 times less than *Xenopus* chromosomes (Poirier *et al*. 2002a), indicating a profound difference in internal structure between unreplicated egg-extract chromatids and prometaphase chromosomes from differentiated cells.

A rod made of a material with a well-defined elastic stretching modulus has a bending stiffness which is proportional to that modulus times the fourth power of the cross-section radius (times a numerical factor close to 1 in value; see Poirier & Marko (2003). Given the numbers for the stretching modulus and the bending stiffness for chromosomes, one can ask whether they are consistent with this uniform-elastic-medium result. The result is that for newt and *Xenopus* chromosomes from tissue culture

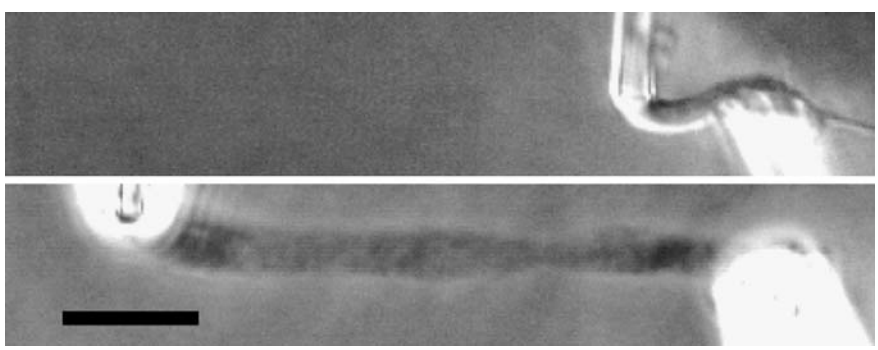

Figure 8. Extreme overextension of newt chromosome leads to swollen chromosome. *Top:* native chromosome before overextension. *Bottom:* chromosome after a series of slow extensions to 40 times native length by a peak force of 16 nN; chromosome is permanently lengthened by approximately 5-fold, and widened approximately 1.5-fold. Bar=10 μm. (Adapted from Poirier *et al*. 2000).

cells, the bending stiffnesses are consistent with their being made of a uniform elastic medium with stretching modulus of 500 Pa (Poirier *et al*. 2002a).

In contrast, the *Xenopus* egg-extract chromatids are thus about 500 times easier to bend than we would expect for a uniform elastic medium, suggesting that egg-extract chromatids have the organization of a halo of chromatin attached to a very thin internal elastic structure, i.e. with no crosslinking in the exterior halo region (Houchmandzadeh & Dimitrov 1999). If two such chromatids were linked together by cohesins as in the prometaphase chromosomes, the resulting structure would be much more difficult to bend, possibly explaning the large difference in bending modulus between egg-extract chromatids and somatic-cell chromosomes.

Observation of bending fluctuations has also been used to estimate stretching modulus in systems where stretching experiments would be very difficult or impossible owing to the small size of the chromosomes involved. Marshall *et al*. (2001) used bending fluctuations of chromosomes in colchicine-poisoned cells to estimate the elastic modulus of mitotic *Drosophila* embryo chromosomes: a value of roughly 10 Pa was obtained, significantly smaller than the 500 Pa measured for amphibian tissue culture cell chromosomes. It would be quite interesting to know the corresponding stretching modulus; recall that *Drosophila* chromosomes are thought to be dominated by highly dynamic condensin I (Oliveira *et al*. 2007).

A more detailed discussion of chromosome mechanics can be found in Poirier & Marko (2003).

Reversible folding and unfolding of mitotic chromosomes by salt

As discussed above, chromatin can readily be made to unfold from 30 nm to 10 nm fibre form by shifting univalent salt concentration to low (10 mM) values at which electrostatic repulsion overwhelms nucleosome stacking interactions (van Holde 1988). This type of experiment can be carried out with whole chromosomes, with dramatic results. Maniotis *et al*. (1997) reported that mitotic chromosomes could be abruptly decondensed and recondensed merely by shifting salt concentration. An older literature concerning this general type of experiment also indicates that mitotic chromosomes can be hypercondensed or greatly decondensed by shifts in salt concentration (Cole 1967, Zelenin *et al*. 1979).

In a series of experiments in which univalent and multivalent salts were sprayed onto newt mitotic chromosomes, not only that mitotic chromosomes could be hypercondensed or decondensed on a few-second timescale, but furthermore chromosome elastic response after such treatments matched the pre-treatment response, suggesting refolding to a near-native state with little or no loss of protein (Poirier *et al*. 2002b). For univalent salt (NaCl), both low-salt (<100 mM) and high-salt (>100 mM) conditions led to chromosome unfolding. Thus, maximum chromosome compaction as a function of NaCl concentration occurred for essentially physiological (100 mM) levels. At low salt, decondensation can be understood in terms of electrostatic repulsion driving adjacent nucleosomes apart, essentially unfolding 30 nm chromatin to the 10 nm form. At high salt, the simplest explanation is that attractive electrostatic interactions favouring chromosome compaction become screened by the high charge density, leading to expansion of the chromosome. This unfolding is dramatic; for 400 mM NaCl, a newt chromosome reaches a volume roughly 5 times larger than its native state.

For divalent salt ($MgCl_2$), different results were found: low divalent concentrations (10 mM) led to compaction of the chromosome (the opposite effect of the univalent salt), possibly due to Mg^{2+}-mediated attractions between single negative charges along chromatin fibres. At high divalent concentrations (100 mM), chromosome expansion was observed, again likely due to screening out of charge interactions. In all cases where univalent and divalent salts were used, the chromosomes rapidly recovered their native elasticity when the flow of ions was stopped.

These experiments indicate that, far from being tightly bound together, chromatin in mitotic chromosomes is greatly compacted by relatively weak electrostatic interactions which can be easily disrupted. The native state can easily be recovered following its disruption. Interestingly, by use of trivalent cations, the volume of a chromosome can be reduced by about a third. Thus, the native state is well below its maximum density; much of the visible mitotic chromatid volume is mobile small molecule species, presumably mostly water. Notably, both the unfolding (expansion) and hypercondensation (contraction) driven by salt were always observed to be isotropic, with length changed by the same factor as width (Poirier *et al*. 2002b).

Molecular connectivity of mitotic chromosomes

Nucleases disintegrate mitotic chromosomes

The elasticity experiments described in the previous section show that mitotic chromosomes can be reversibly extended up to five times their native length, indicating that the molecules holding them together are themselves highly extensible. A main question one can ask is whether this extensibility and elasticity is due to DNA (chromatin) extensibility, or whether chromosome elasticity comes from extensibility of protein structures, e.g. SMCs. A closely related question is whether the chromatin in a mitotic chromosome is folded by being looped or attached to a protein scaffold which is stably connected by protein–protein interactions, or alternately whether non-histone proteins which stabilize mitotic chromatin are essentially disconnected from one another so as to act as chromatin 'crosslinks'.

One way to attack these questions is to use enzyme digestion to determine how the mechanical properties of chromosomes are modified by cleavage of different molecular components. Classic experiments of this type (Callan & Macgregor 1958, Macgregor & Callan 1962) showed that DNAase fragmented amphibian lampbrush chromosomes (meiotic prophase), and that this was not done by RNAase and

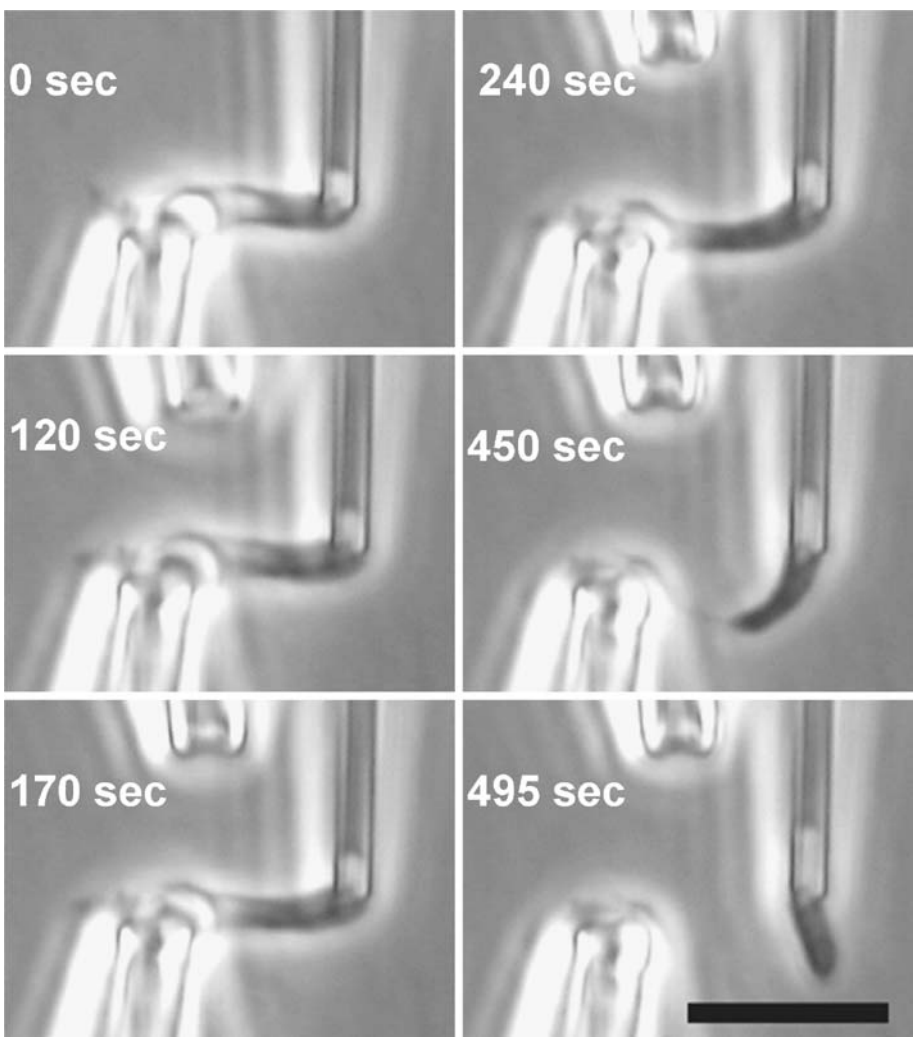

Figure 9. MNase digestion severs mitotic chromosome. Native chromosome rapidly loses elastic stiffness (note bending of chromosome by flow after 240 s of MNase exposure) and is then cleaved. Bar=10 μm. (Adapted from Poirier & Marko 2002c).

proteases. Gall (1963) used DNAase-cleavage experiments to determine that lampbrush chromosomes contained four parallel DNA molecules (i.e. the four chromatids present at meiotic pachytene). Later experiments studied the access of restriction enzymes to loop domains in lampbrush chromosomes (Gould *et al*. 1976).

A few groups have followed this general approach to examine the effect of cutting nucleic acid on mechanical properties of individual mitotic chromosomes. Digestion of DNA has been shown to disrupt mitotic chromatin (Cole 1967, Maniotis *et al*. 1997). We wished to understand the process by which mitotic chromosomes lost their mechanical continuity. Experiments with micrococcal nuclease on a chromosome under low tension (roughly 0.1 nN) revealed that cutting of DNA alone rapidly eliminates chromosome elasticity. Chromosomes become irreversibly extensible after even brief MNase exposure. Sufficient digestion causes cleavage of the whole chromosome (Figure 9), and finally collapse of the remaining chromatin into a spherical droplet, indicating all loss of elasticity and memory of its original shape (Poirier & Marko 2002c).

If light MNase digestion is applied to a chromosome with no tension at all applied to it, one sees little change in chromosome shape or global structure (Figure 10, top panels). However, if the chromosome is then extended, it has no elasticity at all, and extends into a series of domains linked by very thin threads (Figure 10, 0 s), possibly reflecting variations in density of chromatin crosslinking. Restarting light

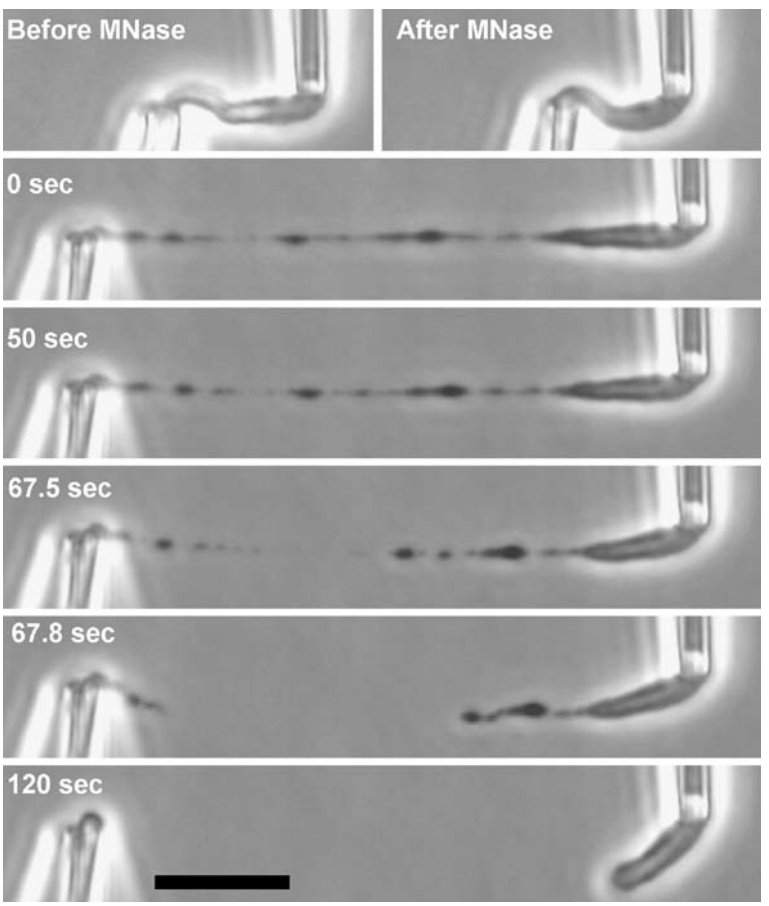

Figure 10. Chromosome unfolding after partial MNase digestion of newt chromosome. *Top left:* native chromosome; *top right:* chromosome after MNase digestion. *Second panel (0 s):* chromosome is extended, and left end breaks up into a series of dense domains linked by thin filaments. *Third through sixth panels (50–12*0 s) show result of MNase spray, which severs extended filament. Note that the remaining segment of chromosome refolds. Bar=10 μm. (Adapted from Poirier & Marko 2002c).

MNase digestion cleaves the chromosome in the middle of the thin fibre (Figure 10, 67.5–67.8 s). The remaining chromatin string then folds back up into a chromosome-like structure (120 s).

Cutting of DNA alone leads to complete dissolution of the mitotic chromosome, and therefore non-histone proteins are not connected together (Poirier & Marko 2002c). Instead, proteins such as topo II and SMC complexes are disconnected from one another, and must act as crosslinkers to form a 'gel' or 'network' of chromatin. Recent experiments on reconstituted *Xenopus* chromatids obtained similar results and conclusions (Almagro *et al*. 2004).

Blunt-cutting restriction enzymes allow a rough estimate of the inter-crosslink distance (Poirier & Marko 2002c). 4-Base-specificity cutters led to the same result as for MNase (Figure 11). However, 6-base-specificity blunt cutters caused no change in chromosome elasticity. Experiments with a restriction enzyme with 5-base specificity led to a reduction of chromosome elastic modulus but not to cleavage of the chromosome. This series of experiments indicates that 4-base specificity is sufficient to entirely disconnect the chromatin inside a mitotic newt chromosome. Given that restriction enzymes are able to efficiently access only linker DNA (Polach & Widom 1995), this corresponds to one cut every ~3 kb. However, 6-base-specificity cutting (one cut every ~40 kb in chromatin) is insufficient to even reduce the elastic modulus. Based on these results, the typical distance between crosslinking elements in the newt chromosome was estimated to be approximately 15 kb.

Proteases gradually expand but do not cleave chromosomes

Maniotis *et al*. (1997) showed that trypsin and proteinase K treatment of whole genomes caused a volume expansion of human mitotic chromosomes.

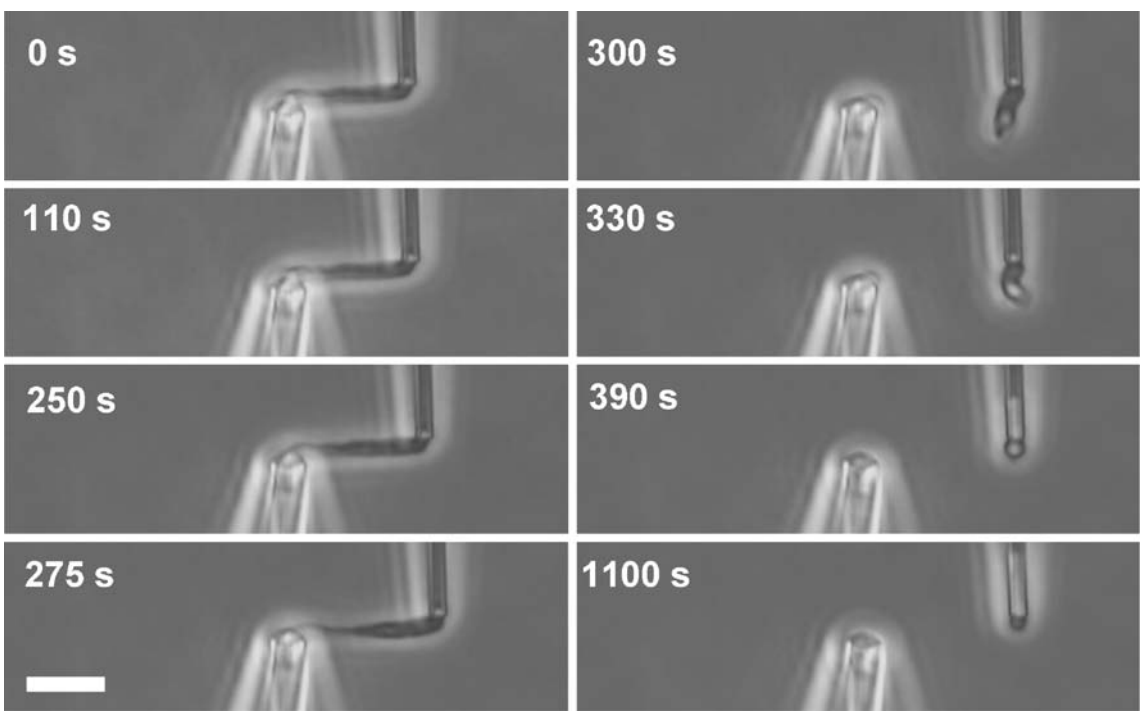

Figure 11. Digestion of newt mitotic chromosome by 4-base-specificity blunt-cutting restriction enzyme *Alu*I. Initial (0 s) image shows native chromosome under low tension (100 pN). As digestion proceeds, the force measuring pipette (right) relaxes, indicating that the chromosome has lost elasticity (250 s). Additional digestion thins (275 s) and cleaves (300 s) the chromosome; additional digestion converts the chromosome to a 'droplet' of chromatin fragments (390 s) and finally eliminates most of the chromosome outside the right pipette (1100 s). Bar=10 μm. (Figures courtesy of M.G. Poirier).

Force-measurement experiments on *Xenopus* reconstituted chromatids (Almagro *et al*. 2004) showed that the elastic stiffness was gradually reduced by protein digestion.

Protease experiments on newt mitotic chromosomes obtained similar results: exposure to either trypsin or proteinase K gradually decondensed and softened chromosomes but without ever entirely eliminating their elastic response or cleaving them (Figure 12) (Pope *et al*. 2006). Protein digestion led to a strongly anisotropic decondensation process, with length increasing by a larger proportion than width. It was also found that partial digestion of mitotic chromosome protein induced sensitivity of the elastic modulus to 6-base-specificity blunt-cutting restriction enzymes. All of these effects are consistent with a network organization of the mitotic chromosome, with a strong degree of anisotropy of folding to allow strong lengthening in response to mild protein digestion (Kireeva *et al*. 2004, Pope *et al*. 2006).

Interchromosome linkers

A feature of chromosome structure that is evident whenever mitotic chromosomes are removed from animal cells is that different chromosomes (replicated chromatid pairs) are connected together by thin, highly extensible filaments. These have been observed in chromosome isolation experiments for many years (Hoskins 1968, Korf & Diacumakos 1978, Maniotis *et al*. 1997) but have always been controversial since they contradict the common wisdom that different chromosomes are separate gene linkage units. Definitive observation of such filaments inside a live cell has not been reported, and observing these filaments outside the cell always invites the criticism that they are an artefact of chromosome isolation (Korf & Diacumakos 1980).

A number of authors have reported that mitotic interchromosome linkers are cut by nucleases (Maniotis *et al*. 1997, Poirier & Marko 2003), and

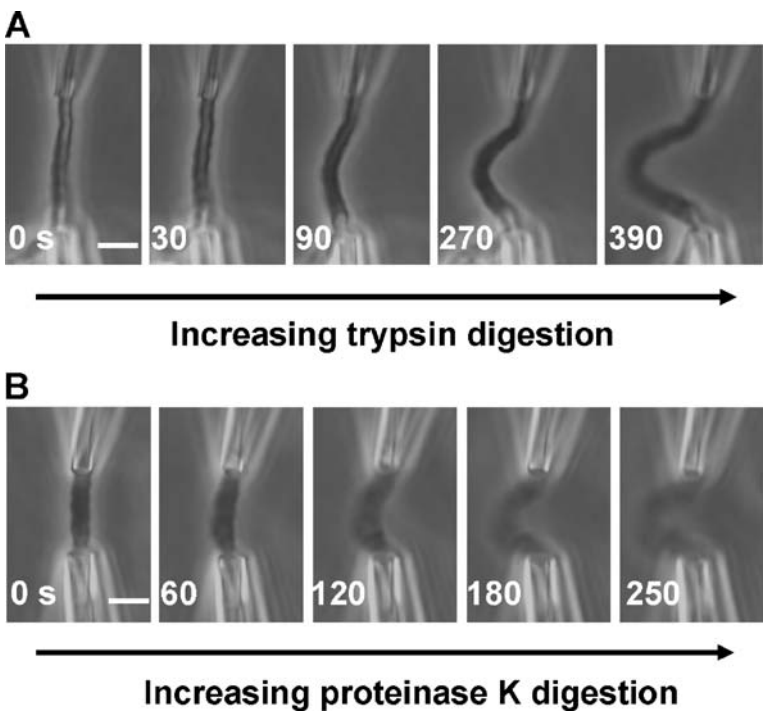

Figure 12. Decondensation driven by digestion of protein in newt mitotic chromosome. **(A)** Progressive lengthening and widening of chromosome resulting from increasing trypsin digestion; digestion time shown in seconds. Expansion is anisotropic, with length increasing more than width. Chromosomes remain elastic during these digestion experiments. **(B)** Similar effects of proteinase K. Bars=5 μm. (Adapted from Pope *et al*. 2006).

therefore that they are based on DNA. In our experiments, we almost always find these fibres between mitotic newt chromosomes (note the chromosome being stretched by an invisible fibre in the lower middle panel of Figure 6); once in every few dozen experiments we observe a loose chromosome free of such linkers. We typically break linkers after firmly attaching a chromosome to two pipettes. Although their mechanical effects are obvious, interchromosome linkers can barely be observed by phase-contrast or DIC indicating that their thickness is in the range of 100–200 nm.

A study of chaffinch (bird) chromosomes revealed filaments containing a centromeric satellite DNA extending between nonhomologous metaphase chromosomes (Saifitdinova *et al*. 2000, 2001). Interchromosome filaments containing centromeric satellite DNA and CENP protein have also been observed in mouse tissue culture cells by Kuznetsova *et al*. (2007). The function of these interchromosome filaments remains an enigma.

Implications for chromosome structure

Mitotic chromosomes are chromatin networks

Biochemical and biophysical results put constraints onto models of how the mitotic chromosome is folded. DNA digestion experiments indicate that the basic organization of the mitotic chromosome is that of a chromatin network or gel with non-DNA crosslinking elements which are not bound to one another (Poirier & Marko 2002c). Note that 'crosslinking' does not necessarily imply covalent binding; the chromatin crosslinkers of interest here may act via non-covalent protein-DNA, protein-protein or even topological interactions (Nasmyth & Haering 2005). It must also be noted that digestion experiments do not rule out an inhomogeneous spatial distribution of crosslinks inside chromatids. However, recent EM studies have observed a surprisingly regular network of chromatin in the interior of egg-extract-assembled chromosomes (Konig *et al*. 2007).

Chromosome elasticity experiments combined with single-chromatin-fibre stretching experiments are consistent with isolated scaffold elements. If the crosslinks were bonded together into a contiguous protein scaffold, one would not expect such a large range of elastic force response, since folded proteins are known to be relatively rigid; For example, condensin-folded structures along single DNAs (Strick *et al*. 2004) require 10 pN forces to be broken, and coiled-coils require even higher 20 pN forces to be uncoiled (Schwaiger *et al*. 2002). The known high degree of extensibility of chromatin fibre (Cui & Bustamante 2000, Bennink *et al*. 2001) can simply explain the large extensibility of mitotic chromosomes at relatively low forces, but only if chromatin crosslinking elements are not bound to one another.

What are the crosslinking elements?

Current data suggest SMC complexes as prime candidates for crosslinkers. Animal condensin units can by themselves condense DNA (Strick *et al*. 2004), and are essential to chromatid condensation in the egg-extract system (Hirano & Mitchison 1994). Depletion of condensins in cells impairs chromosome condensation and causes chromosomes to be mechanically weak (Hudson *et al*. 2003, Ono *et al*. 2003, Hirota *et al*. 2004, Gerlich *et al*. 2006). Finally, estimates for the numbers of condensins on animal chromosomes are consistent with inter-crosslink distances inferred from digestion experiments (Poirier & Marko 2002c).

Cohesins have a chromatin-crosslinking function in mitotic chromosomes, given that they hold sister chromatids together, possibly by a topological mechanisms (Nasmyth & Haering 2005). They appear to be mobile and affected by transcription in yeast (Lengronne *et al*. 2004). Cohesins provide crosslinks between sister chromatids which persist until anaphase.

It is possible that there are other as yet uncharacterized mitotic crosslinking elements, e.g. BAF-1, given that condensin depletion experiments suggest that the cell may have alternatives to condensins to drive chromosome condensation (Hudson *et al*. 2003, Gassmann *et al*. 2004, Hirota *et al*. 2004, Gerlich *et al*. 2006).

SMC-crosslinked-chromatin-network model of mitotic chromosome condensation

The results discussed above, combined with conclusions of Marsden & Laemmli (1979), Losada & Hirano (2001), Lavoie *et al*. (2002), Maeshima & Laemmli (2003), Strukov *et al*. (2003), Kireeva *et al*. (2004), Lavoie *et al*. (2004), Polyakov *et al*. (2006), and Sheval and Polyakov (2006), suggest the follow-

ing scenario for vertebrate chromosome condensation (Figure 13). Numbers are approximate, and apply to the human case.

The first event is loading of cohesin onto unreplicated chromatin. Cohesins are then organized by a process possibly coupled to transcription, into intermittent clusters along replicated sister chromatids, whose positions are programmed by DNA sequence (Glynn *et al*. 2004, Lengronne *et al*. 2004, 2006). This redistribution might also be coupled to DNA replication, which has been suggested to drive condensation and segregation of sister chromatids, e.g. through extrusion of replicated DNA domains (Pflumm 2002, Gotoh 2007). The key point is establishment of well-separated points of cohesion, preceding condensin activity.

Next, during prophase, condensin II is loaded, and acts to condense the parallel sisters (Figure 13A–C). If condensin II acts *in cis* along DNA as observed in single molecule experiments (Strick *et al*. 2004), then crosslinking and potential topological relinking of sisters will not occur. Instead, remnant sister catenation will be pushed out of the condensin-rich regions, to form tight DNA crossings favoured by topo II (Sumner 1996), and generating alternating condensin- and topo II-rich regions (Maeshima & Laemmli 2003).

A plausible mechanism for condensin II to accomplish chromatin condensation *in cis* is for it to initially bind short, contiguous segments of chromatin of length similar to its ~50 nm size (also comparable to the ~30 nm persistence length of chromatin fibre (Cui & Bustamante 2000, Dekker *et al*. 2002)), and then to gradually translocate, or alternately to stimulate binding of additional condensin units at neighbouring chromatin sites, so as to progressively condense chromatin between cohesin 'boundaries' (Lavoie *et al*. 2002, 2004). The outcome would be a series of segregated loop-like chromatin domains, separated by cohesin clusters along the chromatid axis, and a highly contracted chromosome. These loop-like chromatin domains might be folded or interwound by topological effects of condensin (Kimura & Hirano 1997, Kimura *et al*. 1999) or by binding of metal ions (Strick *et al*. 2001). Condensin locations may be programmed: evidence exists supporting defined yeast condensin binding sites spaced by roughly 10 kb (Wang *et al*. 2005).

Local translocation over distances of tens of kilobases would make the condensin II distribution appear stationary at optical scales (Gerlich *et al*. 2006), while still generating a large amount of compaction, and without adding links between sister chromatids. Quite to the contrary, tension built up between adjacent chromatids would drive topo II to gradually segregate them (Marko & Siggia 1997, Maeshima & Laemmli 2003).

This scheme organizes prophase chromatids into a string of rosette-like 'chromomere' structures (Sumner 1991) of size similar to folding intermediates observed by Prusov *et al*. (1983), Zatsepina *et al*. (1983), Belmont *et al*. (1987, 1989), Belmont & Bruce (1994), Kireeva *et al*. (2004), and Belmont (2006). For human chromosomes, these proposed structures contain about 1000 nm of 30 nm fibre (100 kb of DNA), with a condensed volume of roughly 10^6 nm^3, and therefore with roughly a 100 nm diameter (Figure 13C).

These chromomeres can be folded or coiled (e.g. like nucleosomes in 30 nm fibre) only if there is a gradual loss of cohesin along chromatid arms: the cohesins of Figure 13C will oppose longitudinal condensation beyond roughly 1000 bp/nm, with higher compaction factors requiring cohesin removal. Removal of cohesin and further folding gives a further 6-fold compaction, generating a 6000 bp/nm mid-prophase chromatid (Figure 13D; note that the gray balls represent chomomere units of roughly 100 nm diameter).

Volume conservation indicates that the chromosome will become thicker by an amount approximately equal to the square root of the length compaction. For the 60-fold length compaction of chromatin fibre into the human prophase chromatid described above, this is a factor of 8 (times the 30 nm fibre thickness), resulting in segregated prophase chromatids that are 250 nm thick with a condensing II-enriched core region (Kireeva *et al*. 2004).

Then, at NEB, condensin I binds, acting as a highly mobile (Gerlich *et al*. 2006), reversible chromatin crosslinker. Condensin I acting as a reversible crosslinker in the chromosome interior will drive chromatids to adopt a configuration with lower surface area, driving longitudinal compaction and transverse thickening after NEB (Figure 13E) (Kireeva *et al*. 2004). This effect is in exact analogy to surface tension driving the shape of a liquid droplet to be spherical, but for a chromosome, the underlying chromatin network will oppose formation of a sphere, and will maintain an anisotropic shape. The result will be a metaphase chromatid which is

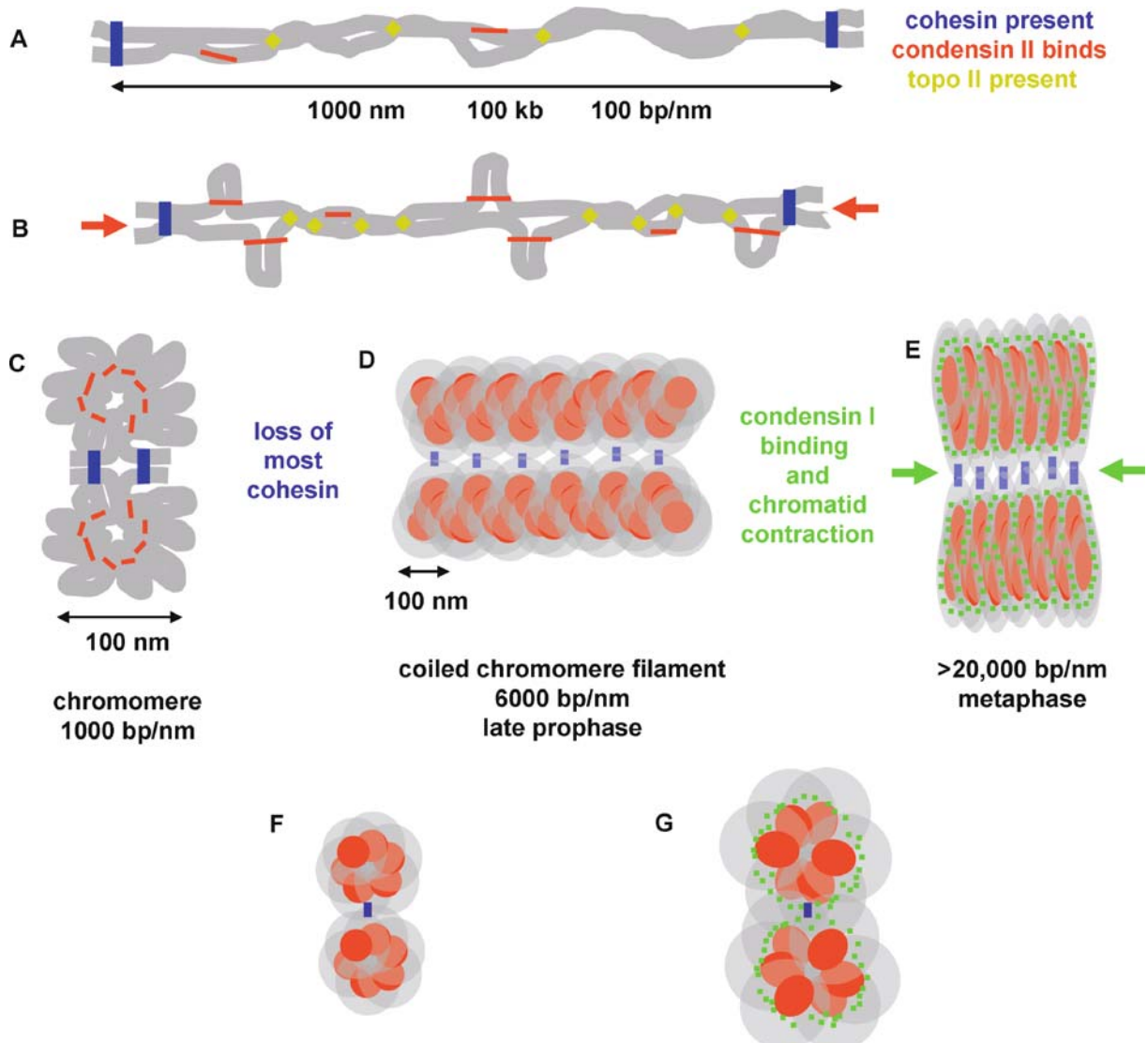

Figure 13. Model of human mitotic chromosome folding. **(A)** Part of the replicated sister chromatids (gray lines represent 30 nm fibre containing 100 bp/nm). Roughly 1000 nm of 30 nm fibre (100 kb of DNA) is between successive cohesin domains (blue bars). **(B)** Binding of condensin II (red bars) during early prophase begins to organize loops of chromatin, gradually shortening the chromosome; topo II (yellow diamonds) binds to and resolves chromatid crossings trapped between condensin-rich regions. **(C)** At end of prophase, the chromatin length between cohesion blocks has been absorbed into condensin-looped regions; the result is a chromatin "rosette" of "chromomere" of roughly 100 nm diameter with a condensin II core. Topo II and adjacent looped regions of chromosome are not shown for clarity. **(D)** Parallel chromatids corresponding to a series of rosette chromomeres organized as in C. Gray balls (100-nm-diameter) correspond to rosette/chomomere structures of C (note change of scale relative to C.). Red balls indicate their condensin II-rich cores. Successive chromomeres are stacked, folded or coiled to achieve a packing density of 6000 bp/nm, about 2000-fold higher than linear in DNA. **(E)** Binding of additional bulk-chromatid-condensing factors including condensin I (green) compresses chromosome along length, increasing its width. Length compaction at this stage is roughly 20 000 bp/nm, approaching 10 000-fold relative to DNA. (**F**) End-on view of prophase chromosome of D.; chromatids contain chromomeres (gray circles) with condensin II-rich cores (red). (**G**) End-on view of metaphase chromosomes of E.; addition of condensin I (green) compresses chromosome lengthwise, increasing chromatid diameters.

shortened and thickened relative to prometaphase, with a condensin II-rich core, covered by a layer of condensin I (Figure 13E).

The final metaphase length to width ratio will be determined by mechanical balance of condensin I condensation versus chromatin network elasticity. Condensin-I-driven longitudinal compaction may force buckling or folding of the central non-histone-protein-rich chromatid axis (Saitoh & Laemmli 1993, 1994). Sufficient crosslinking by condensin I to drive longitudinal compaction will also provide mechanical stabilization: consistent with this, depletion of condensin I has been observed to significantly weaken metaphase chromosomes (Gerlich *et al*. 2006).

In experiments where some condensin subunits are depleted or mutated, condensation might still be driven by partial condensin activity, or even by other proteins (e.g. BAF-1, or direct nucleosome–nucleosome attraction), leading to eventual segregation of adjacent chromatids (Gerlich *et al*. 2006). Note that *trans*-fibre crosslinking factors must bind and unbind, either by being ATP-cycled or by simply binding rather weakly, in order to ensure chromatid segregation (Marko & Siggia 1997).

This model predicts trends across species. The larger is the distance between cohesin domains, the greater will be the length compaction. Metaphase cohesin interdomain distances in vertebrates must be much greater than the 15 kb distance observed in yeast; for *Xenopus* the cohesin density has been estimated to be one per 400 kb (Losada & Hirano 2001). Cohesin interdomain distances should correlate with mitotic loop size, and possibly with convergent transcription domain size (Lengronne *et al*. 2004) and replicon size (Buongiorno-Nardelli *et al*. 1982). Notably, condensin binding sites in yeast have been found to be correlated with DNA replication landmarks (Wang *et al*. 2005).

Stretching and bending elasticity

In this model, cohesin and condensin I and II are not bonded together but act as isolated chromatin crosslinkers, so that a whole chromosome can be stretched simply by affine stretching of chromatin fibres between network nodes. As mentioned above, extension of individual chromatin fibres accounts for the roughly nanonewton force needed to double the length of a vertebrate chromosome. The 5-fold reversible elastic response of whole chromosomes correlates well with the 6-fold extension obtained when chromatin is converted from 10 nm to 30 nm form. Action of condensin I and possibly other crosslinking mechanisms through the body of the chromatid generates the mechanical coupling needed to generate bending elasticity.

Beyond 5-fold extension, one can expect to start to break crosslinking elements, at roughly 20 nN forces (given 2000 fibres in parallel, this corresponds to 10 pN per condensin, the force required for condensin disruption (Strick *et al*. 2004)). The result will be permanent chromosome lengthening and widening, with lengthening predominating owing to the prometaphase condensin I-driven contraction (Poirier *et al*. 2000).

The small bending stiffness of egg-extract-reconstituted chromatids (Houchmandzadeh *et al*. 1997) may be a result of the unreplicated chromatids having regions of individual chromatin fibres between chromomeric domains which can act as ‘hinges’. Consistent with this, under large extension, egg-extract chromatids extend by formation of thin, extended fibres between thicker chromosome domains (Houchmandzadeh & Dimitrov 1999), not seen for comparable extensions of chromosomes from animal cells (Poirier *et al*. 2000). Furthermore, and also supporting this hypothesis, are observations of Almagro *et al*. (2003) that the bending stiffness varies along the egg-extract chromatids.

The difference between the egg-extract and somatic-cell chromosomes may also be a consequence of the embryonic developmental state of the egg extracts. The condensin I to condensin II ratio in egg extracts is about 5:1, while in somatic HeLa cells it is closer to 1:1 (Ono *et al*. 2003). It has been documented that metaphase chromosomes in *Xenopus* embryos are twice as long as and substantially narrower than those in swimming larvae (Micheli *et al*. 1993), the difference in condensation perhaps being due to different ratios of condensin I and II or to cohesin domain or replicon size (Marilley & Gassend-Bonnet 1989).

Effects of cutting and removing molecules

In this model, cutting DNA sufficiently frequently will result in loss of elasticity (due to disconnection of chromatin), with cleavage of the chromosome only if the crosslink elements are not bound together. Insufficient cutting (less than one cut per crosslink) will not change chromosome elasticity.

If protein is cut instead, histone tails and other exposed protein structures along chromatin fibres will be cleaved, causing chromatin fibre unfolding and lengthening. This will drive gradual isotropic expansion, similar to that observed for shifts in univalent salt concentration (Poirier *et al*. 2002b). However, crosslinkers will also be cleaved, possibly causing a less symmetric effect: the hypothesis of a condensin-I-driven longitudinal compaction discussed above can explain the anisotropic unfolding of prometaphase newt chromosomes observed to result from protease treatment (Pope *et al*. 2006). Under the assumption that condensin I is the last major crosslinker added to chromosomes, it will be the crosslinker most exposed to general proteolysis. Cutting condensin I will release constraints that longitudinally compacted the chromosome, leading to longitudinal expansion. Of course general proteolysis will also cut histone tails and other proteins along chromatin which will contribute to the transverse swelling observed during proteolysis of whole chromosomes.

Specific cleavage of condensin (e.g. using engineered kleisins with suitable specific protease sites) on assembled metaphase chromosomes could be instructive. There may be large differences between cleavage of condensin I and condensin II units, given their differing functions in chromosome compaction (Ono *et al*. 2003). Based on the above model, condensin I cleavage should produce lengthening and narrowing, and reduction in elasticity of metaphase chromosomes, while condensin II cleavage should produce little effect (since condensin I acts as a more general stabilizer and crosslinker 'on top' of condensin II). However, simultaneous condensin I plus condensin II cleavage should produce lengthening and widening similar to that seen by forced chromosome unfolding (Poirier *et al*. 2000). Of course, the possibility remains that additional crosslinking elements added during prometaphase (e.g. BAF-1 or other relatively small DNA-crosslinking proteins) may be present in sufficient numbers to maintain some chromosome integrity when condensin is disrupted. An interesting question is how having condensin I or II entirely absent, as in *C. elegans or* yeast respectively, would affect condensin-cleavage experiments.

Network, hierarchical folding and radial loop models are unified by the scenario outlined above. If histones are suddenly removed, there will be release of DNA length and a large degree of swelling of the chromosome, as a more severe and irreversible version of the result of swelling of chromosomes by high or low univalent salt concentrations (Poirier *et al*. 2002b). In the case of histone removal, loop-like domains of DNA will be observed (Paulson & Laemmli 1977), a result of the domain structure of early prophase condensation.

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