Determining the Influence of Histone Tails on Compaction and Transcription Factor Binding

THESIS

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By

Jaime Bravo

Undergraduate Program in Biomedical Engineering

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Thesis Committee:

Gunjan Agarwal, Advisor

Michael Poirier

Abstract

Histone octamer tails play an important role in regulating transcription factor binding and nucleosome compaction. In order to determine the mechanisms by which the histone octamer tails play a role in such processes, the tails were labeled with a spin label, and tracked using electron paramagnetic resonance (EPR). Changes in tail mobility were quantified using EPR and to indicate histone octamer interaction with the DNA and/or with the histone octamer protein itself. EPR spectra of the H3 histone tails were compared before and during compaction. The results of the compaction study were inconclusive, leading to a change in the type of spin label.

This document is dedicated to my mom and dad, who have supported me in all of my endeavors

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Vita

Winter/Spring 2012	Center for Emergent Materials Research Experience for Undergraduates, The Ohio State University
Summer 2012	Materials Physics Research Experience for Undergraduates, University of Florida
Fall 2012 - Present	Capstone in Biomedical Engineering, The Ohio State University
Fall 2012 - Present	Undergraduate Research Thesis in Biomedical Engineering, The Ohio State University

PRESENTATIONS

Denman Undergraduate Research Forum, The Ohio State University, 2012 and 2013

American Physical Societies March Meeting, Baltimore, MD, 2013

FIELDS OF STUDY

Major Field: Biomedical Engineering

Minor Field: Life Sciences

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CHAPTER 1

Introduction

Within a eukaryotic cell, DNA is packaged into a highly conserved structural polymer called chromatin. A nucleosome, the fundamental unit of chromatin [1, 2, 5, 11, 12], consists of 147 base pairs (bp) of DNA wrapped around a histone octamer protein [1, 2, 11, 12]. A histone octamer, Figure. 1.1, consists of an H3-H4 tetramer and two H2A-H2B dimers [2, 12]. The N-terminal tails of histones are long unstructured regions of the histone protein [2]. We aimed to measure the correlation times for the H2A, H2B, and H3 tails using electron paramagnetic resonance (EPR). Measuring the change in correlation times will help to determine how the histone tails are involved in specific processes, including compaction and transcription factor binding.

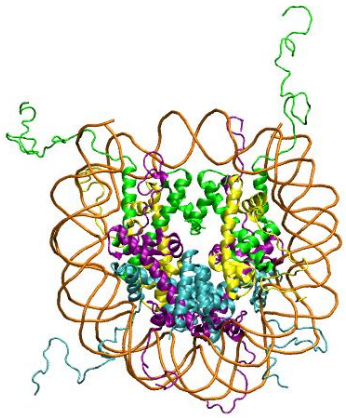

Figure 1.1: Histone octamer structure, modified from [2]

1.1 Electron Paramagnetic Resonance

1.1.1 What is EPR?

EPR is a technique for studying chemical species that have one or more unpaired electrons [6, 16]. Every electron has a spin quantum number s= $\frac{1}{2}$ [16]. In the presence of a magnetic field, B_0, the electron's magnetic moment aligns itself either parallel ($m_s = -\frac{1}{2}$) or antiparallel ($m_s = +\frac{1}{2}$) to the field and the energy difference between the two states, $\triangle$E, depends on the strength of the magnetic field[16] and can be written as

$$\triangle E = gB_eB_0 \tag{1.1.1}$$

B_e is the Bohr magneton and g is the electron spin g-factor. The Bohr magneton is the intrinsic unit of an electron magnetic moment, $B_e = 9.274x10^{-24}JT^{-1}$, and the g-factor of a nitroxide is very close to that of a free electron, $g_e \sim 2.0023$[16].

EPR detects the absorption of microwave photons by the sample at a specific resonant frequency, v. EPR spectra can be generated by either varying the photon frequency incident on a sample while holding the magnetic field constant or doing the reverse; we chose to vary the magnetic field and keep the frequency constant. By increasing an external magnetic field, the gap between the $m_s = -\frac{1}{2}$ and $m_s = +\frac{1}{2}$ energy states is widened until it matches the energy of the microwaves. When the difference between the two energy levels, $\triangle$E, exactly matches the energy of the mirowave radiation ($\triangle E = hv$), transitions between the two states occur, which corresponds to an EPR peak.

1.1.2 Why use EPR?

Nuclear magnetic resonance (NMR) is another method that could be used to measure histone tail dynamics, and has been used for previous studies [19]. NMR is

a technique for studying species containing nuclei with non-zero spins, for example ^{13}C. EPR was a better choice for this experiment for several reasons. NMR requires a large amount of sample, usually in the μM to mM range [4], which makes it less sensitive than EPR. NMR also does not provide information to calculate correlations times, which we need to quantitatively compare any changes in dynamics. However, NMR and EPR are complimentary methods and we chose to use EPR, NMR finding can help to strengthen any findings.

1.1.3 What movements can EPR detect?

There are several types of motion that result in increased line width on an EPR spectra [16], Figure. 1.2. Molecular tumbling, or reorientation of the nucleosome, is the slowest of the three possible motions and therefore contributes very little to the EPR spectrum. The remaining two motions are the movement label around the linker and the actual movement of the nucleosome tail with respect to the nucleosomes. EPR spectra are highly sensitive to the motion of the paramagnetic center (i.e. spin label) and the line width of the EPR spectra will increase as the mobility of the label decreases [6], Figure. 1.2. A spin label is a small molecule ($\sim$100 Da) that contains an unpaired election [16] and can be attached to a protein or DNA. The broadening effect of EPR spectra can be seen in Figure 1.3; the broadening of the peaks shows that the movement has been constricted.

1.2 Aims

1.2.1 Compaction

Nucleosome arrays will compact into higher order structures; however, the details of the higher order structures achieved during compaction are not yet well known. It is believed that the nucleosomes stack and that the histone tails, H3 and H4 in

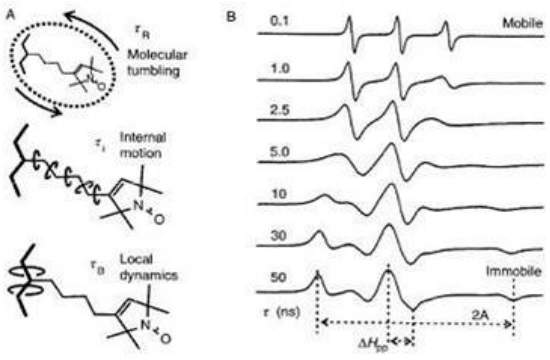

Figure 1.2: Types of EPR motion: (A) The three types of movement that can vary the line width of EPR spectra (B) How the EPR spectrum changes as mobility decreases. Figures A and B taken from [16]

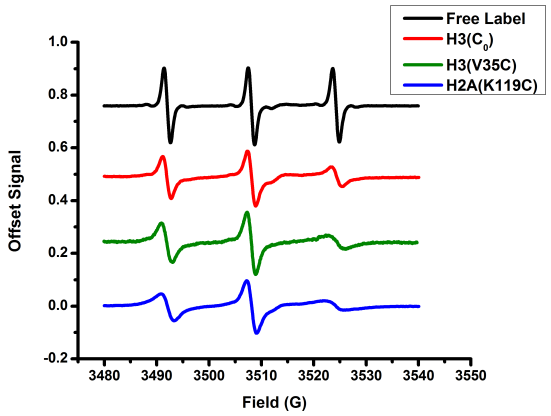

Figure 1.3: An example of EPR spectra showing how the broadens as a result of constrained movement. Free label shows the sharpest peaks, while H3(V35C), which has the label between two gyres of DNA, shows large broadening

particular [5, 18], make contacts with neighboring nucleosomes to stabilize the secondary structure. We hypothesized that the tails become less mobile in a compacted state. Our aim was to use EPR to measure the correlation time of the H2A, H2B, and H3 histone tails in normal mononucleosomes as well as compacted nucleosome arrays. By comparing the correlation times of each tail we could determine if the tails are involved or not.

1.2.2 Transcription factor binding

The mobility of the H3 tail was the focus of this experiment because the H3 tail is believed to play a role in stabilization of the DNA wrapped around the histone octamer [1]. Transcription factors are often sterically occluded from the target site because the site is wrapped into the nucleosome. However, the transcription factor can bind when the DNA transiently unwraps from the histone octamer [7, 14]. We believed that this unwrapping destabilizes the nucleosome; therefore, causing a visible change in H3 mobility during transcription factor binding. We used the transcription factor, LexA, which has been used in a number of previous studies of transcription factor binding within nucleosomes [7, 8, 13, 15].

CHAPTER 2

Methods

2.1 Preparation of nucleosomes

Histone wild type proteins and cysteine mutants will be expressed in E. Coli and purified to $> 95\%$ purity [10]. Histone mutants were made by site directed mutagenesis of the H3 gene to substitute in cysteines at specific locations on the H3 tails for labeling. Histone proteins were refolded into octamer, purified, and labeled in one of two ways. For singly attached labels, octamer was labeled with an MTSL nitroxide spin label. An MTSL spin label was chosen because it is well understood and is commonly used for investigation of intrinsically unstructured proteins. The MTSL spin label also produces a simple EPR spectrum, like those seen in Figure 1.3. For doubly attached labels, octamer was labeled with a conformationally constrained nitroxide spin label. The extra attachment served to improve EPR results by constraining the nitroxide label to the backbone fluctuations. Octamer labeling had about a 60% labeling efficiency; however, extremely high labeling efficiency was not necessary because the unlabeled octamer did not affect the EPR spectra.

In order to compare tail mobilities, both mononucleosomes and nucleosome arrays (17-mer [14]) were prepared. Mononucleosome DNA was prepared by Polymerase Chain Reaction (PCR) amplification from a plasmid containing a high affinity nucleosome positioning sequence and then purified by ion exchange chromography (MonoQ column). Array DNA was prepared by a restriction enzyme digest of plasmids containing a 17-mer sequence. The enzyme DdeI cuts in eight different places on the

plasmid, producing the array DNA (3000 bp) as well as several small fragments (between 150-700 bp). The small pieces function as buffering DNA during reconstitution and prevent binding of additional histones to the nucleosome array. By buffering the reconstitution with the small pieces we were able to achieve fully saturated arrays without aggregation.

Reconstitutions were performed by mixing a ratio of 0.8 histone proteins to DNA in 2 M NaCl, dialyizing down to 500 mM overnight, transferring to 0 M NaCl, and dialyzing again for two changes of buffer lasting 12 hours each. The mononucleosomes required no further purification after reconstitution as excess DNA does not affect EPR spectra. Nucleosome arrays were purified by sucrose gradient to remove buffering DNA and excess octamer.

Once purified, the arrays were checked for saturation. The 17-mer array is structured in a way that the middle positioning sequence has a slightly different sequence than the outer 16 positioning sequences [14]. A MluI restriction enzyme digests in the outer 16, but is blocked when there are nucleosomes present. A BamHI digest targets the center positioning sequence, but is also blocked if a nucleosome is present. The center sequence has a lower affinity to the histone octamer than the outer 16, so if there is a nucleosome there, then there are likely nucleosomes on all sites. Array saturation was checked by running both digests and checking digestion by poly acrylamide gel electrophoresis. Both mononucleosome and array spectra will be measured using a Bruker EMX X-band spectrometer (9.8 GHz).

2.2 Labeling

Purified histone octamer was concentrated until it reached 1.5-3 mg/ml. The concentrated histone octamer and about 500 μL of 1M Tris-HCL, at pH 8,were then

purged under argon for 15-20 minutes at 4℃ to remove O_2. After purging, de-oxygenated Tris-HCl pH 8 was added to the histone octamer sample to 50 mM final concentration. Immediately following the addition of Tris-HCL, 0.01 M solution of label in acetonitrile was added to 20 molar excess in 2 μL additions. In between additions, the sample was mixed via vortex-ing on a low setting. The tube containing the sample was then covered in aluminum foil and allowed to incubate for one hour, at room temperature, on a rotisserie and then overnight at 4℃. Excess label was removed by dialysis into 5 mM Pipes, 2M NaCl buffer at pH 6.1.

Once the labeling reaction was completed, we needed to verify that the label correctly made both connections to the protein. Verification was a two step procedure involving Ellman's reagent and mass spectrometry. Ellman's reagent (5,5'-dithio-*bis*-(2-nitrobenzoic acid)), also known as DTNB, is a versatile water-soluble compound for quantitating free sulfhydryl groups in solution. When a solution of this reagent reacts with sulfhydryls, it produces a measurable yellow-colored product. However, a lack of free sulfhydryl groups does not necessarily mean that our labeling was a success; moreover, if each H3 tail had two labels, instead of one, the Ellman's test would look identical to that of an H3 tail with one label bound twice.

In order to be certain which of the labeling outcomes occurs, we used mass spectrometry on a sample of the labeled protein. Mass spectrometry can distinguish protein with one label from protein with two labels, as each would have their own peak. By comparing the Ellman's reagent test data with the mass spectrometry peaks, we were able to determine if our sample was correctly labeled.

2.3 EPR analysis

The EPR spectrum output was actually the first derivative of the absorption spectrum. The first derivative is obtained through the use of "lock-in" amplification.

The magnetic field was modulated at a frequency of 100 KHz at an amplitude of 1 Gauss. Any signals modulating at the same frequency were amplified, thus reducing the noise. By modulating the field, we are actually measuring the slope of the absorption (i.e. the first derivative) instead of the actual absorption.

The nitrogen-14 nucleus, of the spin label, causes a three line EPR spectra via hyperfine interactions [6, 16]. The three peaks have a corresponding magnetic moment of: m=-1, m=0, and m=1, moving from left to right [6, 16]. For isotropic motion, the line width, $\Delta H(m_I)$ [6], is related to the rotational correlation time, τ_R [17], such that

$$\Delta H(m_I) \propto [\alpha + \beta m_I + \gamma m_I^2]\tau_R + X, \;\; \beta > 0 \tag{2.3.1}$$

$$\tau_R = \frac{4\pi r^3 \kappa \eta}{3 k_B T} \tag{2.3.2}$$

The constants α, β, γ are characteristic of the given spin label and are related to the anisotropy of the g and A tensors, while X represents the contributions to the line width that are not motion dependent [6]. The g tensor represents the Zeeman splitting [6, 16] and the A tensor represents the hyperfine splitting [6, 16]. For Eq.2.3.2, η is the viscosity of the solution, r is the radius of the particle (assumed to be spherical), k_B is Boltzman's constant, T is temperature, and κ is an additional correction factor to account for deviations from sphericity.

When looking for a change in line width, the right peak is the most sensitive because the negative spin state produces a more pronounced change in the line width, Eq. 2.3.1.

CHAPTER 3

Results and discussions

3.1 Nucleosomes with MTSL spin label

EPR was used to measure the mobility of histone tails, modified with an MTSL nitroxide spin label. The label was located in one of five different locations, seen in Figure 3.1; however, there was never more than one location used per nucleosome. By inspection of the mononucleosome data, a clear trend in decreasing linewidth was found when looking at N-terminal tails compared to H2A(K119C), Figure 3.2. Surprisingly, the different N-terminal tails do not show much difference in line-width. The H2A and H2B N-terminal tails are significantly shorter than the H3 N-terminal tails, which is why we expected a larger difference, though all three are very disordered compared to the H2A C-terminal tail.

When trying to determine if the histone tails played a role in compaction, the results were unclear. The EPR spectra of non-compacted and compacted arrays (for H3(C_0)), shown in Figure 3.3, were not distinguishable, which went against our hypothesis. Nucleosomes begin to compact in around 1 mM of magnesium and by 5 mM of magnesium the nucleosomes should be fully compacted. We expected to see a change in tail mobility when moving from a normal state into a compacted state. The negligible change in tail dynamics led us to believe that the spinning of the label about the bonds to the tail may be contributing more motional-narrowing to the spectra than originally believed. Since the correlation time of the nucleosome tail was of primary concern, we decided to switch to a conformationally constrained

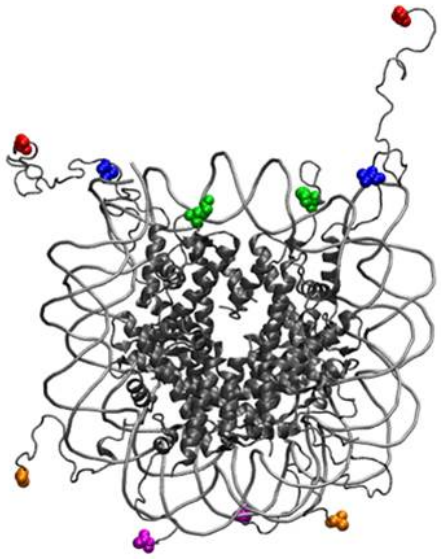

Figure 3.1: Nucleosome showing MTSL spin label locations: H3(C_0) in red, H3(V35C) in blue, H2A(C_0) in yellow, H2A(K119C) in green, and H2B(C_0) in purple)

label [3] so that the movement of the label with respect to the histone tail would not dominate the line width of the EPR spectrum. We hypothesized that the label was too flexible and the EPR spectrum was being influenced by the rotation of the label.

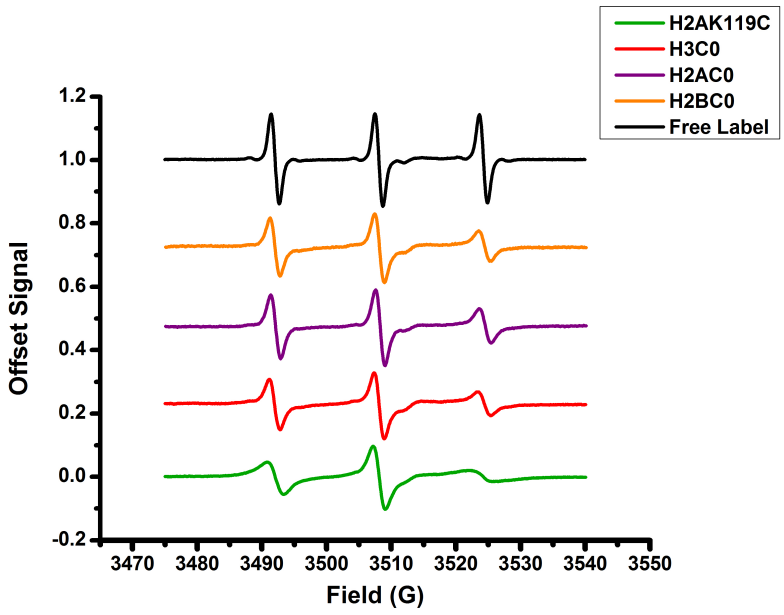

Figure 3.2: EPR spectra of mononucleosomes showing the relative mobilities of each octamer tail.

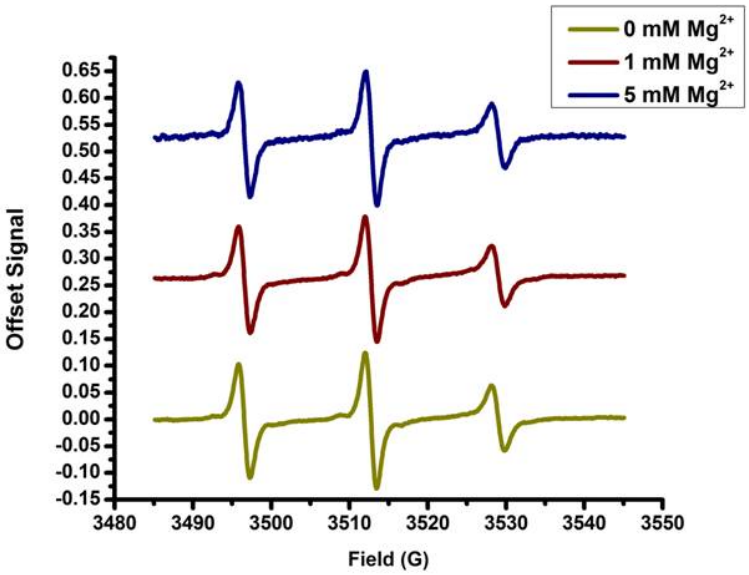

Figure 3.3: Results of previous compaction experiment, which show that there was a negligible change in histone tail mobility during compaction. This went against what we expected.

3.2 Nucleosomes with constrained nitroxide spin label

The position of the label first needed to be changed in order to incorporate the new label. Keeping close to the N-terminus of the H3 tail, we decided to use H3(Q5C,A7C,C110A); the Q5C and A7C side chains are close together and also face the same direction, Figure 3.4, which should make labeling easier. Since the H3(Q5C,A7C,C110A) protein had two cysteins, there was a potential for two separate labels to bind, one to each cystein, instead of one label binding twice. As stated in section 2.2, the Ellman's reagent test could only determine if there where still free thiol groups present. To confirm that the labeling reaction was successful, we used mass spectrometry. Figure 3.5 compares the spectrum of unlabeled H3(Q5C,A7C,C110A), MTSL labeled H3(Q5C,A7C,C110A), and H3(Q5C,A7C,C110A) labeled with the bifunctional label. MTSL labels can only bind once per cystein so the spectrum shows the mass of H3(Q5C,A7C,C110A) with two MTSL labels. The two types of labels have similar masses; therefore, if two bifunctional labels where to bind to H3(Q5C,A7C,C110A) the mass would be similar to that of H3(Q5C,A7C,C110A) with two MTSL labels.

Since the bifunctional label peaks do not reach the peak of the MTSL label, we were confident that the bifunctional label was making two connections. However, when attempting to form nucleosomes the labeled and unlabeled H3(Q5C,A7C,C110) alike were suffering from low yields and excess bands. Figure 3.6 shows a gel of unlabeled H3(Q5C,A7C,C110) with and without TCEP. While the TCEP does help to remove the higher bands, which are most likely connected protein, the nucleosome bands do not look good. There is a large hexosome band, meaning that dimer is falling off somewhere during nucleosome formation.

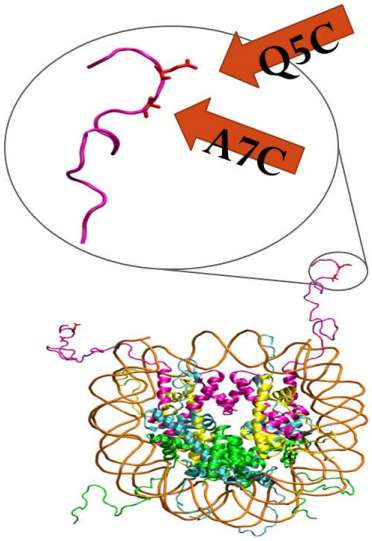

Figure 3.4: Nucleosome highlight showing bifunctional label location. It is important to note the direction in which the side chains of the fifth and seventh amino acid are facing. The direction of the side chains and the proximity of the amino acids were the two main factors when deciding on a new label location

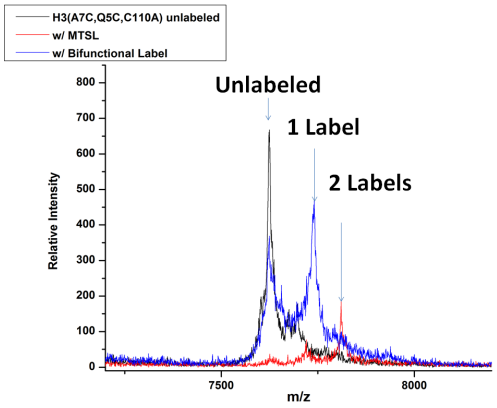

Figure 3.5: Compares the spectrum of unlabeled, MTSL labeled, and constrained nitroxide labeled H3(Q5C,A7C,C110A). MTSL labels can only bind once per cystein so the spectrum shows the mass of H3(Q5C,A7C,C110A) with two MTSL labels. The two types of labels have similar masses; therefore, if two bifunctional labels where to bind to H3(Q5C,A7C,C110A) the mass would be similar to that of H3(Q5C,A7C,C110A) with two MTSL labels.

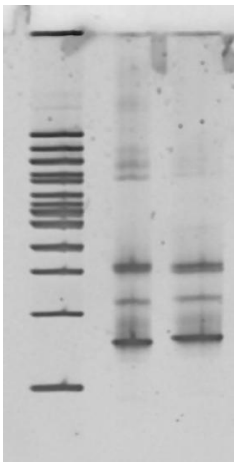

Figure 3.6: Shows a gel of unlabeled H3(Q5C,A7C,C110) with (middle lane) and without (right lane) TCEP. While the TCEP does help to remove the higher bands, which are most likely connected protein, the nucleosome bands do not look good.

3.3 Future work

The formation of good nucleosomes is preventing further progress; however, once we can achieve good formation we can immediately begin taking EPR measurements. Rerunning the nucleosome compaction trials is our first priority following the formation of good nucleosomes. If the results using the bifunctional label match our previous data, then the tails would appear to be highly mobile even in a compacted state; however, if the results conflict, then we were most likely capturing the motion of the label when using the MTSL label.

In addition to the compaction trial, transcription factor binding experiments can also be run as soon as we reach good nucleosome formation. The preparation and purification of LexA was performed as in [9]. For the compaction study, we used a 147 base pair high affinity nucleosome positioning sequence that had been modified to have a single LexA binding site, near one end of the DNA. An additional LexA site was added to the opposite end by standard biochemistry techniques. The addition of a second LexA site ensure that there is a LexA site next to each H3 tail so that a LexA can bind to both sides of the DNA near each of the H3 tails, thus affecting both tails. It is critical that both H3 tails have a LexA site to ensure that the EPR spectra are not skewed due to differences in individual tail mobility. The DNA to be used in the LexA experiment has also already been constructed and once good nucleosome formation is achieved we can also move forward in the transcription factor binding experiment.

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