

**STUDIES OF HISTONE CHAPERONE ASF1 INTERACTIONS
WITH HISTONES H3 AND H4**

A Major Qualifying Project Report

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ABSTRACT

The purpose of this project was to develop an assay to explore the interactions between histone chaperone Anti-Silencing Function 1 (Asf1) and tetramers of histones (H3-H4)₂. A successful assay was developed to work with fluorescein-labeled proteins (Asf1 or histones) and explorations into interactions involving Asf1 protein were begun. Preliminary studies proved the assay was functional with Asf1 to Asf1 interplay assays and could be further used in investigations of histone chaperones and their importance within the life cycle and activity of cells.

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BACKGROUND

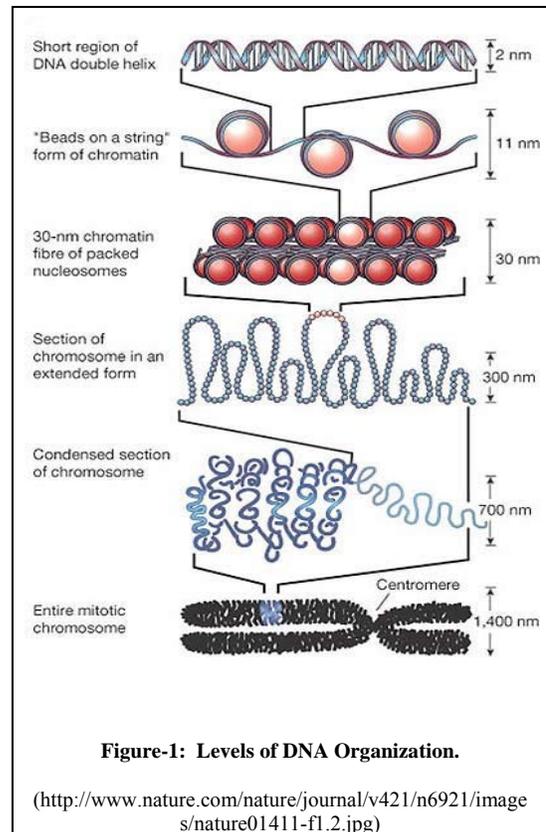
Genetic Organization

The Necessity for Structure

The amount of DNA within a cell is often underestimated; eukaryotic cells contain from 10 million to 100 billion DNA base pairs in a nucleus that is mere microns in diameter (Richmond and Widom, pp.1). When cells undergo mitosis, the genetic information held within the nucleus must be replicated. If lengths of DNA were left uncontrolled, the fragile strands would tangle and break during the life cycle of the cell, especially during mitosis. DNA is packaged into nucleoprotein filaments known as chromatin to protect the DNA from numerous stresses. Chromatin structure allows for control and influence of DNA mediated processes such as replication, transcription, and translation via controlled access to the DNA itself (Kaufman and Almouzni, 2006).

Systematic DNA Organization

The largest level of DNA organization is the chromosome; chromosomes are packaged DNA found in the nucleus of the cell. When unwound, chromosome structure is relatively systematic, as shown in Figure-1. In order to be able to fit all of a cell's genetic information into chromosomes, there are multiple levels of structure and organization.



The first level of organization is the nucleosome- a protein complex around which DNA wraps to form what is known as an 10nm fiber, which takes on a ‘beads of a string’ structure (Figure-1, second row). Nucleosomes then pack together into a 30nm chromatin fiber (Figure-1, third row) that loop and condense to ultimately form the entire mitotic chromosome (Figure-1, lowest row).

Histones

The protein components of nucleosomes are made up of histone proteins. Histones are a well-characterized group of proteins with unique compositions, characteristics, and functions (van Holde, pp. 67). There are multiple classes of histone proteins derived from amino acid composition and sequence; these include histones H1, H2A, H2B, H3, and H4. The sequences of these particular proteins are among the most highly conserved in evolution due to their importance within the cell. Histones H2A, H2B, H3, and H4, known as core histones, have molecular masses of 10-15 kDa (Richmond and Widom, pp. 1). Originally thought to only play roles in the structure of chromatin, histone proteins are now known to have post translational modifications on their C and N-terminal tails. These modified residues participate in genome integrity, transcription, and translation (for review see Peterson and Laniel, 2004).

Core Histones

The evolution of four different core histones probably occurred to provide different stereospecificity of interaction with regards to DNA exit and entry points around the outside edge of the nucleosome core octamer (Richmond and Widom, pp. 4). The term ‘core histones’ derives from the fact that these four histones, together with histone H1 and linker DNA, form nucleosomes around which DNA supercoils. Figure-2 shows a nucleosome core particle with

DNA; the eight protein chains of the core histones can be seen working as an octamer around which the DNA is able to wrap. A complete set of both core proteins and cap histone (H1) protein is required for cell viability. The core histone proteins are found in the same fixed equal-molar stoichiometry, while histone H1 is found in half this amount in all eukaryotes (Richmond and Widom, pp. 2), because each of the core proteins

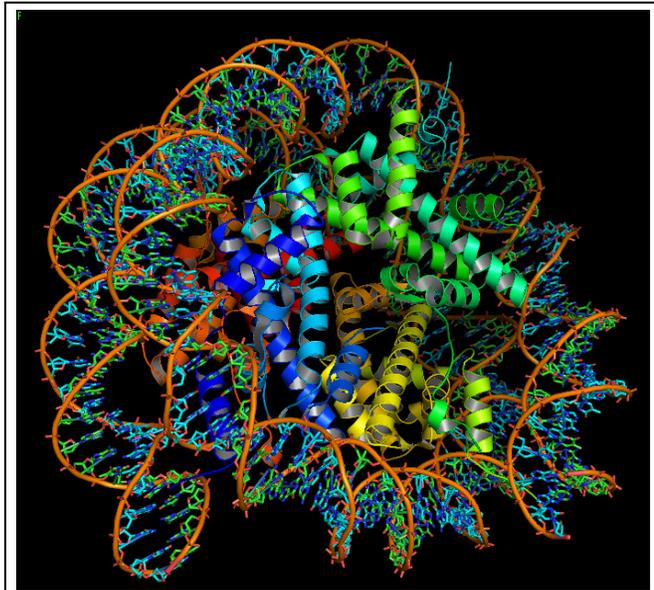


Figure-2: Diagram of a Nucleosome Core Particle. Shown is a 147 bp DNA helix wound around the nucleosome core particle- histone octamer of H2A, H2B, H3, and H4 (Images generated using Pymol with Protein Data Bank entry 1P34)

is needed twice to form the nucleosome core octamer, while the histone H1 cap is needed only once per nucleosome.

Histones H2A and H2B are not as conserved in their sequences as histones H3 and H4 having highly conserved interior sequences and varying lysine-arginine-rich terminal domains (van Holde, pp. 84). This suggests that the N and C terminal domains of histones H2A and H2B do not have as much of a defined and necessary structural role for the protein as the interior region. For structural and functional purposes, histones H2A and H2B exist as heterodimeric pairs (Richmond and Widom, 2000, pp. 3).

Histones H3 and H4 also exist as a heterodimeric pair (Richmond and Widom, 2000, pp. 3). They are also found as tetramers- protein complexes containing two copies each of histones H3 and H4. They play a critical role in determining nucleosome structure, which is suggested by their highly conserved sequences; the sequencing of these proteins in many different organisms

has found that they contain exactly the same number of residues- 102 for histone H4, and 135 for histone H3 (van Holde and Kensal, 1989, pp. 91). This conservation of sequence is maintained across species, with variations of only a few amino acid substitutions between yeast, frog, chicken, and human histones (van Holde and Kensal, 1989, pp.83). For this reason, studying *Xenopus* (frog) histones has a direct impact on understanding human histones.

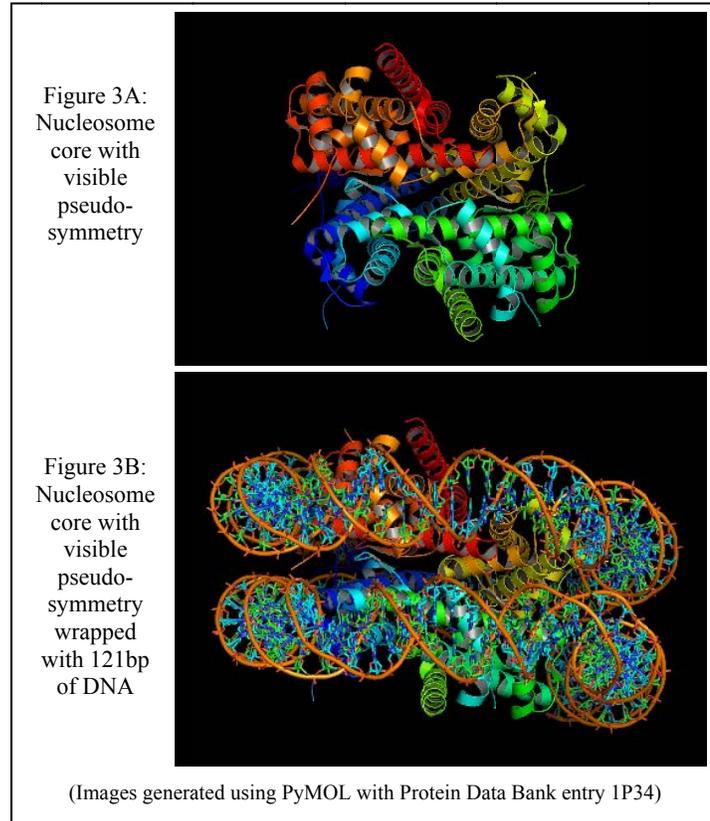
The Nucleosome

Nucleosomes are repeating subunits of chromatin containing an octamer core of histone proteins, linker DNA, and a histone cap. They are formed in step-wise processes mediated by histone chaperones who deposit histones H2A, H2B, H3 and H4 to form the octamer to which the DNA binds.

Structure

The histones of the octamer include two each of histones H2A, H2B, H3, and H4 around which 146 bp of DNA wraps 1.7 times (Luger et al., 1997). The formation of the nucleosome core is such that histones H3 and H4 form a (H3-H4)₂ tetramer wherein two copies of each of the proteins come together through the help of histone chaperones to organize the central approximately 70 bp of DNA. This forms a place for two dimers of H2A/H2B to flank the tetramer. This particular structure is desirable because dimers are more easily removed when they encounter oncoming RNA polymerase II molecules (Kimura and Cook, 2001; Kireeva et al., 2002) or physical tension (Claudet et al., 2005).

Figure-3 shows the nucleosome core particle at an angle such that the symmetry of the octamer is visible. The figure contains two images- the first (Figure-3A) shows solely the protein core while the second (Figure-3B) shows how the same orientation of the core relates to the winding of the DNA helix. There is a slight cleft through the center of the core protein complex that has four histones on either side. Together, the four histone



heterodimeric pairs are arranged in the octamer to yield an overall molecular twofold axis of symmetry, and bind 121 bp of the 146 bp core DNA. However, the central base pair falls on the symmetry axis so that the two-fold symmetry is not quite perfect (Richmond and Widom, 2000, pp. 5).

Function

The nucleosome is essential in genomic organization. As discussed, DNA organizes into chromosomes through compaction of DNA by incorporation into chromatin. Histone complexes, the core particles of nucleosomes, are the first step in compacting DNA over 10,000-fold compared to the straight form, which can stretch almost 2 meters (Richmond and Widom, 2000, pp. 1). The 147 bp DNA within the nucleosome core forms a 1.65-turn left-handed superhelix,

this compression of DNA has important effects on the availability of the transcriptional template, making manipulation of this structure critical to gene expression in eukaryotes (Richmond and Widom, 2000, pp. 5). The rearrangement or removal and subsequent replacement of histones is therefore necessary for processes that require access to the DNA sequence (Linger and Tyler, 2005). In fact, histone deposition onto newly synthesized DNA is almost immediate in order to maintain structure and ensure DNA integrity (for review, see Franco and Kaufman, 2004; Loyola and Almouzni, 2004; Gunjan et al., 2005).

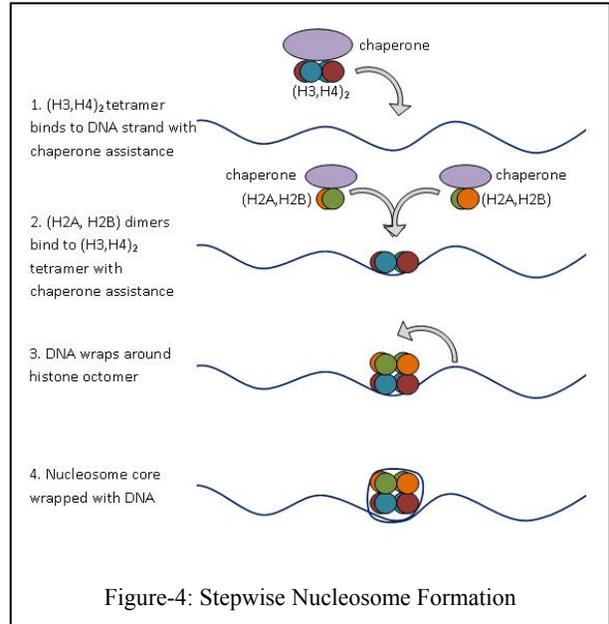
Formation

Nucleosomes are formed quickly on newly synthesized DNA following the passage of polymerase (Kaufman et al, 1997). Electrostatics governs nucleosome formation. Histone heterodimers (H2A-H2B and H3-H4) will combine to form an octamer in the presence of DNA or high salt concentrations, while the absence of DNA or the presence of lower salt conditions will result in histones that exist as tetramers of two histone H3-H4 dimers and dimers of histones H2A and H2B (Richmond and Widom, 2000, pp. 5). The combination of DNA and purified histones, at physiological salt concentrations, results in aggregate formation due to the attraction of the negatively charged DNA to the positively charged histones (for review, see Gunjan et al., 2005). *In vivo*, histone chaperones act to guide nucleosome formation in order to prevent aggregation.

Histones are bound to DNA by noncovalent forces including the important ionic interactions between DNA phosphate and positively charged histone residues (van Holde and Kensal, 1989, pp. 72). Histone (H3-H4)₂ tetramers associate with DNA more avidly than H2A-H2B dimers, the histone (H3- H4)₂ tetramers are the first to associate with the DNA, followed by

the H2A-H2B dimers (Figure-4) (Worcel et al., 1978; Smith and Stillman, 1991; Jackson, 1990).

Although nucleosome assembly proteins control the binding of histones and deposition of the histones onto DNA *in vivo*, nucleosome formation is a two-step pathway which can be mimicked both *in vivo* and *in vitro* (for review, see Franco and Kaufman, 2004; Loyola and Almouzni, 2004; Gunjan et al., 2005). The interaction sites between the histone pairs necessary to form the octamer come from the



$\alpha 3$ helices and C-terminal third of the $\alpha 2$ helices from each histone (Richmond and Widom, 2000, pp. 5). The octamer then contains two of each of the four core histone proteins.

Chaperones

Nucleosome formation must be controlled. Correct histone protein core deposition onto DNA strands impacts compaction of DNA, as well as, ultimately, future control of strand access for transcription, translation, and replication. Nucleosome formation is a highly orchestrated process within eukaryotic cells. As such, many chaperones are used to ensure the proper binding of histone (H3-H4)₂ tetramers and the addition of histone H2A/H2B dimers. Histone chaperones mediate the formation of nucleosome core particles. Two main chaperones of interest include Anti-silencing Function 1 (Asf1) and Chromatin Assembly Factor 1 (CAF-1). CAF-1 and Asf1 work side-by-side during the assembly of newly replicated DNA into chromatin *in vitro* (Tyler et al., 1999; Linger and Tyler, 2005). Deletion of Asf1 or CAF-1 components can lead to global

transcriptional misregulation and an increased DNA damage sensitivity because the lack of histone deposition onto DNA strands ultimately results in improper packaging of the genetic material (Kaufman et al., 1997).

Chromatin Assembly Factor 1

Activated by proteins and/or nucleic acid structures left behind by DNA polymerases, Chromatin Assembly Factor 1 (CAF-1) plays a role in nonhomologous end-joining, homologous recombination and also has an essential role in chromatin assembly (Kaufman, 1996; Linger and Tyler, 2005). CAF-1 is responsible for the placement of histones H3 and H4 onto newly replicated DNA *in vitro* in a replication-dependent manner (Smith and Stillman, 1989; Verrault et al., 1996). A CAF-1 loss can mean multiple types of double stranded DNA (dsDNA) damage (Linger and Tyler, 2005). In yeast, the genes that encode CAF-1 subunits are called Chromatin Assembly Complex (CAC) genes. In yeast, these genes are not essential for cell viability, but deletions result in increased sensitivity to ultraviolet radiation and reduced silencing of genes adjacent to telomeric DNA (Kaufman et al., 1997).

CAF-1 is a three subunit heterotrimeric protein (in humans- p48, p60, p150), and has been highly conserved through evolution (Kaufman et al., 1995; 1997; Tyler et al., 1996; Verreault et al., 1996; Tyler et al., 2001, Linger and Tyler, 2005). The two larger subunits (p60, p150) have been shown to be essential for chromatin assembly *in vitro* (Kaufman et al., 1995). The small subunit (p48) is a member of a subfamily of WD-repeat proteins, and can bind to histone H4 in the absence of the other two CAF-1 subunits- p150 and p60 (Verrault et al., 1996). The p48 subunit can also copurify with all three CAF-1 subunits (p48, p60, p150) and histones H3 and H4 that combine to form a Chromatin Assembly Complex (CAC) (Verrault et al., 1996).

CAC promotes chromatin assembly and is an important intermediate in nucleosome assembly (Verrault et al., 1996). The CAF-1 chaperone has not been crystallized due to the difficulty of expressing the protein in *Escherichia coli*, causing studies to be conducted on CAF-1 after its expression in baculovirus infected insect cells (personal communication- Corey Smith).

Anti-Silencing Function 1

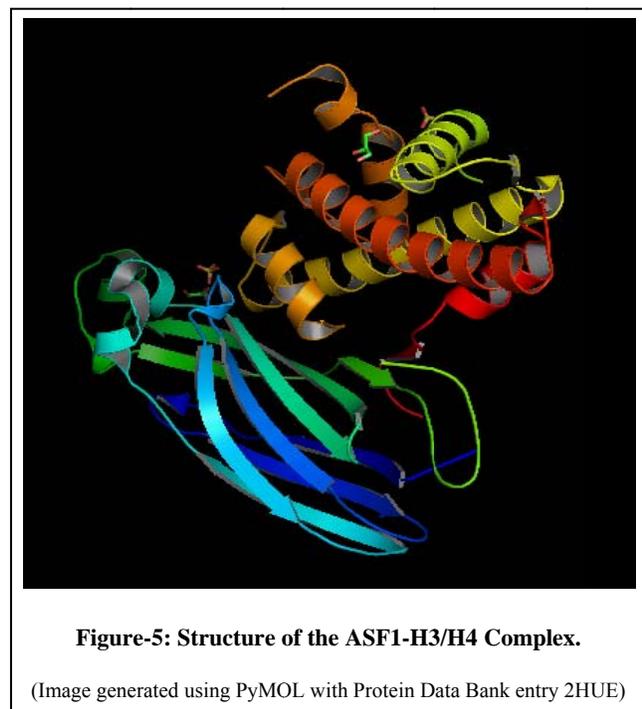
A major fraction of non-DNA bound histones H3 and H4 are bound to Anti-Silencing Function 1 (Asf1), which emphasizes its essential role as a central histone chaperone in eukaryotes (English et al., 2006). Asf1 is a histone chaperone that is a part of both replication-independent and replication-dependent chromatin assembly (Nakatani et al., 2004). Asf1 assembles and disassembles chromatin during repair, replication, and transcription (English et al., 2006). Asf1 is a highly conserved chaperone that interacts with two functional classes of protein- chromatin components such as histone H3, and checkpoint kinases (Mousson et al., 2005). The chaperone has been connected to transcriptional silencing, cellular reaction for repair of DNA damage, and nucleosome formation. It also assists CAF-1 during the assembly of newly synthesized DNA into chromatin *in vitro* (Mello et al., 2002; Smith and Stillman, 1991; Tyler et al., 1999; English et al., 2006). In the absence of Asf1, cells display increased frequencies of DNA mutations, highlighting the protein's importance within the cell. Such mutations include genome rearrangements and non viable cells in the presence of double-strand DNA breaks (Myung et al, 2003; Qin and Parthun, 2002).

The N-terminal domain of Asf1 is the core region of the protein, consisting of three helical linkers on top of a compact immunoglobulin-like β -sandwich fold, and this domain mediates all currently known functions of the full length protein (Daganzo et al., 2003). One of

the important features of Asf1 is a large electronegative surface potential surrounding one side a highly-conserved hydrophobic groove which interacts with histone proteins (Daganzo et al., 2003; Mousson et al., 2005).

The structure of the Asf1-H3-H4 complex (Figure-5) is suspected to create a ‘strand-capture’ mechanism allowing the tail of histone H4 to facilitate chromatin assembly and disassembly (English et al, 2006). The C terminus of histone H4, which is required to the form a mini- β sheet with histone H2A in the nucleosome, undergoes a conformational change upon

binding to Asf1 (English et al, 2006). It is known that Asf1 function both *in vivo* and *in vitro* requires interaction with both histones H3 and H4, however. The relationship between histone H3 and Asf1 is such that the C-terminal helix of the H3 histone binds to Asf1 via the charged residues flanking the hydrophobic groove (Daganzo et al., 2003; Mousson et al., 2005). Asf1 binds the histone (H3, H4) heterodimer by enveloping



the C terminus of histone H3; this action physically blocks the formation of a (H3-H4)₂ heterotetramer (English et al., 2005; English et al, 2006). The association made between histones H3 and H4 and the Asf1 chaperone can be seen in Figure 5. The hydrophobic groove of the chaperone can be seen to cradle the C-terminal helix of the histone H3 protein.

CAF-1 and Asf1 Cooperativity

At present, it is known that both CAF-1 and Asf1 are present during replication to assist in nucleosome core formation and deposition onto DNA. In the presence of CAF-1 during replication, deposition can occur, but with the addition of Asf1, the rate of reaction is greatly increased. This relationship between the presence of both chaperones and an increased rate of nucleosome formation suggests that Asf1 feeds CAF-1 in order to form the nucleosome protein core. However, the mechanism of interplay between the two chaperones during formation and deposition of the core octamer is not yet understood; how these proteins interact with one another is currently a focus of chromatin studies.

PROJECT PURPOSE

The purpose of this project was to develop and apply a method to study interactions between histone (H3-H4) complexes and histone chaperone Asf1. Interactions between histones, DNA, and histone chaperones are only understood at a basic level. The development of this assay would allow an investigation of the affinity of chaperones such as Asf1 for histones or DNA, or the interplay between Asf1 and other histone chaperones like it. Preliminary to applicative experimentation was the development of assay methodologies to use in this study; creation of this assay and early research was the goal of this endeavor.

METHODS

Protein Expression and Purification

Histones

Plasmids containing DNA's for *Xenopus* histones H3 and H4 were transformed into BL21 pLysS *E. coli* cells which contain resistance to ampicillin (AMP) and chloramphenicol (CAM). The transformations were grown overnight at 37°C on LB + CAM (25µg/mL) + AMP (100µg/mL) plates. Large scale growth of the cells was done in 2x YT media (16g Tryptone; 10g Yeast extract; 5g NaCl for 1L- pH7.0) in 1L cultures. Cultures were grown in a shaker at 37°C to an approximate OD₆₀₀ of 0.5, and then induced with 1mM IPTG for an additional two hours at 37°C. The cells were harvested and pelleted using a JLA 10.500 rotor in Beckman Coulter Avanti J-25 Centrifuge for 7 min at 7000rpm. The cell pellet was allowed to drain upside down for a few minutes before being resuspended in 30mL of wash buffer (50mM Tris-HCl, pH7.5; 100mM NaCl; 1nM Na-EDTA; 1mM Benzamidine; 1mM DTT) per 1L culture, flash frozen in liquid nitrogen and stored at -20°C.

The cell pellet suspension was thawed at 37°C and DTT (1mM final) and benzamidine (1mM final) were added. The suspension was placed on ice and sonicated to lyse the cells (to release the inclusion bodies) until no longer viscous using a Branson Sonifier 450 on a constant duty cycle and power setting of '5', in 30 sec bursts, with 5 min resting periods in between. The lysate was spun in a JA 25.50 rotor using a Beckman Coulter Avanti J-25 Centrifuge at 28,000 x g for 20 min to pellet the inclusion bodies containing the expressed proteins, and the supernatant was discarded. The inclusion body pellet was suspended in 30mL TW buffer (wash buffer + 1% Triton X-100) and spun for 15 min at 23,000 x g. A total of three TW suspension and spins were

followed by two more wash buffer suspension and spins. The resulting inclusion body pellet was deep gray in color. The surface of the inclusion body pellet was covered with 0.25mL DMSO for 15 min. The pellet was then minced using a spatula. The minced pellet was soaked for an additional 15 min. 7mL unfolding buffer (7M Guanidine HCl; 20mM Tris-HCl, pH7.5; 10mM DTT (filtered before use)) and a small stir bar were added and the mixture was stirred for 1 hour at room temperature to extract proteins from the inclusion bodies.

A Sephacryl S200 HR gel filtration column was equilibrated with SAU-1000 buffer (7M Urea; 20mM sodium acetate, pH5.2; 1M NaCl; 1mM DTT; 1mM EDTA (filtered before use)) at a rate of 0.3mL/min. Using SAU-1000 buffer, a maximum of 10mL the inclusion body protein was run over the column per run. 4mL fractions were collected. Two peaks appeared on the UV absorption spectra recorded by the AKTA; the second of these peaks was supposed to be the protein while the first was nucleic acid and high molecular weight proteins as determined by the Luger protocol (Luger et al., 1999, pp. 7). Some of the fractions from the first peak and all of the fractions of the second peak were analyzed on a 17% SDS PAGE gel to make sure the histones were present. Histone H3 has a molecular weight of 15,273 and histone H4 has a molecular weight of 11,236, so the molecular weight marker used was a BIO RAD Molecular Weight Standard High and Low Range marker.

The column fractions containing histones were pooled and dialyzed at 4°C into SAU-200 buffer (7M Urea; 20mM sodium acetate, pH5.2; 0.2M NaCl; 1mM DTT; 1mM EDTA (filtered before use)) using three 2L changes of dialysis buffer, the second of which was overnight. The dialyzed material was run over an SAU-200-equilibrated HiTrap SP column with an elution gradient from SAU-200 to SAU-600 (7M Urea; 20mM sodium acetate, pH5.2; 0.6M NaCl; 1mM DTT; 1mM EDTA (filtered before use)) buffer over four column volumes. Fractions of 1.5mL

were collected and analyzed on a 17% SDS PAGE gel. The protein fractions were dialyzed into 4L cold water + 1mM DTT at 4°C with 3 changes of water. The dialyzed proteins were aliquotted into 1.5mL screw-cap tubes. The uncapped tops of the tubes were covered with Parafilm and holes were poked in the film. The tubes were frozen in a dry ice/EtOH bath, lyophilized, and stored at -20°C.

To form tetramers, the lyophilized proteins were resuspended in unfolding buffer and rotated at 4°C for approximately 1-1.5 hours to facilitate pellet dissolution. The concentration of histones H3 and H4 were determined, the proteins were combined in equimolar ratios, diluted to 1mg/mL with unfolding buffer, and dialyzed at 4°C into refolding buffer (2M NaCl; 10mM Tris-HCl, pH7.5; 1mM EDTA; 1mM Na-EDTA) using 3 changes of 2L baths. The resulting tetramers were centrifuged using a Beckman Coulter Optima L-90K Ultracentrifuge and a Ti50 rotor at 33K rpm for 45 min, and then concentrated in a VIVASPIN 6 column to a volume of 1mL. This 1mL of liquid was then run over a refolding buffer-equilibrated Sephacryl S-300 column. Fractions of 1.5mL were collected. The histone tetramer eluted around 65mL. Fractions were analyzed on 17% SDS PAGE gels. The pooled fractions containing histones were stored in 50% glycerol at -20°C for long term storage, or dialyzed into H-histone buffer (25mM Tris-HCl, pH7.5; 1mM EDTA; 10% glycerol; 25mM NaCl; 0.01% NP40; 1mM DTT) for immediate use.

Assay Development

Fluorescence Asf1-H3-H4 binding assays were completed in 100 μ L reactions in a black 96 well plate. Each reaction included [0.1mg/mL final] BSA in addition to fluorescein-labeled Asf1 (Asf1 N-terminal fragment of amino acids 2-155 with an S33C substitution previously prepared in the Kaufman lab) [5nM final], histone (H3-H4)₂ tetramers, and S200 (25mM Tris-HCl, pH7.5; 1mM EDTA; 100mM NaCl; 0.01% NP40; 1mM DTT) reaction buffer. Asf1 concentration was tested at both [2nM final] and [5nM final] concentrations; 5nM was determined to be the more desirable concentration of the chaperone as it yielded a broader, more defined result than 2nM. The concentrations of tetramers were varied to determine the K_d for A34 complexes. For a given run, the concentrations of tetramers used were between 0.025-40 μ M; various concentrations were achieved by performing serial dilutions using S200 buffer.

The total 100 μ L reactions were placed in the wells of the black 96-well assay plate, covered with aluminum foil, and equilibrated for 1.5-4 hours protected from light. It was determined that the intensity of the fluorescence did not degrade over time, meaning 1.5hours was long enough to reach equilibrium. After equilibration, the reactions were analyzed using a PerkinElmer Precisely 1420 Multilabel Counter Victor³ recording fluorescein intensity for 0.3s intervals and fluorescence polarization (FP) 1.0s intervals. The resulting data was analyzed using Microsoft Excel and GraphPad Prism 5.

Fluorescence Experiments

Histone Exchange between Asf1

Application of the assay developed began by testing the ability of chaperones to exchange histone (H3-H4)₂ tetramers between themselves; first, exchange between two Asf1

chaperones was examined. A34 complexes (Asf1-H3-H4 protein complexes previously prepared in the Kaufman lab) were added to 100 μ L reactions containing [5.0nm final] fluorescein-labeled Asf1 and [0.1mg/mL final] BSA. Concentrations of A34 complex between 0.01-20 μ M were added. The reactions were housed in black 96-well assay plates, and equilibrated for 1.5 hours while protected from light. After equilibration, the reactions were analyzed using a PerkinElmer Precisely 1420 Multilabel Counter Victor³ recording fluorescein intensity for 0.3s intervals and fluorescence polarization 1.0s intervals. The result data was analyzed using Microsoft Excel.

RESULTS

The purpose of this project was to develop a method for studying the binding of histone chaperone Anti-Silencing Function 1 (Asf1) to histone (H3-H4)₂ tetramers, and to apply the assay to study interactions between histone chaperones and histone proteins.

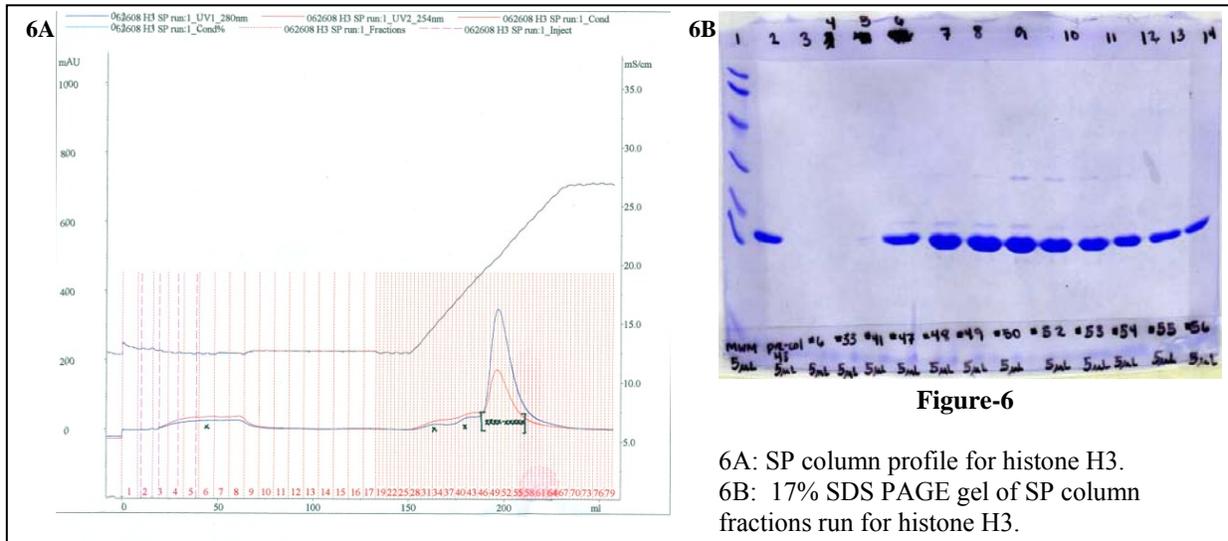
Formation of Histone Tetramers

Histone Expression and Purification

When *Xenopus* histones are expressed in *E. coli*, the proteins aggregate to form inclusion bodies. The histones are recovered from the inclusion bodies by detergent washes. Further purification of the proteins included running the material over S200 and SP columns. Figure-6A shows the profile for the SP run for histone H3; the x's show which fractions were analyzed. In this figure, the gradually sloped line above the protein peak shows the change in conductivity of the eluted sample as the running buffers were switched from SAU-200 to higher osmotic SAU-600. The red peak is the UV spec reading at 254nm, and shows genetic material found in the sample. The blue peak is the UV spec reading at 280nm; it shows the histone protein.

Figure-6B shows the SDS PAGE analysis of the SP column fractions designated in Figure-6A. The first lane is a molecular weight marker, the second lane is a sample of the protein before it was run over the SP column, and the subsequent lanes contain the marked fractions in ascending order. The gel shows clear histone presence in the desired fractions, indicating clean and successful protein purification for histone H3. Only 5μL of material was loaded onto the gel, so the strongly-stained bands indicated at this early point that this preparation of histone H3 was going to have a good yield. Histone H4 (data not shown) yielded

similar results in both column runs and analysis, though not in the same quantities as histone H3; histone H4 generally yielded four to five times lower yields than histone H3.

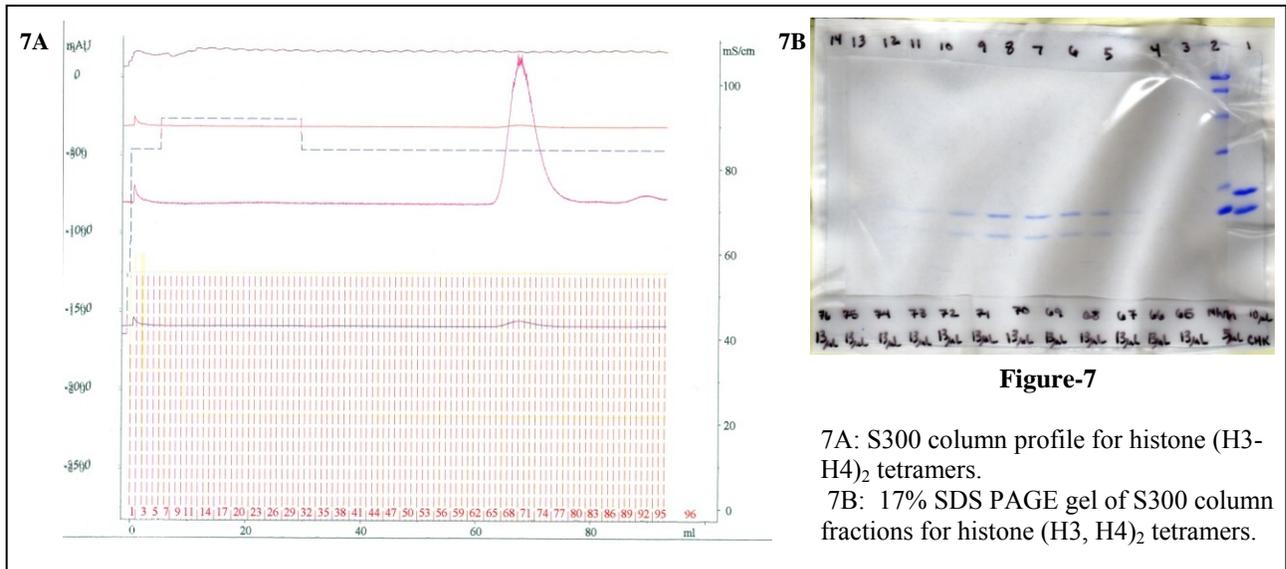


Tetramer Refolding

Figure-7 shows the S300 column profile and a 17% SDS PAGE gel analysis of refolded histone (H3-H4)₂ tetramers. Figure-7A shows the column profile after the tetramers were run over the S300 column in refolding buffer. Fractions of 1.5mL were collected as seen by the dashed vertical red lines. The red peak is a record of the UV spec analysis at 280nm and represents the histone proteins, which eluted at the proper molecular weight- at ~65mL as expected for (H3-H4)₂ tetramers (Luger et al., 1999). Fractions from this run were then analyzed via electrophoresis.

Figure-7B shows the SDS PAGE gel analyzed material taken from fractions after running a concentrated tetramer solution over a S300 column. Having previously analyzed both pure *Xenopus* histone protein pools against chicken histone (H3-H4)₂ tetramers to be certain of their identity, the known tetramer standard was also included on this gel (figure orientation right to left) in lane 1 (right side of 7B). The second lane is the molecular weight marker, and the other

lanes denote the column fractions protein. The histone H3 can be seen running just above the lowest marker band while histone H4 runs just below. This analysis proves that the proteins, which were purified separately, have been successfully unfolded, and refolded as tetramers.



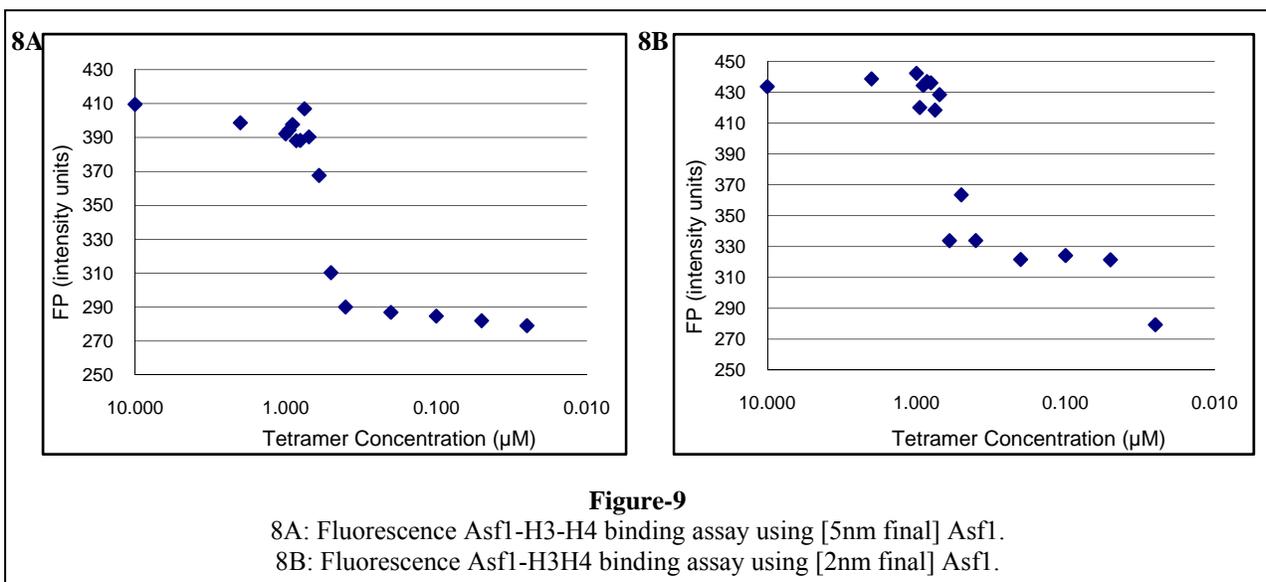
Assay Development

For future studies of interactions between histones, DNA, or other histone chaperones and Asf1, an assay was developed using fluorescent materials. Interactions were analyzed by examining fluorescence polarities of the molecules in question; fluorescence polarization (FP) measurements provide information on orientation of the molecules involved in receptor-ligand interactions. By measuring the changes in FP of fluorescently-labeled Asf1 or histones, association rates for the ligand-receptor interaction were analyzed. Measurable fluorescence is caused by changes in the orientation of the molecule via combination of histones and histone chaperones, which changes the fluorescence polarization of the complex as a whole.

Asf1 Concentration

One of the necessary components of the fluorescence assay development was determining the best concentration of chaperone Asf1 and histones to use in each reaction. First, a broad range of fluorescein-labeled Asf1 concentrations were tested to determine how much labeled protein was needed to get an acceptable fluorescence polarization response. These preliminary experiments indicated that concentrations between 2-5 μM were optimum (data not shown).

Following the initially broad range approach, we further refined the assay. Two different concentrations were tested- [5nm final] and [2nm final] Asf1. It was concluded that [5nm final] Asf1 yielded a better, broader fluorescence curve for determining the K_d of the A34 complex. Figure-8 below shows the results of this comparative study. Figure-8A is the logarithmic curve of the greater concentration of chaperone, while Figure-8B shows the logarithmic curve of the lower concentration of chaperone. From these graphs, it can be seen that, though both yield desirable data, the [5nm final] Asf1 has clearly defined upper and lower limits. However, the consistency of the fluorescence results in both titrations helped to further determine where to focus additional binding studies.

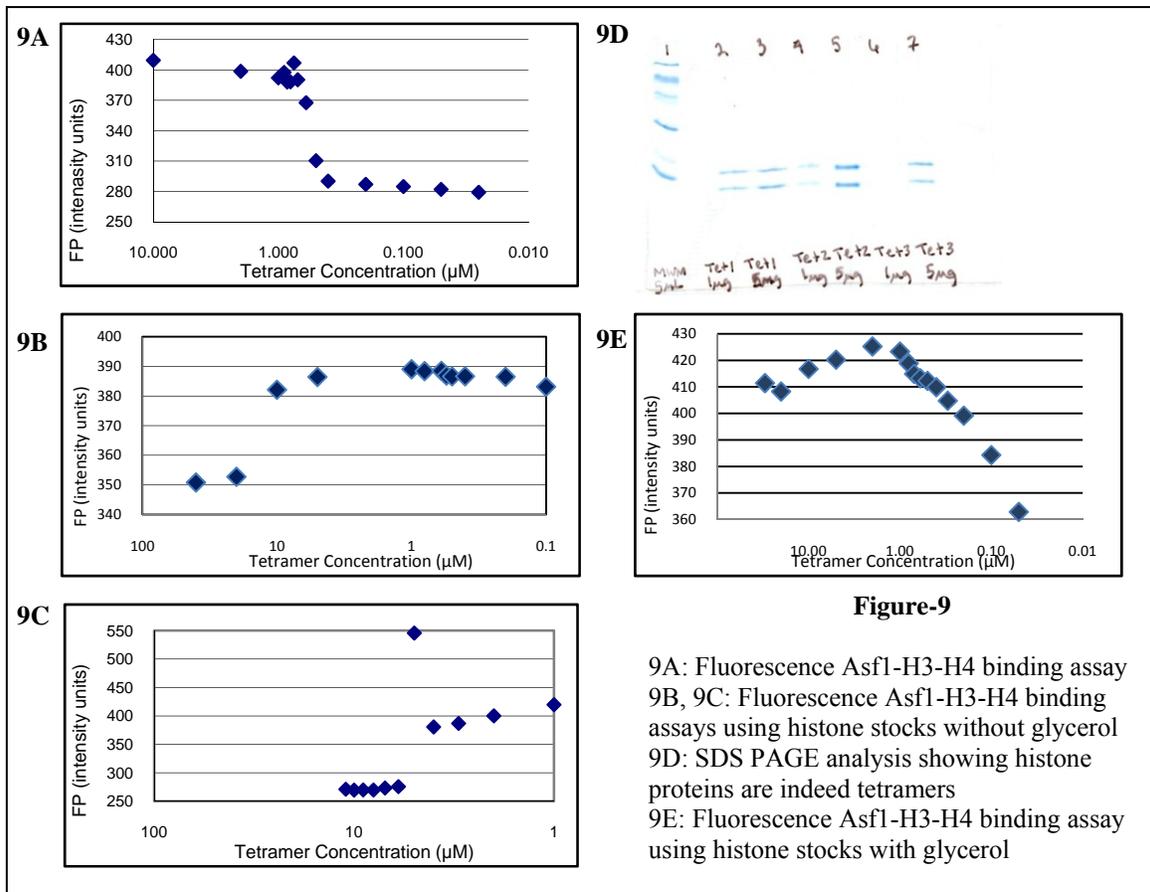


Histone Dialysis

A new dialysis buffer to prepare histone (H3-H4)₂ tetramers for fluorescence binding assays was developed to counteract the aggregation of histone proteins during the rapid freeze-thaw procedures. Figure-9 shows a progression of assay and SDS PAGE gel results used to help find the source of the problem when the assays began to yield unusual results after using a specific batch of refolded and dialyzed tetramers.

Figure-9A is one of the original assay runs showing an expected titration curve for the binding of Asf1 and histone (H3-H4)₂ tetramers. The histones in this run had been dialyzed into 10% glycerol with another batch of proteins. Figures-9B and 9C show the results of the same assay using histone tetramers which had been frozen without glycerol after being dialyzed into S200 buffer. It was originally thought that dialyzing the proteins directly into the assay buffer would better prepare them for experiments, however it is clear that there is something wrong with these particular assays because the titration curves are the reverse of the expectation.

Figure-9D shows the results of an SDS PAGE analysis demonstrating that the tetramers were indeed tetramers. Three different stocks of refolded histone (H3-H4)₂ tetramers were run with 1 μg and 5 μg of protein. Each of these stocks had been dialyzed into S200 buffer. Similar gels were run resulting in confirmation of the identity of the chaperone Asf1. The aggregation of the proteins would have been made null by the heating process prior to gel analysis, meaning it was not found until a thorough search of previous lab notes showed that the initial histone tetramer stocks had been dialyzed into 10% glycerol. Finally, Figure-9E shows the results of an assay after glycerol was added to the dialysis buffer.



Final Assay: Triplicate Data

After determining the proper salt and chaperone Asf1 concentrations, and solving the problem of the aggregating histone proteins, the fluorescence Asf1-H3-H4 binding assay was finally deemed a success via a triplicate run of the assay over a [0.01-20.0 μM] tetramer range. Figure-10 below shows the result of this assay. The equation used to solve for K_d was $Y = B_{\text{max}} * X / (K_d + X) + NS * X + \text{Background}$, where B_{max} is the maximum specific binding in the same units as Y , NS is the slope of nonspecific binding in Y units divided by X units, and the Background is the amount of nonspecific binding with no added ligand. Using the equation given, K_d could be calculated where Y is the intensity of the fluorescence and X is the concentration of histone tetramers; $K_d = 0.1897 \pm 0.042 \mu\text{M}$. Published K_d for peptides of Asf1 (amino acids 1-156) and H3 (amino acids 122-135) binding is $K_d = 100 \pm 20 \mu\text{M}$ (Mousson et al.,

2005). The Asf1 peptide used for this published value is nearly identical to the peptide fragment used to develop this assay, but the shortened H3- which forms the $\alpha 3$ helix necessary for Asf1-H3 binding when bound- would likely contribute to a lower K_d . In addition, the lower published affinity of the shortened H3 was probably caused in part by incomplete folding of the peptides. Using full-length H3 in the experiments leading to Figure-10 would likely provide structural stability necessary for higher Asf1-H3 affinity. The R^2 value for this curve is 0.9257.

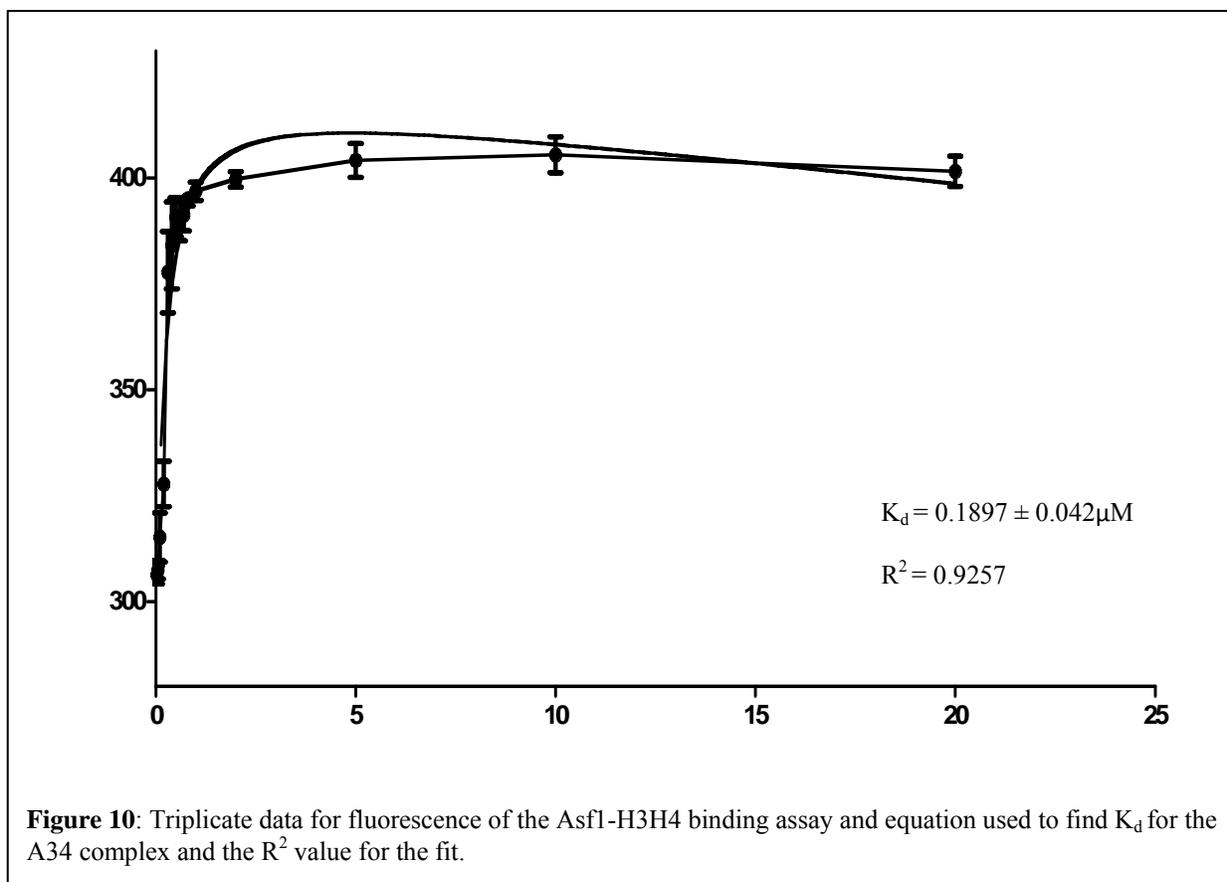
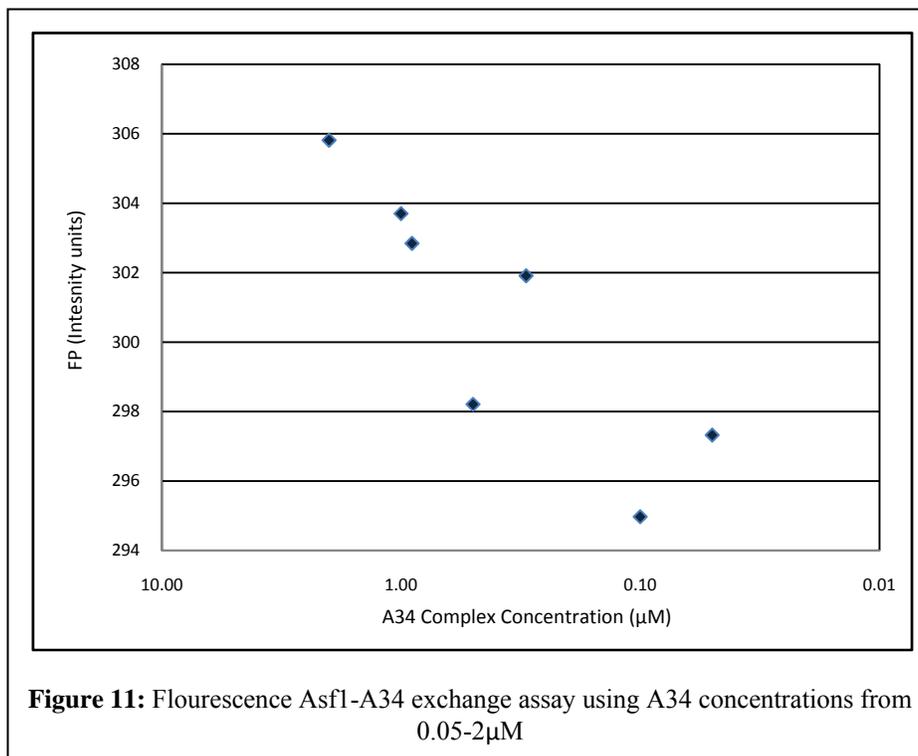


Figure 10: Triplicate data for fluorescence of the Asf1-H3H4 binding assay and equation used to find K_d for the A34 complex and the R^2 value for the fit.

Pilot Fluorescence Experiments on Chaperone Exchange

Histone Exchange Between Asf1 Chaperones

Asf1 and histones H3 and H4 (A34) complexes were titrated into buffer solution containing fluorescein-labeled Asf1 to study whether or not histone exchange can occur between two Asf1 proteins. Figure-11 shows the results of the preliminary study used to establish a base for further experimentation. For this experiment, concentrations of A34 between 0.05-2.0 μ M were used. From the results, it appears that both higher and lower concentrations of the A34 complex need to be tested to conclude whether or not there is exchange between the two Asf1 molecules. However, the beginnings of the standard curves seen in Figures-8A and 8B with histone binding assays can be seen as the concentration of A34 complex was increased.



DISCUSSION

Conclusions

The goal of this project was to develop and use an assay to investigate the interactions between histone chaperones and histone (H3-H4)₂ tetramers. A successful method was developed using fluorescein-labeled proteins and fluorescence polarization (FP) to assess binding between histone tetramers and histone chaperones. It is known that Asf1 breaks (H3-H4)₂ tetramers in order to bind specifically to (H3-H4) heterodimers, the model of which resolved around the C-terminal tail of Asf1 missing from current experiments (English et al., 2005; English et al., 2006). Presumably, this is the interaction seen in the binding assay; combination of the chaperone with (H3-H4) heterodimers via binding between Asf1 and histone H3. $K_d = 0.1897 \pm 0.042 \mu\text{M}$ which is stronger than the published $K_d = 100 \pm 20 \mu\text{M}$ for Asf1-H3 peptide binding (Mousson et al., 2005).

Application of the assay began with preliminary studies of histone exchange between two Asf1 proteins. Although limits in time and prepared material did not allow experiments using more concentrated A34 complexes to be completed, the initial test was helpful in determining the future directions experiments should veer in. Fluorescein-labeled N-terminal fragments of Asf1 should have the same action as full-length Asf1 (Daganzo et al., 2003). However, data generated thus far suggested full-length Asf1 has a slightly higher affinity for histones than the N-terminal fragment leading to the hypothesis that the C-terminal tail of Asf1 plays a role in additional binding capacity of the protein (English et al., 2006).

Experimental Setbacks

During the course of developing optimal conditions for the fluorescence binding assay, it was discovered that histone (H3-H4)₂ tetramers will aggregate when flash frozen without a cryoprotectant, such as glycerol, to ease them between physical states. This was discovered when assays began to have results that did not match earlier studies or follow an expected pattern when a new stock of histone tetramers was used. After making sure all proteins involved (Asf1 and the refolded histone (H3-H4)₂ tetramers) were indeed the expected proteins via SDS PAGE analysis, the cause of the problem initially remained unknown. In time, the lack of glycerol in the original dialysis buffer used to prepare the tetramers for the assays was concluded to be the source of the trouble. H-Histone Buffer was created as a result; the buffer contains 10% glycerol to safeguard the histone proteins as they are frozen for storage or thawed for assay work.

Future Experimentation

A successful assay for histone chaperone exploration has the potential to be used in many studies. The general action of histone chaperones in moderating histone deposition onto DNA strands has been researched, but the specific activity of the proteins is not fully understood. Is there interplay between different or identical chaperones? If there is exchange between chaperones, whether or not a cargo handoff is visible would be an application of the developed assay. Cargo handoff could explain whether a single chaperone contributes to the same nucleosome octamer, or whether each chaperone adds only portions of a single nucleosome core to a DNA strand. Since Asf1 binds histone H3, it may not be readily accessible for exchange to another Asf1 molecule especially the truncated form of the fluorescein-labeled Asf1 used to

create the assay (English et al., 2006). These studies could be completed with Asf1, or with other histone chaperones, such as CAF-1.

Can deposition onto DNA be seen using fluorescently-labeled DNA, and can the affinity of different mixtures of chaperones and histones be measured? The affinity of histones for DNA could be explored by using artificial 10nm fibers, and studying histone on and off rates. By introducing pre-bound A34 complexes to labeled DNA, deposition rates could be explored, and back reactions from histones leaving the DNA could be studied as well. Additionally, how chaperones might accept histones from DNA bound histone substrates could be investigated. Starting with an *in vivo* assembled nucleosomal array (ie. multiple histone tetramers or octamers on a short linear (200-2000bp) strand of DNA as a histone ligand), could fluorescence polarization changes with Asf1 or other chaperones be measured?

In conclusion, a successful method by which to study histone and histone chaperone interactions was developed. The assay can be used in further studies to better understand the reactions and mechanisms for guided nucleosome formation and deposition, as well as protein activity involved in DNA replication and repair.

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