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December 12, 2003

Professor Rob de Ruyter
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Indiana University
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Dear Professor de Ruyter,

I would like to apply for a tenured faculty position in your department. I have sent my CV and a research plan. I also have sent you a list of people who might be possible letter-writers. You have my permission to approach anyone you wish to provide letters of reference; I have not contacted the persons on the list I have sent.

Yours truly,

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Micromechanical study of DNA-protein interactions and chromosome structure

The objective of my research will be to study, using biophysical methods, large-scale organization of DNA by protein-DNA interactions. Three complementary approaches will be used. First, I will focus on experiments on *single-DNA micromanipulation* [1] to directly observe DNA conformational changes driven by protein-DNA interactions. Second, my lab will carry out *whole-chromosome micromanipulation experiments* to study large-scale chromosome folding [2]. Finally, I will supervise *application of statistical mechanics to description of DNA and chromosome structure.* The synthesis of these three approaches to large-scale DNA organization will provide structural and biophysical-chemical information quite complementary to that provided by traditional biochemical approaches (see below), and as a result are likely to have a large impact in fields including physics, biochemistry, molecular biology and biophysics.

All the projects described below will be carried out by Ph.D. and undergraduate students, and postdocs, as part of their training. The people working on these projects will receive highly interdisciplinary training combining biological and physical sciences. They will be highly competitive for the increasing number of faculty positions opening up for biophysical researchers.

1. Experimental single-DNA micromanipulation study of protein-DNA interactions

Traditional biochemical techniques (e.g., solution-phase reactions, gel electrophoretic analyses) analyze ensembles of molecules. To obtain molecular-scale information, biochemists often visualize the macromolecules of interest (by, for example, X-ray diffraction), but such visualizations cannot observe large-scale dynamical fluctuations of molecules. The key advantage of "single molecule biochemistry" is that it allows manipulation of individual macromolecules in solution in real time, allowing direct observation of reaction intermediates and providing data that can discern between competing models of reaction mechanisms.

The last few years have seen productive studies of protein-DNA interactions using micromanipulation techniques. One of the earliest experiments by Finzi and Gelles [3] observed opening and closing of a 300 bp DNA loop mediated by the lac repressor protein. At about the same time, experiments on RNA polymerase demonstrated that enzyme to be a powerful [4]. Leger et al [5] subsequently observed the elongation of the double helix driven by RecA polymerization. These experiments proved that it was practical to carry out biochemical reactions on micromanipulated DNAs, that protein-DNA interactions generated large DNA stresses, and that force could modulate biochemical reactions. More recent DNA micromanipulation experiments have studied topoisomerase activity [6], DNA helicase activity [7,8], random insertion by Mu transposase [9], compaction of DNA by IHF protein [10], and assembly of individual chromatin fibers [11,12]. A major component of this field has been development of theoretical models to interpret these experiments. I have written a number of basic papers in this field, including work which has established the basic theory for polymer elasticity of single DNAs [13-17], as well as theories of folding of DNAs by proteins [18-20].

a. Preliminary results*:* My group has recently focused on development of methods for the study of biochemical reactions on DNA molecules tethered between a support (e.g., a microscope slide or a bead mounted on a micron-scale pipette) and a paramagnetic particle (Fig. 1). By adjusting a magnetic field gradient, precisely controlled forces can be applied to the magnetic bead and thus to the DNA molecule itself. This general technique was developed by Bustamante and co-workers [21], and later developed by Strick et al [22,23]. This approach allows precise control of forces applied to a DNA over a range of 0.01 to 100 pN, i.e., from forces too weak to stretch a DNA up to forces able to

denature DNA and disrupt protein-DNA interactions. The extensions of DNAs are observed optically, so distance measurement is usually limited by a combination of the amount (time) of averaging done, the tension in the DNA, and one's ability to locate the center of a colloidal particle. The positional resolution can be less than 10 nm. Note this does not imply imaging resolution, i.e., the ability to resolve detail, at this scale; resolution is diffraction-limited to at best 100 to 200 nm.

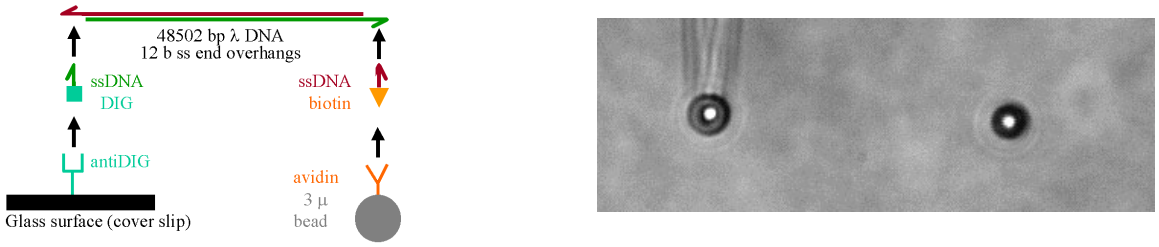

Fig.1: *Left:* Scheme for attachment of single DNA molecules to solid supports. Short ssDNAs carrying digoxigenin and biotin are ligated to the ends of a > 10 kb DNA; this allows binding of a DNA to an antidigoxigenin-coated surface (either a cover slip, or a colloidal particle), and to a streptavidin-coated paramagnetic bead. A permanent magnet can be used to apply controlled tensions to a single DNA. *Right:* A 32-micron λ-DNA dimer between 3-micron-diameter beads, the left (antidig-coated) one held in a micropipette, the right paramagnetic one being pulled with a ~1 pN force generated by a magnet about 10 mm to the right.

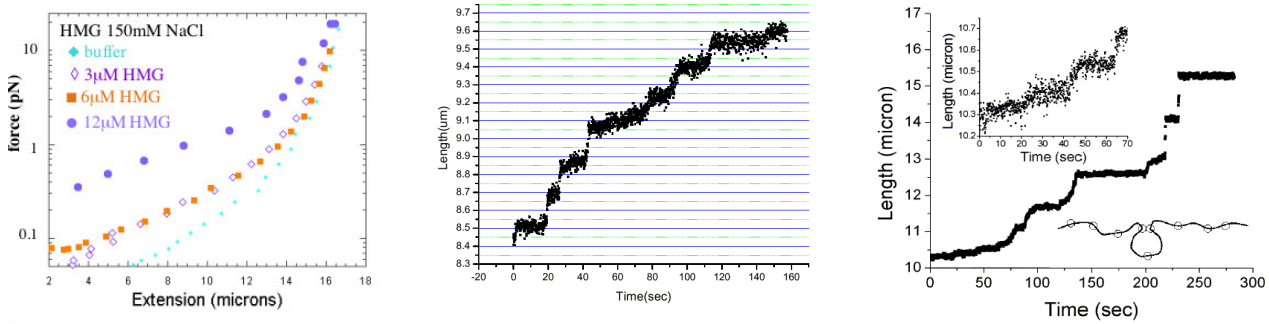

Fig. 2: *Left:* Force-extension data for DNA in presence of HMGB1, 20 mM HEPES, 150 mM NaCl, pH 7.5, 25 C. As the HMGB1 concentration is increased, compaction of DNA occurs, i.e. extension of DNA is reduced at each force. *Center:* Extension as a function of time during nucleosome removal from chromatin fiber; a series of steps are observed at constant force ~15 pN. *Right:* Opening of a series of BspMI-mediated loops along a single λ-DNA; 10 nM BspMI, phosphate buffer, 140 mM NaCl, pH 7.5, 25 C.

Fig. 2 left shows an example of an experiment with the DNA-bending protein HMGB1, which binds to and bends DNA. In buffer we observe the standard DNA force-extension curve; these data indicate the expected DNA bending persistence length of 50 nm [14]. The addition of HMGB1 causes its compaction; as higher concentrations of HMGB1 are added and more bending occurs, the DNA requires more force to be extended. High forces (10 pN) straighten out the bends, recovering the 16.3 micron length of the 48.5 kb λ-DNA.

Protein-DNA interactions can also driven with force, and monitored in real time with high precision. Fig. 2 center shows an experiment where nucleosomes are being removed by force, following assembly of chromatin fiber using Xenopus egg extracts, following the method of Bennink et al [11]. A fixed force of 15 pN results in successive nucleosome removals; jumps of multiples of the

~60 nm nucleosome length are clearly seen. The Brownian noise (fast fluctuations) in this signal have amplitude ~30 nm. Note that the slower smooth motion is not instrumental drift (this is eliminated by measuring the distance between molecule ends from a single video frame), but rather a slow and continuous change in chromatin fiber length. Our current setups produce raw data with roughly 20 nm length resolution, corresponding to about 60 bp. This resolution can be improved by a combination of instrument stabilization and data analysis.

Fig. 2 right shows an experiment where the restriction enzyme BspMI has been used with 100 uM Ca2+, conditions under which it binds two copies of its recognition sequence without cutting [24]. For a single molecule, this will result in sequence-specific DNA looping (Fig 2, right,lower inset). In the experiment shown, after incubation, a steady force of 2 pN was applied, and a series of step events were observed, due to opening of a series of DNA loops. λ-DNA has 41 BspMI binding sites with a typical spacing of 1 kb (300 nm). The 15 steps observed in the run shown have an average step size of roughly 300 nm; small loop-opening events of as little as 30 nm are directly observable in the raw data (Fig. 2 right, upper inset).

b. Proposed research*:* Having developed both theoretical and experimental techniques for single-DNA experiments, we are poised to carry out a wide variety of studies of DNA-organizing proteins. These fall into a few categories:

i. Nonspecifically interacting DNA-folding proteins: Our experiments on HMG-type proteins have shown that it is practical to study proteins which bind nonspecifically to DNA using single-molecule techniques. DNA-bending proteins play an important role in a wide variety of in vivo processes. We will continue to study the HMG proteins (HMGB1, HMGB2, NHP6A) using single-molecule techniques, and also study bacterial analogues (notably HU). We have already found that irreversible, cooperative binding is common to NHP6A, HMGB1 and HU. A new collaboration with Prof. S. Ben-Yehuda (Hebrew Univ., Jerusalem) will start study of the 20kD nonspecifically binding protein RacA which appears to play a central role in nucleoid condensation [25]. No biochemical work has yet been done on this important protein.

A new technique that we will develop as part of this project is combining single-molecule micromanipulation with fluorescence microscopy. Our collaborator (Prof. R. Johnson, UCLA Medical School) is working on gfp fusion versions of NHP6A and HMGB1. This will allow us to visualize, and eventually count, the number of proteins bound to the single DNAs that we are studying. Since we expect >500 proteins to be bound per λ-DNA it should be straightforward to observe them, given a state-of-the-art camera and using scanned-laser-spot illumination. Improvement of this experiment to the 10-protein sensitivity level can then follow for application in other experiments.

ii. In vitro-assembled chromatin: In collaboration with Prof. R. Heald (UC Berkeley, MCB) we are starting a large project to study the biochemical dynamics of the chromatin fiber, the basic DNA-protein element of eukaryote chromosomes. Currently we are studying the biochemical dynamics of the nucleosome assembly reaction itself, and the micromechanical properties of the resulting chromatin fiber. It should be noted that reliable experiments on chromatin fiber mechanical properties have not been done; measurement of compaction factor, stretching and bending properties of the interphase-like chromatin assembled by egg extracts is a current goal.

Following this, we will study how chromatin fiber assembly is affected by a number of biochemical factors. First, we will study how ATP level affects the reaction, using ATP depletion via apyrase and ATP addition to native extracts. We will directly observe how ATP levels affect assembly, disassembly, and physical properties of chromatin. We will also study how extracts prepared to assemble mitotic instead of interphase chromatin differ. Finally, we will use antibodies to

a number of proteins to deplete specific factors from the extracts, providing 'chemical knockout' experiments to study specific protein functions.

The above experiments will be done on 'λ-chromatin', nucleosomes assembled onto λ-DNA. Data of Bennink et al [11] and our lab agree that the number of nucleosomes assembled onto λ-DNA is remarkably close to the ~200 bp per nucleosome which is known to occur in vivo. However, there are bound to be differences between λ-chromatin and 'real' chromatin, probably structural, and certainly functional. Therefore we will carry out experiments to assemble chromatin starting with single yeast chromosome DNA. This can be purified and end-labeled using our technique, via insertion of λ cos sites into the telomere regions of a specific chromosome (collaboration with Prof. J. Haber, Brandeis University, Biology). We have already worked with 200 kb tetramers of λ-DNA, similar in length to the yeast chromosomes that we will use. This will lead to experiments on whole chromosomes, interphase and metaphase, using a nearly purely biochemical approach, that will eventually allow study e.g. of how initiation of transcription of specific genes affects large-scale chromatin structure.

iii. Sequence-controlled DNA interactions in cis: We have begun to study DNA looping by proteins which bind specific sequences (Fig. 2, right). I have also spent some time studying this problem theoretically [18-20]. I propose to continue working on restriction enzymes which loop DNA, but also to expand our work on looping into the domain of site-specific recombination. At present we are developing systems to study the Tn5 transposon (collaboration with Prof. W. Reznikoff, University of Wisconsin Madison) and V(D)J recombination (collaboration with D. Roth, New York University).

In each of these cases the procedure will be to clone two specific sites into E. coli plasmids, and then to extract and purify the plasmids. Then the plasmids are cut open, labeled oligos ligated, and the repurified to yield linear, end-labeled DNA as in Fig. 1, left. We have followed this procedure for 15 kb plasmids carrying Tn5 sites, with the help of the Reznikoff lab. Once linear labeled molecules carrying two specific sites can be manipulated, we will introduce the site-binding enzyme and observe loop formation.

The first objective of these experiments will be to measure the 'communication time', or time until the loop complex forms. Then, we will study the stability of the looped complexes at a number of applied forces; in each case the length of the DNA tether will provide the 'read out' for the loop. Further experiments will examine the effect of DNA supercoiling (introduced using double-strand tethers and rotating magnets [22,23]) on the synapse formation. Eventually our micromanipulation assay can be used to compare mutants of e.g. Tn5 or the V(D)J RAG proteins.

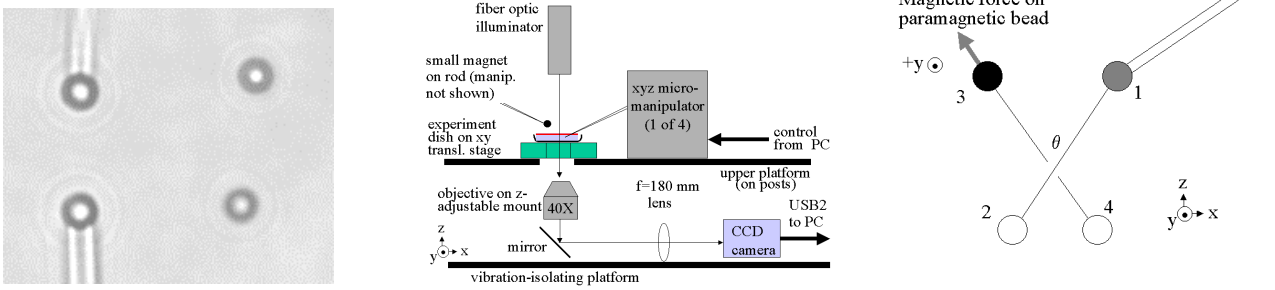

Fig. 3: Single-DNA manipulation for in trans experiments. *Left:* two λ-DNAs attached to pipettes (left) and being pulled to the right by a magnetic field gradient. The two molecules are about 10 microns apart. *Middle:* sketch of setup to allow double-molecule experiments, using a combination of pipette and magnetic manipulation of beads. *Right:* Sketch of topo II experiment: forces and positions of DNAs will be monitored during the strand passage event.

iv.DNA interactions in trans: A major new thrust of my group will be to develop techniques to manipulate two separate DNA molecules, in order to study enzyme-mediated interactions acting on two molecules. Combining micropipettes to grab beads with magnetic manipulation, we are already able to control two nearby DNAs at once (Fig. 3, left). A new setup will be built to facilitate such experiments (Fig. 3, middle) and just as one example, to carry out experiments such as passing one DNA through another, using topoisomerase II (Fig. 3, right). Experiments on topoisomerase II will allow us to directly study the strand passage event, using force as a bias for the enzyme.

2. Experimental micromechanical-biochemical study of large-scale chromosome structure

My lab has previously developed techniques for the isolation, micromanipulation, and micron-scale biochemical modification of single, mitotic chromosomes [2]. I propose to continue this project to study the structure of the mitotic chromosome of higher eukaryote cells, for a few model systems. The main approach will be to use biophysical force measurements (Fig. 4) to assay structural changes in mitotic chromosomes that are introduced biochemically. In this way, the role of specific types of molecules in defining mitotic chromosome structure can be quantitatively studied.

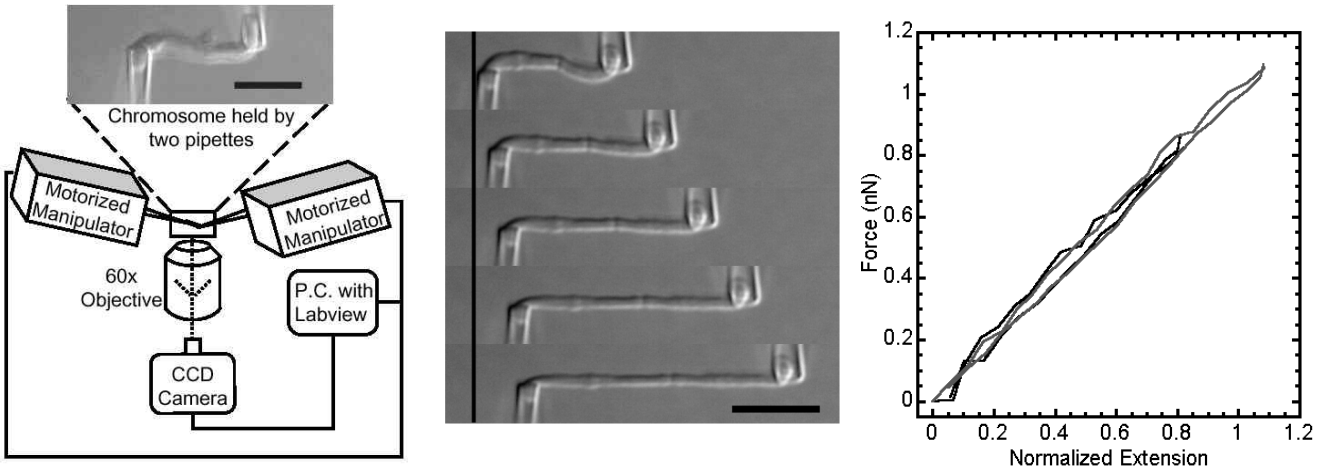

Fig. 4: *Left:* Basic mitotic chromosome manipulation setup. Cells and extracted chromosomes are imaged through a 60x contact objective, while manipulation is done from above using micropipettes. DIC photo shows a TVI chromosome held between two pipettes as in a force-extension experiment. An additional pipette (not shown) is used to 'spray' reactants in biochemical-micromechanical experiments. Bar is 10 μm. *Center:* Typically, one pipette is moved with a manipulator, allowing forces to be measured by monitoring the deflection of the other, stationary, pipette. Bar is 10 μm. *Right:* Force-extension data (extension and retraction) for a single chromosome showing elastic 'baseline'; the mitotic chromosome is an elastic solid with a force constant ~ 1 nN, corresponding to an effective modulus of ~300 Pa. Extension is given as a fraction of relaxed chromosome length, so extension of 1 corresponds to doubling of native length.

Preliminary results for newt chromosomes using DNA-cutting enzymes combined with micromanipulation suggest that DNA itself (i.e. chromatin) provides the structural integrity of the folded mitotic chromosome (Fig. 5) [26]. These results challenge the classical 'protein scaffold' model of mitotic chromosome structure, and indicate the value of our approach in ruling out specific models of chromosome structure. A first objective is further study of DNA connectivity in the mitotic

chromosome in this way, and to extend these measurements to *Xenopus* chromosomes. Additional enzyme experiments will study the role of RNA and protein in mitotic chromosome structure.

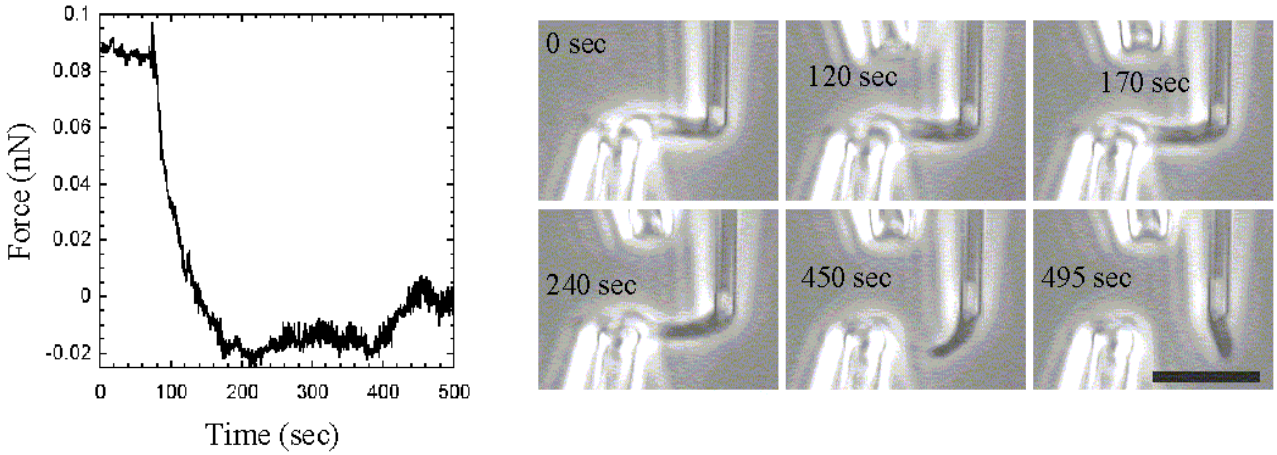

Fig. 5: *Left:* Time course of tension in a chromosome, and chromosome morphology, during digestion by 1 nM MNase, with initial tension 0.1 nN [26]. Spraying starts at 80 sec; force decays after 30 sec; chromosome is cut after 450 sec. *Right:* Images of chromosome morphology during this experiment show its complete disconnection and then dissolution by the nuclease. The spray pipette can be seen in the upper center of the t>120 sec frames.

My second objective is to characterize the relation between mitotic chromosomes from somatic cells and chromosomes reconstituted using *Xenopus* egg extracts. Preliminary results indicate strong differences in physical properties of cell-derived and egg-extract-derived chromosomes, which we will examine using our micromechanical-biochemical assays. This work will be in close collaboration with Prof. R. Heald (UC Berkeley) who is an expert egg-extract experimenter. This collaboration will permit experiments on the physical role of the condensin chromosome-organizing proteins.

The intellectual merit of these proposed activities is based on their focus on fundamental questions of chromosome structure. The proposed study of the question of whether or not there is a contiguous protein skeleton inside the mitotic chromosome directly addresses a basic and open topic in cell biology. Understanding the relation of reconstituted chromosomes to chromosomes in cells is extremely important given the general assumption that the former system is an accurate model of chromosomes. Characterization of the function of the condensin SMCs [27] in mitotic chromosome structure is also an open and important basic problem.

3. Theoretical statistical mechanics of DNA conformation and chromosome organization

a. DNA micromechanics and distortion of double helix by protein-DNA interactions: I have established statistical-mechanical models for description of double-stranded DNA (dsDNA, the Watson-Crick double helix) molecules under tension [14] and twisting stress [15]. These theoretical works were essential to interpretation of experiments on single DNA molecules (for example [13,22]). Some of my recent work has been on models for the remarkable structural transitions that occur in the double helix under high tensions and torques [16,17]. Development of our understanding of dsDNA elasticity prompted me to study the microscopic double helix fluctuations [28] responsible for dsDNA elasticity. These turn out to be much larger than anticipated in the DNA structure literature. These

fluctuations play a role in the mechanism for distortion of DNA structure by dsDNA-binding proteins, as was made concrete for the case of RecA-dsDNA binding in one of my collaborative works [5].

Another theoretical project that I have focused on is description of `unzipping' of the two single strands in a dsDNA. This is relevant to our understanding of the forces that hold the double helix together, and in understanding the cell machinery that takes DNA strands apart (as occurs, for example, during DNA replication). My group has analyzed unzipping initiation [29], and dynamics [30,31]. This work has led us to understand how the kinetics of opening-closing of even 10-bp-long molecules could be on the 1 sec time scale, as has been recently experimentally observed [32]. There are many molecular-biological problems related to the opening of the double helix which our group will be able to analyze starting from our results from unzipping. Examples include the analysis of the role of strand separation in force-driven structural transitions of the double helix [33], and the role of transient single-stranded DNA 'bubbles' in double helix bending.

In the near-term my group will work theoretically on protein-DNA interactions, particularly on those which lead to DNA distortions [18,19.20]. These include DNA-bending proteins as well as proteins which nonspecifically trap DNA 'loops'. I am currently also working on description of processes of communication between distant sequences and on the sequence-matching interactions associated with site-specific DNA recombination. These projects fit closely with the experimental single-molecule projects described above.

b. Control of topology and packaging of chromosomal DNAs: One of the biophysical problems that I am working on is the description of the cell processes which package the centimeter-long chromosomal DNAs, and keep them from becoming hopelessly entangled with one another inside the nucleus of roughly micron diameter. This work is currently aimed at understanding the mechanisms which fold chromosomes into their active interphase and condensed metaphase forms, and in understanding how modulation of chromosome folding is coupled to disentanglement of chromosomes during cell division.

Eukaryote chromosomes are folded in a heirarchical fashion, with the DNA first folded around histone proteins to form a series of `nucleosome' structures which then folds into the `chromatin fiber'. This DNA-protein fiber is then folded by other proteins which are in the process of being studied in a number of laboratories [27]. My past work on statistical mechanics of the nucleosome fiber [17] has been useful to experimental groups [34,35] and is a starting point for polymer-physics descriptions of chromosomes [36].

At present we are at work on description of the `SMC' proteins which fold the chromosome into its compact mitotic chromatid form. These proteins appear to stabilize a coiled form of the chromatin fiber [37,38] and have been shown to be essential to maintain the structure of mitotic chromosomes [39]. My theoretical interests are in description of the SMC-mediated condensation of chromatin fiber, and in understanding how this condensation is connected to disentanglement of the duplicate chromosomes from one another. Preliminary results show that either the tethering of a polymer to itself, or the gradual local compaction and associated stiffening of a polymer, can drive out entanglements at progressively larger length scales.

4. Funding Plan

At present my research program is funded by two NSF grants. Theoretical research is funded through NSF-DMR Materials Theory; support via a CAREER award from this program (1997-2002) has been renewed as a regular grant (2002-6) Experimental research on chromatin structure is currently being funded by a grant from NSF-BIO Eukaryote Genetics (2003-2006).

I am actively pursuing further funding. A joint R21 proposal with Prof. David Roth (NYU Medical School) to the NIH received a priority score of 136 in October 2003. This score puts our proposal in the top 10%, making it extremely likely to receive funding; a funding decision will come early in 2004. Finally, my other single-DNA preliminary results will lead to a submission to NSF-PHY in spring 2004.

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