Stability of DNA Four-Way Junctions and
Characterization of Binding to Integration Host Factor

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Table of Contents

Table of Contents

List of Figures

List of Tables

Chapter 1: Introduction

1.1 Project Description

1.2 DNA Four-Way Junctions – Holliday Junctions

1.3 Architectural Proteins – HU and Integration Host Factor

1.4 IHF Sequence Specificity

1.5 IHF-Induced Bending in DNA

1.6 Project Goals

Chapter 2: Theory, Methods, Practice

2.1 Oligonucleotide Purification

2.1.1 Theory of Oligonucleotide Purification

2.1.2 Oligonucleotide Purification

2.2 Oligonucleotide Annealing

2.2.1 Theory of Oligonucleotide Annealing

2.2.2 Oligonucleotide Annealing

2.3 Protein Purification

2.3.1 Theory of Protein Purification

2.3.2 Growth and Preparation of IHF for Purification

2.3.3 Protein Purification

2.3.4 Protein Assessment by Gel Electrophoresis

2.4 Binding Assays in Gel: Gel Mobility Shift Assay (GMSA)

2.4.1 Theory of Gel Mobility Shift Assay

2.4.2 Gel Mobility Shift Assay

2.5 Binding Assays in Solution: Fluorescence Spectroscopy

2.5.1 Theory of Steady-State Fluorescence Spectroscopy

2.5.2 Labeling of DNA with External Fluorescent Probes

2.5.3 Theory of Anisotropy, Intensity, Stoichiometry

2.5.4 Solution Binding Fluorescence Assays: Intensity, Anisotropy, Stoichiometry
2.6 Förster Resonance Energy Transfer (FRET) ------------------------------69
  2.6.1 Theory of Förster Resonance Energy Transfer ---------------69
  2.6.2 Four-Way Junction Labeling Designs --------------------72
  2.6.3 Ion Binding Monitored by FRET--------------------------74
  2.6.4 IHF Binding Monitored by FRET--------------------------75
  2.6.5 FRET Analysis------------------------------------------76
2.7 Time-Resolved Fluorescence Spectroscopy------------------------76
  2.7.1 Theory of Time-Resolved Fluorescence Spectroscopy---------76
  2.7.2 Time-Resolved FRET--------------------------------------78
2.8 DNA Four-way Junction Thermodynamics ------------------------81
  2.8.1 Theory of Nucleic Acid Thermodynamics-------------------81
  2.8.2 Labeling of DNA with Internal Fluorescent Probes--------86
  2.8.3 Junction Melting Experiments-----------------------------89

Chapter 3: Results --------------------------------------------------------91

3.1 Purification of IHF ---------------------------------------------------91
  3.1.1 Assessment of Purification by SDS-PAGE---------------------91
  3.1.2 IHF Activity Check – Binding of IHF to Consensus Sequence
     Duplex DNA-----------------------------------------------------96
3.2 IHF Binding to JH1 ---------------------------------------------------97
  3.2.1 GMSA IHF and JH1 ------------------------------------------100
  3.2.2 Fluorescence Intensity--------------------------------------101
  3.2.3. Fluorescence Anisotropy----------------------------------102
  3.2.4 Stoichiometry of Binding------------------------------------103
3.3 Ion-Induced Junction Conformation ----------------------------------105
  3.3.1 FRET--------------------------------------------------------106
  3.3.2 Time-Resolved FRET------------------------------------------111
3.4 Protein-Induced Junction Conformation -----------------------------115
  3.4.1 FRET--------------------------------------------------------116
  3.4.2 Time-Resolved FRET------------------------------------------118
3.5 DNA Four-way Junction Stability and Thermodynamic Properties 122
  3.5.1 Design of Holliday Junctions-----------------------------123
  3.5.2 Junction Melting------------------------------------------133
Chapter 4: Discussion

4.1 JH1 Binding Interactions with Architectural Proteins

4.2 Geometric Characterizations of Junctions

4.3 Thermodynamic Properties of Junctions

4.4 Future Directions

Chapter 5: References

Chapter 6: Appendices

6.1 Buffer Recipes

6.2 Gel Recipes

6.3 Worksheets

   6.3.1 GMSA Worksheet
   6.3.2 Fluorescence Intensity Worksheet
   6.3.3 FRET worksheet
List of Figures

Figure 1: DNA four-way junction. An illustration of a DNA four-way junction composed of four homologous single strands. 3

Figure 2: Model of Holliday’s homologous recombination. A model proposed by Robin Holliday illustrating the formation of Holliday junctions upon strand invasion and branch migration during homologous recombination (P. S. Ho & Eichman, 2001). 4

Figure 3: Open and stacked conformations of four way junctions. DNA four-way junctions can exist in open or stacked conformations depending on the ionic concentrations of the local environment. In the absence of divalent ions, such as magnesium, junctions exist in a planar open conformation (a). Upon the addition of magnesium, junctions undergo a folding process such that the helical arms become coaxially stacked. The stacked structure can adopt a parallel or antiparallel geometry, but antiparallel strands have been observed exclusively by crystallography (b) (Lilley & White, 2001). 5

Figure 4: Conversion of four-way junction. DNA four way junctions can experience conversions between two possible antiparallel, stacked conformations – Iso I and Iso II. The colors represent the strands of the junction structure: blue for X, green for R, yellow for B, and red for H (W. Wang et al., 2016). 7

Figure 5: Ribbon diagram of HU-DNA complex, TR3. The protein homodimer is in gold and orange. Top view of HU-DNA complex (b) (K. K. Swinger, Lemberg, Zhang, & Rice, 2003). 15

Figure 6: Crystal structure of IHF bound to nicked duplex DNA containing the consensus sequence. The alpha subunit of IHF is shown in white, beta subunit in pink. The consensus sequence is highlighted in green (Phoebe A. Rice et al., 1996) 18

Figure 7: Interactions between IHF alpha subunit arm and conserved bases, bp 33 - 38. Two arginines reach into the minor groove to make hydrogen bonds with the conserved bases, yet the interactions do not fully explain the sequence specificity. The sequence specificity of IHF seems to rely on a large number of small interactions (Phoebe A. Rice et al., 1996). 22

Figure 8: Close up view of the tip of the alpha subunit arm of IHF. Proline 65 residue (yellow) intercalates between DNA base pairs and induces a kink in the DNA. Consensus sequence bases are green (Phoebe A. Rice et al., 1996). 25
Figure 9: A Jablonski diagram illustrating the energy levels involved in absorption and emission of light in a fluorescent molecule (Lakowicz, 2006). 55
Figure 10: Schematic diagram of a spectrofluorometer (Lakowicz, 2006). 56
Figure 11: Structures of external fluorescent dyes: 5-carboxyfluorescein succinimidyl ester (FAM) (a), 5-Carboxytetramethylrhodamine succinimidyl ester (TAMRA) (b). 58
Figure 12: Two labeling schemes to model the two antiparallel conformation of four-way junctions. In the first construct, the B strand (yellow) is labeled with TAMRA (pink star) and the X strand (blue) is labeled with FAM (orange star). In the second construct, the R strand (green) is labeled with TAMRA and the X strand is labeled with FAM. The labeling schemes are shown in the stacked Iso I and Iso II conformations to depict the differences in the positioning of the fluorophores. 73
Figure 13: A typical UV melting curve showing the transition from the junction structure to random coils as a function of temperature (a). The melting curve is normalized and converted to the alpha vs. temp profile (b). 85
Figure 14: SDS-PAGE gel to assess expression of IHF during growth phase of E. coli, before and after IPTG induction. The first lane following the marker is a sample of previously purified IHF used as a standard. The proceeding lanes show the expression profiles after every hour of growth starting with the fifth hour in the second lane, and ending by the eleventh hour in the eighth lane. The culture was induced by addition of IPTG after the seventh hour (lane 4). Upon IPTG induction, increased production of IHF was observed. 92
Figure 15: SDS-PAGE gel to assess Polymin P and ammonium sulfate precipitation steps in extracting IHF from cell lysate. Lane 1 is the sample of lysed cells. lane 2: Polymin P precipitation supernatant, lane 3: Polymin P precipitation pellet, lane 4: 0.5M NaCl wash pellet, lane 5: 0.5M NaCl wash supernatant, lane 6: supernatant of ammonium sulfate precipitation (0-50% cut), lane 7: pellet of ammonium sulfate precipitation (0-50% cut), lane 8: supernatant of ammonium sulfate precipitation (50-75% cut), lane 9: supernatant of ammonium sulfate precipitation (50-75% cut), lane 9: final dialysis. 93
Figure 16: SDS-PAGE gels assessing the fractions eluted from HiTrap Heparin HP column. Lane 1 in (A) is the same loaded onto the HiTrap HeparinHP column. Lane 2 in (A) is the pellet found after dialysis following the precipitation steps.
Lanes 3 to 8 in (A) are fractions collected as IHF was loaded onto the column. Lanes 9 to 14 in (A) are Buffer A washes that proceed the IHF application. In gels (B), (C), and (D), every lane shows the assessment of every other fraction collected from the HiTrap Heparin HP column. Lanes 3 to 6 in (D) shows the elution of IHF at approximately 900 mM NaCl. Fractions containing IHF were pooled, concentrated, and dialyzed to obtain pure protein product (E).

Figure 17: (a) Polyacrylamide gel of IHF binding to IHF consensus sequence duplex H134. Increasing protein concentrations of protein, from 0 M (first lane) to a final concentration 900 nM (last lane), are incubated with a constant 10 nM concentration of H134. As more DNA is bound by protein, the complex formation increases, resulting in a shift in the gel. The lower band represents free DNA while the upper band represents bound DNA. (b) Binding curve analysis of free DNA as a function of protein concentration. Quantification of the intensity of pixel volumes of free DNA bands was plotted with increasing protein concentrations and revealed a Kd of 28.9 ± 5.6 nM.

Figure 18: The immobile Holliday junction containing the IHF consensus sequence (JH1). The J3 junction template was modified to contain the IHF consensus sequence (red) at the center of the junction.

Figure 19: Polyacrylamide gel of IHF binding to the Holliday junction containing the IHF consensus sequence. Increasing concentrations of IHF was titrated into a constant 10 nM concentration of JH1. (B) Binding curve analysis of the free DNA as a function of IHF concentration.

Figure 20: Analysis of binding curve between the interaction of IHF and JH1 measured by changes in fluorescence intensity. Average of three trials.

Figure 21: Analysis of JH1+IHF binding curve between the interaction of IHF and JH1 measured by changes in fluorescence anisotropy.

Figure 22: Stoichiometry binding of IHF to 100 nM JH1 monitored by change in fluorescence intensity. The transition from the linear fit of the binding curve to the linear fit of the saturation curve refers to the molar ratio of IHF to JH1, 1:1 stoichiometry.

Figure 23: Native gel of JH1 junctions. Lane 1 is the unlabeled JH1 junction, used as a control. Lane 2 is the donor only junction, JH1X-FAM. Lane 3 is the acceptor only junction of the BX construct – JH1B-TAM. Lane 4 is the doubly labeled junction of the BX construct – JH1X-FAM_B-TAM. Lane 5 is the acceptor only
of the RX construct – JH1R-TAM. Lane 6 is the doubly labeled junction of the RX construct – JH1X-FAM_R-TAM. Less than 5% single stranded DNA was detected for all junctions.  

Figure 24: Plot of FRET efficiency of JH1 BX construct, FAM labeled on the X arm (blue) and TAMRA labeled on the B arm (yellow), as function of potassium ion concentration. FRET efficiencies were calculated by fluorescence decrease in donor signal of the JH1 junction.

Figure 25: Plot of FRET efficiency of JH1 RX construct, FAM labeled on the X arm (blue) and TAMRA labeled on the R arm (green), as function of potassium ion concentration. FRET efficiencies were calculated by fluorescence decrease in donor signal of the JH1 junction.

Figure 26: Fluorescence lifetime decay curves of donor emissions of JH1 singly labeled with FAM and doubly labeled with FAM and TAMRA on the X and R strands, respectively, in low salt (A) and high salt (B). Quenching at high salt concentrations is observed.

Figure 27: Plot of FRET efficiency of JH1 BX construct, FAM labeled on the X arm and TAMRA labeled on the B arm, as a function of protein concentration under stoichiometric conditions. FRET efficiencies were calculated from the decrease in fluorescence donor signal of the doubly-labeled JH1 junction relative to the donor only junction.

Figure 28: Plot of FRET efficiency of JH1 RX construct, FAM labeled on the X arm and TAMRA labeled on the R arm, as function of protein concentration under stoichiometric conditions. FRET efficiencies were calculated from the decrease in fluorescence donor signal of the doubly-labeled JH1 junction relative to the donor only junction.

Figure 29: Fluorescence lifetime decay curves of the donor emission of JH1 singly labeled with FAM or doubly labeled with FAM and TAMRA on the X and R strands, respectively, in high salt, in the absence (A) or presence of IHF (B).

Figure 30: Illustration of junction arms. XR arm: 5’ end of the X strand and 3’ end of R strand. BX arm: 5’ end of the B strand and 3’ end of the X strand. HB arm: 5’ end of the H strand and 3’ end of the B strand. RH arm: 5’ end of the R strand and 3’ end of the H strand.

Figure 31: Junction J3_R28-6MAP. Immobile 34 base pair Holliday junction containing the 6MAP internal fluorophore at the 28th position on the R strand.
The 6MAP, indicated as the red “F”, is located in the middle region of the XR arm, at precisely 11 base pairs away from the center of the junction. Figure 32: Junction J3_X28-6MAP. Immobile 34 base pair Holliday junction containing the 6MAP internal fluorophore at the 28th position on the X strand. The 6MAP, indicated as the red “F”, is located in the middle region of the BX arm, at precisely 11 base pairs away from the center of the junction. Figure 33: Junction J3_B25-6MAP. Immobile 34 base pair Holliday junction containing the 6MAP internal fluorophore at the 25th position on the R strand. The 6MAP, indicated as the red “F”, is located in the middle region of the HB arm, at precisely eight base pairs away from the center of the junction. Figure 34: Junction J3_R7-6MAP. Immobile 34 base pair Holliday junction containing the 6MAP internal fluorophore at the 7th position on the R strand. The 6MAP, indicated as the red “F”, is located in the middle region of the RH arm, at precisely 11 base pairs away from the center of the junction. Figure 35: Junction J40_X25-6MAP. Immobile 40 base pair Holliday junction containing the 6-MI internal fluorophore at the 25th position on the R strand. The 6-MI, indicated as the red “F”, is located towards the center of the junction, at precisely five base pairs away from the center of the junction. Figure 36: Junction J40_H18-6MAP. Immobile 40 base pair Holliday junction containing the 6-MI internal fluorophore at the 18th position on the H strand. The 6-MI, indicated as the red “F”, is located towards the center of the junction, at precisely three base pairs away from the center of the junction. Figure 37: Temperature dependence of the extent of melting (α) by UV absorption. These curves were derived from fits of the temperature dependence of absorbance at 260 nm of the immobile 34 bp J3 Holliday junction. Figure 38: Temperature dependence of the extent of melting (α) as measured by fluorescence intensity. These curves were derived from fits of the temperature dependence of fluorescence intensity of the immobile 34 bp J3 Holliday junction. Figure 39: Temperature dependence of the extent of melting (α) by UV absorbance and fluorescence intensity. These curves were derived from fits of the temperature dependence of either the UV absorbance or the fluorescence intensity of the immobile 40 bp J3 Holliday junction containing the 6MI probe.
List of Tables

Table 1 DNA substrates used for binding assays. IHF consensus sequence in red. -- 36
Table 2: Junctions used for melting experiments. F indicates the position of labeling
--------------------------------------------------------------- 87
Table 3: Parameters derived from fitting of time-resolved fluorescence decay curves
for JH1 under low and high salt conditions. -------------------------- 111
Table 4: Evaluation of structural populations of doubly labeled sample, JH1_X-
FAM_R-TAM, under high salt conditions. ----------------------------- 114
Table 5: Parameters derived from global fitting of time-resolved fluorescence decay
curves for JH1 under high salt conditions in the absence and presence of IHF.
-------------------------------------------------------------------- 119
Table 6: Table of thermodynamic parameters of immobile 34 bp Holliday junction J3
by UV absorption. The average molar enthalpy (ΔH) and entropy (ΔS) changes
of the denaturation, or helix to random coil transition, of the four-way junction
were determined by van’t Hoff analysis. ------------------------------- 135
Table 7: Table of thermodynamic parameters of immobile 34 bp Holliday junction J3
by fluorescence intensity. The average molar enthalpy (ΔH) and entropy (ΔS)
changes of the denaturation, or helix to random coil transition, of the four-way
junction were determined using a van’t Hoff analysis. ------------------- 138
Table 8: Table of thermodynamic parameters of immobile 40 bp junction by UV
absorption and fluorescence intensity. ------------------------------- 140
Chapter 1: Introduction

1.1 Project Description

This project examines the Holliday junction, a DNA four-stranded junction, or 4WJ. We specifically examine binding interactions with architectural proteins, the global conformational geometry, and thermodynamic properties. An immobile DNA Holliday junction containing the consensus sequence (JH1) is used to characterize the binding interactions with the architectural protein, Integration Host Factor (IHF). A DNA binding- and bending-protein, IHF interacts with DNA to facilitate the formation of higher-order DNA-protein complexes important for gene regulation, DNA replication, repair, and recombination. IHF is known to bind with nanomolar affinity and induce a 160-degree bend in duplex DNA containing the consensus binding sequence. Interestingly, we have shown that IHF binds Holliday junctions that do not contain the consensus sequence (J3) with nanomolar affinity and 1:1 stoichiometry. In this study, we investigate whether the presence of the IHF consensus sequence influences binding to DNA four-way junctions. We utilize gel shift and fluorescence binding assays to measure affinity and have observed high affinity binding for Holliday junction containing the IHF consensus sequence (JH1).
This study focuses on the effects of protein binding. Steady state and time-resolved Förster Resonance Energy Transfer (FRET) methods allow us to look at the geometry of JH1 under different protein and ion concentrations. These assays measure the efficiencies and distances between the arms of the junction and determine the conformational populations of the junction. This study also focuses on the stability of junctions. Understanding the unfolding process as a function of temperature provides energetic information on their genetic functions and structures. Theoretical predictions suggested that melting of junction arms is asymmetric. By studying the protein-DNA interactions, resulting geometries of junctions, and stability of junctions, we will be able provide insight into the mechanism by which IHF recognizes and binds to JH1.

1.2 DNA Four-Way Junctions – Holliday Junctions

DNA, more commonly found as double stranded helices, can also take the form as four-stranded junctions, under certain physiological conditions. One type of junction, the Holliday junction, is formed during recombination and repair events. This junction has a branched structure, with four double-helical segments extending
out from the center (Figure 1). From this branch point, the four arms are connected by the covalent continuity of the strands. Essentially, the four-way junction is an interchange between four DNA single strands (Lilley & White, 2001).

Figure 1: DNA four-way junction. An illustration of a DNA four-way junction composed of four homologous single strands.

Four-way junctions were first recognized as a means to introduce genetic diversity in cells. In 1964, Robin Holliday described four-way junctions as part of his homologous recombination model, which occurs between DNA molecules that contain regions of close homology (Duckett et al., 1988). The homologous recombination begins with two nicked homologous DNA molecules that undergo strand invasion and branch migration to form what is now known as the Holliday
junction (Figure 2). Thus, Holliday junctions are intermediates that allow for the crossing-over between the two homologous duplexes to exchange genetic information. Recently, Holliday junctions have been recognized in an increasing number of important processes, including λ phage integrative site-specific recombination, double-stranded break repair, and maintenance of the genomic integrity (P. S. Ho & Eichman, 2001).

Figure 2: Model of Holliday’s homologous recombination. A model proposed by Robin Holliday illustrating the formation of Holliday junctions upon strand invasion and branch migration during homologous recombination (P. S. Ho & Eichman, 2001).
As a highly charged molecule, DNA is highly sensitive to the electrostatic properties of the local environment with regards to its folding and junction formation. The Holliday junction can exist in different conformations depending on the electrostatic properties of the local environment. The most commonly encountered configurations of the Holliday junction are the “open” and “stacked” conformations (Figure 3). The “open” conformation is found at low ionic concentrations, whereas the “stacked” conformation is induced under high ionic concentrations (Clegg, Murchie, & Lilley, 1994).

Figure 3: Open and stacked conformations of four way junctions. DNA four-way junctions can exist in open or stacked conformations depending on the ionic concentrations of the local environment. In the absence of divalent ions, such as magnesium, junctions exist in a planar open conformation (a). Upon the addition of magnesium, junctions undergo a folding process such that the helical arms become coaxially stacked. The stacked structure can adopt a parallel or antiparallel geometry, but antiparallel strands have been observed exclusively by crystallography (b) (Lilley & White, 2001).
The open conformation of the Holliday junction is characterized by the open central region, from which the four arms are directed towards corners of a square. The structure exhibits a flat, square planar geometry with four-fold geometry. Because the arms of the junctions are spread out, the repulsion between the negatively charged phosphates, which are concentrated near the intersection of the helical arms, are minimized (P. S. Ho & Eichman, 2001).

The stacked conformation of the Holliday junction is described by the stacked-X-structure, which is formed by the pairwise coaxial stacking of the helical arms and a right-handed rotation of the axes (Lilley & White, 2001). In this stacked form, the high repulsion between the arms is effectively shielded by the addition of cations, allowing pairs of arms to be in close proximity. Unlike the open conformation, the stacked conformation adopts an antiparallel orientation, lowering the symmetry to a two-fold geometry. In solution assays and crystal structures, the resulting stacked-X-structure is found to have two continuous strands that run the length of the stacked helices and two exchanging strands that cross over from one pair of stacked helices to the other. Holliday junctions are thought to swap between two possible stacked antiparallel configurations depending on the stacking partners.
(Figure 4). One conformer, Isoform I, the B arm coaxially stacks with the H arm, while in the other conformer, Isoform II, the B arm coaxially stacks with the X arm (Clegg et al., 1994).

Figure 4: Conversion of four-way junction. DNA four way junctions can experience conversions between two possible antiparallel, stacked conformations – Iso I and Iso II. The colors represent the strands of the junction structure: blue for X, green for R, yellow for B, and red for H (W. Wang et al., 2016).
Extensive studies have shown that different junctions exhibit biases towards one conformer. This conformer bias is essentially a balance between stacking interactions, electrostatic repulsion and sequence-specific contacts (Declais & Lilley, 2008). Evidence from crystal structures of Holliday junctions reveal that the preference for a particular stacked conformer is due to specific interactions, stacking energies, and steric hindrance at the branch point. The intersection of the helical segments can be very compact, consisting of strong van der Waals, hydrogen bonding and stacking interactions. The local DNA sequence at the junction is also found to determine the stability of different conformers. The four base pairs at the branch point are critical because the specific combination of bases establishes sequence-specific hydrogen bonds that would minimize the steric clashes between the base pairs. The second base pairs beyond the branch point are also considered crucial by maintaining specific phosphate-base hydrogen bonds to prevent destabilization of the junction structure (Ortiz-Lombardia et al., 1999). Additional evidence from crystal structures also reveals that the sequence-specific interactions between arms further away from the branch point also seem to be critical in determining the geometric features of the junctions (Eichman, Ortiz-Lombardia, Aymami, Coll, & Ho, 2002). This junction
conformer bias is important in creating two different sides in the structure that are essential for recognition by proteins that interact with junctions and those involved in recombination.

To study the geometry and interactions with proteins, a stabilized form of the junction was developed - an immobilized model of junctions. Immobile junctions were first constructed because naturally-occurring, transient Holliday junction structures were unstable; the sequence symmetry would quickly resolve the junction structure to double helices (Kallenbach et al., 1983) (Seeman & Kallenbach, 1983). Immobile junctions are characterized by asymmetrical sequences that prevent the junction from branch migration, with a fixed intersection point. The immobile structure is assumed to be a snapshot of the naturally-occurring junction, and therefore reflects the structural characteristics and protein-DNA interactions of mobile junctions. Because the fixed structure eliminates the numerous conformational possibilities, the immobile junction is very useful for studying the geometry, stabilizing forces, and dynamics of DNA Holliday junctions (Altona, Pikkemaat, & Overmars, 1996).
Studying the geometries and dynamics of Holliday junction structures is important for understanding the mechanism behind Holliday’s model of genetic recombination. In particular, the stacking orientation is found to influence the recognition by junction-resolving enzymes and the resulting resolution of the junction. Extensive studies have shown that stacking orientation can regulate the range of genetic information exchange, whether it is over short or long ranges of DNA (McKinney, Declais, Lilley, & Ha, 2003). Thus, studying the structures of Holliday junctions would elucidate interactions between proteins and DNA, and potentially reveal recognition mechanisms involved in protein-DNA interactions.

In addition, studying thermodynamic parameters and stability of the Holliday junction is important for understanding the formation of the geometric conformations and preferences for certain conformations. Early studies of thermal denaturation behavior of DNA duplex structures reflect the “zipper” model, rather than the all-or-none transitions (Vamosi & Clegg, 2008), (Husler & Klump, 1994). The two-state all-or-none model refers to the reaction mechanism in which DNA duplexes transition from complete helices to random coils occurs in a single step. The more complex “zipper” model assumes that the melting of DNA duplexes begins at ends of the
helices and proceeds similarly to the opening of a zipper, towards the other end of the
helix until the bimolecular duplex structure is completely “unzipped” as random coils.
Because arms of the Holliday junction are thought to reflect the duplex structure and
exhibit identical helical properties as duplex DNA, the “zipper” model for helix to
random coil transition may more accurately describe the denaturation of junction
arms, as opposed to the all-or-none model. When considering the “zipper” model,
there are two possible events, either the “unzipping” from the ends of the extended
arms of the junction, or “unzipping” of the helical arms from the center of the
junction. Understanding the events during denaturation of DNA four way junctions
would provide information about the stability of the junction structure, its formation,
and its preference of conformation. In addition, the “zipper” model may provide an
explanation for the existence of pre-formed Holliday junction structures found during
genetic recombination.
1.3 Architectural Proteins – HU and Integration Host Factor

Architectural proteins orchestrate the three-dimensional organization of the genome through a multitude of interactions with DNA. Their ability to coordinate long-range contacts between DNA sequences have been implicated in DNA manipulation processes, such as replication, repair, recombination, and gene expression regulation (Pombo & Dillon, 2015). Architectural proteins also facilitate the assembly, while enhancing the stability and activity, of higher order structures. The formation of multiprotein-DNA complexes often requires a remodeling of the DNA structure to bring other DNA-bound proteins into close spatial proximity. This task can be readily achieved by architectural proteins that create conformational changes to linear DNA. One such class of proteins is the prokaryotic type II-DNA binding protein, which includes HU and Integration Host Factor (IHF), both found in high abundance in Escherichia coli (Travers, 1997). HU and IHF have been shown to induce extreme deformations in the locally inflexible double-stranded DNA structure (Becker & Maher, 2015). They are also involved in interacting with a variety of DNA substrates that arise from the formation of higher order complexes (Bewley, Gronenborn, & Clore, 1998).
A wide array of architectural proteins are also present in eukaryotes, most notably in *Drosophila* and humans (Cubenas-Potts & Corces, 2015). Some examples include RNA polymerase III-associated factor protein (TFIIIC) and CCCTC-binding factor protein (CTCF), which colocalizes accessory proteins, such as cohesin and condensin, to mediate communications between functional and topological domains of the 3D chromatin structure (Gomez-Diaz & Corces, 2014). The degree to which architectural proteins are important to cellular processes merits investigation due to their abundance and ubiquitous presence in prokaryotic and eukaryotic systems.

HU is a small and naturally abundant architectural protein found in *E. coli*. HU was first proposed to be a histone-like protein, therefore named, “H” for histone-like, and “U” for the *E. coli* strain U93 (Kamashev, Balandina, Mazur, Arimondo, & Rouviere-Yaniv, 2008). HU exists as a dimer of closely related subunits of approximately 10 kDa. Due to its basic properties and ability to bend and wrap DNA into nucleosome-like structures, HU is often referred to as a histone-like protein (Oberto, Drlica, & Rouviere-Yaniv, 1994). Various studies suggest that HU acts to indirectly stabilize nucleosome-like DNA structures by promoting processes that
increase global supercoiling. Mutagenesis experiments reveal that HU affects the stimulation of gyrase and suppression of topoisomerase I, which suggests that HU plays a role in chromosomal compaction and negative supercoiling (Malik, Bensaid, Rouviere-Yaniv, & Drlica, 1996). High resolution protein-mapping techniques in vivo show that the loss of HU is correlated with a global unrestrained negative superhelical density, which strongly suggests that the presence of HU is required for the formation of supercoiled structures (Becker & Maher, 2015). Molecular simulations show that HU can populate DNA nonspecifically and distort the DNA randomly along its pathway (Wei, Czapla, Grosner, Swigon, & Olson, 2014). In addition, co-crystal structures of HU and DNA reveal that HU induces or stabilizes DNA into different bend angles. Due to the flexibility in its mechanism of action, HU may contribute to the formation of higher order protein-DNA complexes that require different bend angles (K. K. Swinger & Rice, 2004).
Figure 5: Ribbon diagram of HU-DNA complex, TR3. The protein homodimer is in gold and orange. Top view of HU-DNA complex (b) (K. K. Swinger, Lemberg, Zhang, & Rice, 2003).
Most importantly, HU is characterized by its non-specific binding to duplex DNA. In other words, HU binds to duplex DNA in a structurally-specific manner, such that it would recognize the global molecular shape rather than chemical features of the DNA base pairs. Interestingly, co-crystal structure analysis and EMSA experiments have shown that HU binds preferentially to DNA four-way junctions and certain damaged or distorted DNA structures containing nicks, gaps, or loops with high affinity (Kerren K. Swinger & Rice, 2007).

Integration Host Factor (IHF) is another small and naturally abundant architectural protein found in E. coli. IHF exists as a 20 kDa heterodimer composed of two closely related (~30% homology), yet nonidentical subunits. These two subunits, alpha subunit (11 kDa) and beta subunit (9 kDa), are joined to form one compact body composed of alpha helices, with two long beta ribbon arms extending from each subunit (Phoebe A. Rice, Yang, Mizuuchi, & Nash, 1996). IHF was originally discovered as a required component, more specifically, a host factor, for integrative recombination of bacteriophage λ (Lorenz, Hillisch, Goodman, & Diekmann, 1999). Early studies demonstrated that IHF acts to regulate a broad range
of cellular functions (Friedman, 1988). It has been shown that IHF plays the role as a “general regulator”, influencing approximately 120 *E. coli* genes (K. K. Swinger & Rice, 2004). Most commonly, IHF has been found to bind to upstream regions to stimulate transcription of genes, such as the $\sigma^{54}$ and $\sigma^{70}$ promoters in some Gram-negative bacteria (Hoover, Santero, Porter, & Kustu, 1990). IHF is also known to be involved in the stabilization of repressor binding and enhancement of transposition, and influence initiation of DNA replication (Freundlich, Ramani, Mathew, Sirko, & Tsui, 1992). Like HU, IHF also belongs to a class of histone-like proteins that are capable of bending and wrapping DNA into condensed structures (Oberto et al., 1994). The bends induced by IHF bring DNA binding sites into close proximity, thereby allowing transcriptional machinery to access the DNA. IHF is most noted for its site-specific interactions at H1, H2, and H’ sites on the *att*P sequence, and the resulting severe bend to facilitate the the formation of the three-dimensional DNA-protein complex required for site-specific recombination (Ellenberger & Landy, 1997).
Figure 6: Crystal structure of IHF bound to nicked duplex DNA containing the consensus sequence. The alpha subunit of IHF is shown in white, beta subunit in pink. The consensus sequence is highlighted in green (Phoebe A. Rice et al., 1996)
Crystallography data reveal that IHF is structurally identical to HU, such that the superimposed images of the two proteins result in a root-mean-square deviation of 1.0 Angstrom. The subunits in IHF are found to share a 40% sequence homology to each of the subunits of HU. Interestingly, although the crystal structures reveal structural similarities between HU and IHF, the two architectural proteins exhibit different modes of DNA recognition - sequence-nonspecific interactions for HU, and both sequence-nonspecific interactions and sequence-specific interactions for IHF (Rafferty et al., 1996).

Sequence specific IHF-DNA interactions are crucial for regulation and formation of protein-DNA complexes, while nonspecific interactions provide organization and stabilization of the nucleoid structure (Holbrook, Tsodikov, Saecker, & Record Jr, 2001). Nonspecific IHF binding to duplex DNA occurs at low affinities and may have higher stoichiometry, leading it to outcompete specific binding interactions at high molar excess of IHF to DNA ratios. However, due to the low affinity binding, nonspecific interactions span over shorter lengths of contact and do not produce the characteristic DNA bend as seen in specific binding (Aeling et al., 2006).
1.4 IHF Sequence Specificity

The two general models of sequence specific protein-DNA recognition are classified as direct and indirect readout. Direct readout describes the interaction in which proteins recognize specific sequences based on the unique chemical signatures of the DNA bases. This recognition is mediated by the formation of hydrogen bonds or hydrophobic contacts between amino acids and bases. Indirect readout describes the interaction in which proteins rely on sequence-specific structural aspects of DNA (Rohs et al., 2010). Varying aspects of the local structure of DNA include the intrinsic curvature, topology of major and minor grooves, ordered water structures, and local geometry of backbone phosphates, and flexibility or deformability (Aeling et al., 2006).

IHF relies on the indirect readout mechanism to recognize sequence-dependent physical characteristics in the DNA. Early studies using alkylation of guanine and adenine to alter the major and minor groove surfaces revealed that IHF preferentially interacts with the minor groove. Hydroxyl radical protection assays revealed that IHF preferentially protects specific clusters of sugar residues (Yang & Nash, 1989). Recently, co-crystal structures of IHF bound to nicked duplex DNA
demonstrate that IHF makes no contacts with the major groove of the DNA, but instead makes contacts with the minor groove. Of the approximately 35 contacts IHF makes with duplex DNA, 26 contacts are made with the phosphate backbone, while only three hydrogen bonds are made with base-pairs in the minor grooves, and none with unique features of DNA bases in the major groove. Intriguingly, IHF seems to make minimal contacts with the minor groove, perhaps suggesting a more complex mechanism for specificity (K. K. Swinger & Rice, 2004).
Figure 7: Interactions between IHF alpha subunit arm and conserved bases, bp 33 - 38. Two arginines reach into the minor groove to make hydrogen bonds with the conserved bases, yet the interactions do not fully explain the sequence specificity. The sequence specificity of IHF seems to rely on a large number of small interactions (Phoebe A. Rice et al., 1996).

More specifically, IHF relies on the indirect readout mechanism to recognize a specific sequence of DNA. Footprinting methods demonstrate that IHF selectively protects three binding segments at the phage attachment site, \textit{attP} in \textit{E. coli} (Craig & Nash, 1984). These three binding segments are found to share a common sequence. Comparisons and alignment of these sequences, along with the evaluation of over 170
known binding sites, reveal the critical feature for specific IHF-DNA interactions: the IHF consensus sequence - WATCAANNNTTR (Yang & Nash, 1989). The consensus sequence contains three small clusters of conserved bases (Aeling et al., 2006). Two of these clusters, the WATCAA element and TTR element, are highly conserved. The WATCAA element (W=A/T) is located at the center of the binding site, and the TTR portion is located four base pairs in the 3’ direction from WATCAA. The third element is a poly(A)-tract that contains four to six adenines, located eight to nine base pairs in the 5’ direction from the WATCAA element (S. Wang, Cosstick, Gardner, & Gumport, 1995). Upon recognition of the consensus sequence in duplex DNA, IHF binds to sites containing the three elements very tightly, on the order of 1 nM binding affinity, and induces a severe bend (S. Wang et al., 1995).

1.5 IHF-Induced Bending in DNA

Generally, minor groove binding proteins establish interactions with DNA by widening the narrow groove, producing bends in the DNA such that the DNA is
deformed away from the protein (Rohs et al., 2010). As a minor groove binding protein, IHF induces large bends using two main strategies - hydrophobic intercalation and asymmetric neutralization. Co-crystal structures demonstrate that IHF inserts a hydrophobic residue, proline, located at the tips of each beta-ribbon arm, between base pairs on the minor groove side of the DNA. The two hydrophobic residues, proline65 on the alpha subunit and proline64 on the beta subunit are absolutely conserved among all members of HU and IHF (Bewley et al., 1998). The intercalation introduces kinks in the DNA, such that the roll angle at the two positions increases to approximately 57 degrees, resulting in an extremely shallow and wide minor groove. This distortion to the DNA structure induces an overall bend angle of approximately 160 degrees, which is centered at the 5’ end of the WATCAA element, such that the TTR element is positioned on one side while the poly(A)-tract element is positioned on the other. The IHF-DNA interaction is often described by a “double embrace”, which is the result of IHF tightly curling its arms around the DNA to target specific kink locations nine base pairs apart, and the DNA tightly wrapping around the IHF (P. A. Rice, 1997).
Figure 8: Close up view of the tip of the alpha subunit arm of IHF. Proline 65 residue (yellow) intercalates between DNA base pairs and induces a kink in the DNA. Consensus sequence bases are green (Phoebe A. Rice et al., 1996).

To stabilize the large distortion of the DNA structure, the alpha helical body of IHF makes extensive contacts with the phosphate backbone of the DNA. The basic surface of the IHF protein achieves stabilization by neutralizing the phosphate charges of the peptide backbone on the inside of the bend. The interaction with the phosphate backbone is facilitated by the amino termini of the alpha helices from both subunits, which bind to opposite sides of the minor groove, forming a clamp. The contacts made by the individual subunits are roughly symmetrical, but as a whole, the
intertwined alpha helical dimer body contacts are asymmetrical. One side of the body makes intimate interactions with the conserved TTR bases. This coordination is achieved, surprisingly, by only three side chains, all of which are arginines that make direct contacts with the bases in the minor groove. The other side of the dimer body makes no contact with DNA bases, but rather, makes mostly exclusive electrostatic interactions, even solvent-mediated interactions (Ellenberger & Landy, 1997). Thus, as a minor groove binding protein, IHF not only induces large bends, but also displays specificity using the indirect readout mechanism to probe for local features of the sequence-dependent DNA structure.
1.6 Project Goals

HU and IHF, exhibiting similar functions and structural compositions, belong to the same DNA binding protein family. They both exist as heterodimers in *E. coli* and directly affect gene expression across the bacterial genome. Interestingly, these proteins also exhibit very distinct characteristics. HU and IHF interact with DNA differently, and play different roles during different time points of the cell life cycle. HU binds nonspecifically to duplex DNA while having preference for atypical DNA structures, such as junctions, forks, and nicked DNA. IHF binds with varying levels of sequence specifically, most importantly to a consensus sequence and induce bends greater than 160 degrees. Immunoprecipitation techniques used to determine binding characteristics of HU and IHF during different phases of growth demonstrate that the two proteins exhibit different effects on global gene expression in *E. coli*. Genomics analysis show that HU has global and uniform changes on expression, while IHF has differential expression on only a small number of genes, resulting in very little to almost no observable global effects on gene expression. Thus, HU is thought to stabilize the overall chromosome structure and perhaps play a role in DNA repair, while IHF is thought to exhibit combinatorial regulation, as having a possible role in
facilitating the binding of other transcription factors to upstream regions of target
genes (Prieto et al., 2012).

While HU and IHF binding to duplex DNA are studied at high resolution,
more binding studies with atypical DNA structures are necessary to pinpoint the
preferential binding behavior. For example, would HU or IHF interact with Holliday
junctions, and structures leading up to the final formation of the junction, often
described in the genetic recombination model? The binding behavior with atypical
DNA structures may suggest a new set of diverse roles outside of transcriptional
regulation. The recognition of these structures is also another key element. How do
HU and IHF recognize atypical DNA structures? Several X-ray and NMR structures
have provided new insights about the arrangement of the protein heterodimers and
contacts made with duplex DNA. Förster Resonance Energy Transfer (FRET)
measurements have also provided tools for studying the geometrical conformation of
DNA substrates upon binding. Further experiments would be necessary to address the
mechanism behind the nonspecific recognition of HU and sequence-specific
recognition of IHF.
Previous work in the Mukerji lab has characterized the binding interactions of HU and IHF with various DNA substrates. Laura Nocka determined that HU binds tightly to mobile and immobile Holliday junction with a random sequence (Nocka, 2013). Olga Buzovetsky (Buzovetsky, 2010) and Suzanne Ho (Ho, 2013) determined that IHF binds tightly, in the nanomolar range, to an immobile model of Holliday junction with a random sequence (J3). Veronica Birdsall has shown tight binding of IHF to a smaller junction (J20), and to certain single-stranded forks (Birdsall, 2015). The next step would be to determine the recognition mechanism that allows IHF to bind to these DNA structures with nanomolar affinity. Does sequence play a role in the recognition? How can IHF recognize and bind with high affinity to substrates with and without the consensus sequence? Is the recognition mediated by indirect readout? How does IHF distinguish the local structural features of these DNA substrates? Would the junction conformation and orientation affect IHF binding? Upon binding, does IHF also induce severe bends to these DNA structures? What would the resulting junction conformation look like?

The first part of my thesis focuses on the binding interaction of IHF to another DNA substrate - Holliday junction containing the consensus sequence (JH1). Since
IHF is found to bind with high affinity to duplex DNA containing the consensus sequence and to four way junction without the consensus sequence. Will IHF induce severe bending in JH1, as seen in duplex DNA? Which conformation would the junction have upon binding? Finally, the thermodynamic quality and stability of the junction structure will be assessed. Energetic information to their genetic function and structure may reveal the flexibility and local structures that are important for contacts made by IHF. This thesis will attempt to answer these questions through the study of IHF interactions with immobile Holliday junction containing the consensus sequence with the goal of understanding the various elements required to promote IHF recognition.
Chapter 2: Theory, Methods, Practice

2.1 Oligonucleotide Purification

2.1.1 Theory of Oligonucleotide Purification

Oligonucleotide purification by denaturing urea polyacrylamide gel electrophoresis separates the desired oligomer from incorrectly synthesized DNA particles based on differences in charges. Urea is used as a denaturing agent in the polyacrylamide gel to eliminate any inter- and intra-molecular secondary structures. The negatively charged DNA will migrate through the gel towards the positive terminal. Larger DNA particles will migrate slower through the gel while smaller DNA particles will migrate faster through the gel. Usually, the majority of synthesized DNA is considered pure. Thus, the darkest band, and usually the largest band is assumed to be the desired DNA oligomer. The pure DNA oligomer can be excised and extracted from the gel matrix by electroelution (Summer, Gramer, & Droge, 2009).
2.1.2 Oligonucleotide Purification

The 20% urea denaturing gel solution containing urea, TBE and bis/acrylamide was prepared in a flask. The urea solution was dissolved by running the flask under warm tap water, not exceeding 55°C, for several minutes until the urea was fully dissolved. The solution was degassed and sonicated for 10 minutes to remove bubbles.

In the meantime, the gel plates were set up. Spacers that were 0.2 cm thick were placed on the bottom, right and left edges of a 20 cm by 22 cm gel plate with plenty of petroleum jelly. A second gel plate of smaller size, 20 cm by 20 cm, was placed over the spacers and aligned with the bottom of the first gel plate. Additional petroleum jelly was added to the bottom and side edges of the plates to ensure a seal.

Upon completion of degas and sonication, 200 µl of 10% APS was added to the polyacrylamide solution and mixed gently by swirling the flask. To ensure the gel plates would not leak, the small plug, containing 3 ml of polyacrylamide solution and 10 µl of TEMED was first poured into the gel plates. Once the plug solidified and the plates showed no signs of leakage, the remaining solution was mixed with 40 µl of
TEMED and poured into the plates. Two well combs were inserted into the plates. The gel was allowed to polymerize for at least an hour.

Once the gels solidified, the plates were assembled in the gel apparatus containing 1X TBE. A syringe with a bent needle was used to remove trapped air bubbles along the bottom edges of the plates. A pasteur pipette was used to remove any excess urea in the wells. The gel was prerun at 400 Volts for 30 minutes at room temperature.

The DNA sample was prepared using equal volumes of synthesized DNA and 2X formamide loading buffer (900 µl of formamide and 100 ul of 10X TBE). A minimum concentration of 100 nmol of DNA sample was used in order to visualize DNA bands by UV shadowing. The DNA sample was mixed very well and placed in a 90°C water bath for three minutes. Immediately after heating, the sample was spun down and placed on ice. DNA was loaded onto the gel, alongside Bromophenol Blue as a marker. The gel was run for approximately seven hours at 400 Volts at room temperature. During this time, the temperature of the plates should be monitored in the case of an overheated system. The gel can be stopped when the marker has traveled three fourths of the way down the gel. The gel was removed from the plates.
carefully and visualized using UV shadowing. The thickest and usually the slowest band, assumed to be the desired oligomer, was excised with a clean razor.

The desired oligomer can now be extracted from the gel matrix by electroelution using the Whatman Elutrap. This electroelution apparatus consists of horizontal electrophoresis chambers in which the DNA can be placed. The ends of the chamber were sealed with a BT1 membrane that prevents DNA from leaving the chambers. At the positive end of the chamber, an additional membrane, BT2, is placed interior to BT1 to form a well where the recovered DNA sample can be collected. The electroeluter was filled with 1X TBE. Once the electroeluter apparatus was ready, the DNA sample was prepared. The excised gel band containing the desired DNA was diced into small pieces to maximize the surface area and allow for efficient recovery of DNA. These pieces were placed into the reservoir of the chamber and the electroeluter was run at 100 V at room temperature. After two hours, the flow of current was reversed for one minute to release any DNA that was trapped in the BT1 membrane. The entire volume of eluted DNA was collected from the well. At least five fractions were collected every two hours. The DNA samples were concentrated using a 3,500 MWCO spin column at 4°C. Residual acrylamide was
removed from the sample via ethanol precipitation and dialysis against water overnight.

2.2 Oligonucleotide Annealing

2.2.1 Theory of Oligonucleotide Annealing

Isolated oligonucleotides were annealed to form duplexes and junctions used for *in vitro* experiments. Duplexes and junctions were formed using equimolar amounts of corresponding single strands to prevent unwanted single stranded impurities. The process of annealing involves the heating and slow cooling of complementary DNA single strands. The heating process breaks hydrogen bonds between base-pairs and prevents the formation of secondary structures in single strands. It is important to heat the DNA sample slightly above the melting point of the desired construct to ensure removal of any unwanted products. Once heated for several hours, the DNA sample is slow cooled to room temperature to allow the annealing process to proceed enthalpically. Over this long period of time, unpaired single strands hydrogen bond perfectly with their complementary strands to form the
most energetically favorable construct. Two single strands are used to anneal duplex DNA, and four are used to anneal junctions.

### 2.2.2 Oligonucleotide Annealing

Table 1: DNA substrates used for binding assays. IHF consensus sequence in red.

<table>
<thead>
<tr>
<th>DNA Structure</th>
<th>Sequences 5’ – 3’</th>
<th>Extinction Coefficient</th>
</tr>
</thead>
</table>
| H134 34mer IHF sequence | *HA:* TAT GCA GTC ACT ATG AAT CAA CTA CTT AGA TGG T  
                         | *HB:* ACC ATC TAA GTA GTT GAT TCA TAG TGA CTG CAT A | 542880 |
| JH1 34mer IHF sequence | *JXihf:* CCA GAC TGC AGT AAT CAA CTA CTT AGG ACG GAG G  
                          | *JBihf:* CCT CCG TCC TAA GTA GTG GCT GCT ACC GGA AGG G  
                          | *JRihf:* CCT TCA ACC ACC GCT CAT GAT TAC TGC AGT CTG G  
                          | *JHihf:* CCC TTC CGG TAG CAG CCT GAG CGG TGG TTG AAG G | 1027520 |
DNA duplexes and junctions were formed using equal molar amounts of corresponding single strands and annealing buffer (10 mM Tris-HCl pH 7.6, 0.1 mM EDTA pH 7.4, 300 mM NaCl) that contains high salt concentrations (Table 1). The DNA constructs were annealed in an eppendorf tube wrapped with parafilm and secured with a cap to ensure that water does not escape or evaporate from the tube. The sample was immersed into a pre-heated 70°C large water bath. The sample was kept at the constant temperature of 70°C for four hours. The sample was then slowly cooled to room temperature overnight by turning the water bath off and allowing it to equilibrate back to room temperature.

DNA annealing efficiency was assessed on a 6.5% native polyacrylamide gel and visualized using GE Healthcare’s Typhoon Trio. In preparation of making the gel, a gel apparatus was set up with 10 cm by 8 cm plates and 1.5 mm spaces. The gel solution was poured between the plates and the gel comb was inserted into the solution. The gel was allowed to polymerize for approximately two hours. The gel was then set up in the gel-running apparatus and pre-run at 150 V for 30 minutes. After the gel was pre-run, samples with 2% ficoll were loaded onto the gel alongside the marker. Immediately after loading, to avoid diffusion of the DNA, the voltage was
changed to 110 V and run for 1 hour, or until the dye front of the marker reaches \( \frac{3}{4} \) of
the plate. After the gel was run, the gel was stained with Sybr Green I dye and
scanned using GE Healthcare’s Typhoon Trio. In the scanner program, the
fluorescence mode was selected with the acquisition PMT at 400. The scan
wavelength was in the blue region with an excitation of 450 and emission of 520 nm.
The gel was scanned to 200 microns and analyzed with ImageQuant TL. Quantifying
the band density of the samples assessed the annealing efficiency.

The integrity of the junctions was confirmed on a native gel by densitometry.
2.3 Protein Purification

2.3.1 Theory of Protein Purification

To isolate our protein of interest, IHF, extensive protein purification procedures were performed to achieve the highest yield and activity. *E. coli* cells that have been transformed with an IHF overexpression vector were grown and induced to overproduce IHF protein. Upon reaching the middle to late log phase of the bacterial growth, the cells are lysed. To isolate IHF from the other cytosolic components, a series of purification steps, including Polymin P precipitation, ammonium sulfate precipitation, and affinity exchange column chromatography were used. Polymin P precipitation separates IHF from nucleic acids of the cell. Ammonium sulfate precipitation alters the solubility of proteins to selectively extract IHF. Finally, affinity exchange column chromatography isolates IHF from many other proteins based on its affinity for the column material in varying concentrations of salt (Filutowicz, Grimek, & Appelt, 1994).
2.3.2 Growth and Preparation of IHF for Purification

IHF was purified using the *E. coli* strain 1084B, which had been transformed with an IHF overexpression vector. LB containing antibiotic ampicillin was streaked with a stab from a deep freeze glycerol stock at 70°C and incubated overnight at 37°C. A single colony was isolated and re-streaked on a new LB-Amp plate and incubated overnight at 37°C. To grow a starter culture, a single colony from the second LB-AMP plate was isolated and added to 10 mL of autoclaved LB medium solution with 50 µg/ml of sterile ampicillin. The starter culture was incubated overnight at 37°C, shaking at 220 RPM.

Ten ml of the starter culture was transferred to one liter of LB medium containing 50 µg/ml of ampicillin and incubated for five hours at 37°C. After five hours, the optical density or absorbance of the culture was monitored at 650 nM every hour, until an optical density of 0.9 was reached. This optical density signifies the beginning of the exponential stage of growth. At this point, the culture was induced with 0.04 mM of IPTG (isopropyl-beta-D-thiogalactopyranoside), which is a highly stable synthetic analog of lactose that inactivates the lac repressor and induces the expression of cloned genes under control of the lac operon. The optical density at
650nm was continually monitored every hour until an optical density of 2.6 was reached. Due to the non-linear response of the instrumentation at high densities, above 1.0, the sample was diluted. The cell culture was distributed evenly among autoclaved 250 mL tubes and centrifuged at 8K RPM for 1 hour in RC5C Sorval Superspeed Centrifuge, using a Ti60-rotor at 4 °C. The supernatant was decanted and the cell paste was resuspended in Buffer A, which contains 50 mM Tris-HCl pH 7.4, 20 mM NaCl, 1.0 mM EDTA, 3.0 nM BME, and 10% glycerol. The amount of buffer that is required to resuspend the pellet was calculated by measuring the weight of the cells.

To open up the cells and separate protein from DNA, the following was added to the solution: 1/100 volume of 25 mg/mL lysozyme, ¼ volume of 4M KCl, and 1 nM PMSF. Lysozyme breaks the bacterial cell walls, potassium chloride separates or breaks bonds between protein and DNA, and PMSF was used to deactivate proteases from digesting proteins of interest after cell lysis. This mixture was incubated on ice for 30 minutes. To further open up cells, high-pressure homogenization was performed. The sample was centrifuged at 5K RPM for 1 hour at 4°C to separate the supernatant from the pellet. The supernatant now contains the desired protein. The
supernatant was dialyzed four times against 4 L of Buffer A to remove the high concentration of KCl that was previously used to separate and break bonds between protein and DNA. To further isolate protein from DNA, Polymin P Precipitation was carried out. Polymin P is used to precipitate nucleic acids and separate DNA from protein. The dialyzed supernatants were combined, stirred on ice, and treated with a total concentration of 0.5% Polymin P over 20 minutes such that one mL was added every five minutes. After all the Polymin P was added, the sample was stirred for an additional 20 minutes. After the solution was well mixed, the sample was centrifuged at 5K RPM for 40 minutes. The first Polymin P supernatant contains the protein. The pellet, contains a mixture of both protein and DNA. To retrieve the remaining protein, the pellet was resuspended in 35 mL of Buffer A with 0.5M NaCl and centrifuged at 5K RPM for 30 minutes to break up the pellet. The resulting supernatant and pellet were obtained. The supernatant now contains the desired protein. The supernatant from first Polymin P procedure and the supernatant from the NaCl wash were combined and preceded to the ammonium sulfate precipitation. Ammonium sulfate granules were ground in a mortar and pestle until fine and powdery. An initial 0.242 g/ml of ammonium sulfate was added to the supernatant over 20 minutes while
stirring. After adding all of the required ammonium sulfate, the supernatant was stirred for an additional 30 minutes to ensure that all of the ammonium sulfate was mixed well. The mixture was centrifuged at 5K RPM for 40 minutes. The supernatant now contains the protein, since IHF is soluble at low concentrations of ammonium sulfate. Additional ammonium sulfate was added, 0.322 g/mL, to a final ratio of 0.564 g/mL slowly over a period of 20 minutes. The sample was allowed to mix for an additional 1.5 hours on ice. The mixture was centrifuged at 5K RPM for 40 minutes. The pellet now contains the protein, since the high concentration of ammonia precipitates IHF.

An aliquot sample was taken at each stage, including both supernatants and pellets samples. These samples were run through an SDS-PAGE gel to ensure that IHF was extracted at corresponding steps, and maintained in the final ammonium sulfate pellet, and that it was not lost in previous steps. SDS-PAGE, polyacrylamide gel electrophoresis, denatures the protein such that the proteins are separated as it travels through the gel. The pellet of the ammonium sulfate treatment was resuspended in 5 mL of Buffer A and dialyzed four times against 4 L of Buffer A to
remove all salts that may interfere with further purification procedures. After dialysis, the sample was centrifuged at 5K RPM to separate larger molecules from the protein.

### 2.3.3 Protein Purification

The fully automated AKTA purification system and a pre-packed HiTrap Heparin HP column was used for purification. The column was first equilibrated with 10 column volumes of Buffer A at a flow rate of 1.0ml/min. The protein was applied onto the column. The column was then washed with 10 column volumes of Buffer A (50 mM NaCl) to rid the column of any molecules that did not attach. A 20-column-volume gradient of Buffer A containing 0.1 M NaCl to Buffer A containing 1.7M NaCl was applied at a flow rate of 1ml/min. The elute was collected in 1 ml fractions and later accessed on a SDS-PAGE gel to determine fractions that contained pure IHF. These fractions were pooled together and dialyzed into Buffer A (without BME) overnight to rid of the high salt concentrations that were required to elute the protein. The dialyzed sample was then concentrated using a stir concentrator. Once a small enough volume was obtained, the sample was centrifuged at 7K RPM for 15 minutes to rid of any aggregates that may have formed.
To verify that the protein did not contain any contaminants, a sample of high concentration of IHF was run on a SDS-PAGE gel again and silver-stained to observe for signs of degradation.

Once pure protein was obtained, the protein concentration was measured via Bradford Protein Assay and verified with Beckman UV-Vis spectroscopy. In the Bradford Assay, pre-determined protein standards were compared with the desired protein. It is important to note that the absorbance reading for protein strands are only accurate between 0.2 and 0.8. In addition, the assay is only accurate if the linear fit generated by the standard measurements results in a $R^2$ value greater than 0.995.

Purified IHF were aliquotted and stored in the -20°C freezer in 50% glycerol. A small aliquot for immediate usage was stored in the 4°C fridge in binding buffer containing 10% glycerol. This glycerol amount is crucial to maintain the activity of IHF and prevent IHF from sticking to the eppendorfs.

The activity of the protein is accessed using the gel mobility shift assay with a well-characterized DNA substrate – duplex H134.
2.3.4 Protein Assessment by Gel Electrophoresis

Gel electrophoresis, specifically SDS-PAGE, was used to assess the protein expression from purification procedures. The top part of the gel corresponds to the stacking gel, which is a protein-denaturing gel that separates protein polypeptide chains based on size. Smaller polypeptides migrate faster down the gel than larger chains. The bottom part of the gel corresponds to the running gel, or resolving gel. Since we know the molecular weight of the protein, we can determine where it appears on the gel and clear bands would indicate pure protein.

The gel-making apparatus consists of gel plates with a width of 10 cm and a height of 8 cm, and 1.5 mm spacers were used. The running solution was poured into the apparatus first. Approximately 1 ml of isopropanol was pipetted on top of each running solution to attain a straight surface and remove bubbles formed when the running solution was poured. The running solution was allowed 30 minutes for polymerization. Once the running gel polymerized, the isopropanol was poured out and the gel was allowed to dry for a few minutes. The stacking solution was added until it filled to the top of apparatus and the gel comb was inserted into the stacking
solution between the gel plates. The stacking solution was allowed approximately two hours to fully polymerize.

Protein growth, purification, and isolation samples were prepared for the electrophoresis. 16 µl of the protein sample was combined with 4 µl of the 5X gel-loading buffer. These samples were mixed very well and boiled at 90°C for five minutes. The samples were mixed again and loaded on the gel along with a Kaleidoscope Precision Plus Protein Dual Color Standards marker for polypeptide length reference. Gels were run at room temperature in 1X SDS buffer at 100V until the marker reached the very bottom of the gel. Gels stained with Coomassie Brilliant Blue dye and incubated for 45 minutes on a rocking platform. Coomassie Brilliant Blue stain visualizes the protein by forming complexes with proteins via non-covalent electrostatic interactions with the amine groups. The gel was destained overnight to rid excess dye. Gels were visualized using visible light. Protein bands were assessed by comparing their sizes in reference to the bands on the marker, along side a known pure sample of IHF protein.
2.4 Binding Assays in Gel: Gel Mobility Shift Assay (GMSA)

2.4.1 Theory of Gel Mobility Shift Assay

Gel Mobility Shift Assays (GMSA), also known as gel electrophoresis mobility shift assay (EMSA), was used to detect interactions between IHF and DNA substrates (Hellman & Fried, 2007). GMSA allows for the visualization of DNA in a non-denaturing, native polyacrylamide gel matrix. Similar to that of the SDS-PAGE electrophoresis, GMSA gel also separates molecules based on its weight, charge, and shape, as the system is subjected to electrophoresis. Because the DNA backbone is negatively charged due to the phosphate functional groups, the DNA will travel towards the positive electrode located at the bottom of the gel. Smaller DNA molecules will migrate further down while larger molecules will not migrate as quickly. Similarly, DNA as part of a complex with protein are also be subjected to electrophoresis in the same manner. Free DNA, without any protein, would travel the fastest, and run predictably lower than DNA in complex with IHF. The difference in weight produces the separation, and therefore shift of bands. These bands are fluorescently visualized by a dye that binds to DNA and quantified by its pixel volume. Thus, strategically combining and monitoring solutions of varying
concentrations of IHF to a constant concentration of DNA yields a binding curve to describe the binding interactions between IHF and DNA substrates (Carey, 1991).

Binding interactions and binding affinities are described by the equilibrium dissociation constant, $K_d$, which evaluates the strength of binding of protein to its ligand. The dissociation constant can be interpreted as the protein concentration at which half of the DNA is bound. Thus, the binding constant can be determined by quantifying the amount of free DNA as a function of protein concentration in a one step process.

$$DNA + Protein \rightleftharpoons DNA \times Protein$$

$$K_D = \frac{[DNA][Protein]}{[DNA \times Protein]}$$

$$f_f = \frac{[DNA]}{[DNA]_T} = \frac{[DNA]_T - [DNA \times Protein]}{[DNA]_T} = 1 - \frac{[DNA \times Protein]}{[DNA]_T}$$

$$[DNA]_T = [DNA] + [DNA \times Protein] = f_f [DNA]_T + [DNA \times Protein]$$

$$[DNA \times Protein] = (1 - f_f) [DNA]_T$$

$$[Protein] = [Protein]_T - [DNA \times Protein] = [Protein]_T - (1 - f_f) [DNA]_T$$

$$K_D = \frac{[f_f [DNA]_T ([Protein]_T - (1 - f_f) [DNA]_T))}{(1 - f_f) [DNA]_T}$$

$$f_f = -\frac{(K_D + [Protein]_T - [DNA]_T + \sqrt{K_D + [Protein]_T - ([DNA]_T)^2 - 4 [DNA]_T K_D}}{2 [DNA]_T}$$

Equation 1: Determination of the dissociation constant, $K_d$, from the molar fraction of DNA bound, $f_f$ (Buzovetsky).
In addition to characterizing the binding interactions between IHF and DNA substrates, this technique was used to assess activity of freshly isolated and purified IHF.

2.4.2 Gel Mobility Shift Assay

A 6.5% polyacrylamide native gel was used for gel binding assays. Upon the addition of each ingredient, the solution was gently mixed to avoid bubbles. Before the addition of TEMED, the gel apparatus was set up with plates of 10 cm by 8 cm with 1.5 mm spacers. Once TEMED was added, the solution was mixed very well and quickly poured into the assembled gel apparatus. A 1.5 mm 15 well comb was inserted into the gel, and allowed to polymerize at room temperature for least one hour. Once the gel solidified, the gel was pre-run at 100 V in 1X TBE at 4°C for a minimum of 20 minutes. In the meantime, solutions of protein and DNA were combined.

Protein-DNA complexes were prepared by adding increasing concentrations of protein to a constant concentration of DNA, 10 nmol. A spreadsheet was used to establish the amount of protein and DNA that was required to perform the titration in
order to determine the binding constant (Appendix 6.3.1 GMSA Worksheet). The samples were prepared in GMSA Binding Buffer specific to IHF, which includes, 5 mM Tris-HCl pH 7.5, 0.1 mM EDTA, 70 mM KCl, and 0.5% ethylene glycol. Ficoll was added to each loading sample to a final concentration of 2% to ensure that the DNA did not diffuse out of the wells upon loading. Once the samples were prepared, they were incubated for one hour at 4°C. The marker was prepared with 16 µl ddH2O, 2 µl gel loading dye, 0.5 µl DNA size marker, and 2 µl 20% Ficoll. Efficient loading techniques were exercised to shorten the amount of time of DNA freely floating in the wells. Often, the samples were loaded while the current was on. Additional care was taken to ensure exact amounts of corresponding protein and DNA were loaded because quantification and analysis depended highly on the amount of DNA in each sample. The gel was run at 100 V for approximately one hour, or until the dye front reached ¾ of the gel. Careful consideration was taken in determining the buffer and voltage used because the heat generated by the system may denature DNA substrates, and create smears in the gels that would make it difficult for quantification and analysis. For DNA substrates that were less stable, the voltage was reduced to 80 V.
After the gel was run, the gel can be treated in order to highlight the DNA bands. The gel was removed from the apparatus and immediately stained with a fluorescent dye, Sybr Green I (10 µl Sybr Green dye and 70 ml of 1X TBE buffer) for 20 minutes at room temperature. Sybr Green is diluted in TBE buffer because Sybr Green reagent is pH sensitive and is less stable in water. To ensure maximal staining sensitivity, the staining container was protected from light by covering it with aluminum foil and placed on a shaker. After 20 minutes, the gel was washed thoroughly with ddH2O.

Once the staining was complete, the gel can be visualized and analyzed. The gel was scanned using the Typhoon PhosphorImager 840, set to the fluorescence mode with acquisition PMT at 400, resolution of 100 microns, and scan wavelength in the blue region (488 nm). The software ImageQuant 5.2 was used to quantify the intensity of the DNA bands, stained by Sybr Green, in terms of pixel volume. Assuming the intensity of the DNA bands correlate with the concentration of DNA, the pixel volume, though in arbitrary units, reflect the amount of DNA in each band. Boxes of equal sizes were drawn around the bands to capture the bands representing free DNA and bound DNA. The resulting pixel volumes, or DNA intensity bands,
were plotted against corresponding protein concentrations, and fitted to a 1:1 binding model to calculate a dissociation constant using the analysis software Origin v 9.0.

2.5 Binding Assays in Solution: Fluorescence Spectroscopy

2.5.1 Theory of Steady-State Fluorescence Spectroscopy

Fluorescence results from the relaxation of molecules to the ground state through emission of a photon, after being electrically excited by a particular wavelength of light (Figure 9). The fluorescence process consists of three phases: excitation, excited state, and emission, which all occur on different timescales. The excitation of a molecule, occurring on the scale of femtoseconds, is the absorption of light at a particular wavelength, specific to the molecule. When an incoming photon of light interacts with a molecule, the molecule absorbs the energy, thereby promoting the valence electrons of the molecule from their ground state to higher energy level orbitals, or into the excited state. In this excited state, the molecule undergoes vibrational and conformational relaxation from a higher energy level to the lowest energy level on the scale of picoseconds. In the final phase, the emission of light,
usually at a longer wavelength, is the return of the molecule from excited singlet states to the ground state. This process occurs on a relatively longer timescale of nanoseconds, yet still very rapidly and is comparable to the nanoscale lifetime of fluorophores. The fluorescence process is thus the re-emission of light at a longer wavelength after the molecular absorption of light energy at a shorter wavelength (Boyer, 1993).

The phenomenon of fluorescence depends on fluorescent compounds, also known as fluorophores, which typically contain aromatic groups. Fluorophores are molecules that have the ability to absorb energy of a specific wavelength and re-emit energy at a different wavelength. Examples of fluorophores include Fluorescein, Tetramethylrhodamine, 6-MI, and 6-MAP. Each of these molecules have unique absorption and emission profiles due to differences in the chemical construct and structure. Fluorescence allows for the highly sensitive detection of these profiles, and therefore characterizations of these molecules. Changes in the local environment of the fluorophore would result in changes in the fluorescence intensity of the signal. By attaching fluorophores to a system of interest, such as a DNA molecule, any changes to the DNA molecule can be detected by monitoring the fluorescence intensity of the
attached fluorophore. Thus, the phenomenon of interactions between light and matter, has not only provided a tool for the characterization of binding, structure, and environment of molecules, but has also formed the basis for the expansive fields of fluorescence spectroscopy (Lakowicz, 2006).

Figure 9: A Jablonski diagram illustrating the energy levels involved in absorption and emission of light in a fluorescent molecule (Lakowicz, 2006).

The intensity of fluorescence of a particular molecule is closely monitored using an intricate instrument - a spectrofluorometer. The setup of a spectrofluorometer contains a light source, two monochromators, sample holders, a detector, and other components to enhance optical detection. Light sources, such as
xenon lamps, provide high intensity excitation at all wavelengths. The two monochromators enable the selection of excitation and emission wavelengths that pass through the sample sitting in the sample holder. The detector, or photomultiplier tubes, detects and records the fluorescence emitted by the molecule. The fluorescence signal is quantified and represented in a graphical form of spectra (Lakowicz, 2006).

Figure 10: Schematic diagram of a spectrofluorometer (Lakowicz, 2006).
One type of fluorescence measurement is a steady-state measurement, which involves a continuous beam of light at which the intensity or emission spectrum is recorded. Steady-state observations performed in solution report on equilibrium conditions. Because steady-state emission is on the nanosecond timescale, equilibrium can be reached almost immediately upon exposure to exciting light.

2.5.2 Labeling of DNA with External Fluorescent Probes

In order to perform fluorescence experiments in solution, JH1 was labeled with external fluorescent probes. For binding characterizations, one strand of the junction was modified to allow for the association with a fluorescent dye. The modified single strand, known as the linker strand, contains a 5’ amino modifier C6 linker. Fluorescent probes containing active succinimidyl esters, such as carboxyfluorescein (FAM) or carboxytetramethylrhodamine (TAMRA), were conjugated to the primary amine of the modified oligomer via formation of covalent amide bonds (Wilson & Kool, 2006).
The Molecular Probes Hand book protocol optimized for labeling of 100 µg of an 5’-amine-modified oligonucleotide was followed to label JXihfL strands with FAM, and JRihfL strands and JBihfL strands with TAMRA.

For the labeling reaction, 200 µg fluorescent dye powder, either FAM or TAMRA, were weighed and dissolved immediately into 14 µl anhydrous dimethylsulfoxide (DMSO) in an eppendorf tube. Once FAM or TAMRA were completely dissolved, 100 µg purified linker strand, 7 µl ddH2O, and 75 µL 0.1 M (0.038 g/ml) sodium tetraborate pH 8.5 were added to the eppendorf. The vial was
tapped gently to allow the reagents to mix and covered in aluminum foil to shield away from any light. The reaction was allowed to incubate on a rotating shaker at low speed for at least six hours at room temperature. For the first two hours, the vial was tapped gently every half hour to ensure the reaction remained well mixed.

Following the reaction, unincorporated dye was removed by multiple rounds of ethanol precipitation and washing. Removal of any excess dye was especially crucial. 1/10 volume of 3 M NaCl and 2.5 volumes of cold absolute ethanol was added to the reaction mixture. The vial was mixed very well and was incubated in the -20°C freezer for a minimum of three hours. After incubation, the mixture was centrifuged at 12g for 45 minutes in the cold room. For low concentrations of DNA, longer centrifugation time of 45 minutes, or more, is highly recommended to recover the maximum amount of DNA. Loss of sample usually occurs if the centrifugation is not long enough. The supernatant, containing the excess dye, was carefully removed. In order to remove the high concentration of salt, the pellet was rinsed twice with cold 70% ethanol. In 70% ethanol, DNA should still not be soluble, so it should precipitate out, whereas the salt is more soluble and should go into solution. After applying 70% ethanol, the solution was centrifuged at 12,000X g for 15 minutes in the cold room.
The supernatant was removed and the final pellet was allowed to air dry for 5 to 10 minutes. The dry pellet is dissolved in water. Upon completion of the 70% ethanol wash, the DNA sample should be clean of excess dye and salt.

The amount of excess dye was assessed to ensure successful removal of all excess dye. The supernatant from ethanol precipitation was checked on the FluoroMax. Minimal fluorescence activity of the supernatant indicated the successful removal of excess dye. If high fluorescence activity was observed, then additional rounds of ethanol precipitation were repeated.

Sometimes, excess dye was removed via water washes in 3,500 molecular weight cut-off (MWCO) VivaSpin columns following ethanol precipitation.

To further ensure the purity of the DNA sample, dialysis against water overnight may help remove residual small particles.

Once JXihf-FAM, JBihf-TAMRA, and JRihf-TAMRA singly-labeled strands were acquired, the concentrations of the singly-labeled strands were determined. Using the Beer-Lambert law, the approximate number of dye molecules per DNA molecules can be calculated,
\[ A = \varepsilon \times l \times [DNA] \]

where \( A \) is the absorption at the excitation maxima of the DNA, extinction coefficient is the measure of capacity in which DNA absorbs light, and path length is the length in centimeters of the cuvette.

Because dyes also contribute to the absorbance at 260 nm, a correction factor was used to calculate an accurate DNA absorbance reading. The correction factor was experimentally obtained by comparing the following relationship,

\[ \text{Correction Factor} = \frac{A_{260\text{free dye}}}{A_{\text{max em free dye}}} \]

where \( A_{260\text{free dye}} \) is the absorbance of the free dye measured at 260 nm, and \( A_{\text{max em free dye}} \) is the absorbance measured at the peak excitation wavelength of the free fluorophore in solution – FAM at 494 nm and TAMRA at 555 nm. The DNA absorbance at 260 nm can be corrected to account for the additional absorbance of the fluorophore

\[ A_{260\text{corrected}} = A_{260} - (A_{\text{max em free dye}} \times CF_{\text{max free dye}}) \]

By rearranging and substituting the corrected absorbance expression into the Beer-Lambert Law, the total concentration of the single-stranded DNA can be calculated,

\[ [\text{labeled single strand}] = \frac{A_{260} - (A_{\text{max free dye}} \times CF_{\text{max free dye}})}{\varepsilon \times l} \]
where \( E \) is the extinction coefficient of the DNA single strand.

Once the corrected concentration of the labeled single strand was determined, the singly-labeled junctions were annealed. In order to determine how much of the junction was labeled, the degree of labeling of the junction was determined.

Optimization of the labeling efficiency is important to achieve desired results in fluorescence experiments. The efficiency of the labeling reaction can be quantified by comparing the concentration of labeled DNA relative to the total DNA concentration.

The Beer-Lambert law can also be used to determine the concentration of the labeled DNA,

\[
[Labeled \ DNA] = \frac{A_{dye}}{\varepsilon_{dye} \times l}
\]

where \( \varepsilon_{dye} \) is the extinction coefficient of the fluorophore.

Now the concentration of the labeled DNA can be compared with the total amount of DNA

\[
Labeling \ Efficiency = \frac{Concentration \ of \ Labeled \ DNA}{Total \ Concentration \ of \ DNA} \times 100\%
\]
2.5.3 Theory of Anisotropy, Intensity, Stoichiometry

Fluorescence anisotropy measurements provide information about the size, shape, orientation, and flexibility of molecules. In solution, unbound DNA is tumbling, or freely rotating and has a high average rotational rate. On the contrary, bound DNA, due to its increased size, exhibits slower tumbling dynamics. By detecting the differences in the rotational dynamics of macromolecules, properties such as binding affinity of protein-DNA interactions can be determined. In titration experiments, as more protein is titrated into a sample containing DNA, more DNA is bound. The increasing concentration of protein-DNA complexes results in a change – usually a decrease – in the average rotational movement. Thus, fluorescence anisotropy measurements detect changes in the average rotational rate which can be used to characterize protein binding to DNA substrates (Lakowicz, 2006).

The average rotational rate can be detected by monitoring the changes between excited and emitted polarization. When a molecule, such as a fluorophore, is excited with polarized light, it can also emit partially polarized light. However, when exposed to polarized light, not all fluorophores absorb the polarized light. Only fluorophores that have their absorption transition moment oriented parallel to the
electric vector of the incident light are preferentially excited. This phenomenon is called photoselection, which results in partially polarized fluorescent emission. The extent of emission polarization can be described in terms of anisotropy, 

\[ r = \frac{I_{\text{parallel}} - GI_{\text{perpendicular}}}{I_{\text{parallel}} + 2GI_{\text{perpendicular}}} \]

where \( I_{\text{parallel}} \) is the observed intensity when the emission polarizer is oriented parallel to polarized excitation, \( I_{\text{perpendicular}} \) is the observed intensity when the emission polarizer is oriented perpendicular to the excitation polarizer, and \( G \) is the correction factor discussed below (Lakowicz, 2006).

In a typical experimental set-up, the path of the light follows an L-shaped format. The excitation light, generated by the light source, propagates along the x-axis to the sample, and the emission is detected along the y-axis. A vertical polarizer is placed between the excitation source, such that the sample can be excited with vertically polarized light that is oriented parallel to the vertical, or z-axis. In the excited state, and before the emitted polarized light is captured by the detector, the excited molecule experiences rotational movements, or angular displacements. These movements reorient the axis of the emitted polarized light. Because DNA oligomers and proteins are relatively large molecules, and rotate within the fluorescence lifetime
in the nanosecond timescale, the angular displacements can be detected. This angular displacement can be calculated by taking the difference between the vertically polarized emission, $I_{\text{parallel}}$, and horizontally polarized emission, $I_{\text{perpendicular}}$. The difference between the parallel and perpendicular polarizations is normalized to the total intensity, $I_{\text{total}} = I_{\text{parallel}} + 2I_{\text{perpendicular}}$, which is the sum of intensities along the x, y, and z axes.

Usually, a correction factor, the G-factor, is calculated to account for the differences in relative efficiency of vertically polarized and horizontally polarized light traveling through the monochromator. The G-factor is measured using horizontally polarized excitation. In this orientation, the vertically polarized light and horizontally polarized light should be equivalent and proportional to $I_{\text{perpendicular}}$. Any difference between $I_{\text{parallel}}$ and $I_{\text{perpendicular}}$ would be due to the detection instrumentation.

$$G \text{ factor} = \frac{I_{HV}}{I_{HH}}$$

The anisotropy equation can be adjusted to include the G factor, 

$$r = \frac{I_{VV} - G I_{VH}}{I_{VV} + 2GI_{VH}}$$
where $I_{VV}$ is the same as $I_{\text{parallel}}$ and $I_{VH}$ is the same as $I_{\text{perpendicular}}$. The first subscript corresponds to the polarization of the excitation and the second subscript corresponds to the polarization of the emission.

By plotting the anisotropy values against protein concentration, a binding curve can be generated. Upon titration of increasing amounts of protein into a DNA sample, protein-DNA complexes are formed, which are bulkier, and exhibit slower rotational movement. This slower movement leads to more polarized light, increasing the ratio of polarized components relative to the total intensity. Thus, the increase in anisotropy is proportional to the increasing formation of protein-DNA complexes.

Fluorescence intensity, or the signal of fluorescence, measures the amount of photons emitted by the sample molecules after excitation. The fluorescence intensity observed depends on a variety of factors, such as the fluorophore used and the changes in size and conformation of the DNA with the fluorophore attached. Thus, the binding event of protein to DNA can be characterized using fluorescence intensity by quantifying the signal of the fluorophore. Fluorescence intensity changes can be detected upon the addition of protein to a sample containing DNA. The increased average number of protein-DNA complexes formed can result in changes in size and
conformation of the DNA molecule. Thus, fluorescence intensity can be used to
report on protein binding to DNA. Similar to gel experiments, fluorescence intensity
values recorded after titration can be plotted against increasing protein concentration
to generate a binding curve.

Stoichiometry of binding measures the number of proteins bound to DNA, or
the molar ratio of protein to DNA in a complex. Stoichiometric conditions require
high concentrations of DNA, at least ten-fold higher than the dissociation constant, to
ensure that the binding affinity does not contribute to the stoichiometric results. In
addition, titrations of at least 3:1 molar ratio of protein-DNA complex is required to
ensure saturation of protein-DNA binding.

2.5.4 Solution Binding Fluorescence Assays: Intensity, Anisotropy, Stoichiometry

Solution steady-state fluorescence experiments by measuring intensity and
anisotropy were performed using the Horiba Jobin Yvon Fluoromax-4
spectrofluorometer. IHF binding characterizations with fluorescently-labeled DNA
four-way junctions were performed in the IHF binding buffer (same buffer used for
GMSA assays). The experiment begins with a sample of free DNA, to which increasing concentrations of protein are titrated to generate a binding curve. A spreadsheet (Appendix 6.3.2 Fluorescence Intensity Worksheet), was used to establish the amount of protein required to perform the titration.

Sample preparation for steady-state fluorescence solution experiments were optimized to achieve high detection of fluorescence intensity and maintain a high signal to noise ratio. DNA samples fluorescently-labeled with FAM or TAMRA were prepared in 5 nm by 5 nm glass cuvettes with a starting volume of 600 µl. The samples were placed in the sample chamber holder set to 12°C. During the experiment, the sample chamber was purged with nitrogen to reduce condensation. Fluorometer slits were set to 5 nm bandpass in excitation and emission.

Prior to each measurement, samples were incubated, while stirring, for five minutes. Different settings were used to detect fluorescence intensity of fluorescent probes used. Fluorescein was excited at 490 nm and detected at its emission maximum at 520 nm. TAMRA was excited at 555 nm and detected at its emission maximum at 580 nm. For anisotropy measurements, polarizers set at VH (0°, 90°), VM (0°, 54.7°), and VV (0°, 0°) were used.
Upon completion of the solution experiment, the fluorescence data was analyzed using Origin v. 9.0. A binding curve was plotted, comparing the fluorescence intensity as a function of increasing protein concentration and the binding constant was determined.

2.6 Förster Resonance Energy Transfer (FRET)

2.6.1 Theory of Förster Resonance Energy Transfer

Förster resonance energy transfer is another application of fluorescence that reveals the distance-dependent interaction between two fluorophores by monitoring the transfer of energy from a donor molecule to a nearby acceptor molecule. The fluorophores used to label a macromolecule are referred to as the donor fluorophore and acceptor fluorophore. Energy transfer occurs when the emission spectrum of a donor molecule overlaps with the excitation spectrum of the acceptor molecule. This process occurs non-radiatively, such that in the transfer, the donor does not actually emit a photon and the acceptor does not absorb a photon - only the energy is transferred. Detection of energy transfer is an important tool for making spatial
measurements and examining the changes in molecular proximity on the scale of nanometers, comparable to the dimensions of biological molecules.

In the process of FRET, a donor fluorophore absorbs energy upon excitation and transfers this excitation energy to a nearby acceptor. As a result of the transfer, the donor fluorophore experiences a loss of energy, while the acceptor fluorophore experiences a gain in energy. The efficiency of the energy transfer, \( E \), can be determined based on the \((\text{Ratio})_D\), which is the ratio of the extracted donor fluorescence intensity in the presence \( (F_{DA}) \) and the absence \( (F_D) \) of the acceptor:

\[
E = 1 - \frac{F_{DA}}{F_D}
\]

The donor emission is extracted from the doubly-labeled DNA and compared with the donor emission from the donor-only labeled DNA. Incomplete labeling of the DNA samples can be corrected by accounting for the efficiency of labeling of the acceptor on the doubly-labeled DNA \((a^+)\):

\[
E = \left( 1 - \frac{F_{DA}}{F_D} \right) \frac{1}{a^+}
\]

Another method to determine the energy transfer is based on the ratio of increased acceptor fluorescence to directly excited acceptor fluorescence. This ratio is defined as \((\text{Ratio})_A\):
\[(Ratio)_A = \frac{F_{em}^{DA}(v_1, v')}{F_{em}^{A}(v_2, v'')}\]

where \(F_{em}^{DA}(v_1, v')\) represents the extracted donor of acceptor emission spectra excited by the donor at \(\lambda_{max}(v')\), and \(F_{em}^{A}(v_2, v'')\) represents the emission of acceptor excited by the acceptor at \(\lambda_{max}(v'')\). The efficiency of energy transfer can be calculated using \((Ratio)_A\) :

\[E = \left[ (Ratio)_A \times \left( \frac{a_1^+}{a_1^+} \right) - 1 \right] \times \left[ \frac{\varepsilon^A(v')}{\varepsilon^D(v')} \right] \times \frac{1}{d^+}\]

where \(\varepsilon^A(v')\) and \(\varepsilon^D(v')\) are extinction coefficients of the donor and acceptor at the donor excitation wavelength, \(d^+\) is the fractional labeling of the donor fluorophore on the doubly-labeled DNA, \(a_1^+\) is the fractional labeling of acceptor fluorophore on the acceptor-only DNA, and \(a_1^+\) is the fractional labeling of acceptor fluorophore on the doubly-labeled DNA (Clegg, 1992), (Ho, 2013).

The calculated efficiency of energy transfer is directly related to the distance between the two fluorophores. The efficiency depends on the inverse sixth power of the distance between the donor and acceptor pair \((r)\),

\[E = \frac{R_0^6}{R_0^6 + r^6}\]

where \(R_0\) is the Förster radius at which half of the excitation energy of the donor is transferred to the acceptor fluorophore. In order words, the Förster radius is referred
to as the distance at which the efficiency of energy transfer is 50% (Vitoc & Mukerji, 2011); (Clegg, 1992).

Changes in the distance between the FRET dye pair result in changes in the efficiency of energy transfer. Thus, FRET is a high resolution tool, which provides accurate spatial measurements, and is often referred to as the “spectroscopic or molecular ruler” in biological systems.

### 2.6.2 Four-Way Junction Labeling Designs

In preparation for FRET experiments, JH1 junctions with specific labeling schemes were designed. These labeling schemes included donor only labeled, acceptor only labeled, and doubly-labeled junctions. To differentiate the two isomer conformations, Iso I and Iso II, two labeling schemes for JH1 were constructed (Figure 12). For the first labeling scheme, the 5’ end of the B strand containing a linker was labeled with TAMRA and X strand was labeled with FAM. For the labeling scheme, the R strand was labeled with TAMRA and the X strand was labeled with FAM.
Figure 12: Two labeling schemes to model the two antiparallel conformations of four-way junctions – Iso I and Iso II. In the first construct, the B strand (yellow) is labeled with TAMRA (pink star) and the X strand (blue) is labeled with FAM (orange star). In the second construct, the R strand (green) is labeled with TAMRA and the X strand is labeled with FAM. The labeling schemes are shown in the open and stacked Iso I and Iso II conformations to depict the differences in the positioning of the fluorophores.
2.6.3 Ion Binding Monitored by FRET

Ion-induced changes in junction conformation were monitored by Förster Resonance Energy Transfer. These experiments were performed as titration assays in 5 mM TRIS-HCl pH 7.6, 0.1 mM EDTA, and 0.5% ethylene glycol, with increasing potassium ion concentrations, and a fixed DNA concentration of 100 nM DNA. Direct titration methods were employed to yield greater accuracy and efficiency, while requiring smaller amounts of protein and DNA. For these assays, increasing amounts of protein were titrated into the sample in the cuvette. During the experiment, it was important to maintain a constant DNA concentration and monitor the volume increase of the sample. For the direct titration, high concentrations of protein were titrated directly into the sample in the cuvette, ensuring that the total volume of the sample did not exceed 10%. This method was performed with larger cuvettes, 5 mm by 5 mm. The larger volume size made it possible to maintain the volume change under 10% of the total volume.

Prior to the experiment, DNA samples were dialyzed against 5 mM NaCl solution to remove the high salt concentrations required for annealing. Since low salt concentrations were known to induce an open conformation in junctions, the 5 mM
NaCl concentration was chosen strategically to ensure that the junctions did not fall apart. For each experiment, three samples were required: FAM-labeled DNA (donor only), TAMRA-labeled DNA (acceptor only), and doubly-labeled DNA with both FAM and TAMRA. The doubly-labeled and acceptor only samples were excited at the donor and acceptor wavelengths, 490 nm and 555 nm. The donor only sample was excited at the donor wavelength, 490 nm. Fluorimeter slits were set at a 5 nm bandpass for excitation and emission to optimize the signal-to-noise ratio. The samples were incubated for five minutes at 12°C prior to each measurement.

2.6.4 IHF Binding Monitored by FRET

Protein-induced Forster Resonance Energy Transfer experiments were performed under the same conditions as the ion-induced FRET experiments. These experiments were performed immediately after the ion-induced FRET experiments to ensure consistency of the conformation of junction at high salt concentrations. In addition, longer incubation times of eight minutes were used to allow for equilibration at each titration point.
2.6.5 FRET Analysis

One method of determining the efficiency of energy transfer is the detection of decreasing donor fluorescence. Emission spectra were analyzed using the software GRAMS AI. The doubly-labeled spectra were corrected by subtracting the spectra of the acceptor only sample excited at the donor absorption maximum. By removing the acceptor contribution or fluorescence from the doubly-labeled spectra, only the donor fluorescence is extracted. This allows us to monitor any changes in donor fluorescence relative to the total donor fluorescence intensity. The corrected donor only and extracted donor spectra were integrated across the peak of emission, 500 nm to 540 nm for FAM. Ratio D, an indicator of the enhanced donor emission intensity resulting from FRET, is calculated (Chapter 2.6).

2.7 Time-Resolved Fluorescence Spectroscopy

2.7.1 Theory of Time-Resolved Fluorescence Spectroscopy

Another type of fluorescence measurement is the time-resolved measurement, which measures the excited state decay of a molecule to obtain information about the fluorescence lifetime, or the average time a fluorophore remains in the excited state.
immediately following excitation. The nanosecond measurement of intensity decays provides additional molecular information from fluorescence, such as presence of multiple populations of conformations or distances of a DNA structure in a heterogeneous mixture. These features are generally lost or convoluted in steady-state processes when the average of the ensemble is taken. Time-resolved measurements can be made by the time-domain method, in our case, using time-correlated single-photon counting (TCSPC). Instead of continually exciting the sample and collecting the constant emission of a steady-state system, the time-resolved fluorescence relies on pulses of light to excite the sample. To achieve resolution for this measurement, the time width of the pulse must be shorter than the decay time of the fluorophore. Due to the short timescale of fluorescence, the measurement of time-resolved emission requires sophisticated and high speed detection instrumentation (Lakowicz, 2006).

In a TCSPC setup, a laser diode is used to excite the sample with a pulsed wavelength of light. The detection rate is adjusted to approximately one photon per 100 excitation pulses to avoid biases towards shorter lifetimes. The excited state decay of the fluorophore is constructed from fluorescence photons detected by the
photomultiplier tube, where the signal is passed through a constant function
discriminator (CFD), and converted from time to voltage with the time-to-amplitude
converter (TAC). The data is recorded in the form of a histogram, which shows the
number of photons detected as a function of time and essentially describes the photon
probability distribution at time intervals between the excitation pulse and the
fluorescence pulse. There are numerous strategies for analyzing and deconvoluting
the TCSPC data. Some examples include nonlinear least squares analysis and
maximum entropy method.

\[ 2.7.2 \textbf{Time-Resolved FRET} \]

For time-resolved FRET experiments, samples of donor only and doubly
labeled samples were prepared in the same manner as for the steady-state FRET
experiments. High and low salt conditions in absence and presence of IHF were used
to detect the preferences of conformational populations of JH1. Samples were
prepared in 3 mm by 3 mm siliconized glass cuvettes with a stir bar. To optimize the
signal, DNA samples of 300 nM were prepared in a 1:1 stoichiometry with protein.
Prior to measurements, the samples were bubbled with helium and incubated in the sample holder for 10 minutes for temperature equilibration to 12°C.

The PTI Time Master instrument was used to acquire TR-FRET data. The 375 nm diode laser was used to excite the FAM label. Emission was detected at 520 nm for FAM, with a 490 nm cut-off filter. The excitation and emission slits were both set to a 15 nm bandpass. The intensity decay data were collected to 20,000 counts in the peak channel with a total width of 4,092 channels and using an emission polarizer set to the magic angle of 54.7 degrees.

The intensity decay data was modeled using the software Globals WE and FargoFit. A simple intensity decay can be described by a single exponential

$$I(t) = I_0 e^{-t/\tau}$$

where $t$ is time, $I_0$ is the intensity at $t = 0$, immediately following the excitation pulse, and $\tau$ is the lifetime in nanoseconds. To evaluate FRET exhibited in the doubly labeled sample, the donor only and doubly labeled decays were globally fit to a model of a sum of exponentials. The expression for multi-exponential model is given by the sum of individual single exponential decays

$$I(t) = \sum_i \alpha_i e^{-t/\tau}$$
where $\alpha_i$ is the pre-exponential factor that represents the amplitudes of individual components. The results were evaluated by the goodness of fit and the chi-squared parameter, $\chi^2$. Upon identification of individual components that exhibit distinct fluorescence lifetimes, the fractional contribution of the intensity is determined by

$$f_i = \frac{\alpha_i \tau_i}{\sum_j \alpha_j \tau_j}$$

The fraction population of individual components is determined by

$$f_p = \frac{\alpha_i}{\sum \alpha_j}$$

Finally, the amplitude-weighed mean lifetime can be determined for the decay.

$$\tau_{mean} = \sum_i \alpha_i \tau_i$$
2.8 DNA Four-way Junction Thermodynamics

Two melting behaviors were hypothesized for the helix to coil transition of four-way DNA junctions. The first thermodynamic model assumes that the junction melts in the “all-or-none” manner, in which the transition from a fully formed junction to random coils occurs in a single step. The second thermodynamic model assumes that the melting process of the junction structure mimics a zipper, starting with one junction arm dissociating from the rest of the junction structure, such that the transition occurs in multiple steps. Two possibilities arise from this model, the “unzipping” from the distal ends of the helical arms, or the “unzipping” from the center of the junction (Vamosi & Clegg, 2008).

2.8.1 Theory of Nucleic Acid Thermodynamics

Nucleic acid thermodynamics studies the effect of temperature on the global and local DNA structure. When DNA is exposed to high temperatures, the DNA structure undergoes the helix to coil conformational transition. This transition is the result of broken hydrogen bonds between base pairs, causing the DNA structure to be less stable and eventually fall apart into single-stranded oligonucleotides. This
process is known as DNA denaturation, or DNA melting. The temperature of melting (Tm) is defined as the temperature at which 50% of the DNA structure has converted to single-strands.

The melting temperature depends on various factors, including the length of the molecules, sequence-specific local structures, dynamics of the molecule, and other conditions, such as salt concentration and DNA concentration. In particular, the guanine-cytosine (GC) content of the DNA greatly affects the melting temperature. Guanine-cytosine base pairs are linked by three hydrogen bonds, while the adenine-thymine base pairs are linked by two hydrogen bonds. Because more energy is required to break three hydrogen bonds, as compared to two hydrogen bonds, guanine-cytosine base pairs are more stable. Thus, DNA substrates containing higher GC content will melt at a higher temperature than DNA substrates with low GC content.

The transition from an intact DNA helical structure to single-stranded coils can be measured by monitoring the absorbance of DNA at 260 nm, or the fluorescence of internal probes at the maximum emission wavelength. Absorbance melting experiments provide information about the global stability of the DNA
substrate in a particular conformation by determining the overall melting temperature of the junction ensemble. Fluorescence melting experiments provide information about the local stability of the DNA substrate by determining the melting behavior at a particular location on an individual arm, determined by the location of the fluorescent probe.

For absorbance melting experiments, the absorbance of the DNA structure transition relies on the hypochromicity effect, which results from the fact that DNA duplexes absorb less light than single-stranded DNA (Devoe & Tinoco, 1962). Base stacking in a well-formed DNA structure decreases the overall absorbance, while the denaturation of the DNA structure results in the increase of absorbance. This increase occurs because bases in denatured DNA are able to absorb more ultraviolet light than stacked bases. Thus, the increase in absorbance as a function of temperature, also known as the melting curve, can be used to calculate the melting temperature of DNA substrates.

For fluorescence measurements, changes to shape and size influence stacking and will be reflected in the intensity values. When the junction arms are in the duplex state, the local dynamics of the internal probe will be restricted by the formation of
hydrogen bonds to the complementary strand. When the junction arms have melted, the local dynamics of the internal probe will be very different - much more flexible. Thus, the change in the fluorescence intensity as a function of temperature can be used to determine the melting temperature at a specific location along one junction arm.

Under equilibrium conditions, several equations are used to analyze the melting profile of DNA structures. First, the absorbance vs. temperature profile, and the fluorescence vs. temperature profile, are normalized and converted to Alpha vs. temperature profiles, in which alpha is defined as the fraction of single strands in the junction. This conversion assumes that the change in absorbance, and change in fluorescence, at any temperature results from the melting reaction. The alpha value normalizes the curve by adjusting the upper and lower baseline of the melting profile,

$$\alpha = \frac{\left[ (m_l T - b_l) - A_t \right]}{\left[ (m_l T - b_l) - (m_u T - b_u) \right]}$$

where $T$ is the temperature, $m_u$ is the slope of the upper baseline of the graph, $b_u$ is the y-intercept of the upper baseline, $m_l$ is the slope of the lower baseline, $b_l$ is the y-intercept of the lower baseline, and $A_t$ is the absorbance or fluorescence at time $t$. The melting temperature is thus defined as the temperature at which $\alpha = 0.5$. 
Figure 13: A typical UV melting curve showing the transition from the junction structure to random coils as a function of temperature (a). The melting curve is normalized and converted to the alpha vs. temp profile (b).

In order to determine the melting temperature from the normalized and converted melting curve, the alpha vs. temperature profile was fit to a Boltzmann function,

\[ y = A_2 + \frac{A_1 - A_2}{1 + e^{\frac{x-x_u}{x}}}. \]
where $A_1$ is the initial absorbance, $A_2$ is the final absorbance, $x$ is the input temperature, $x_0$ is the melting temperature, and $y$ is the calculated absorbance value.

The thermodynamic properties can be extracted from melting curves by taking advantage of the van’t Hoff equation. The van’t Hoff transition enthalpy, $\Delta H_{vH}$, at the $T_m$ in which $\alpha = 1/2$ for a junction formed by four single strands is

$$
\Delta H_{vH} = (2 + 2n)RT_m^2 \left( \frac{\delta \alpha}{\delta T} \right)_{T=T_m}
$$


2.8.2 Labeling of DNA with Internal Fluorescent Probes

To assess the coarse-grain modeling that suggested asymmetric melting of junction arms, several junctions containing internal probes, 6-MI and 6MAP, at various locations were designed. As nucleoside analogues, internal probes make excellent internal reporter of local DNA stability and dynamics. 6-MI is a fluorescent guanine analogue that is able to hydrogen bond to cytosine in duplex DNA, and 6-MAP is a fluorescent adenine analogue that is able to hydrogen bond to thymine in duplex double-stranded DNA.
Table 2: Junctions used for melting experiments. *F* indicates the position of labeling.

<table>
<thead>
<tr>
<th>Junction</th>
<th>Sequence</th>
</tr>
</thead>
</table>
| J34 R28-6MAP | **JX34**: CCA GAC TGC AGT TGA GTC CTT GCT AGG ACG GAG G  
               | **JB34**: CCT CCG TGG TAG CAA GGG GCT GCT ACC GGA AGG G  
               | **JR28-6MAP**: CCT TCA ACC ACC GCT CAA CTC AAC TGC FGT CTG G  
               | **JH34**: CCC TTC CGG TAG CAG CCT GAG CGG TGG TTG AAG G     |
| J34 X28-6MAP | **JX28-6MAP**: CCA GAC TGC AGT TGA GTC CTT GCT AGG FCG GAG G  
               | **JB34**: CCT CCG TGG TAG CAA GGG GCT GCT ACC GGA AGG G  
               | **JR34**: CCT TCA ACC ACC GCT CAA CTC AAC TGC AGT CTG G  
               | **JH34**: CCC TTC CGG TAG CAG CCT GAG CGG TGG TTG AAG G     |
| J34 B25-6MAP | **JX34**: CCA GAC TGC AGT TGA GTC CTT GCT AGG ACG GAG G  
               | **JB25-6MAP**: CCT CCG TGG TAG CAA GGG GCT GCT FCC GGA AGG G  
               | **JR34**: CCT TCA ACC ACC GCT CAA CTC AAC TGC AGT CTG G  
               | **JH34**: CCC TTC CGG TAG CAG CCT GAG CGG TGG TTG AAG G     |
| J34 R7-6MAP  | **JX34**: CCA GAC TGC AGT TGA GTC CTT GCT AGG ACG GAG G  
               | **JB34**: CCT CCG TGG TAG CAA GGG GCT GCT ACC GGA AGG G  
               | **JR7-6MAP**: CCT TCA FCC ACC GCT CAA CTC AAC TGC AGT CTG G  
               | **JH34**: CCC TTC CGG TAG CAG CCT GAG CGG TGG TTG           |
| J40 H18-6MI | JX40: AGT CTA GAC TGC AGT TGA GTC CTT GCT AGG ACG GAT CCC T  
|            | JB40: AGG GAT CCG TCC TAG CAA GGG GCT GCT ACC GGA AGC TTC T  
|            | JR40: AGG AAT TCA ACC ACC GCT CAA CTC AAC TGC AGT CTA GAC T  
|            | JH40: AGA AGC TTC CGG TAG CA F CCT GAG CGG TGG TTG AAT TCC T |
| J40 X25-6MI | JX40: AGT CTA GAC TGC AGT TGA GTC CTT FCT AGG ACG GAT CCC T  
|            | JB40: AGG GAT CCG TCC TAG CAA GGG GCT GCT ACC GGA AGC TTC T  
|            | JR40: GG AAT TCA ACC ACC GCT CAA CTC AAC TGC AGT CTA GAC T  
|            | JH40: AGA AGC TTC CGG TAG CAG CCT GAG CGG TGG TTG AAT TCC T |
| J34 unlabeled | JX34: CCA GAC TGC AGT TGA GTC CTT GCT AGG ACG GAG G  
|            | JB34: CCT CCG TGG TAG CAA GGG GCT GCT ACC GGA AGG G  
|            | JR34: CCT TCA ACC ACC GCT CAA CTC AAC TGC AGT CTG G  
|            | JH34: CCC TTC CGG TAG CAG CCT GAG CGG TGG TTG AAG G |
| J40 unlabeled | JX40: AGT CTA GAC TGC AGT TGA GTC CTT GCT AGG ACG GAT CCC T  
|            | JB40: AGG GAT CCG TCC TAG CAA GGG GCT GCT ACC GGA AGC TTC T  
|            | JR40: AGG AAT TCA ACC ACC GCT CAA CTC AAC TGC AGT CTA GAC T  
|            | JH40: AGA AGC TTC CGG TAG CAG CCT GAG CGG TGG TTG AAT TCC T |
2.8.3 Junction Melting Experiments

Absorbance DNA melting experiments were performed on the Beckman DU 650 Spectrophotometer and fluorescence experiments were performed on FluoroMax 4. Immobile junctions were slowly heated from 10°C to 90°C, and cooled to ambient room temperature. During this process, the absorbance and fluorescence intensities were monitored closely at 260 nm for absorbance measurements and at 430 nm for fluorescence intensity measurements of 6-MI and 420 nm for 6-MAP. For absorbance melting experiments, the ramp rate was set to 1°C per minute and the absorbance was recorded every 0.5°C. For fluorescence melting experiments, the fluorescence intensity was recorded every two degrees Celsius. A 2°C increment was used instead of a 1°C increment due to the limitations of the temperature controller of the temperature bath.

The preparation of the samples required a few steps. Samples were prepared in buffer containing a salt concentration of 200 mM NaCl. To avoid formation of oxygen gas bubbles during the heating process, the samples were bubbled with helium gas to remove the oxygen gas from the sample solution. Once the samples were prepared and put into the sample cuvette, the cuvette was sealed with Teflon
tape to prevent evaporation of the sample solution, which would alter the concentration of the sample. The change in concentration would affect the melting curve. The chamber holding the cells was purged and flooded with nitrogen to prevent condensation during the heating process.

The melting curve was corrected to calculate alpha. Alpha was graphed as a function of temperature to obtain a melting point.
Chapter 3: Results

3.1 Purification of IHF

3.1.1 Assessment of Purification by SDS-PAGE

IHF was isolated from the *E. coli* strain that overexpresses the protein following an established protocol and techniques described by Olga Buzovetsky and Veronica Birdsall (Filutowicz et al., 1994); (Buzovetsky, 2010); (Birdsall, 2015). The growth phase of *E. coli* was closely monitored every hour. Pure IHF is observed as a doublet between 10 and 15 kD bands of the BioRad Precision Plus Protein Standards marker, consistent with previous results and the expected molecular weight of the protein subunits (Figure 14).
Figure 14: SDS-PAGE gel to assess expression of IHF during growth phase of *E. coli*, before and after IPTG induction. The first lane following the marker is a sample of previously purified IHF used as a standard. The proceeding lanes show the expression profiles after every hour of growth starting with the fifth hour in the second lane, and ending by the eleventh hour in the eighth lane. The culture was induced by addition of IPTG after the seventh hour (lane 4). Upon IPTG induction, increased production of IHF was observed.

IHF was extracted from the bacterial cell lysate by Polymin P precipitation and ammonium sulfate precipitation. Aliquot samples of supernatants and pellets were taken from each step of the precipitation protocol and assessed on a denaturing SDS-PAGE gel to determine the efficiency of each step (Figure 15).
Figure 15: SDS-PAGE gel to assess Polymin P and ammonium sulfate precipitation steps in extracting IHF from cell lysate. Lane 1 is the sample of lysed cells. lane 2: Polymin P precipitation supernatant, lane 3: Polymin P precipitation pellet, lane 4: 0.5M NaCl wash pellet, lane 5: 0.5M NaCl wash supernatant, lane 6: supernatant of ammonium sulfate precipitation (0-50% cut), lane 7: pellet of ammonium sulfate precipitation (0-50% cut), lane 8: supernatant of ammonium sulfate precipitation (50-75% cut), lane 9: supernatant of ammonium sulfate precipitation (50-75% cut), lane 9: final dialysis.

IHF was purified on a HiTrap Heparin HP column prepacked with Heparin Sepharose High Performance on a chromatography system, AKTA design. The simple automated system efficiently eluted IHF with gradient of salts. Upon application to the column, IHF binds to the column material, while other proteins
from cell lysate were removed during the column wash. The gradient of salt, ranging from 100 mM to 2 M NaCl, was applied to selectively elute IHF from all other proteins. IHF was eluted from the column at approximately 900 mM NaCl. Fractions collected from column were assessed on SDS-PAGE gels (Figure 16).
Figure 16: SDS-PAGE gels assessing the fractions eluted from HiTrap Heparin HP column. Lane 1 in (A) is the same loaded onto the HiTrap HeparinHP column. Lane 2 in (A) is the pellet found after dialysis following the precipitation steps. Lanes 3 to 8 in (A) are fractions collected as IHF was loaded onto the column. Lanes 9 to 14 in (A) are Buffer A washes that proceed the IHF application. In gels (B), (C), and (D), every lane shows the assessment of every other fraction collected from the HiTrap Heparin HP column. Lanes 3 to 6 in (D) shows the elution of IHF at approximately 900 mM NaCl. Fractions containing IHF were pooled, concentrated, and dialyzed to obtain pure protein product (E).

3.1.2 IHF Activity Check – Binding of IHF to Consensus Sequence Duplex DNA

The activity of the newly purified protein was examined by gel mobility shift assay (GMSA) and compared with the previously determined known binding constant (Buzovetsky, 2010). The binding interaction between IHF and H134, a 34 bp duplex containing the consensus sequence, is well characterized – the binding constant for the interaction is ~30 nM (Buzovetsky, 2010). Figure 17 shows a gel mobility shift assay of the newly purified protein and H134. The purified protein was active and has a binding constant of 28.9 ± 5.6 nM for H134, consistent with the previously reported values.
Figure 17: (a) Polyacrylamide gel of IHF binding to IHF consensus sequence duplex H134. Increasing protein concentrations of protein, from 0 M (first lane) to a final concentration 900 nM (last lane), are incubated with a constant 10 nM concentration of H134. As more DNA is bound by protein, the complex formation increases, resulting in a shift in the gel. The lower band represents free DNA while the upper band represents bound DNA. (b) Binding curve analysis of free DNA as a function of protein concentration. Quantification of the intensity of pixel volumes of free DNA bands was plotted with increasing protein concentrations and revealed a Kd of 28.9 ± 5.6 nM.

3.2 IHF Binding to JH1

The binding affinity of IHF and Holliday junction containing the IHF consensus sequence, JH1, was assessed to determine whether the presence of the consensus sequence plays a role in IHF recognition and binding to DNA junctions. IHF is known to recognize and bind specifically with nanomolar affinity to its
consensus sequence in duplex DNA. Interestingly, IHF also exhibits nanomolar affinity binding to four way junctions without the consensus sequence. Previous work in the Mukerji lab (Birdsall, 2015); (Buzovetsky, 2010); (S. Ho, 2013) has characterized the binding interactions of IHF with an immobile DNA four way junction – J3, a 34 bp junction containing a random sequence, based on the junction design originally created by Lilley and co-workers (Duckett et al., 1988). The high affinity binding between IHF and a random sequence junction suggests that in this case, the consensus sequence is not a primary recognition mechanism for IHF. To further investigate the whether the consensus sequence affects binding to junctions, JH1, a 34 bp junction containing the IHF consensus sequence, derived from the J3 construct, was developed and characterized (Figure 18). The IHF consensus sequence was placed in middle of junction as it was believed that IHF binds to the center of the junction, similar to the HU protein. The X strand was modified to contain the sequence 5’ AATCAACTACTTA 3’ in the middle of the junction. Portions of the R strand and B strand were also modified to ensure base pairing with the consensus sequence, while the H strand was unchanged. These oligomers were annealed to
assemble a four-way junction with 17 bp arms. Formation of the junction was verified by non-denaturing gel electrophoresis.

Figure 18: The immobile Holliday junction containing the IHF consensus sequence (JH1). The J3 junction template was modified to contain the IHF consensus sequence (red) at the center of the junction.
3.2.1 GMSA IHF and JH1

The binding characterization by gel mobility shift assay revealed that IHF binds with nanomolar affinity to JH1 (Figure 19). The stacked conformation of the junction was induced under high concentrations of salt at 70 mM KCl. Three replications were performed and the calculated average Kd value is 65 +/- 8.2 nM.

Figure 19: Polyacrylamide gel of IHF binding to the Holliday junction containing the IHF consensus sequence. Increasing concentrations of IHF was titrated into a constant 10 nM concentration of JH1. (B) Binding curve analysis of the free DNA as a function of IHF concentration.
3.2.2 Fluorescence Intensity

Binding between IHF and JH1 were determined in solution by monitoring fluorescence intensity. The fluorescence intensity depends on the interactions of fluorophores with the surrounding solvent molecules, such that changes in fluorescence intensity reflect changes in the surroundings of the fluorophore after complex formation. When increasing concentrations of IHF were titrated into a sample of DNA, the fluorescence intensity decreased and reached saturation, taking the shape of a binding curve (Figure 20). Three replications yielded an average Kd of 9.19 +/- 2.01 nM.

Figure 20: Analysis of binding curve between the interaction of IHF and JH1 measured by changes in fluorescence intensity. Average of three trials.
3.2.3. **Fluorescence Anisotropy**

Fluorescence intensity measurements were made in conjunction with fluorescence anisotropy measurements for IHF binding to JH1 junction. Anisotropy measurements provide additional information about the size, shape, and orientation of the molecules. These measurements also confirm the observed changes in fluorescence intensity did not solely arise from effects of the fluorophore, such as quenching due to salt concentrations, but rather reflect the interaction between IHF and JH1. Upon titration of protein into the DNA sample, the anisotropy change is expected to increase due to the slower average rotational rate of the larger macromolecule or complex (Figure 21). The dissociation constant measured by anisotropy for IHF binding to JH1 junction is less than 7.6 nM.

![Fluorescence Anisotropy](image)

Figure 21: Analysis of JH1+IHF binding curve between the interaction of IHF and JH1 measured by changes in fluorescence anisotropy.
3.2.4 Stoichiometry of Binding

When extracting dissociation constants from binding curves, it is necessary to know the stoichiometry of binding. The stoichiometry determines the number of proteins that bind to one DNA molecule. To ensure the model used to describe the binding curve is accurate, the stoichiometry of binding of IHF to JH1 was determined. In addition, IHF binding assays with JH1 in gel indicated additional rows of bound DNA bands that migrated slower (Figure 19), suggesting the binding of different conformations, or a binding stoichiometry greater than the 1:1 molar ratio.

To determine the stoichiometry of binding between IHF and JH1, binding assays under stoichiometric conditions were performed in solution by monitoring fluorescence intensity. Since the dissociation constant measured for IHF binding to JH1 by fluorescence intensity was approximately 10 nM, the concentration of DNA used for stoichiometric measurements was 100 nM. The ten-fold concentration was used to ensure that changes in fluorescence intensity reflected the number of proteins bound, and were not the consequence of protein binding affinity.
Figure 22: Stoichiometry binding of IHF to 100 nM JH1 monitored by change in fluorescence intensity. The transition from the linear fit of the binding curve to the linear fit of the saturation curve refers to the molar ratio of IHF to JH1, 1:1 stoichiometry.

Stoichiometric analysis of IHF binding to JH1 confirmed that only one IHF protein was binding to a single DNA molecule (Figure 22). This information was incorporated into the mathematical model used to describe the binding curves (Section 2.4.1).

In summary, binding assays demonstrated that IHF binds to a Holliday junction containing the consensus sequence with nanomolar affinity and a 1:1 binding stoichiometry.
3.3 Ion-Induced Junction Conformation

Prior to studying the conformational effect of JH1 upon IHF binding, it was necessary to first determine JH1 conformations in the presence of varying potassium ion concentrations. Potassium ion was chosen to optimize the binding properties of the IHF protein. Previous studies have demonstrated that the J3 Holliday junction primarily adopts the Iso II conformation (~80%) under high salt concentrations. More specifically, Vitoc and Mukerji monitored the structural conformations of J3 as a function of ionic strength with FRET measurements, and have shown that by 70 mM KCl, the J3 junction adopted the stacked Iso II conformation (Vitoc & Mukerji, 2011). Although J3 and JH1 junctions are essentially the same junction, the difference in the central sequence determine the preference for the stacked conformation. Therefore, it was necessary to determine if the differential sequence in JH1 affected the structural conformations in presence of ions.
3.3.1 FRET

Fürster Resonance Energy Transfer measurements of JH1 as a function of potassium ion concentrations were monitored to determine the structural conformation and isoform preference of JH1. These experiments were performed under the same conditions as the fluorescence intensity binding assays. By monitoring the fluorescence intensity of the donor only, acceptor only, and doubly-labeled junctions, the transfer of energy can be determined. In particular, the FRET efficiency trends of decreasing donor fluorescence intensity, or increasing acceptor fluorescence intensity, can provide clues regarding the conformation of the junction. Comparisons between the two labeled schemes, BX and RX, would determine the preference for the stacking isoforms.

The preparation of the labeled junctions was assessed in a native, or non-denaturing, gel. The gel allowed for the visualization and quantification of the integrity of the annealed junctions used for fluorescence experiments.
Figure 23: Native gel of JH1 junctions. Lane 1 is the unlabeled JH1 junction, used as a control. Lane 2 is the donor only junction, JH1X-FAM. Lane 3 is the acceptor only junction of the BX construct – JH1B-TAM. Lane 4 is the doubly labeled junction of the BX construct – JH1X-FAM_B-TAM. Lane 5 is the acceptor only of the RX construct – JH1R-TAM. Lane 6 is the doubly labeled junction of the RX construct – JH1X-FAM_R-TAM. Less than 5% single stranded DNA was detected for all junctions.

The native gel confirmed the integrity of each junction; each junction was successfully annealed with minimal single strand concentrations (Figure 23).
Figure 24: Plot of FRET efficiency of JH1 BX construct, FAM (orange star) labeled on the X arm (blue) and TAMRA (pink star) labeled on the B arm (yellow), as function of potassium ion concentration. FRET efficiencies were calculated by fluorescence decrease in donor signal of the JH1 junction.
Figure 25: Plot of FRET efficiency of JH1 RX construct, FAM (orange star) labeled on the X arm (blue) and TAMRA (pink star) labeled on the R arm (green), as function of potassium ion concentration. FRET efficiencies were calculated by fluorescence decrease in donor signal of the JH1 junction.
At low concentrations of potassium ion concentrations, the relatively high efficiency values for the BX labeling scheme and the relatively low efficiency values for the RX model are consistent with the characterization for open and planar junction conformation. The average initial efficiency values for both labeling constructs at close to 0 mM KCl were consistent with efficiency values reported by Vitoc for the J3 junction in 10 mM KCl (Vitoc & Mukerji, 2011). The increase of potassium ion concentrations resulted in a decrease in efficiency values for the BX scheme (Figure 24), while the efficiency values for the RX scheme increased (Figure 25). The average efficiency values of the RX labeling scheme at high potassium ion concentrations is also consistent with the efficiency value reported by Vitoc for the J3 junction in 60 mM KCl. The decrease in efficiency values suggested that the distance between the labeled B and X arms are increasing. The increase in efficiency values observed for the RX labeled junction further suggested that the distance between the labeled R and X arms was decreasing, and forming the coaxial stacking conformation. Information from the two labeling schemes indicates that at relatively high potassium ion concentrations, the junction favors the Iso II conformation.
3.3.2 Time-Resolved FRET

Figure 26: Fluorescence lifetime decay curves of donor emissions of JH1 singly labeled with FAM and doubly labeled with FAM and TAMRA on the X and R strands, respectively, in low salt (A) and high salt (B). Quenching at high salt concentrations is observed.

Table 3: Parameters derived from fitting of time-resolved fluorescence decay curves for JH1 under low and high salt conditions.

<table>
<thead>
<tr>
<th></th>
<th>DNA</th>
<th>$\alpha_1$</th>
<th>$\tau_1$, ns</th>
<th>$\alpha_2$</th>
<th>$\tau_2$, ns</th>
<th>$\alpha_3$</th>
<th>$\tau_3$, ns</th>
<th>Amplitude-Weighted $\tau_{mean}$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Low salt</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JH1 X-FAM</td>
<td></td>
<td>11.64</td>
<td>0.99</td>
<td>5.46</td>
<td>2.49</td>
<td>1.68</td>
<td>4.44</td>
<td>1.73</td>
</tr>
<tr>
<td>JH1 X-FAM R-TAMRA</td>
<td>4.41</td>
<td>2.25</td>
<td>10.57</td>
<td>2.24</td>
<td>2.31</td>
<td>4.45</td>
<td>2.54</td>
<td></td>
</tr>
<tr>
<td><strong>High salt</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JH1 X-FAM</td>
<td></td>
<td>15367</td>
<td>0.27</td>
<td>15327</td>
<td>2.37</td>
<td>3823.2</td>
<td>5.79</td>
<td>1.81</td>
</tr>
<tr>
<td>JH1 X-FAM R-TAMRA</td>
<td>19837</td>
<td>0.11</td>
<td>10102</td>
<td>1.00</td>
<td>13333</td>
<td>3.72</td>
<td>1.43</td>
<td></td>
</tr>
</tbody>
</table>
Time-resolved FRET experiments were performed to compare the conformational populations of the JH1 junction in the presence of low and high ion concentrations. The fluorescence decays indicated that at low ion concentrations, there was little to no change in fluorescence lifetime decay curves between the donor only sample and doubly-labeled sample. At high ion concentrations, the fluorescence lifetime decay curve for the doubly-labeled sample was more quenched than the decay curve of the donor only sample (Figure 26). Global fitting of the decay curves and calculations of efficiency of transfer from amplitude-weighted mean lifetimes were performed using the following equation:

\[
Efficiency = 1 - \frac{\tau_{doubly}}{\tau_{donor}}
\]

These analyses revealed results consistent with the steady-state fluorescence intensity experiments. Under low salt conditions, the efficiency of transfer is small, giving a negative value, suggesting that the junction exhibits an open, planar structure. Under high salt conditions, the efficiency calculated from the amplitude-weighted mean lifetime is 0.21. This efficiency value indicates that there is transfer of efficiency between the FRET pair, suggesting the junction adopts the Iso II stacked conformation where the probes are in close proximity.
These analyses also reveal the specific components that exhibit the FRET behavior. The presence of energy transfer can be determined by a large relative change in the fluorescence lifetime. Comparisons of the fluorescence lifetimes between the donor only and the doubly labeled sample under the high salt condition reveal that the first and second component exhibit the greatest amount of energy transfer, while the third component does not (Table 3). The first component, on the fast timescale, has a relatively large fluorescence lifetime change, from $\tau = 0.27$ ns in the donor only sample to $\tau = 0.11$ ns in the doubly labeled sample. The second component, the mid-range lifetime, also exhibits a relatively large change, from $\tau = 2.37$ ns in the donor only sample to $\tau = 1.00$ ns in the doubly labeled sample. The last component, or the slowest component, changing from $\tau = 5.79$ ns in the donor only sample to $\tau = 3.72$ ns in the doubly labeled sample, does not exhibit as large a change proportionally, indicating that the energy transfer is minimal or not reflected in this component. The slow component most probably arises from the dye in the relatively unquenched state as the lifetime is very close to that of the FAM monomer. Thus, the large relative changes observed in the fluorescence lifetimes in the first and second
components, which correspond to high efficiencies of energy transfer, suggest that the JH1 junction is induced into the stacked conformation under the high salt condition.

Once the components exhibiting the FRET behavior have been identified, the fractional populations of structural conformations of the JH1 junction can be determined. Since the fast and mid-range components both exhibit FRET, these components are summed, and compared with the slow component. These components of the doubly-labeled sample under the high salt condition were evaluated based on the weighted-amplitudes, or pre-exponential factors.

Table 4: Evaluation of structural populations of doubly labeled sample, JH1_X-FAM_R-TAM, under high salt conditions.

<table>
<thead>
<tr>
<th>JH1 X-FAM_R-TAMRA High salt</th>
<th>Component #1</th>
<th>Component #2</th>
<th>Component #3</th>
<th>% of Populations</th>
<th>% Iso II Population exhibiting FRET</th>
<th>% Iso I Population exhibiting No FRET</th>
</tr>
</thead>
<tbody>
<tr>
<td>19837</td>
<td>0.46</td>
<td>0.23</td>
<td>0.31</td>
<td>69%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10102</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>% Iso II Population exhibiting FRET</td>
<td></td>
</tr>
<tr>
<td>13333</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>% Iso I Population exhibiting No FRET</td>
<td></td>
</tr>
</tbody>
</table>
It is estimated that approximately 69% of the total population is exhibiting energy transfer, while 31% is not (Table 4). Because we are probing the X and R arms specifically, the proximity of the probes allowing the FRET behavior indicates that approximately 69% of the junction is in the stacked Iso II conformation and 31% is in the stacked Iso I conformation.

3.4 Protein-Induced Junction Conformation

Once the junction conformations were determined as a function of potassium ion concentration, IHF effects on the JH1 junction structure were studied. Previous studies indicated that upon binding to the stacked conformation of Holliday junction J3, IHF induced the junction to adopt the open conformation. To determine the resulting IHF-induced structural changes of JH1, FRET experiments were performed as a function of protein concentration. These experiments were performed under the same conditions as the ion-induced FRET experiments and analyzed in a similar manner.
3.4.1 FRET

FRET measurements were performed under stoichiometric conditions to determine the IHF-induced structural changes of the JH1 junction at the salt concentration of 70 mM KCl.

Figure 27: Plot of FRET efficiency of JH1 BX construct, FAM (orange star) labeled on the X arm (blue) and TAMRA (pink star) labeled on the B arm (yellow), as a function of protein concentration under stoichiometric conditions. FRET efficiencies were calculated from the decrease in fluorescence donor signal of the doubly-labeled JH1 junction relative to the donor only junction.
Figure 28: Plot of FRET efficiency of JH1 RX construct, FAM (orange star) labeled on the X arm (blue) and TAMRA (pink star) labeled on the R arm (green), as function of protein concentration under stoichiometric conditions. FRET efficiencies were calculated from the decrease in fluorescence donor signal of the doubly-labeled JH1 junction relative to the donor only junction.
The addition of IHF into a sample of JH1 junction at high salt concentrations of 70 mM KCl resulted in an increase in efficiency for the BX scheme (Figure 27), in contrast to the decrease in efficiency values for the RX scheme (Figure 28). Starting from the stacked Iso II conformation, the change in FRET efficiency upon IHF binding for both schemes reflected a protein-induced opening of the junction.

3.4.2 Time-Resolved FRET
Figure 29: Fluorescence lifetime decay curves of the donor emission of JH1 singly labeled with FAM or doubly labeled with FAM and TAMRA on the X and R strands, respectively, in high salt, in the absence (A) or presence of IHF (B).

Table 5: Parameters derived from global fitting of time-resolved fluorescence decay curves for JH1 under high salt conditions in the absence and presence of IHF.

<table>
<thead>
<tr>
<th>Condition</th>
<th>DNA</th>
<th>$\alpha_1$</th>
<th>$\tau_1$, ns</th>
<th>$\alpha_2$</th>
<th>$\tau_2$, ns</th>
<th>$\alpha_3$</th>
<th>$\tau_3$, ns</th>
<th>Amplitude-Weighted Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>No IHF</td>
<td>JH1 X_FAM</td>
<td>15357</td>
<td>0.266</td>
<td>15327</td>
<td>2.374</td>
<td>3823.2</td>
<td>5.789</td>
<td>1.81</td>
</tr>
<tr>
<td></td>
<td>JH1 X-FAM R-TAMRA</td>
<td>19837</td>
<td>0.106</td>
<td>10102</td>
<td>1.00</td>
<td>13333</td>
<td>3.716</td>
<td>1.43</td>
</tr>
<tr>
<td>With IHF</td>
<td>JH1 X-FAM</td>
<td>15088</td>
<td>0.269</td>
<td>12960</td>
<td>2.067</td>
<td>5990.3</td>
<td>5.024</td>
<td>1.79</td>
</tr>
<tr>
<td></td>
<td>JH1 X-FAM R-TAMRA</td>
<td>15261</td>
<td>0.220</td>
<td>11411</td>
<td>1.639</td>
<td>8729.3</td>
<td>4.444</td>
<td>1.72</td>
</tr>
</tbody>
</table>

Time-resolved FRET experiments were performed to compare the conformational populations of the JH1 junction in absence and presence of IHF at high ion concentrations (Figure 26). Global fitting of the decay curves deconvoluted three components, a fast component with a lifetime of approximately 0.1 to 0.3 ns, a mid-range component with an approximately 1 to 2 ns lifetime, and a slow
component greater than 3 ns (Table 5). When these components are averaged to
determine the amplitude-weighted lifetime, and calculated for the efficiency of
energy transfer, the resulting efficiency is 0.21 and 0.04 in the absence and presence
of IHF, respectively. As described above, large changes in the fluorescence lifetimes
of the fast and mid-range components confirm the presence of high energy transfer
under the high salt conditions in the absence of protein, suggesting the preference for
the stacked Iso II conformation. However, upon addition of the IHF protein, the
efficiency of energy transfer decreases significantly to 0.04, suggesting that the
protein induces formation of the open junction conformation.

The analysis of individual components in the presence of the IHF protein
confirms the low efficiency values obtained from the amplitude-weighted mean
lifetimes. Comparisons of the first and second components in the presence of IHF,
which had exhibited large fluorescence lifetime changes in the absence of IHF, reveal
a smaller relative change in fluorescence lifetimes, corresponding to a diminished
amount of energy transfer. The first component of the doubly labeled sample changes
from $\tau = 0.269$ ns in the absence of IHF to $\tau = 2.20$ ns in the presence of IHF. The
second component of the doubly labeled sample changes from $\tau = 2.067$ ns in the
absence of IHF to $\tau = 1.639$ ns in the presence of IHF. The last component also exhibits a small relative change, from $\tau = 5.024$ ns in the absence of IHF to $\tau = 4.444$ ns in the presence of IHF. Thus, relatively small changes in fluorescence lifetimes and low efficiency values of the doubly labeled sample in the presence of the IHF protein further confirms that the JH1 junction is in the open conformation.

In summary, the efficiency of energy transfer determined by time-resolved FRET experiments are consistent with steady-state FRET experiments, in which the junction is induced into the stacked Iso II conformation under the high salt conditions, and converted into the open conformation by the IHF protein.
3.5 DNA Four-way Junction Stability and Thermodynamic Properties

Alongside binding characterizations and determination of JH1 global conformational geometry, this thesis examines the thermodynamic and structural properties of DNA Holliday junctions by evaluating the stability of each arm. As part of Holliday’s model of genetic recombination, Holliday junctions are thought to adopt various orientations. Extensive studies have shown that many factors influence the structures of the Holliday junction. Evidence from crystallographic studies reveal that under X-ray crystallographic conditions, structures of Holliday junctions in the absence of protein adopt the stacked antiparallel orientation. Experimental studies utilizing fluorescence spectroscopy reveal that ionic concentrations of the local environment play a role in biasing the junctions into open and closed conformations. Holliday junctions also interconvert between two possible stacked antiparallel conformations, Iso I and Iso II. Recently, simulations by 3SPN.2 model, a coarse-grained representation of DNA was used to predict thermodynamic and structural properties of DNA Holliday junctions (W. Wang et al., 2016). Preliminary modeling further suggested that the melting of junction arms was asymmetric, as opposed to the
“all-or-none” model for dissociation of the junction. In addition, there is a possibility that junction arms may dissociate either from the ends of the arms, or from the center of the junction, as observed in three-way junctions (Husler & Klump, 1994). Thus, evaluating the stability of junctions in certain conformations is critical in understanding the events in genetic recombination, particularly branch migration, and the structural basis of DNA recognition by IHF.

3.5.1 Design of Holliday Junctions

To investigate the model predictions experimentally, the stability of individual arms of the junctions as well as the stability at different locations along the arms of the junctions were evaluated. In order to accomplish this, the immobile 34 bp J3 junction, originally designed by Lilley and co-workers, was modified to contain fluorescent base analogs. The incorporation of these analogs, the 6-MAP and 6-MI fluorophores, within the junction arm at distinct locations allowed for precise monitoring of strand dissociation at the location of the probe.
The thermodynamic profiles and the stability of individual arms were examined by UV absorbance and fluorescence. The four arms of the junction have the following compositions:

Figure 30: Illustration of junction arms. XR arm: 5’ end of the X strand and 3’ end of R strand. BX arm: 5’ end of the B strand and 3’ end of the X strand. HB arm: 5’ end of the H strand and 3’ end of the B strand. RH arm: 5’ end of the R strand and 3’ end of the H strand.
The 6MAP probe was placed strategically in the middle of each arm. Because 6MAP is an adenine analog and can only replace the adenine base, the positioning of the 6MAP probe in the middle of each arm was approximate. The possibility of altering the sequence of the junction such that the probe can be placed exactly in the middle of each arm was not considered because maintaining the J3 template sequence was crucial – the local sequence-specific interactions may play roles in the stability of the arms. Thus, the probes were placed in the middle regions of each arm at the following locations:

XR arm: 11th base pair from the center of the junction (Figure 31)  
Junction J3-R28_6MAP

BX arm: 11th base pair from the center of the junction (Figure 32)  
Junction J3-X28_6MAP

HB arm: 8th base pair from the center of the junction (Figure 33)  
Junction J3-B25-6MAP

RH arm: 11th base pair from the center of the junction (Figure 34)  
Junction J3-R7-6MAP
Figure 31: Junction J3_R28-6MAP. Immobile 34 base pair Holliday junction containing the 6MAP internal fluorophore at the 28th position on the R strand. The 6MAP, indicated as the red “F”, is located in the middle region of the XR arm, at precisely 11 base pairs away from the center of the junction.
Figure 32: Junction J3_X28-6MAP. Immobile 34 base pair Holliday junction containing the 6MAP internal fluorophore at the 28\textsuperscript{th} position on the X strand. The 6MAP, indicated as the red “F”, is located in the middle region of the BX arm, at precisely 11 base pairs away from the center of the junction.
Figure 33: Junction J3_B25-6MAP. Immobile 34 base pair Holliday junction containing the 6MAP internal fluorophore at the 25th position on the R strand. The 6MAP, indicated as the red “F”, is located in the middle region of the HB arm, at precisely eight base pairs away from the center of the junction.
Figure 34: Junction J3_R7-6MAP. Immobile 34 base pair Holliday junction containing the 6MAP internal fluorophore at the 7th position on the R strand. The 6MAP, indicated as the red “F”, is located in the middle region of the RH arm, at precisely 11 base pairs away from the center of the junction.
Next, to test for the zipper model, junction dissociation is monitored with internal fluorophores placed at different locations along junction arms to evaluate their relative stability. Two additional junctions were designed to probe the stability of the junction arms near the center of the junction. These junctions directly probe for the possibility of the zipper model for junction dissociation occurring from the center of the junction. An internal probe was placed at the following locations of the junction arms:

- BX arm: 5th position from the center of the junction (Figure 35)
  Junction J40-X25_6MI

- HB arm: 3rd position from the center of the junction (Figure 36)
  Junction J40-H18_6MI

These junctions are composed of 40 bp strands, 20 bp arms. These junctions are also based on the sequence of the immobile J3 junction template. The 6MI probe was incorporated three base pairs from the center of the junction.
Figure 35: Junction J40_X25-6MAP. Immobile 40 base pair Holliday junction containing the 6-MI internal fluorophore at the 25\textsuperscript{th} position on the R strand. The 6-MI, indicated as the red “F”, is located towards the center of the junction, at precisely five base pairs away from the center of the junction.
Figure 36: Junction J40_H18-6MAP. Immobile 40 base pair Holliday junction containing the 6-MI internal fluorophore at the 18th position on the H strand. The 6-MI, indicated as the red “F”, is located towards the center of the junction, at precisely three base pairs away from the center of the junction.
3.5.2 Junction Melting

DNA melting can be used as a tool to characterize thermodynamic and structural properties of junction stability. Comparing the melting point of the ensemble junction to those of individual arms and at specific locations within the arms yield new information regarding junction stability. Absorbance melting experiments were performed to determine the global melting temperature and stability of the junction. In this method, only the average stability of the overall structure can be probed. To determine the melting point at specific locations, fluorescence melting experiments monitoring the internal probe and its interactions in solution are performed.

To ensure that incorporation of the probe did not alter junction stability and therefore the thermodynamic profile, absorbance measurements were made for each junction with and without the internal probe (Figure 37).
Figure 37: Temperature dependence of the extent of melting ($\alpha$) by UV absorption. These curves were derived from fits of the temperature dependence of absorbance at 260 nm of the immobile 34 bp J3 Holliday junction.
Table 6: Table of thermodynamic parameters of immobile 34 bp Holliday junction J3 by UV absorption. The average molar enthalpy ($\Delta H$) and entropy ($\Delta S$) changes of the denaturation, or helix to random coil transition, of the four-way junction were determined by van’t Hoff analysis.

<table>
<thead>
<tr>
<th>Junction</th>
<th>Tm, Celsius</th>
<th>$\Delta H$, kcal/mol</th>
<th>$\Delta S$, kcal/(K*mol)</th>
<th>$\Delta G$, kcal/mol</th>
</tr>
</thead>
<tbody>
<tr>
<td>J3 unlabeled</td>
<td>61.7 ± 0.99</td>
<td>394.1 ± 77.3</td>
<td>1.3 ± 0.2</td>
<td>36.3 ± 0.1</td>
</tr>
<tr>
<td>J3_R28-6MAP</td>
<td>63.1 ± 1.5</td>
<td>361.0 ± 63.6</td>
<td>1.2 ± 0.2</td>
<td>36.4 ± 0.2</td>
</tr>
<tr>
<td>J3_X28-6MAP</td>
<td>64.0 ± 0.8</td>
<td>403 ± 132.2</td>
<td>1.3 ± 0.4</td>
<td>36.5 ± 0.1</td>
</tr>
<tr>
<td>J3_B25-6MAP</td>
<td>61.4 ± 2.5</td>
<td>416.5 ± 109</td>
<td>1.3 ± 0.3</td>
<td>36.2 ± 0.3</td>
</tr>
<tr>
<td>J3_R7-6MAP</td>
<td>58.1 ± 2.6</td>
<td>268.6 ± 72.7</td>
<td>0.9 ± 0.2</td>
<td>35.9 ± 0.3</td>
</tr>
</tbody>
</table>

Melting experiments monitoring absorbance of DNA at 260 nm revealed that all the junctions have comparable melting temperatures and do not differ significantly from each other, indicating that the probes do not alter junction stability.

Comparisons were made between labeled junctions and unlabeled junctions. All four of the labeled junctions have comparable melting temperatures to the unlabeled junction (Table 6). Thus, it is sufficient to conclude that these five junctions have similar stability profiles. The absorbance measurements provide a control for
observations made in fluorescence melting measurements, such that any changes
detected in fluorescence measurements would indicate differences in the
thermodynamic profiles at specific locations occupied by the probe, and not artifacts
from the replacement of an analogue to the original base.

Fluorescence melting experiments of the four junctions containing probes in
the middle of the arms suggested that the denaturation of the Holliday junction was
not accurately described by the all-or-none model, and thus might be better described
by the zipper model for junction dissociation (Figure 38). Upon evaluation of the
thermodynamic profiles of individual junction arms, the XR arm, probed by the
J3_R28-6MAP junction, revealed a significantly lower melting temperature at T_m =
65.0 ± 1.1 °C (Table 7). The remaining three junction arms, BX arm, HB arm, and
RH arm, all have similar melting temperatures at approximately T_m = 68 °C. The
differences in the melting temperatures between the XR arm and the other three
junction arms indicated that the XR arm is less stable. The lower stability in one
junction arm in comparison to the other junction arms suggests that the junction does
not denature as a cooperative unit, but rather in steps with intermediates. Thus, the
one step all-or-none model does not accurately describe the denaturation of the
Holliday junction, and our results indicate that the “zipper” model is a better description of the transition from the junction structure to random coils.

Figure 38: Temperature dependence of the extent of melting ($\alpha$) as measured by fluorescence intensity. These curves were derived from fits of the temperature dependence of fluorescence intensity of the immobile 34 bp J3 Holliday junction.
Table 7: Table of thermodynamic parameters of immobile 34 bp Holliday junction J3 by fluorescence intensity. The average molar enthalpy ($\Delta H$) and entropy ($\Delta S$) changes of the denaturation, or helix to random coil transition, of the four-way junction were determined using a van’t Hoff analysis.

<table>
<thead>
<tr>
<th>Junction</th>
<th>Tm, Celsius</th>
<th>$\Delta H$, kcal/mol</th>
<th>$\Delta S$, kcal/(K*mol)</th>
<th>$\Delta G$, kcal/mol</th>
</tr>
</thead>
<tbody>
<tr>
<td>J3_R28-6MAP</td>
<td>65.0 ± 1.1</td>
<td>236.7 ± 39.6</td>
<td>1.2 ± 0.2</td>
<td>35.3 ± 0.09</td>
</tr>
<tr>
<td>J3_X28-6MAP</td>
<td>67.7 ± 0.3</td>
<td>296.1 ± 59.9</td>
<td>1.3 ± 0.4</td>
<td>35.5 ± 0.03</td>
</tr>
<tr>
<td>J3_B25-6MAP</td>
<td>67.5 ± 0.4</td>
<td>387.2 ± 47.3</td>
<td>1.3 ± 0.3</td>
<td>36.2 ± 0.04</td>
</tr>
<tr>
<td>J3_R7-6MAP</td>
<td>68.1 ± 0.5</td>
<td>341.2 ± 25.8</td>
<td>0.9 ± 0.2</td>
<td>35.6 ± 0.07</td>
</tr>
</tbody>
</table>

The fluorescence melts suggest that the dissociation of the Holliday junction more closely follows the “zipper” model; the two possibilities of the “zipper” model, dissociation beginning from the ends the junction arms or from the center of the junction, were assessed. In particular, positions towards the center of the junction on two different junction arms were studied. Similarly, melting experiments by UV absorption were performed to confirm that the incorporation of internal probes near the center of the junction did not perturb the stability of the junction ensemble (Figure 39). For these assessments, an available immobile 40 bp four way junction was used.
The internal fluorophore, 6-MI was incorporated on two junction arms towards the center of the junction.

Figure 39: Temperature dependence of the extent of melting ($\alpha$) by UV absorbance and fluorescence intensity. These curves were derived from fits of the temperature dependence of either the UV absorbance or the fluorescence intensity of the immobile 40 bp J3 Holliday junction containing the 6MI probe.
Table 8: Table of thermodynamic parameters of immobile 40 bp junction by UV absorption and fluorescence intensity.

<table>
<thead>
<tr>
<th>Junction</th>
<th>Tm, Celsius UV Absorption</th>
<th>Tm, Celsius Fluorescence Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>J40 unlabeled</td>
<td>62.0 ± 2.1</td>
<td>N/A</td>
</tr>
<tr>
<td>J40_X25-6MI</td>
<td>65.3 ± 1.9</td>
<td>55.96 ± 3.99</td>
</tr>
<tr>
<td>J40_H18-6MI</td>
<td>66.3 ± 1.0</td>
<td>68.34 ± 0.74</td>
</tr>
</tbody>
</table>

The thermal melting profiles by UV absorption of the labeled junctions and unlabeled junctions revealed that the incorporation of 6MI near the center of the junction did not perturb the stability of the overall junction structure. The average melting temperate at approximately $T_m = 65 \, ^\circ C$ of the two labeled junctions is comparable with the average melting temperature at $T_m = 62 \, ^\circ C$ of the unlabeled junction measured by UV absorption (Table 8).

Fluorescence melting experiments of these two junctions revealed that the dissociation of junction arms commences from the center of the junction. Fluorescence intensity as a function of thermal energy was compared between junctions J40_X25-6MI and J40_H18-6MI containing the 6MI probe at five base
pairs on the BX arm and three base pairs on the HB arm, respectively, from the center of the junction. The J40_X25-6MI junction, probing the BX arm has a significantly lower melting temperature, at $T_m = 55.96 \pm 3.99 \degree C$, indicating that the position at five base pairs from the center of the junction on the RH arm is less stable. This observation not only confirms the “zipper” model, but also strongly suggests that the junction may dissociate from the center of the junction, such that the zipper proceeds from the center of the junction to the distal ends of each junction arm.

However, the J40_H18-6MI junction, probing the HB arm, yielded a comparable melting temperature at $T_m = 68.34 \pm 0.74 \degree C$ to that of the unlabeled junction. Having probed two positions along the HB junction arm – at the middle with J3_B25-6MAP junction and towards the center of the junction with J40_H18-6MI junction – and found that the melting temperature is comparable, $T_m = 67.5 \pm 0.4 \degree C$ and $T_m = 66.3 \pm 1.0 \degree C$ respectively, the two positions are thought to have similar stability or dissociate under the same thermal conditions. It seems that the dissociation behavior for the HB arm does not mirror the “zipper” pattern starting from the center of the junction, as the BX arm has shown. Additional thermal stability information at additional positions along the HB arm will be needed to assess whether
the junction could potentially dissociate following a sequential “zippering” mechanism. In summary, fluorescence spectroscopic measurements monitoring internal probes has provided thermodynamic parameters at the level of single base resolution and yielded novel information regarding the relative stabilities of the junction structure.
Chapter 4: Discussion

4.1 JH1 Binding Interactions with Architectural Proteins

This project examined the binding interactions between architectural protein IHF and JH1, the DNA Holliday junction containing the IHF consensus sequence.

Architectural proteins have important roles in directly affecting gene expression across the bacterial genome by facilitating three-dimensional organization of the genome. As part of the family of architectural proteins, IHF is capable of inducing extreme deformations in the double-stranded nucleic acid structure. IHF also has the unique ability to bind DNA with high affinity due to its recognition and binding to a consensus sequence. IHF shares high structural and sequence homology with another DNA-binding protein, HU. Interestingly, both of these proteins have high preference binding to atypical or discontinuous DNA structures, such as DNA four-way junctions, or the Holliday junction, an important intermediate observed in genetic recombination. However, the mechanisms of interactions differ greatly between these two architectural proteins – HU binds nonspecifically with a 2:1 stoichiometry, while IHF binds sequence-specifically with a 1:1 stoichiometry. In an attempt to understand
these unique binding interactions, the binding interactions and structural influences of IHF to Holliday junctions were compared with HU.

Binding assays in gel and solution show that IHF binds tightly to Holliday junction containing the IHF consensus sequence. The nanomolar dissociation constants determined for IHF binding to JH1 are comparable with those determined between IHF and H134, a duplex DNA containing the IHF consensus sequence. Because both DNA substrates, duplex and four-way junction, contain the IHF consensus sequence, one can deduce that high binding specificity of IHF is driven by the presence of the IHF consensus sequence. Perhaps this is not entirely surprising because IHF has been known for its sequence-specific interactions with DNA.

However, the nanomolar binding affinities for IHF binding to JH1 are also comparable to those between IHF and J3, the Holliday junction containing a random DNA sequence. The high binding specificity between IHF and DNA four way junctions appears to be independent of sequence. This result raises questions about the specificity of the binding interactions between IHF and DNA four way junctions and its subsequent implications in biological processes. These investigations begin to
describe another mechanism for recognition by these DNA-binding and –bending proteins – proteins that exhibit a duality of sequence and structural recognition.

4.2 Geometric Characterizations of Junctions

Structural parameters of DNA substrates are shown to play crucial roles in the recognition of architectural proteins. HU and IHF have been demonstrated to recognize different structural conformations of the J3 Holliday junction. HU has been demonstrated to recognize and bind to an open, planar junction conformation, and induce the junction into the stacked form, prohibiting branch migration and recombination. Interesting, IHF has been demonstrated to recognize and bind to the stacked Iso II J3 junction conformation and induce the transition from the stacked form to the open form, perhaps exhibiting a contrasting role to that of the HU protein.

Characterization of the structural parameters of the JH1 junction are similar to the effects of ions and IHF on the J3 junction. Using two labeling schemes, the examination of the JH1 junction by FRET revealed the transitions from open to stacked conformations, or vice versa. Under low ion conditions, the JH1 junction remains in the open, planar form. While under high ion conditions, JH1 is induced
into the stacked Iso II form. Interestingly, protein binding has the opposite effect. Starting from a stacked conformation under high salt conditions, the junction is opened and stabilized into the open conformation by increasing concentrations of IHF.

Based on these results, the differences in mechanisms of IHF and HU recognition and binding to Holliday junctions are accentuated. While both proteins bind tightly, they recognize and induce opposite conformations upon binding – HU induces and stabilizes the stacked conformation while IHF induces and stabilizes the open conformation. Thus, the inverse roles exhibited by IHF and HU suggest a more intimate interplay between these two proteins, perhaps as a means to balance regulatory roles in DNA replication, recombination, and repair processes in the cell.

### 4.3 Thermodynamic Properties of Junctions

Finally, this thesis presents the novel observation of DNA four way junctions exhibiting features of the “zipper” model, as opposed to the all-or-none model, for DNA structure dissociations. By evaluating the thermal stability of the junction structure as an ensemble of four annealed strands and each strand individually by
monitoring the UV absorption and fluorescence intensity of site-specific internal probes, the sequential transition from bimolecular doubly helices to random coils can be monitored. Intriguingly, these results revealed that one junction arm, the XR arm, melted or dissociated from the junction at a lower temperature than the other junction arms. Thus, the weaker stability of one junction arm relative to the other junction arms provides evidence for the “zipper” model, in which the junction falls apart in a sequential manner with appearances of intermediate structures before reaching the complete random coil state.

In addition, the melting behavior suggests the preference for the “zippering” or dissociation to start from the center of the junction. The stability of one junction arm, the BX arm, revealed that the position at five base pairs from the center of the junction, as compared to the middle region of the junction arm, at approximately 11 base pairs from the center of the junction, was less stable. Thus, this suggests that the melting begins at the center before moving to the middle of the junction arm. To verify and track the sequential events of dissociation along the four individual arms, additional evaluations of thermal stability at other locations along junction arms are required.
4.4 Future Directions

To further our understanding of the recognition mechanism of IHF to various DNA structures, especially atypical structures, such as Holliday junctions and forks, investigations in the role of the IHF consensus sequence is required. IHF is found to recognize the immobile 34 base pair Holiday junction with nanomolar affinity without any preference of sequence. The recognition mechanism of IHF to random sequence structures seems to rely on structural properties. In order to determine the preference of recognition, it is important to investigate atypical DNA structures that also include the consensus sequence. In JH1, the consensus sequence was placed in the middle of the junction because IHF was previously found to bind preferentially to the microstructures of the center of the junction. Additional assays would test whether IHF would still exhibit high affinity binding to the center of the junction when the consensus sequence is placed along different locations of the arms. Ultimately, X-ray co-crystal structures of architectural proteins bound by Holliday junctions would provide visualization of the intricate interaction and allow us to understand the recognition mechanism of DNA substrates during genetic recombination events in the cell.
Finally, a deeper understanding of the four-way junction structure would provide additional information about the stability of different conformational preferences and during the interaction with architectural proteins. Future studies involve investigating the thermodynamic properties along the tips of junction arms, as well as all other positions along junction arms. Another component to explore would be the stability of the IHF consensus sequence, and its stability at various locations of the junction. Future exploration of junction stability may also involve structures found during the formation, such as pre-Holliday junction structures, or intermediate structures found during branch migration.
Chapter 5: References


Vitoc, C. I., & Mukerji, I. (2011). HU binding to a DNA four-way junction probed by Forster resonance energy transfer. Biochemistry, 50(9), 1432-1441. doi:10.1021/bi1007589


Chapter 6: Appendices

6.1 Buffer Recipes

Annealing buffer

10 mM Tris-HCl pH 7.6
0.1 mM EDTA pH 7.4
300 mM NaCl

Buffer A

50 mM Tris-HCl pH 7.4
20 mM NaCl
1.0 mM EDTA
3.0 nM BME
10% glycerol

IHF Binding Buffer (used for GMSA and fluorescence experiments)

5 mM Tris-HCl pH 7.5
0.1 mM EDTA
70 mM KCl
0.5% ethylene glycol

6MI/6-MAP Buffer (used for melting experiments)

5 mM Tris-HCl pH 7.6
0.1 mM EDTA
200 mM NaCl
6.2 Gel Recipes

6.5% native polyacrylamide gel
1.36 ml of ddH2O
3.9 ml of 40% acrylamide
0.6 ml of 10X TBE
120 µl of 10% APS
30 µl of TEMED

16% denaturing gel (running gel)
4.9 ml of ddH2O
3.75 ml of 1.5 M Tris-HCl pH 8.8
6 ml of 40% acrylamide
75 µl of 20% SDS
150 µl of 10% APS
15 µl of TEMED

4% denaturing gel (stacking gel)
5.8 ml of ddH2O
0.94 ml of 1 M Tris-HCl pH 6.8
0.76 ml of 40% acrylamide
0.038 µl of 20% SDS
75 µl of 10% APS
7.5 µl of TEMED

20% urea denaturing gel
25.2 g of Urea
6 ml of 10X TBE
31.6 ml of 38% acrylamide / 2% bis solution (solution prepared with
38 g of acrylamide and 2 g of bis into 100 mls)
200 µl 10% APS
50 µl TEMED
### 6.3 Worksheets

#### 6.3.1 GMSA Worksheet

<table>
<thead>
<tr>
<th>Sample #</th>
<th>fraction bound</th>
<th>[DNA]</th>
<th>[Protein]</th>
<th>moles IHF</th>
<th>Vol IHF ul</th>
<th>Stock</th>
<th>Vol DNA ul</th>
<th>Vol Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>free DNA</td>
<td>1.00E-08</td>
<td>0</td>
<td>0.00E+00</td>
<td>0.000</td>
<td>none</td>
<td>4.292</td>
<td>13.71</td>
</tr>
<tr>
<td>2</td>
<td>0.08</td>
<td>1.00E-08</td>
<td>6.02E-09</td>
<td>1.20E-13</td>
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<th>vol buffer</th>
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DNA 55.794 100 1 of original 99
6.3.2 Fluorescence Intensity Worksheet

### Intensity IHF + JH1

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<td>Total Volume</td>
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<th>Vol needed, uL</th>
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1:100 dilution
1:25 dilution
1:5 dilution
### 6.3.3 FRET worksheet

#### FRET Stoich conditions - JH1X_FAM-R_TAMRA high salt

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