1-1-2006

Characterization of the DNA-binding Properties of Silent Information Regulator 3 Protein

Cotteka Nichisha Johnson

Follow this and additional works at: http://mds.marshall.edu/etd

Part of the Biological Phenomena, Cell Phenomena, and Immunity Commons, and the Cell and Developmental Biology Commons

Recommended Citation

Characterization of the DNA-binding Properties of Silent Information Regulator 3 Protein

Thesis submitted to
the Graduate College of
Marshall University

In partial fulfillment of
the requirements for the degree of
Master of Science in
Biological Sciences: Molecular Biology

by

Cotteka Nichisha Johnson

Dr. Philippe Georgel Ph.D., Committee Chairperson
Dr. Eric Blough, Ph.D.
Dr. Richard Niles, Ph.D.
Dr. Guo-Zhang-Zhu, Ph.D.

Marshall University
July 2006
ABSTRACT

Characterization of the DNA-binding Properties of Silent Information Regulator 3 Protein

By Cotteka Nichisha Johnson

The eukaryotic genome is organized into distinct domains with discrete regulatory potential, influenced by the variation in local chromatin structure. An example of chromatin-mediated consequences on gene expression is mating type determination of yeast *Saccharomyces cerevisiae*, which depends on silencing of specific gene cassettes. Silencing in yeast involves key proteins, amongst which the Silent Information Regulator (Sir) proteins are essential. Sir1p, Sir2p, and Sir4p are recruited to nucleate silencing events, while Sir3p serves as the pivotal factor in spreading the silent state. This investigation characterizes the DNA binding properties of Sir3p using DNA fragments with varying degrees of inherent flexibility, a characteristic that mimics nucleosomal and linker DNA. Sir3p binds to all DNA tested, but displays a higher affinity for linear DNA fragments, and even promotes formation of distinct DNA-protein complexes. Cumulatively, these results suggest the involvement of a DNA component in spreading of the silent state in *Saccharomyces cerevisiae*. 
Acknowledgements

I humbly acknowledge my advisor Dr. Philippe Georgel for his addition to this thesis in terms of illustrative figures and for his persistent assistance in the completion of this study. I express sincere gratitude to my colleague Nicholas Adkins for his role as a resource for lab etiquette and techniques. I thank Dr. Eric Blough, Dr. Richard Niles and Dr. Guo-Zhang Zhu for their participation on my committee and their insightful comments and suggestions that served to enhance the quality of both my research and this written work.

“Scientists have already cast much darkness upon the subject, and if they continue their investigations we shall soon know nothing at all about it.”

Mark Twain
# Table of Contents

Abstract...........................................................................................................................................ii  
Acknowledgement.........................................................................................................................iii  
Index of Figures..............................................................................................................................v  
Index of Tables...............................................................................................................................vii  
Chapter One: Introduction..............................................................................................................1  
Chapter Two: Experimental Design and Procedures.................................................................33  
Chapter Three: Results..................................................................................................................43  
Chapter Four: Discussion..............................................................................................................67  
Conclusion and Perspectives........................................................................................................80  
Appendix.........................................................................................................................................74  
References.......................................................................................................................................78
## Index of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1:</td>
<td>Basic DNA structure</td>
<td>2</td>
</tr>
<tr>
<td>Figure 2:</td>
<td>The Nucleosome Core Particle</td>
<td>3</td>
</tr>
<tr>
<td>Figure 3:</td>
<td>Histone Proteins</td>
<td>5</td>
</tr>
<tr>
<td>Figure 4:</td>
<td>Chromatin Architecture</td>
<td>7</td>
</tr>
<tr>
<td>Figure 5:</td>
<td>Model or Sir Protein Assembly</td>
<td>10</td>
</tr>
<tr>
<td>Figure 6:</td>
<td>Sir3p and Histone Tail Interaction</td>
<td>13</td>
</tr>
<tr>
<td>Figure 7:</td>
<td>Current Sir3p binding partners</td>
<td>15</td>
</tr>
<tr>
<td>Figure 8:</td>
<td>Linker Accessibility: EcoR1 Digestion Assay</td>
<td>17</td>
</tr>
<tr>
<td>Figure 9:</td>
<td>Model of Sir3p DNA binding activity</td>
<td>18</td>
</tr>
<tr>
<td>Figure 10:</td>
<td>Sir3-DNA Binding: Experimental Design</td>
<td>20</td>
</tr>
<tr>
<td>Figure 11:</td>
<td>Sir3p and Nucleosomal DNA 1</td>
<td>21</td>
</tr>
<tr>
<td>Figure 12:</td>
<td>Sir3p and Nucleosomal DNA 2</td>
<td>22</td>
</tr>
<tr>
<td>Figure 13:</td>
<td>DNA Sequences&lt;br /&gt;(p29-p58, PX199, 208-1 and 4WJ)</td>
<td>23</td>
</tr>
<tr>
<td>Figure</td>
<td>Title</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>--------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>14</td>
<td>Origin of DNA fragments</td>
<td>24</td>
</tr>
<tr>
<td>15</td>
<td>Electrophoretic Mobility Shift Assay Sir3p Depletion of p29-p58 DNA</td>
<td>36</td>
</tr>
<tr>
<td>16</td>
<td>Electrophoretic Mobility Shift Assay Sir3p Depletion of PX199 DNA</td>
<td>39</td>
</tr>
<tr>
<td>17</td>
<td>Electrophoretic Mobility Shift Assay Sir3p Depletion of 208-1 DNA Fragment</td>
<td>41</td>
</tr>
<tr>
<td>18</td>
<td>Four-way Junction (4WJ) Construction</td>
<td>43</td>
</tr>
<tr>
<td>19</td>
<td>Four-way Junction Migration</td>
<td>45</td>
</tr>
<tr>
<td>20</td>
<td>Electrophoretic Mobility Shift Assay Sir3p Depletion of 4WJ DNA</td>
<td>46</td>
</tr>
<tr>
<td>21</td>
<td>Electrophoretic Mobility Shift Assay Sir3p-p29-p58 Complexes</td>
<td>50</td>
</tr>
<tr>
<td>22</td>
<td>Electrophoretic Mobility Shift Assay Sir3p-PX199 Complexes</td>
<td>51</td>
</tr>
<tr>
<td>23</td>
<td>Electrophoretic Mobility Shift Assay Sir3p-208-1 Complexes</td>
<td>52</td>
</tr>
</tbody>
</table>
| 24     | Southern Blot: p29-p58 Probe  
**p29-p58** + PX199 + 208-1 Sir3p Competition | 55   |
| 25     | Southern Blot: PX199 Probe  
**PX199** + p29-p58 + 208-1 Sir3p Competition | 56   |
| 26     | Southern Blot: 208-1 Probe  
**208-1** + p29-p58 + PX199 Sir3p Competition | 57   |
<p>| 27     | Sir3p Primary Amino Acid Sequence                            | 63   |</p>
<table>
<thead>
<tr>
<th>Figure</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 28:</td>
<td>Sir3p-DNA Hierarchies</td>
<td>68</td>
</tr>
<tr>
<td>Figure 29:</td>
<td>Model of Sir3p-DNA</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>Activity in Silencing</td>
<td></td>
</tr>
<tr>
<td>Figure 30:</td>
<td>Intensity Test and Values 1</td>
<td>74</td>
</tr>
<tr>
<td>Figure 31:</td>
<td>Graph of Intensity Values vs. Internal Control DNA Masses</td>
<td>75</td>
</tr>
<tr>
<td>Figure 32:</td>
<td>Calculating Sir3p DNA Depletion</td>
<td>76</td>
</tr>
<tr>
<td>Figure 33:</td>
<td>Graph of Sir3p DNA Depletion</td>
<td>77</td>
</tr>
</tbody>
</table>
Index of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sir3p DNA Depletion Values</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>p29-p58 DNA Fragment</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Sir3p DNA Depletion Values</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>PX199 DNA Fragment</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Sir3p DNA Depletion Values</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>208-1 DNA Fragment</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Sir3p DNA Depletion Values</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>4WJ DNA Fragment</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Sir3p DNA Depletion</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>A Comparison of Four DNA Fragments</td>
<td></td>
</tr>
</tbody>
</table>
Chapter One

Introduction

DNA and Chromatin - Background

The fundamental unit of life is DNA, which contains hereditary biological information that must be correctly copied and transmitted from generation to generation each time the cell divides. Using X-ray diffraction, biologists have been able to determine and characterize the three dimensional structure of DNA (Watson, Crick, Wilkins, and Franklin, 1953; Siden, 1994). DNA is composed of two complementary polynucleotide chains, each composed of four types of nucleotide subunits, and held together by hydrogen bond interactions (Figure 1). The nucleotides are composed of a five carbon sugar, a phosphate group, and one of four bases: adenine, cytosine, guanine or thymine (Figure 1). Covalent interactions link the base, sugar and phosphate groups together to form a stable, yet flexible structure, with alternating sugar and phosphate groups forming a backbone around the bases. The arrangement of the nucleotides creates a gently twisted, double helical, polar structure that is conducive to DNA-associated activity within the nucleus of the cell (Crick, 1954; Siden, 1994).

The majority of DNA within the eukaryotic cell is sequestered within the confines of the nucleus which occupies a mere 10% of total cellular volume (Siden, 1994 and Lilley, 1992). Since the length of naked DNA exceeds the space delimited by the nuclear membrane, DNA must be condensed to fit into the nucleus. The shortest human chromosome contains $\sim 4.6 \times 10^7$ bp of DNA, and this is $\sim 10$ times the length of the Ecoli (Escherichia coli) bacterial genome (Siden, 1994; Lilley, 1992; and Kindt et al.,

This length is equivalent to 14,000 µm of extended DNA and when condensed this chromosome is approximately 2 µm long, a packaging ratio of about 7000. This degree of compaction allows the entire genome, 3 x 10^9 bp in length, to fit snugly into the nuclear space, a diameter of ~ 10^{-5} m (Lowary and Widom, 1977; Kornberg, 1997).

**Figure 1: Basic DNA Structure.**

DNA is the basic unit of life, containing hereditary information that must be transmitted precisely from generation to generation. DNA is structured as two anti-parallel, complementary polynucleotide strands, composed of 4 types of nucleotide subunits. The nucleotides are composed of a five carbon or pentose sugar, one or more phosphate groups and one of four bases; Adenine (pink), Thymine (yellow), Guanine (green), and Cytosine (blue). The bases are contained by the two polymer chains and interact with each other by means of hydrogen bonds. Covalent interactions between alternating phosphates and bases, forms a negatively charged, stable yet flexible backbone that is conducive to DNA function within the cell. (Figure from Raul et al, 2004)
To achieve this level of packing, a number of DNA-associated proteins (primarily histones) are used to compact the DNA into several hierarchical levels of organization and ultimately into its native conformation, termed chromatin.

The basic, repeating unit of chromatin is the nucleosome core particle, which is composed of ribbons of DNA, 146 bp in length, wrapped in a left-handed manner, 1.75 times around an octamer core of Histone proteins (Kornberg and Thomas, 1974; Kornberg, 1977). The histones making up the nucleosome core particle (Figure 2) are H2A, H2B, H3 and H4 and they are highly conserved and well-suited for the critical role they play in shaping chromatin architecture (Figure 3).

![nucleosome core particle](image)

Figure 2: The nucleosome core particle
The Nucleosome is the basic unit of chromatin, and is comprised of ribbons of DNA, 146 bp in length, wrapped in a left-handed manner 1.7 times around an octamer of histone proteins (2 each of H2A, H2B, H3 and H4). The histone proteins have highly basic tails that associate tightly with the negatively charged DNA molecule. Nucleosomes are regularly spaced and repeat every 180-200 bp along the DNA and they represent the first order of DNA folding and compaction referred to as nucleosomal arrays. Nucleosomal arrays are 10nm fibers and are further folded to form a 30nm fiber by the addition of histone protein, H1. (Figure from Luger et al. 1997, Nature).
These core proteins are small (10-14 kDa), highly positively charged, basic proteins that associate tightly with the negatively charged phosphate backbone of the DNA molecule to form stable, nucleosomal units (Kornberg and Thomas, 1974; Kornberg, 1977; Lowary and Widom, 1997). Core histone affinity for DNA is conferred by the presence of lysine and arginine amino acid residues, especially within the N-termini of the histone proteins. In fact, the composition of the core histone proteins is at least 20% lysine and arginine, and these basic residues have a strong preference for the negatively-charged double helical DNA structure (Kornberg and Thomas, 1974; Lowary and Widom, 1997).

Furthermore, these lysine and arginine residues are susceptible to post-translational chemical modifications such as acetylation, phosphorylation, methylation, ubiquitination and ADP-ribosylation, all of which influences chromatin organization by affecting either the stability or accessibility to regulatory factors (Kornberg, 1977; Luger and Hansen, 2005).

When the linker DNA is added, nucleosomes (Figure 4B) form regularly spaced, beads-on-the-string structures or nucleosomal arrays (Figure 4C). Nucleosomes in these arrays are evenly spaced along the DNA forming a 10 nm nucleofilament and allow a packaging ratio of about 6 compared to naked non-histone associated DNA (Widom, 1991a; Widom, 1991b; Luger et al., 1997). Nucleosomal arrays are referred to as the primary structure of chromatin (Luger et al., 1997; Woodcock and Dimitrov, 2001). Folding of the array and the addition of linker histones, H1 (Figure 4D) promotes nucleosome-nucleosome interactions to form a condensed 30 nm, stably folded secondary chromatin structure (Yao et al., 1993; Zlatanova and Holde, 1996; Luger et al., 1997; Woodcock and Dimitrov, 2001).
Figure 3: Histone proteins

Histones are small (10-14 kDa) basic proteins that are critical for packaging of eukaryotic DNA into its native chromatin structure within the nucleus. An octamer of the histone proteins (2 each of H2A, H2B, H3 and H4) forms the core of the nucleosome core particle (shown in inset). The core histones are highly positively charged and are able to interact tightly with the negatively charged phosphate backbone of DNA molecules. The core histone proteins contain a central region of approximately 70 amino acids that make up the histone fold domain important for histone assembly. This domain allows the histones to dimerize and eventually form the octamer core. The histone fold-motif also makes connections with the DNA adding to the stability of the nucleosome. Histones can be posttranslationally modified by enzymes typically on their lysine and arginine-rich N-terminal tails, but also in their globular domains. The usual chemical modifications include methylation, acetylation, phosphorylation, ubiquitination, and ADP-ribosylation and these affect histone function, chromatin architecture and ultimately gene activity. All four core histones are required for cell viability. (Figure from Wheeler et al., 2005)
Further folding events and interactions between the secondary structures (long range fiber-fiber interactions) lead to higher-order tertiary chromatin conformations. This level of folding and compaction increases the packing ratio to approximately 40 (Widom, 1991a; Yao et al., 1993; Zlatanova and Holde, 1996; Luger et al., 1997; Woodcock and Dimitrov, 2001). Chromatin compaction and folding beyond the 10 nm and 30 nm fibers, produce condensed conformations or higher-order structures that result in a 10,000 fold decrease in naked DNA length as seen in the formation of mitotic chromosomes (Schwarz and Hansen, 1994; Wolfe, 1998;). While the 10 nm primary structure and the 30 nm secondary structure chromatin intermediates are fairly well-characterized, very little is known about the chemical properties and function of tertiary structure chromatin at the higher order level of compaction. An important concept for gene regulation is the fact that chromatin is not a rigid or static structure. Even though the DNA is so thoroughly packaged, and also assumes higher-order chromatin conformations by means of internucleosomnal contacts, and through association with core and linker histones and other non-histone factors, chromatin has been demonstrated to have a very malleable conformation. In fact, it is the substrate for a number of gene activities including DNA replication, repair, recombination and transcription (Marshall et al., 1997; Wolffe, 1998). To accommodate these gene regulatory events, DNA within the nucleus is arranged into distinct domains with discrete regulatory potential, where genes can be differentially regulated; some genes can be turned on and upregulated at a specific stage of development, while others are simultaneously down-regulated, even repressed or silenced (Alberts and Sternglanz, 1990; Elgin, 1996). Silencing prevents the build up of stable transcripts from specific genes in the eukaryotic genome (Alberts and Sternglanz, 1990; Elgin, 1996). Silenced or repressed chromatin in higher eukaryotes is referred to
as heterochromatin. The establishment of heterochromatin depends on the activity of a number of chromatin binding factors, in yeast these are primarily the family of Sir (Silent Information Regulator) proteins (Grunstein, 1998; Gartenberg, 2000). Sir3p has been relatively well characterized and is known for its strong preference for the tails of nucleosome core histones, specifically histones H3 and H4 (Hecht et al., 1995; Grunstein, 1998). However little has been investigated regarding Sir3p DNA binding properties.

Figure 4: Chromatin Architecture: Structure, Folding, and Compaction.
DNA is packaged to fit into the confined space of the nucleus through interaction with the highly charged, basic histone proteins, H2A, H2B, H3 and H4. A, Naked or free DNA is negatively charged and interacts tightly with positively charged, Lysine and Arginine rich histones to form B, nucleosomes. C, Nucleosomes are evenly spaced along the DNA forming a 10nm nucleofilament or nucleosomal array referred to as the primary structure of chromatin. D, Folding of the array and the addition of linker histones promotes nucleosome-nucleosome interactions to form a condensed 30nm, stably folded secondary chromatin structure. Further folding events and interactions between the secondary structures (long range fiber-fiber interactions) lead to E, higher-order tertiary chromatin conformations, of which little is known about structure or function. (Figure adapted from Hansen et al. Biochem. 1989).
In this study we discuss the Sir3 protein (Sir3p), with a focus on its DNA binding abilities. We hypothesize that Sir3p is a protein with promiscuous DNA-binding properties, having the ability to bind DNA molecules with differences in sequence, curvature and bendability. Since Sir3p is known to bind histones within the nucleosome, we initially hypothesized that it may also preferentially interact with nucleosomal or linker DNA and this Sir3p-DNA interaction may play a role in chromatin silencing.

**Gene Silencing**

Silencing refers to the organization of specialized chromatin regions into structures that prevent the access of remodeling factors, recombination machinery and other gene activating proteins such as transcription factors, and the blocking of these factors prevents gene expression within the silenced region (Laurenson and Rine, 1992; Lowell and Pillus, 1998). Silencing was initially characterized in *Saccharomyces cerevisiae* and defined as the process that represses transcription of duplicated copies of the mating type loci HML and HMR (Herskowitz et al., 1991; Laurenson and Rine, 1992, Donze et al., 1999). Silencing is different from gene-specific repression in that it is more than simply the repression of individual genes under the control of one specific promoter. Instead silencing involves the complete repression of most of the genes within a specific chromosomal region. Silencing in yeast is accomplished by the activity of cis-acting regulatory regions known as ‘silencers’. These silencers can interact with trans-acting factors such as silencing proteins and can repress the expression of target genes at distal regions of the chromosome. (Loo and Rine, 1994; Holmes and Broach, 1996).

Since many of the structural and biochemical properties of heterochromatin still remains to be characterized, silenced chromatin in *Saccharomyces cerevisiae* has been used as
a model system of study to gain insight into the properties of silenced chromatin in higher eukaryotes. Using genetic studies researchers have clearly identified several silenced regions in the yeast chromosome and these include rRNA encoding DNA (rDNA), the telomeric regions, and the most thoroughly characterized, the two silent mating type loci (HMR and HML) (Loo and Rine, 1994; Holmes and Broach, 1996). Silenced chromatin, in Saccharomyces cerevisiae, displays a number of heterochromatic features (Lowary and Widom, 1989). These include a tangible reduction in expression levels, hypoacetylated histone tails, specifically H3 and H4, and the involvement of trans-acting proteins that bind cis-acting elements (silencers) to establish the silenced state (Lowary and Widom, 1989; Loo and Rine, 1994; Holmes and Broach, 1996, Wang, 2004). Among the primary trans-acting, silencing protein factors in yeast are Rap1 (Repressor Activator Protein), ORC (Origin of replication complex), and Silent Information Regulators Sir1p, Sir2p, Sir3p and Sir4p (Figure 5).

The ORC is conserved throughout eukaryotes and is essential for DNA replication initiation, while Rap1 binds to \([C_{(1-3)}A]_n\) repeats at the ends of chromosomes and assist in the regulation of telomere length (Giesman et al., 1991; Bell et al., 1993, Bell et al., 1995; Buck and Shore, 1995) Both Rap1 and ORC have been shown to play a critical role in the establishment of silent chromatin in yeast, by acting to specifically recruit and concentrate other silencing proteins, such as the Sir proteins, at the silent loci (Kurtz and Shore, 1991; Hardy et al., 1992; Foss et al., 1993; Fox et al., 1995).
Figure 5: Model of assembly of silent information regulator (Sir) proteins.
Sir1p binds first to a complex of proteins called the origin of recognition complex (ORC) that associates with the silencer complex. Sir4p is then recruited to the silencer by the action of Sir1p and repressor activator protein 1 (Rap1). Sir2p is probably targeted to the silencer as part of a heterodimer with Sir4p. Sir3p is also recruited to silencer by Sir4p and associates with both Sir4p and Rap1. Cooperative interactions promote the spread of silencing information throughout the locus.

The SIR Proteins

The four Sir proteins were identified as a result of null mutational studies, in cells with complete knockouts of the Sir genes. In cells lacking the Sir proteins, the silent mating type genes controlled by these proteins were activated (Laurenson and Rine, 1992; Grunstein, 1994; Grunstein, 1997; Gartenburg, 2000). Sir2p, Sir3p, and Sir4p have been shown to be essential for the establishment of silent states, because of the critical structural roles they play as scaffolding and dimerization partners for the other silencing
factors (Lustig, 1998; Gartenburg, 2000). Sir1p, on the other hand, has been characterized as an accessory factor that helps in the recruitment of the other Sir proteins to target genes, its activity is not essential (Laurenson and Rine, 1992; Lustig, 1998; Chien et al., 1993). Sir2p is an NAD$^+$-dependent deacetylase that is conserved in both higher eukaryotes and bacteria. The NAD$^+$-dependent deacetylation reaction carried out by Sir2p has a free energy of hydrolysis that is similar to that of ATP, therefore a remarkable amount of energy is released in Sir2-dependent silencing at the yeast mating type HMR and HML loci (Moazed, 2001a; Ghidelli et al., 2001, Guarente, 2000; Landry et al., 2000) Studies have provided evidence that suggest that this energy is used to induce conformational changes in the DNA and protein factors necessary for the organization of silenced chromatin structures (Landry et al., 2000; Chen and Widom, 2004). In contrast to Sir2p, Sir4p has no currently known enzymatic activity; instead Sir4 has been characterized as a structural protein, serving as a mediator for the recruitment of other silencing protein factors. Sir4p heterodimerizes independently with either Sir2p or Sir3p, and targets them to gene areas to engage in repression (Laurenson and Rine, 1992; Cockell et al, 1995). Studies have shown that Sir4p also associates with stationary components on the periphery of the nucleus, such as the nuclear matrix, and this helps to restrict its activity at the silenced chromatin domains (Laurenson and Rine, 1992; Moazed, 2001a). Sir4 has also been shown to associate with two ubiquitin hydrolases and studies have demonstrated that the removal of ubiquitin from gene targets enhances silencing activity (Palladino et al. 1993; Moazed, 2001a).


Silent Information Regulator Protein 3

Sir3p has been defined as the seminal factor in the spreading of silent chromatin in *Saccharomyces cerevisiae* (Park *et al*., 1998) It is a 113 KDa protein and like Sir4p, Sir3p does not display any enzymatic activity; instead Sir3p plays a structural role, mediating protein-protein interactions (Hecht *et al*., 1996; Liu and Lustig, 1996; Lustig *et al*., 1996) Using a number of genetic mutational assays and the two-hybrid system, researchers have been able to identify a large pool of Sir3p binding partners (Figure 7). Sir3p partners include not only members of the Sir family or other silencing factors such as ORC and Rap1. Additionally, Sir3p also interacts with an important DNA repair factor called RAD7 (Stone and Pillus, 1998). This implies that Sir3 protein may have roles that extend beyond its activity as a silencing protein. Sir3p possesses a 214 amino acid region referred to as a bromo-homology domain that is thought to be a key interaction site for Sir3p-Sir3p homodimerization and for interaction with other silencing factors (Park *et al*, 1998). *In vivo* studies have demonstrated that Sir3p is hyperphosphorylated on a number of Serine residues, in response to heat shock, starvation and other stresses and these changes correspond to changes in silenced states (Hecht *et al*., 1996; Lustig *et al*., 1996; Park *et al*, 1998). However, it has not been determined whether the phosphorylation of Sir3p regulates its binding or spreading activity at silent gene targets. It has been suggested that the activity of all the members of the Sir family may be mediated by their binding to histones, with Sir3p interacting primarily with the N-termini of histones H3 and H4 (Figure 6) (Thompson *et al*., 1994; Moazed, 2001a).
Figure 6: Sir3p and histone protein-protein interactions.
Sir3p activity at the yeast HMR and HML silent loci is in part dependent on its ability to form a heterodimer with Sir4p and also on the NAD+ dependent deacetylase activity of Sir2p. Sir4p recruits and targets Sir3p to the loci and Sir2p deacetylates the N-termini of histone tails, specifically histones H3 and H4 creating high affinity sites for Sir3p to bind. Sir2p deacetylation activity disrupts DNA histone contacts and allows Sir3p-histone protein-protein interactions. Sir3p also acts in tandem with repressor activator protein 1. The activity of Rap1, Sir2p and Sir4, initiates silencing and Sir3p acts to spread and maintain the silent state at the yeast silenced non-mating type loci and telomeres. No DNA component is shown as part of Sir3p silencing activity. (Figure from Hecht et al., 1995, Cell).

Model of Assembly of SIR Proteins

The current model of Sir proteins assembly is depicted in figure 5. Sir proteins are targeted to silencers through an ordered, sequential series of protein-protein interactions (Vendetta et al., 2000; Wang et al., 2004; Chen and Widom, 2004). Sir1p can be recruited to the silencer in the absence of any of the other Sir proteins. It binds directly to the ORC at the silencer and when tethered to the ORC, initiates the assembly of the essential Sir proteins (Sir2p, Sir3p, and Sir4p). Sir1p and Rap1 recruit Sir4p to the silencer. Sir2p heterodimerizes with Sir4p and is thought to be targeted to the silent locus...
by its association with Sir4p. Sir3p is also recruited to the silent locus by forming a heterodimer with Sir4p. Sir2p NAD\(^+\)-dependent deacetylase catalytic activity creates hypoacetylated H3 and H4 tails (Figure 6) generating the high affinity sites for Sir3p/Sir4p heterodimer recruitment. (Vendetta et al., 2000; Wang et al., 2004; Chen and Widom, 2004). Sir3p also binds Sir4p, Rap1p and probably Sir1p (Gardner and Fox, 2001) and Sir2p (Strahl-Bolsinger et al., 1997), although it has a stronger preference for Sir4p (Gardner and Fox, 2001; Carmen et al., 2002; Chen and Widom, 2004). The recruitment and activity of Sir1p, Sir2p, Sir4p and Rap1p are thought to initiate silencing while Sir3p is the pivotal factor that enhances interactions between all the protein factors and cause the spreading of the silent state to distal regions of the chromosome (Laurenson and Rine, 1992; Vendetta et al., 2000; Chen and Widom, 2004). Despite the fact that Sir3p has been shown to bind to DNA in vitro (Georgel et al., 2001; Ghidelli et al., 2001), the DNA role in Sir3p-dependent silencing remains unclear.
Figure 7: Sir3p binding partners.
The two hybrid protein-protein interaction assay, in vitro analyses and other biochemical techniques such as immunoprecipitation were used to determine sir3p binding partners. Sir3p has been seen to associate with all the other Sir family members at the yeast HMR and HML silenced loci and telomeres. Sir3p may also influence DNA replication since it interacts with the ORC which is responsible for DNA replication initiation. Sir3p also binds to and influence the activity of Rap1, a common transactivator in yeast, responsible for the expression of hundreds of different genes. Sir3p has been implicated in DNA repair since it can associate tightly with the protein product of the RAD7 gene, a key component involved in DNA repair. Finally, Sir3p also homodimerizes and this may play a role in concentrating sir3p protein at the silencer. (Figure adapted from Stone and Pillus. BioEssays, 1998.)
**Thesis Goals**

Recently published data from our lab demonstrated that Sir3p binds to 208-12 DNA, the tandemly-repeated, nucleosome positioning, 208-bp 5S rDNA sequence from *Lytechinus variegatus* (Sea urchin), albeit with lower affinity than its interaction with chromatin (Georgel, *et al*., 2001). Georgel *et al*, tested linker DNA accessibility to Sir3p in a chromatin context. An Eco R1 restriction digestion assay (Figure 8) was done using a 208-12 nucleosomal array, with Eco R1 restriction sites located within each of the 11 linker DNA regions. The nucleosomal array was incubated with increasing molar ratios of Sir3p and then subjected to the Eco R1 restriction enzyme digestion (Figure 8). The rationale was that if Sir3p binds to the linker DNA then it would protect the Eco R1 restriction sites from digestion. However, if Sir3p binds elsewhere (nucleosomal DNA) then the linker region would remain accessible for partial or complete restriction and the result would be digestion of the 208-12 nucleosomal array to form mononucleosomes (Figure 8). Results showed that Sir3p did not protect the linker from restriction, suggesting that Sir3p does not bind linker DNA or is not sufficient to protect the Eco R1 restriction sites from digestion. This suggests that Sir3p interacts elsewhere perhaps with DNA in close contact with the core histones (Georgel *et al*., 2001). The aim of my thesis is to characterize the DNA-binding properties of Sir3 protein. Based on Georgel *et al*’s study, several scenarios can be envisioned. One can rationalize that Sir3p might bind to distorted regions, (non-uniform regions of DNA around the histone octamer that is sharply bent or has deviated from the usual B-DNA structure) wrapped around the histone octamer. Another possibility is that Sir3p might bind to the DNA at the entry and exit point of DNA around the octamer core (Figure 9).
Figure 8: Linker accessibility using Eco R1 assay.

The experimental design used to test Sir3p-linker DNA associations involved the use of Eco R1 enzyme, since the 208-12 nucleosomal array was constructed with Eco R1 restriction sites within each of the 11 linker DNA regions between the 12 nucleosomes of the 208-12 nucleosomal array. The rationale is, linker DNA seems the most readily available for protein binding, and if Sir3p preferentially binds to the linker DNA region then it will protect the Eco R1 site from restriction. However, if Sir3p binds to DNA in closer contact with the histone core then this would leave the Eco R1 sites within the linker unprotected and accessible to the enzyme. The product of the restriction would be mononucleosomes. (Figure prepared by Dr. Philippe Georgel and results of EcoR1 assay has been published by Georgel et al. PNAS, 2001).
Figure 9: Model of Sir3p-DNA interaction within chromatin.
Based on Eco R1 restriction map of the 208-12 Nucleosomal array, the proposed mode of Sir3p DNA binding activity is that Sir3p binds not just to the histone proteins (specifically the N-termini of Histone H3 and H4) but also to different regions of the 146 bp superhelical DNA within the nucleosome core particle. Eco R1 sites (red arrows) within the linker were not protected by Sir3p (green ovals) binding and this suggests that Sir3p, in addition to binding high affinity sites, H3 and H4 N-termini, must preferentially bind to nucleosomal DNA rather than linker DNA. (Figure adapted from Georgel et al. PNAS, 2001).

Using an Electrophoretic Mobility Shift Assay as my main experimental tool (Figure 10), I wanted to compare Sir3 protein’s affinity for DNA with differences in sequence, which would also mean differences in curvature and bendability (Figures 11, 12 and 13). These intrinsic structural properties were selected with the goal of determining whether Sir3p preferentially binds one structural DNA conformation (straight,
curved, bent or four-way junctions) over another. To address this question we selected four fragments. The p29-p58 DNA fragment which is a 212 bp fragment prepared from the promoter region of the heat shock protein 70 gene of *Drosophila melanogaster* using two primers (p29 forward and p58 reverse, see sequence in Tsukiyama *et al.*, 1994). The PX199 DNA fragment is a 199 bp fragment prepared from a plasmid (pPol I 208-4) encompassing a binding site for the RNA polymerase 1 gene from *Ancanthameoba histolitica* (Figure 13), (Georgel, and Robert, 2001). The PX199 fragment was cleaved from the plasmid by Pvu II and Xba restriction. The 208-1 DNA fragment is a 208 bp fragment from the 5s rDNA tandemly repeated gene of *Lytechinus variegatus* (Figure 13) (Georgel, and Robert, 2001). The four-way junction (Lilley, 1993; Lilley, 1994) is a four stranded DNA structure synthesized using 4-synthetic single stranded oligonucleotides, each approximately 36bp in length. The 3-D structures of the DNA templates were modeled using the Trifonov DNA bending algorithm (Figure 14) and Mathematica™ was used to calculate the affinities used to determine the mode of binding. (Trifonov and Mengeritsky, 1987; Trifonov, 1991). Even though these experiments examine Sir3p-DNA binding, we wanted to stay within the context of chromatin and therefore we selected DNA templates that mimic the DNA that would be found associated with the histone core; nucleosomal DNA. The p29-p58 fragment is relatively straight DNA and was used as a mimic of the linker DNA. The PX199 fragment displays a somewhat sharp bend about 40bp from its 3′ end of the sequence, and mimics distorted regions of DNA curvature at superhelical location (SHL) +/- 1.5 and +/- 4.5 (as defined by Luger *et al.*,1997) that is typically found in the nucleosome (Beutel and Gold, 1992; Luger *et al.*,1997; Richmond and Davey, 2003). The 208-1 DNA has 2 slight curves centrally located within the fragment, that resembles DNA wrapped more uniformly around the histone octamer
(Hagerman, 1988; Luger et al, 1997; Richmond and Davey, 2003, Cloutier and Widom, 2005). The four-way junction (4WJ), four stranded DNA structure, synthesized using four synthetic single stranded oligonucleotides, was used as a mimic of the entry and exit site of nucleosomal DNA. (Lilley, 1993; Lilley, 1994; Kadrmas et al., 1995; Richmond and Davey, 2003).

While these fragments are not perfect representations of the native structure of DNA within the nucleosomes, they provide an in vitro model that can be used as a starting point to characterize the DNA binding properties of the Sir3 protein to chromatin.

**Figure 10: Experimental design for Sir3p-DNA interaction.**

A simple three step experimental procedure was followed for the duration of the characterization of Sir3p DNA binding properties. Purified Sir3 protein was incubated with specific DNA sequences, for a maximum of 30 minutes at room temperature, to allow binding activity. Incubated samples were then electrophoresed on a 1% agarose gel (1x TAE) and gels were stained, imaged and nucleoprotein structures were analyzed. Electrophoretic Mobility Shift Assay (EMSA) is a well established method for studying DNA-protein interaction.
Figure 11: Modeled DNA fragments and the nucleosomal regions they mimic.

Three fragments were selected to test Sir3p DNA binding ability. The p29-p58 DNA fragment is relatively straight or linear DNA and in this study serves as a paradigm for linker DNA. DNA within the nucleosomal core particle must bend and curve to wrap around the histone octamer core. The 208-1 fragment serves as a mimic of curved DNA within the nucleosome and the PX199 mimics sharply bent DNA, which is also typical of DNA within the nucleosome core particle. Fragments were modeled using the Trifonov bending algorithm (Figure prepared by Dr. Philippe Georgel, 2005, Marshall University).
Figure 12: Four-way junction mimics nucleosomal DNA entry and exit region.
DNA within the nucleosome particle intersects as it enters and exits the nucleosome to connect with the adjacent nucleosome within the nucleosomal array or chromatin fiber. A four-way junction DNA sequence was used as a mimic of the entry and exit site of nucleosomal DNA, to test Sir3p ability to interact with such a conformation. The four-way junction was synthesized using 4 synthetic, single-stranded oligonucleotides, ~35-40 bp in length (Figure adapted from Panyutin et al. 1995, Embo. Journal).
All fragment had DNA sequences that were approximately 200 bp in length (-/+ 20 bp). Templates were PCR amplified and concentrations were checked using a Nanodrop system. Integrity of the DNA templates was checked on a native 1% agarose gel. All fragments migrated to expected molecular weights when compared to 1 kb+ DNA markers. Standard DNA mass of 50ng of each DNA fragments was used in each sample throughout this study.

**p29-p58 DNA fragment**

TCGAGAAATTTCCTGGCCTTATTCTCTATTCTGTTTTGTGACTCTCCCTCTCTGTACT ATTGCTCTCTCACTCTGTCGACAGTAAACGCGACTCTATTCTCGTTTGCTTCGAGAG AGCGGCCCTCGAATGTTCCGCAGAAAAGAGCGGGCCGGAGATATAATAGAGGCGCT CGTCCAGAGGGACGTCAATTCTAATCIIAACAAGCAAAGTGACACATCGCGAA

**PX199 DNA Fragment**

TGGCGAAAGGGGATGTGCTGCAAGGCAGTTAAGTTGAGTACGCAGCCAGGG TTTTCCCAGTCACGACTTTGTAAAACGACGGCCAGTCCGAAGCTTTTGCTGC AGGATCCCAGCGAAAACCAGCAGACCGGCGAGCAGTTTTCTGCACTAA ACTGGTCGGACCCTCGCGAAAGTATATATATATAAAAGGGACGGGTCCGCGCG

**208-1 DNA Fragment**

GGAATTCCAAGCAATAACTTCCAGGAGATTATAAGCCGATGACGTCATAACA TCCCCGCCCTTAAATACCCTTTACTTTCTACGCAAGCACGCCTACGACCATAAC CATGCTGAATATACCGGTCTGTCGATCGATCGAATATCAGAGGTC TCGTTAGTACTTTGGATGGAGACCGCGCTTGGGAATACCGAATTCCCCGA

**Four-way Junction Oligonucleotides**

4WJ 01: ACCATGCTCGAGATTACGAGCAGCTTCGATCGTCG 4WJ 02: AATTGCAGACGTCAAGCTGAATACGTGAGGCCTAGG 4WJ 03: TTTCGCATGCATGCGATATCGAATATCGAATATCGAGCATGG 4WJ 04: GATCCTAGCGTACGTATTATATCGATGATGC

Figure 13: Sequence of DNA templates.
Figure 14: Origin of DNA templates.
The three DNA templates used were selected based on their structure, conformation and inherent bendability and curvature. The p29-p58 fragment is relatively straight and was restricted (Xba 1) from a plasmid (pd hsp xx 3.2 (Tsukiyama et al., 1994)) containing the 3.2 Kb promoter and some flanking regions of the heat shock protein (hsp 70) gene in *Drosophila melanogaster*. The PX199 fragment is sharply bent and was prepared from a plasmid (pPol1 208-4 (Georgel and Robert, 2002)) containing the promoter region of the RNA polymerase I gene in *Acanthameoba histolitica*. The 208-1 fragment was prepared by digesting the 208-12 5S rDNA containing 12 tandem repeats of the 5S gene from *Lytechinus Variegatus* (Sea urchin). Sir3p interacted with all fragments albeit with different affinity (Figure prepared by Dr. Philippe Georgel, 2005 Marshall University.)
Chapter Two

Experimental Design

The objective of this work was to measure Sir3p affinity for DNA, with the goal of determining whether or not a specific DNA conformation would enhance or hinder Sir3p DNA binding properties or abilities. All Sir3p-DNA binding studies were performed using the Electrophoretic Mobility Shift Assay (EMSA). EMSA is used as a measure of protein-DNA interaction. In each experiment 50 ng aliquots of DNA fragments (p29-p58, PX199, 208-1 and 4WJ) were used in the presence of increasing molar ratios of Sir3 protein. DNA depletion at each level of Sir3 protein was measured using an intensity test, where the depletion of free DNA was determined by comparing the density of free DNA bands on a 1% agarose gel. A titration of DNA was used to create an internal calibration curve and to serve as a negative control by serving as a standard to compare the density of free DNA under sample conditions. The internal calibration curve consisted of 50 ng, 37.5 ng, 25 ng, and 12.5 ng of each DNA fragment. Experimental Design is further depicted below and described in detail under experimental procedures.
Experimental Procedures

Preparation of Sir3 protein:

The Sir3p was purified and characterized according to the protocol described in the literature (Georgel et al., 2001). Briefly, Sir3p was harvested from SF9 insect cells that were infected with recombinant baculovirus expressing Sir3p. Harvested Sir3p was purified using a combination of methods including ammonium sulfate fractionation, Ni affinity chromatography, and Q-sepharose chromatography. The integrity and concentration of the purified Sir3 protein was determined using SDS/PAGE. Known concentrations of BSA were used as a standard. Recombinant Sir3 protein migrated on the gel with an apparent molecular mass of approximately 120 kDa, which is within 7 kDa of the native protein molecular mass of 113 kDa. Sir3p was prepared by Dr. Steve McBryant of Dr. Jeffery Hansen’s laboratory, Colorado State University. The final Sir3 protein preparation was dialyzed against a buffer containing 10 mM Tris, 2 mM NaCl, 0.2 mM beta-mercaptoethanol, and 0.25 mM EDTA. Sir3p was stored in aliquots of 10 µl at negative 80 °C (note: aliquots were only submitted to one cycle of freeze/thawing to avoid damage to the protein).

DNA Fragments: Selection, Modeling and Preparation:

Preparation of the p29-p58 DNA Fragment:

The p29-p58 fragment was prepared from the plasmid pdHSPXX3.2 containing the promoter region of the HSP70 gene from Drosophila melanogaster. Forward primer in the polymerase chain reaction was primer p29, and the reverse primer was p58 (Primer sequences: p29- TCG AGA AAT TTC TCT GGC CG and p58- TTC GCG ATG TGT TCA
CCT). The PCR product was ethanol precipitated using 10 µl of 5 M Sodium Acetate and 275 µl of 100 % ethanol, centrifuged at 13000 g and supernatant was removed from DNA pellet. 1 ml of 70% ethanol was added to pellet and it was spun again at 13000 g. Finally, the supernatant was removed and discarded and the p29-p58 pellet was re-suspended in 30 µl of milli-Q H₂O. Final concentration of the p29-p58 product was determined using the Nanodrop Nucleic Acid Analysis software. Based on the results of the Trifonov DNA Curvature algorithm, the p29-p58 DNA is a relatively straight or linear DNA template.

Preparation of the PX199 DNA Fragment:

The PX199 DNA fragment was prepared from the plasmid pPol I 208-4 (Georgel et al, 1993), containing the binding region for Acanthameoba histolitica RNA polymerase I. The plasmid pPol I 208-4 was previously digested (Georgel et al, 1993) using the restriction enzymes Pvu II and Xba I, and this restriction digest resulted in a 199 bp fragment and two other fragments (a 1080 bp fragment comprising of 4 copies of the 208 5S positioning sequence and a 2320 bp fragment from the pUC 8 plasmid sequence). The PX199 fragment was amplified using 40 cycles of PCR using primers PX199-100 bottom and PX199-100 top (Primer sequences: PX199-100 bottom- CGC TCG TTT TAC AAC GTC and PX199-100 top- CCG CAC AGA TGC GTA AGG). The PCR product was ethanol precipitated using (add volumes) 10 µl of 5 M Sodium Acetate and 275 µl of 100% ethanol, centrifuged at 13000 g, supernatant was removed from DNA pellet, and 1 ml of 70% ethanol was added and pellet was spun at 13000 g. Finally, supernatant was removed and discarded and the PX199 pellet was resuspended in 30 µl of milli-Q H₂O.
Final concentration of the PX199 product was determined using the Nanodrop Nucleic Acid Analysis software.

Preparation of the 208-1 DNA Fragment:

The 208-1 fragment was previously prepared (Georgel et al., 1993) by the digestion of a 12-mer tandemly repeated DNA sequence of the 5S gene of Sea urchin. This 12-mer DNA sequence was subjected to Ava I restriction enzyme digestion with overnight incubation at 37 °C. The restriction product was verified by gel electrophoresis on a 3.5% polyacrylamide gel. The gel was stained with Ethidium Bromide for 15 minutes, washed three times with milli-Q H2O and imaged. The 208-1 fragment was amplified using 40 cycles of PCR and the product was ethanol precipitated where the DNA using 10 µl 5 M Sodium Acetate and 275 µl 100 % ethanol, centrifuged at 13000 g. Supernatant was removed from DNA pellet, and 70% ethanol was added and the DNA was spun again at 13000 g. Finally, supernatant was removed and discarded and the 208-1 pellet was resuspended in 30 µl of milli-Q H2O. Final concentration of the 208-1 product was determined using the Nanodrop Nucleic Acid Analysis software.

Conformational DNA structure analysis:

The sequence of the p29/p58, PX199 and the 208-1 fragments were analyzed to determine their 3-D conformation using the Trifonov CURVATURE program (http:hydra.icgeb.trieste.it/~kristian/dna/) which utilizes the dinucleotide bending analysis method and uses a given window of approximately 80 bp to calculate the global bend of the DNA molecule (Trifonov and Mengeritsky, 1989; Trifonov, 1991). Based on the results of the Trifonov DNA Curvature algorithm/program the p29-p58 DNA is a relatively
straight or linear DNA template, the PX199 fragment is a somewhat sharply bent sequence ~40 bp from the 3’end, and the 208-1 fragment has two slight curves centrally located within the fragment.

Four-way Junction Construction

Four single stranded oligonucleotides were prepared by Integrated DNA Technologies Incorporated (O1: ACCATGCTCGAGATTACGAGCAGCTTTCGATCGTCG, O2: AATTCGA CGATCGAAGCTGAATACGTGAGGCCTAGG, O3: TTTCGCATGCATGCATCGATATCT CGTAATCTCGAGCATGG and O4: GATCCTAGGCCTCACGTATTATATCGATGCATG CG) A four way junction DNA configuration was assembled by sequentially annealing equimolar amounts of the four single stranded oligos at 65 °C. Oligonucleotides 1 and 2 were incubated in standard TE buffer (10 mM Tris HCl and 1 mM EDTA) at 65 °C, and slow-cooled to 10 °C to allow the formation of DNA duplexes. Oligonucleotides 3 and 4 were subjected to the same treatment (incubation at 65 °C and slow-cooled to 10 °C. The two intermediate DNA products (duplexes) were incubated again at 65 °C and slow cooled to 10 °C. The formation of the four way junction sequence was visualized on a 10% acrylamide gel and run adjacent to known masses (50 ng) of the two intermediate DNA duplexes (O1-O2) and (O3-O4) and known masses (50 ng) of the single stranded oligos (O1, O2, O3, O4). Based on an intensity test analysis, 90% of the single stranded oligos were incorporated into four way junction.
Sir3 Protein and DNA Binding Studies:

A master mix was prepared, containing 300 ng of DNA (50 ng per sample of p29-p58 or PX199 or 208-1), plus 6 µl of glycerol (1 µl per sample). Six samples were prepared in the presence of increasing masses of Sir3p (0.062, 0.125, 0.25, 0.5, 0.75, 1 (µg)) corresponding to Sir3p molar ratios \((r^{\text{Sir3}})\) of 1.25, 2.5, 5, 10, 15 and 20 respectively. Final volumes were adjusted to 10 µl per reaction mix using TE buffer. Sir3p-DNA samples were incubated for 30 minutes at room temperature and subsequently electrophoresed using a 1% agarose gel in 40 mM Tris, 20 mM Acetic Acid and 1mM EDTA (TAE) as running buffer. Gels were run for 1 hour at 100 volts, and Sir3p-p29-p58, Sir3p-PX199, and Sir3p-208-1 complexes were visualized after SYBR green staining and imaged. The amount of depleted DNA by Sir3 protein binding was determined using an intensity test. DNA depletion values were used to determine Sir3p affinity.

Sir3p-DNA: Competition of p29-p58, PX199, and 208-1 for Sir3p binding.

Using a master mix of the three DNA templates combined (p29-p58, PX199, and 208-1) 300 ng per template (total DNA was 900 ng, 150 ng per reaction) plus 6 µl of glycerol (1 µl per sample), 6 samples were prepared and increasing masses of Sir3p (0.183, 0.375, 0.75, 1.5, 2.25, and 3.0 (µg)) were added, corresponding to molar ratios \((r^{\text{Sir3}})\) of 3.66, 7.5, 15, 30, 44, and 60 for samples 1-6 respectively. Final volume was adjusted to 10 µl per reaction mix using TE buffer. Sir3p-DNA samples were incubated for 30 minutes at room temperature and subsequently electrophoresed using a 1% agarose gel in 1X TAE as running buffer. Gel was run for 1 hour at 100 volts, and Sir3p-p29-p58, Sir3p-PX199, and Sir3p-208-1 complexes were visualized after SYBR green staining and imaged. Sir 3 binding affinity was determined as described for the previous
DNA fragments. To quantify DNA depletion for all DNA fragments using the same gel, a sequential Southern blot procedure was utilized where the blots were stripped and reprobed with a labeled DNA probe specific to each fragment (p29-p58, PX199, and 208-1).

**Southern Transfer:**
After electrophoresis and staining, gels were thoroughly washed twice in Denaturing buffer of 0.5 M NaOH, 1.5 M NaCl at 15 minutes per wash with vigorous shaking. Gels were subsequently washed twice at 15 minutes per wash with vigorous shaking in Neutralization buffer of 0.5 M Tris pH 7.5, and 1.5 M NaCl. Gels were rinsed briefly in 10X SSC (1.5 M Sodium Chloride, 0.6 M Sodium Citrate) and an overnight Southern transfer apparatus was set up. After transfer, the membranes were briefly rinsed in 2X SSC and probed with labeled DNA probes specific to each fragment (p29-p58, PX199, and 208-1) and UV cross-linked, to ensure that DNA remained bound to membranes.

**Probe Preparation:**
Alkphos Direct™ Detection kit was used to create labeled DNA probes of each DNA fragment (p29-p58, PX199, and 208-1). Before labeling, 100 ng of each DNA fragment was denatured by boiling for 5 minutes and rapid cooling on ice for 5 minutes. Denaturation allows probes to bind by complementary base pairing with the DNA UV-cross-linked onto a Southern blot membrane. Labeled probes were used immediately or stored at -20 °C in 50% glycerol.
**Stripping Southern Blots:**

To perform competition assay, blots were stripped and reprobed with each labeled DNA probe (p29-p58, PX199, and 208-1). To strip the probes, blots were incubated at 60 °C in 0.5% SDS solution for 60 minutes using a hybridization oven (60 rotations per minute). Blots were washed in standard TE buffer for 1 hour and tested for stripping efficiency. Stripping efficiency was checked by treating the blots with chemiluminescent CDP-Star detection reagent and exposing them to film. Blots were stored in a 0.1% SDS solution at 4 °C between each hybridization treatment.

**Probe Hybridization:**

Blots were probed using Alkphos Direct™ hybridization buffer supplemented with 0.5 M NaCl and 4% Alkphos Direct™ blocking buffer. Blots were first pre-hybridized in probe-free hybridization buffer for 30 minutes in a 55 °C hybridization oven (60 rotations per minute). The labeled probe was then added to the hybridization buffer and blots were allowed to hybridize overnight in a 55 °C hybridization oven (60 strokes per minute). Blots were then subjected to post hybridization washes using a primary wash buffer (2 M Urea, 0.1% SDS, 50 mM Na Phosphate pH 7.0, 150 mM NaCl and 0.2% Alkphos Direct™ blocking reagent). Blots were washed twice for 10 minutes to remove unbound probe. Blots were subsequently washed in Secondary buffer (50 mM Tris Base, 50 mM NaCl, 2 mM MgCl₂) and the signal was detected by autoradiography (using Kodak BioMax™ film).
Signal Generation and Detection:
Chemiluminescent signal generation and detection reagent (CDP-Star™) was used to generate signal on the Southern blots. Blots were incubated for five minutes in the presence of 3 ml detection reagent (30 µl/cm²). Excess reagent was removed, and blots were wrapped in plastic wrap and exposed to X-ray film (Kodak BioMax™) in cassette under dark room conditions and developed. Exposure times ranged from 1 minute to 30 minutes depending on the intensity of the signal.

Staining and Imaging:
SYBR green Nucleic Acid gel stain™ (Invitrogen Detection Technologies), was used to stain all gels. Gels were placed on shaker in 75 ml of 1X TAE supplemented with 0.75 µl of SYBR green DNA stain. Gels were stained for 30 minutes and imaged using AlphaEase FluorChem 9900™ imaging system with a SYBR green filter.

Intensity Test and DNA Depletion Analysis
Images of SYBR green-stained gels or Southern blots were scanned and the determined peak areas were integrated to generate DNA depletion values. These values were used to quantify Sir3p affinity for each DNA fragment (p29-p58, PX199, 208-1). Intensity test values were acquired by selecting the signal of the remaining free DNA and determining the surface area of the integrated peaks. Using intensity values and the known masses of DNA of the internal calibration curve or control, a Y= mx formula was created. Intensity values represented the y-variable requirement and the X-variables were calculated to give the DNA mass of un-depleted DNA for each fragment (p29-p58, PX199, 208-1). The graphs were interpreted to determine the 50% DNA depletion mark for each fragment.
(what molar ratio of Sir3p is required to half-deplete each DNA fragment). Intensity tests without a more thorough mathematical analysis do not permit the determination of an exact affinity value for each fragment, but they allow for a semi-quantitative determination of Sir3p DNA conformation preference.
Chapter Three

Results

Sir3 Protein and its affinity for DNA:

Sir3 has been thoroughly characterized as a chromatin binding protein, interacting with the N-termini of histone proteins, specifically with lysine and arginine residues within the tails of histones H3 and H4 (Laurenson and Rine, 1992; Moazed, 2001a; Santos-Rosas et al., 2004). In order to characterize the DNA binding abilities of Sir3 protein we used Electrophoretic Mobility Shift Assay (EMSA) to quantify Sir3p-DNA interactions with DNA fragments that resemble either linker or nucleosomal DNA. DNA depletion (initial mass of DNA loaded minus mass of DNA bound to Sir3p) is used as an indication of Sir3p-DNA interaction and is also used to estimate the relative affinity of Sir3p for each fragment.

Sir3 protein binds all tested dsDNA conformations and four-way-junction DNA.

Eukaryotic DNA, in its native state, takes on a number of straight, curved and sharply bent conformations due to the sequence of the DNA (Hagerman et al., 1992; Cloutier and Widom, 2004). Transient four-way junction conformations are also observed in eukaryotic DNA as intermediates during recombination (Lilley and Clegg, 1993, Lilley, 1994, Grosschedl et al., 1994). Six samples, each containing 50 ng of the 212 bp p29-p58 relatively linear DNA fragment (used as a substitute for linker DNA), were incubated with increasing amounts of Sir3 protein corresponding to molar ratios ($r^{\text{Sir3}}$) of 1.25, 2.5, 5, 10, 15 and 20 and free DNA depletion was monitored. Even though Sir3p depletion of DNA was not detectable at lower $r^{\text{Sir3}}$ (1.25, 2.5, 5), significant depletion was seen as
molar ratios increased (10, 15 and 20) (Fig. 15). It required $r^{\text{Sir3}}$ of 10 to deplete half the free p29-p58 DNA (See Table 1). Ratios ($r^{\text{Sir3}}$) of 15 and 20 resulted in complete depletion of the free p29-p58 DNA fragment (Fig.15, lanes 6 and 7). These results confirm Sir3p protein is DNA binding protein and gives evidence that it has an affinity for straight DNA.

Figure 15: DNA binds and depletes free p29-p58 DNA.
Aliquots of purified Sir3 protein, and PCR amplified, ethanol precipitated p29-p58 DNA was incubated in TE buffer at room temperature for 30 minutes. Samples were subsequently electrophoresed using a 1.0 % agarose gel (1X TAE) as described under experimental procedures. A 4-increment titration of p29/p58 DNA served as an internal negative control, depicted in lanes 8, 9, 10, and 11 (no Sir3p). Partial depletion of the p29/p58 is observed at a molar ratio ($r^{\text{Sir3}}$) of 10 (lane 5) and complete depletion of free p29/p58 is observed $r^{\text{Sir3}}$ of 15 and 20 (lanes 6 and 7).

Sir3p titration or increasing masses corresponded to Sir3p/DNA molar ratios of 1.25, 2.5, 5, 10, 15, and 20 (Lanes 2-7). Lane 1 contains 1 Kb+ DNA markers (GIBCO/BRL).
Table 1: Sir3p DNA Depletion: p29-p58 DNA Fragment

<table>
<thead>
<tr>
<th>Lanes</th>
<th>DNA</th>
<th>Sir3</th>
<th>Final Volume</th>
<th>Intensity</th>
<th>Average DNA Depletion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lane 1</td>
<td>50 ng</td>
<td>0.0625 µg</td>
<td>10 µl</td>
<td>145</td>
<td>1</td>
</tr>
<tr>
<td>Lane 2</td>
<td>50 ng</td>
<td>0.125 µg</td>
<td>10 µl</td>
<td>142</td>
<td>4</td>
</tr>
<tr>
<td>Lane 3</td>
<td>50 ng</td>
<td>0.250 µg</td>
<td>10 µl</td>
<td>139</td>
<td>7</td>
</tr>
<tr>
<td>Lane 4</td>
<td>50 ng</td>
<td>0.500 µg</td>
<td>10 µl</td>
<td>131</td>
<td>25</td>
</tr>
<tr>
<td>Lane 5</td>
<td>50 ng</td>
<td>0.750 µg</td>
<td>10 µl</td>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td>Lane 6</td>
<td>50 ng</td>
<td>1.0 µg</td>
<td>10 µl</td>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td>Internal Control 1</td>
<td>50 ng</td>
<td>0</td>
<td>10 µl</td>
<td>146</td>
<td>-</td>
</tr>
<tr>
<td>Internal Control 2</td>
<td>37.5 ng</td>
<td>0</td>
<td>10 µl</td>
<td>137</td>
<td>-</td>
</tr>
<tr>
<td>Internal Control 3</td>
<td>25 ng</td>
<td>0</td>
<td>10 µl</td>
<td>131</td>
<td>-</td>
</tr>
<tr>
<td>Internal Control 4</td>
<td>12.5 ng</td>
<td>0</td>
<td>10 µl</td>
<td>126</td>
<td>-</td>
</tr>
<tr>
<td>Background</td>
<td>120</td>
<td></td>
<td></td>
<td></td>
<td>-</td>
</tr>
</tbody>
</table>

The p29-p58 DNA was depleted by Sir3p in a Sir3p molar ratio dependent manner. DNA was subjected to increasing molar ratios of Sir3p and DNA depletion at each Sir3p/DNA ratio was measured. Note: depletion is imperceptible at lower Sir3p/p29-p58 ratios. However, as Sir3p/p29-p58 ratios increased the mass of p29/p58 DNA depleted by Sir3p binding also increased suggesting a cooperative mode of binding. It required Sir3p/DNA ratio of 10, to deplete 50 % of the initial free DNA mass. Sir3p ratios of 15 and 20 completely depleted free p29-p58. The DNA component (50ng of p29-p58) remained the same in each sample. Internal control, was a 4-increment DNA titration of 50, 37.5, 25, and 12.5 (ng) of the p29-p58 fragment. This internal control and the intensity values were used for an intensity test analysis to gain p29-p58 DNA Depletion Values. DNA Depletion Values given are averages of 3 experiments and are rounded to the nearest tenth. Depletion Values for DNA given in the table are nanogram masses.
Previous studies have demonstrated that DNA containing inherent bends conferred by their sequences is more likely to form stable complexes with their cognate DNA binding proteins (Hagerman et al., 1992; Cloutier and Widom, 2004). To measure Sir3p ability to bind to DNA containing bends inherent to the DNA sequence, we incubated the 199 bp sharply bent PX199 DNA fragment (used as a substitute for distorted nucleosomal DNA) with increasing amounts of Sir3p corresponding to \( r^{\text{Sir3}} \) of 1.25, 2.5, 5, 10, 15 and 20. At low \( r^{\text{Sir3}} \) (1.25, 2.5 and 5) no detectable Sir3p-PX199 interaction or depletion of the free PX199 fragment by Sir3p was observed (Fig. 16, lanes 2, 3, and 4). It required a \( r^{\text{Sir3}} \) of 13 to half-deplete of the free DNA (See Table 2). Sir3/PX199 molar ratios \( (r^{\text{Sir3}}) \) of 15 and 20 completely depleted the free PX199 fragment (Fig. 16, lanes 6 and 7). Based on \( r^{\text{Sir3}} \) values observed for half-depletion, the PX199 DNA fragment shows a lower binding affinity than that of p29-p58 for Sir3p (See Table 5).

Eukaryotic DNA must remain pliable or yielding so as to curve around the histone octamer and accommodate the multiple histone-DNA interactions needed to stabilize nucleosomes, (Luger et al, 1997; Hagerman et al., 1992; Cloutier and Widom, 2004). To characterize Sir3p binding affinity for intrinsically curved DNA (curves conferred by the sequence and steric interactions in the absence of any altering factors such as temperature or pH) we incubated the 208-1 DNA fragment with increasing amounts of Sir3p resulting in Sir3p/DNA molar ratios \( (r^{\text{Sir3}}) \) of 1.25, 2.5, 5, 10, 15, 20. No significant Sir3p/208-1 binding was detected at \( r^{\text{Sir3}} \) up to 10 (Fig. 17, lanes 2, 3, 4, and 5). Only at \( r^{\text{Sir3}} \) of 15, did we start to observe depletion of the free 208-1 DNA by Sir3p binding (Fig. 17, lane 6). Complete depletion of the 208-1 DNA required a \( r^{\text{Sir3}} \) of 20 (Fig. 17, lane 7). These results suggest that Sir3p also has a lower affinity for intrinsically curved DNA than it does for linear or bent DNA.
Cumulatively, the results of the Sir3p/DNA affinity studies for each fragment confirm Sir3p as a DNA binding protein and show that it can differentiate between various double-stranded DNA conformations. Although exact affinities have not yet been determined, our results suggest that, in this *in vitro* system, Sir3p has a higher affinity for the straight p29-p58 DNA fragment, than for the sharply bent Px199 and the slightly curved 208-1 fragments.

**Figure 16: DNA binds and depletes free PX199 DNA.**

Aliquots of purified Sir3 protein, and PCR amplified, ethanol precipitated, PX199 DNA were incubated in TE buffer at room temperature for 30 minutes. Samples were subsequently electrophoresed using a 1.0 % agarose gel (1X TAE) as described under experimental procedures. A 4-increment titration of PX199 DNA was used an internal negative control (no Sir3p), (lanes 8, 9, 10, and 11). Complete depletion of the PX199 DNA fragment is observed at molar ratios $r_{\text{Sir3}}$ of 15 and 20.

Increasing masses of Sir3p were used, corresponding to molar ratios ($r_{\text{Sir3}}$) of 1.25, 2.5, 5, 10, 15, and 20 (Lanes 2-7). Lane 1 contains 1 Kb+ DNA markers (GIBCO/BRL).
Table 2: Sir3p DNA Depletion: PX199 DNA Fragment

<table>
<thead>
<tr>
<th>Lanes</th>
<th>DNA</th>
<th>Sir3</th>
<th>Final Volume</th>
<th>Intensity</th>
<th>Average DNA Depletion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lane 1</td>
<td>50 ng</td>
<td>0.0625 µg</td>
<td>10 µl</td>
<td>153</td>
<td>0.5</td>
</tr>
<tr>
<td>Lane 2</td>
<td>50 ng</td>
<td>0.125 µg</td>
<td>10 µl</td>
<td>152</td>
<td>2.0</td>
</tr>
<tr>
<td>Lane 3</td>
<td>50 ng</td>
<td>0.250 µg</td>
<td>10 µl</td>
<td>152</td>
<td>2.0</td>
</tr>
<tr>
<td>Lane 4</td>
<td>50 ng</td>
<td>0.500 µg</td>
<td>10 µl</td>
<td>147</td>
<td>20.0</td>
</tr>
<tr>
<td>Lane 5</td>
<td>50 ng</td>
<td>0.750 µg</td>
<td>10 µl</td>
<td>0</td>
<td>50.0</td>
</tr>
<tr>
<td>Lane 6</td>
<td>50 ng</td>
<td>1.0 µg</td>
<td>10 µl</td>
<td>0</td>
<td>50.0</td>
</tr>
<tr>
<td>Internal Control 1</td>
<td>50 ng</td>
<td>0</td>
<td>10 µl</td>
<td>152</td>
<td>-</td>
</tr>
<tr>
<td>Internal Control 2</td>
<td>37.5 ng</td>
<td>0</td>
<td>10 µl</td>
<td>143</td>
<td>-</td>
</tr>
<tr>
<td>Internal Control 3</td>
<td>25 ng</td>
<td>0</td>
<td>10 µl</td>
<td>134</td>
<td>-</td>
</tr>
<tr>
<td>Internal Control 4</td>
<td>12.5 ng</td>
<td>0</td>
<td>10 µl</td>
<td>125</td>
<td>-</td>
</tr>
</tbody>
</table>

PX199 DNA was depleted in a molar ratio dependent manner. PX199 DNA was subjected to increasing ratios of Sir3p and DNA depletion was monitored. Note: depletion was imperceptible at lower Sir3p/PX199 ratios. As Sir3p/DNA ratios increased Sir3p binding and depletion of PX199 DNA also increased. At Sir3p/DNA ratios of 15 and 20, we observed complete depletion of free PX199 DNA, suggesting a cooperative mode of Sir3p binding to the PX199 fragment. The PX199 DNA component remained the same in each sample (50 ng). Internal control, was a 4-increment DNA titration of 50, 37.5, 25, and 12.5 (ng) of the PX199 fragment. This internal control and the intensity values were used for an intensity test analysis to gain DNA Depletion Values. DNA Depletion Values given are averages of 3 experiments and are rounded to the nearest tenth. Depletion Values given in the table are nanogram masses.
Figure 17: DNA binds and depletes free 208-1 DNA.

Aliquots of purified Sir3 protein, and PCR amplified ethanol precipitated 208-1 DNA were incubated in TE buffer at room temperature for 30 minutes. Samples were subsequently electrophoresed using a 1.0 % agarose gel (1X TAE) as described in under experimental procedures. A 4-increment 208-1 DNA titration was prepared to serve as an internal negative control as depicted in lanes 8, 9, 10, and 11 (no Sir3p). Partial depletion of the 208-1 DNA by Sir3p binding is observed at $r_{Sir3}^3$ of 15, and complete depletion at $r_{Sir3}^3$ 20.

Sir3p masses incubated with the 208-1 DNA resulted in molar ratios ($r_{Sir3}^3$) of 1.25, 2.5, 5, 10, 15, and 20 (Lanes 2-7). Lane 1 contains 1 Kb+ DNA markers (GIBCO/BRL).
Table 3: Sir3p DNA Depletion: 208-1 DNA Fragment

<table>
<thead>
<tr>
<th>Lanes</th>
<th>DNA</th>
<th>Sir3</th>
<th>Final Volume</th>
<th>Intensity</th>
<th>Average DNA Depletion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lane 1</td>
<td>50 ng</td>
<td>0.0625 µg</td>
<td>10 µl</td>
<td>158</td>
<td>0</td>
</tr>
<tr>
<td>Lane 2</td>
<td>50 ng</td>
<td>0.125 µg</td>
<td>10 µl</td>
<td>157</td>
<td>1</td>
</tr>
<tr>
<td>Lane 3</td>
<td>50 ng</td>
<td>0.250 µg</td>
<td>10 µl</td>
<td>157</td>
<td>1</td>
</tr>
<tr>
<td>Lane 4</td>
<td>50 ng</td>
<td>0.500 µg</td>
<td>10 µl</td>
<td>156</td>
<td>4</td>
</tr>
<tr>
<td>Lane 5</td>
<td>50 ng</td>
<td>0.750 µg</td>
<td>10 µl</td>
<td>150</td>
<td>20</td>
</tr>
<tr>
<td>Lane 6</td>
<td>50 ng</td>
<td>1.0 µg</td>
<td>10 µl</td>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td>Internal Control 1</td>
<td>50 ng</td>
<td>0</td>
<td>10 µl</td>
<td>157</td>
<td>-</td>
</tr>
<tr>
<td>Internal Control 3</td>
<td>37.5 ng</td>
<td>0</td>
<td>10 µl</td>
<td>148</td>
<td>-</td>
</tr>
<tr>
<td>Internal Control 4</td>
<td>25 ng</td>
<td>0</td>
<td>10 µl</td>
<td>137</td>
<td>-</td>
</tr>
<tr>
<td>Internal Control 2</td>
<td>12.5 ng</td>
<td>0</td>
<td>10 µl</td>
<td>129</td>
<td>-</td>
</tr>
</tbody>
</table>

Background 122 -

208-1 DNA was depleted in a Sir3p molar ratio dependent manner. DNA was incubated with increasing molar ratios (r\text{Sir3}) of Sir3p (1.25, 2.5, 5, 10, 15, and 20). Sir3p depletion of the unbound 208-1 DNA at the lower Sir3p/208-1 ratios (1.25, 2.5, 5, and 10), was imperceptible. Note: It requires a molar ratio of 20, corresponding to the largest Sir3p mass used, to deplete 100% of the unbound 208-1 DNA, suggesting Sir3p binds to 208-1 DNA in a cooperative manner. DNA component was 50 ng and remained the same in each sample. Internal control was a 4-increment 208-1 DNA titration of 50, 37.5, 25, and 12.5 (ng). This internal control and the intensity values was used for an intensity test analysis to gain DNA depletion Values. Depletion Values given are averages of 3 experiments and are rounded to the nearest tenth. Depletion Values for DNA are given in the table as nanogram masses.
Sir3p and Four-Way Junction (4WJ) Binding Ability:

Recent studies have made use of the four-way junction as an architectural substitute for the nucleosomal DNA entry and exit site (see Figure 12) (Lilley and Clegg, 1993, Lilley, 1994, Grosschedl et al., 1994). The four-way junction, constructed as described in the material and method section was used as binding template (Fig. 18, A, B and C).

![Figure 18: Four-way junction construction.](image)

Four-way junctions have been used in several studies to mimic different confirmations that occur transiently in the cell such as Holliday junctions. Here it mimics the entry and exit point of nucleosomal DNA. Aliquots of equal amounts of 4 synthetic oligonucleotides of similar length (~ 35-40 bp) were re-suspended in Milli Q H₂O. A, Oligo 1 and oligo 2 were incubated together, and oligo 3 and 4 together, for 10 minutes at 65 °C and allowed to anneal by slow cooling from 65 °C to 10 °C. B, The O₁-O₂ duplex was incubated with the O₃-O₄ duplex at 65 °C and slow cooled to 10 °C to form the four stranded four-way junction. C, shows the confirmation and overall structure of the four-way junction used in this study (O₁, labeled red; O₂, labeled blue, O₃, labeled pink and O₄ labeled green; see Figure 13 for actual sequence of the four-way junction oligonucleotides, (Figure adapted from Panyutin et al. 1995, Embo. J).
First, to determine the integrity of the four-way junction, 50 ng aliquots of the single stranded oligonucleotides, the DNA duplexes generated as intermediates of the four-way junction (See Figure 18B), and the constructed four-way junction, were electrophoresed adjacent to each other on a 10% acrylamide gel (Fig. 19). The four-way junction electrophoretic migration was lower, compared to that of the DNA duplexes (intermediate mobility) and the single stranded oligonucleotides (Fig. 19, lanes 2-4). By monitoring free or partially complexed DNA we determined that 90% of the single oligos were incorporated into the four-way junction.

To define Sir3p affinity for the 4WJ DNA, six samples of the constructed four-way junction were incubated with increasing molar ratios ($r^{Sir3}$) of 3.7, 7.5, 15, 30, 44, and 60 (Fig. 20). Using EMSA, we monitored Sir3p-4WJ interactions as a function of Sir3p/DNA molar ratio. At the Sir3p-4WJ molar ratios of 3.7, 7.5, 15, and 30, no detectable Sir3p-4WJ binding is observed (Fig. 20, lanes 2, 3, and 4). At Sir3p/DNA molar ratios (44 and 60) we started to detect some Sir3p-4WJ binding; however even at the highest Sir3p-4WJ ratio of 60, 4WJ DNA was not even half-depleted. Sir3p does not appear to strongly discriminate between the double–stranded DNA conformations tested but clearly binds poorly to four-way junctions. When compared to the affinity for the other double-stranded DNA templates tested, the 4WJ displays a much lower ability to form a defined complex with Sir3p.
Figure 19: Migration of a Four-way junction.
The integrity and structure of the four-way junction (O₁-O₂-O₃-O₄) confirmed by comparing migration patterns of the four-way junction to that of the duplexes (2WJ; (O₁-O₂ and O₃-O₄)), and single stranded oligonucleotides (O₁, O₂, O₃, and O₄). Aliquots of the four-way junction (lane 2), two duplexes (lane 3 and 4) and four single stranded oligos (lanes 5-8) were electrophoresed using a 10% acrylamide gel as described under experimental procedures. The four-way junction is a high molecular weight structure compared to the duplexes and the single oligos. This migration pattern confirms the successful incorporation of the 4 single oligonucleotides (O₁, O₂, O₃, and O₄) into the four-way junction conformation. Intensity test analysis confirms that 90% of the oligos were incorporated into the four-way junction.
Figure 20: Sir3p and four-way junction binding activity at high local Sir3p concentration.

Four-way junctions are transient DNA conformations that occur at different times within the nucleus, usually during DNA repair and recombination. The 4WJ DNA template was used as a mimic of the entry and exit site of nucleosomal DNA. Equal aliquots of 4WJ DNA were incubated for 30 minutes at room temperature, with increasing masses of Sir3p. The samples were subsequently electrophoresed on a 1% agarose gel (1X TAE), stained and imaged as described under experimental procedures. Lanes 2-5 shows no perceptible 4WJ-Sir3p binding activity at Sir3p/4WJ ratios ($r_{Sir3}^*$) of 3.7, 7.5, 15, and 30. Lanes 6 and 7 shows smears of slight 4WJ-Sir3p interaction at Sir3p/4WJ molar ratios of 44 and 60. A four-increment titration of DNA (50, 37.5, 25, 12.5 (ng)) was used as an internal, negative control (Lanes 8-11).

Sir3p titration corresponded to Sir3p/4WJ ratios of 3.7, 7.5, 15, 30, 44, and 60. (Lanes 2-7)).

Lane 1 contains 1 Kb+ DNA size markers (GIBCO/BRL).
Table 4: Sir3p DNA Depletion: 4WJ DNA Fragment

<table>
<thead>
<tr>
<th>Lanes</th>
<th>DNA</th>
<th>Sir3</th>
<th>Final Volume</th>
<th>Intensity</th>
<th>Average DNA Depletion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lane 1</td>
<td>50 ng</td>
<td>0.183 µg</td>
<td>10 µl</td>
<td>168</td>
<td>0</td>
</tr>
<tr>
<td>Lane 2</td>
<td>50 ng</td>
<td>0.375 µg</td>
<td>10 µl</td>
<td>168</td>
<td>0</td>
</tr>
<tr>
<td>Lane 3</td>
<td>50 ng</td>
<td>0.750 µg</td>
<td>10 µl</td>
<td>168</td>
<td>0</td>
</tr>
<tr>
<td>Lane 4</td>
<td>50 ng</td>
<td>1.500 µg</td>
<td>10 µl</td>
<td>165</td>
<td>4</td>
</tr>
<tr>
<td>Lane 5</td>
<td>50 ng</td>
<td>2.250 µg</td>
<td>10 µl</td>
<td>159</td>
<td>10</td>
</tr>
<tr>
<td>Lane 6</td>
<td>50 ng</td>
<td>3.000 µg</td>
<td>10 µl</td>
<td>159</td>
<td>10</td>
</tr>
<tr>
<td>Internal Control 1</td>
<td>50 ng</td>
<td>0</td>
<td>10 µl</td>
<td>169</td>
<td>-</td>
</tr>
<tr>
<td>Internal Control 3</td>
<td>37.5 ng</td>
<td>0</td>
<td>10 µl</td>
<td>163</td>
<td>-</td>
</tr>
<tr>
<td>Internal Control 4</td>
<td>25 ng</td>
<td>0</td>
<td>10 µl</td>
<td>156</td>
<td>-</td>
</tr>
<tr>
<td>Internal Control 2</td>
<td>12.5 ng</td>
<td>0</td>
<td>10 µl</td>
<td>151</td>
<td>-</td>
</tr>
</tbody>
</table>

Very little Sir3p/4WJ binding was observed even at high molar ratios. 4WJ DNA was incubated with increasing masses of Sir3p resulting in Sir3p/4WJ molar ratios of ratios of 3.7, 7.5, 15, 30, 44, and 60. Sir3p did not deplete free 4WJ DNA at the lower Sir3p/4WJ ratios (3.7, 7.5, 15 and 30). Note: It requires $r^{Sir3}$ values of 44 and 60, corresponding to the largest Sir3p masses used, to deplete 20% of the of the unbound 4WJ DNA suggesting Sir3p has a very low affinity for the 4WJ compared to the other DNA sequences (p29-p58, PX199, and 208-1). DNA component was 50 ng and remained the same in each sample. Internal control was a 4-increment 4WJ DNA titration of 50, 37.5, 25, and 12.5 (ng). This internal control and the intensity values were used for an intensity test analysis to gain DNA depletion Values. Depletion Values given are averages of 3 experiments and are rounded to the nearest tenth. Depletion Values for DNA are given in the table as are nanogram masses.
Table 5: Comparison of Sir3p-DNA Interaction for Four DNA fragments

<table>
<thead>
<tr>
<th>DNA Fragments</th>
<th>Sir3p mass for 50% DNA depletion</th>
<th>r\textsuperscript{Sir3} for 50% DNA depletion</th>
<th>r\textsuperscript{Sir3} for DNA/protein Distinct complex</th>
</tr>
</thead>
<tbody>
<tr>
<td>P29/p58</td>
<td>0.5ug</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Px199</td>
<td>0.65ug</td>
<td>13</td>
<td>15</td>
</tr>
<tr>
<td>208-1</td>
<td>0.8ug</td>
<td>16</td>
<td>15</td>
</tr>
<tr>
<td>4WJ</td>
<td>&gt; 3.0ug</td>
<td>&gt; 60</td>
<td>—</td>
</tr>
</tbody>
</table>

All DNA fragments had an affinity for Sir3 protein. Exact affinities have not been calculated to date; however, based on DNA depletion values from triplicate experiments per fragment, Sir3p seems to have a higher affinity for the straight p29-p58 DNA fragment. It required Sir3p/p29-p58 molar ratio of 10 to half-deplete free p29-p58 DNA. In contrast it required r\textsuperscript{Sir3} of 13, to deplete 50% of the PX199 fragment and r\textsuperscript{Sir3} of 16 for Sir3p to deplete 50% of the 208-1 DNA. The four-way junction, used to mimic the entry and exit conformation of DNA in the nucleosome, did not show much interaction with Sir3p compared to the other DNA fragments used. The highest ratios used in this study did not half-deplete the 4WJ. In terms of Sir3p DNA binding or affinity sequences can now be ranked as p29/58, PX199, 208-1, and 4WJ, listing with a decreasing order of affinity.
**Sir3p Can Form Defined High Molecular Weight Complexes with DNA:**

Chromatin architectural proteins (CAP) have been shown to bind to either DNA, histones and/or other CAPs promoting formation of tertiary chromatin structure (Bustin and Reeves, 1996). In the process of characterizing Sir3p DNA binding properties, we found that DNA binding occurs at relatively low \( r^{\text{Sir3}} \) and that higher \( r^{\text{Sir3}} \) are required to form distinct DNA-protein complexes (as seen by EMSA). In these experiments Sir3p masses were three times the masses used in the previous Sir3p-DNA binding affinity experiments. To maintain molar ratios, DNA masses were also tripled.

Six samples were prepared each containing 150 ng of DNA (either p29-p58), PX199 or 208-1) and were incubated with increasing Sir3p molar ratios of 1.25, 2.5, 5, 10, 15, 20. At \( r^{\text{Sir3}} \) up to 5, very little distinct complex Sir3p-p29-p58 was observed (Fig. 21, lanes 2, 3, and 4) as indicated by the presence of free or un-depleted DNA. However, as \( r^{\text{Sir3}} \) was increased, Sir3p/p29-58 distinct complex formation also augmented (Fig. 21, lanes 5-7).

Similarly, Sir3p formed high molecular weight complexes with the PX199 DNA fragment. At lower \( r^{\text{Sir3}} \), up to 5, distinct complex Sir3p/PX199 was not detectable (Fig. 22, lanes 2, 3, and 4) as determined by the presence of free or un-depleted PX199 DNA (Fig. 22, lanes 2, 3, and 4). At \( r^{\text{Sir3}} \) of 10 we observe some tangible DNA-protein interaction formation of an indistinct, Sir3p-PX199 complex that is of lower electrophoretic mobility than the Sir3p/p29-p58 complex formed at the same \( r^{\text{Sir3}} \) of 10 (Fig. 22, lane 5). As \( r^{\text{Sir3}} \) increases Sir3p/PX199 distinct complex is amplified (Fig. 22, lanes 6 and 7).

Sir3p also organized 208-1 DNA into high mobility complexes. At lower \( r^{\text{Sir3}} \), up to 5, no Sir3p-208-1 distinct complex is tangible (Fig. 23, lanes 2, 3, and 4), as indicated by the free or un-depleted 208-1 DNA (Fig. 23, lanes 2, 3, and 4). At \( r^{\text{Sir3}} \) of 10, we observe
some Sir3p/208-1 interaction which resulted in depletion of the free 208-1 as indicated by the smears in the gel, but no distinct Sir3p/208-1 complex is formed (Fig. 23, lane 5). As \( r^{\text{Sir3}} \) increases, distinct Sir3p-208-1 complex is augmented at \( r^{\text{Sir3}} \) of 15 and 20 (Fig. 23, lanes 6 and 7).

**Figure 21: Sir3p-p29-p58 complex formation at high Sir3p molar ratio.**

Samples each containing 50 ng of p29-p58 DNA was incubated for 30 minutes at room temperature with increasing masses of Sir3 protein corresponding to molar ratios of 1.25, 2.5, 5, 10, 15, and 20. Southern blots showed that Sir3p assembled p29-p58 DNA into well-defined, distinct DNA-protein complexes at molar ratios (rSir3) of 10, 15, and 20. Lanes 2-4 show free or un-depleted p29-p58 DNA and no Sir3p/p29-p58 complexes are formed at these lower rSir3 values (1.25, 2.5, and 5). Lanes 5-7 show 3 distinct Sir3p/p29-p58 high molecular weight complexes (circled red). Lane 1 contains 1 kb+ DNA size markers, but does not show up in the southern blot image since southern was probed with labeled p29-p58 DNA probe.

Sir3p masses incubated with the p29-p58 DNA resulted in molar ratios (rSir3) of 1.25, 2.5, 5, 10, 15, and 20 (Lanes 2-7).
Figure 22: Sir3p-PX199 complex formation at high Sir3p molar ratio.

Samples, each containing 50ng of PX199 DNA were incubated for 30 minutes at room temperature with increasing masses of Sir3 protein corresponding to ratios ($r_{Sir3}$) of 1.25, 2.5, 5, 10, 15, and 20. Southern blots show that at Sir3p $r_{Sir3}$ of 15 and 20, Sir3p assembles PX199 DNA into DNA-protein complexes. Lanes 2-4 show unbound or un-depleted PX199 DNA, at these lower $r_{Sir3}$ (1.25, 2.5, and 5) no Sir3p/PX199 complex is visible. Lane 5 ($r_{Sir3}$ of 10) shows a slightly shifted DNA-protein low mobility complex and lanes 6 and 7 show 2 distinct Sir3p-PX199 high molecular weight complexes (circled red). Lane 1 contains 1 kb+ DNA size markers, but does not show up in the southern blot image since southern was probed with labeled PX199 DNA probe.

Sir3p masses incubated with the PX199 DNA resulted in molar ratios ($r_{Sir3}$) of 1.25, 2.5, 5, 10, 15, and 20 (Lanes 2-7).
Figure 23: Sir3p-208-1 complex formation at high Sir3p molar ratio.

Sir3p/208-1 samples were prepared and incubated for 30 minutes at room temperature with increasing masses of Sir3 protein corresponding to ratios ($r^{\text{Sir3}}$) of 1.25, 2.5, 5, 10, 15, and 20. Southern blots show Sir3p/208-1 complexes formed at $r^{\text{Sir3}}$ of 15 and 20. Lanes 2-4 show free or un-depleted 208-1 DNA ($r^{\text{Sir3}}$ values of 1.25, 2.5, and 5) Lanes 5 shows a smear, where all 208-1 DNA has been depleted but no tangible DNA-protein complex has been formed ($r^{\text{Sir3}}$ 10). Lanes 6 and 7 show 2 Sir3p-208-1 high molecular weight complexes (circled red). Lane 1 contains 1 kb+ DNA size markers, but does not show up in the southern blot image since southern was probed with labeled 208-1 DNA probe.

Sir3p masses incubated with the 208-1 DNA resulted in molar ratios ($r^{\text{Sir3}}$) of 1.25, 2.5, 5, 10, 15, and 20 (Lanes 2-7).

These results suggest that Sir3p displays a highly cooperative mode of binding, requiring higher molar ratios to induce formation of Sir3p-DNA complexes with any of the fragments. We again suggest an order of binding affinity, where Sir3p protein may have a higher affinity for straight (p29-p58) DNA than for the bent (PX199) fragment or the
curved (208-1). It required higher molar ratios of Sir3p (higher number of molecules of Sir3p per DNA molecule) to induce formation of DNA-protein complexes with the bent PX199 fragment and the curved 208-1 than the $r^{\text{Sir3}}$ values that were required for the straight p29-p58 sequence to form complexes with Sir3p. Furthermore, the Sir3p/p29-p58 complexes had lower electrophoretic mobilities than Sir3p/PX199 complexes and Sir3p/208-1 complexes. Sir3p did not form complexes with the four-way junction at the $r^{\text{Sir3}}$ values used in this study. However, we did observe some Sir3p-4WJ binding albeit at much lower ratios than those observed for the other DNA templates (p29/p58, PX199, and 208-1). These results are based on triplicate experiments for each fragment.

**Competition Studies:**

To compare the binding affinity of Sir3p for each DNA fragment under conditions that would reflect *in vivo* situation (multiple DNA fragments and conformations available simultaneously) we developed a competition assay. Six samples were prepared, each containing 50 ng of each DNA fragment combined (50 ng p29/p58, 50 ng PX199 and 50 ng 208-1) for a total of 150 ng of mixed DNA per sample. Samples were incubated with increasing amounts of Sir3 protein corresponding to Sir3p/DNA molar ratios of 1.25, 2.5, 5, 10, 15, and 20. These are the same Sir3p/DNA ratios used in the initial Sir3p-DNA affinity experiments. Since each sample contains 50 ng of each DNA fragment, (p29/p58, PX199, and 208-, for a total of 150 ng of DNA per sample), the Sir3p masses were adjusted to maintain an identical Sir3p/DNA molar ratios range. It is important to note that although the Sir3p mass has increased per sample, volumes remained the same and therefore Sir3p molarity was increased three-fold.
Similarly to what was observed at lower molarity, the p29/p58 DNA fragment maintains its binding affinity for Sir3p even in the presence of the other DNA fragments (PX199 and 208-1) over the same $r^{\text{Sir3}}$ range. At $r^{\text{Sir3}}$ up to 5, no detectable Sir3p/p29-p58 binding is observed (Fig. 24, lanes 2, 3 and 4). The main difference in Sir3p binding to the DNA fragment in this experiment versus the previous experiments (independent of the other two DNA fragments) was the formation of a distinct complex at $r^{\text{Sir3}}$ of 10 and above (Figure 24 lanes 5-7), while the prior similar experiments at lower molarity resulted in depletion of DNA but the complexes formed were undefined or indistinct (smears in the gels, see Figure 21).

Similar results were observed with the PX199 fragment, where even with the addition of the two other fragments (p29/p58 and 208-1) Sir3p completely depletes free PX199 DNA at similar ratios (15 and 20) as those seen in the initial Sir3p/PX199 binding experiments, at lower Sir3p molarities (p29/p58 and 208-1) fragments were absent) (Fig. 25, lanes 5 and 6). The difference in the Sir3p/PX199 binding is the formation of distinct or well defined complexes (Fig. 25, lanes 5 and 6) as a result of an increase in Sir3p molarity, where as in the prior experiments, complexes formed were indistinct or undefined (smears in gel, see Figure 22). Even in direct competition for Sir3p binding, the molar ratios at which we observe Sir3p/PX199 binding and complex formation are still higher than those at which we observe Sir3p/p29-p58 binding and complex formation. These results imply that Sir3p affinity for PX199 is intermediate compared to Sir3p affinity for p29-p58.
Figure 24: Competition for Sir3p Binding: Probing with a p29-p58 DNA probe.

Competition studies confirmed Sir3p DNA binding preferences and showed the effect of molarity on Sir3p/p29-p58 binding. Six samples each containing equal masses of each DNA fragment (p29/p48, PX199, and 208-1) were incubated with increasing $r^{Sir3}$. Southern blots show that even in direct competition with the other DNA fragments (PX199 and 208-1) Sir3p binds to and forms complexes with p29-p58 DNA at $r^{Sir3}$ of 10, 15 and 20. The presence of the other DNA fragments (PX199 and 208-1) did not out-compete p29/p58 DNA for Sir3p binding or hinder Sir3p-p29-p58 complex formation. In competition, the p29/p58 fragment is still depleted by Sir3p at lower $r^{Sir3}$ than those observed for the PX199 and 208-1 fragments, and Sir3p still forms complexes with p29/p58 at lower $r^{Sir3}$ than those observed for the PX199 and 208-1 fragments. Sir3p molarity has increased and we see the formation well-defined complexes (Compare Figures 21 and 24).

Sir3p masses incubated with the p29-p58 DNA resulted in molar ratios ($r^{Sir3}$) of 1.25, 2.5, 5, 10, 15, and 20 (Lanes 1-6). Lanes 7-10 contains increasing masses of p29-p58 DNA in the absence of Sir3p, serving as an internal control.
Figure 25: Competition for Sir3p Binding: Probing with a PX199 DNA probe.

Six samples, each containing equal masses of each DNA fragment (p29/p48, PX199, and 208-1), were incubated for $r^{\text{Sir3}}$. Even in competition with two other DNA fragments, (p29/p48 and 208-1), we observe that at $r^{\text{Sir3}}$ of 10, 15 and 20, the PX199 DNA template is able to bind enough Sir3p to form well defined complexes (lanes 4, 5, and 6). Sir3p molarity is higher in this experiment compared to the previous Sir3p/PX199 studies, in the absence of the competing fragments, therefore Sir3p/PX199 complexes are formed at lower $r^{\text{Sir3}}$ (10) and complexes are also more distinct of defined (Compare Figures 22 and 25)

Sir3p masses incubated with the PX199 DNA resulted in molar ratios ($r^{\text{Sir3}}$) of 1.25, 2.5, 5, 10, 15, and 20 (Lanes 1-6). Lanes 7-10 contains increasing masses of PX199 DNA in the absence of Sir3p, serving as an internal control.
Sir3p completely depletes and forms distinct complexes with 208-1 DNA at molar ratios of (15 and 20) even in the presence of the competitors, p29-p58 and PX199 (Fig. 26, lanes 5 and 6). These molar ratios mirror those at which we observed Sir3p/208-1 binding in the previous experiments, in the absence of the competing fragments. The difference here is the formation of distinct Sir3p-208-1 complexes (Fig. 26, lanes 5 and 6) at higher Sir3p molarities, where as the lower molarities of Sir3p in the prior experiments resulted in undefined complexes (smears in gel), see Figure 23.

![Figure 26: Competition for Sir3p Binding: Probing with a 208-1 labeled probe.](image)

Samples containing equal masses of each DNA fragment (p29/p48, PX199, and 208-1) were incubated with increasing Sir3p molar ratios ($r^{\text{Sir3}}$) of 1.25, 2.5, 5, 10, 15, and 20. Southern blot showed that even in direct competition with the other DNA fragments, the Sir3p binds, depletes and forms complexes with the 208-1 sequence at $r^{\text{Sir3}}$ values of 10, 15, and 20 (lanes 4-6). Sir3p molarity has increased in this experiment compared to previous Sir3p/PX199 experiments that were done in the absence of the other two fragments (p29-p58 and PX199), therefore we also see more defined complexes at lower $r^{\text{Sir3}}$ (Compare Figures 23 and 26).

Sir3p masses incubated with the 208-1 DNA resulted in molar ratios ($r^{\text{Sir3}}$) of 1.25, 2.5, 5, 10, 15, and 20 (Lanes 1-6). Lanes 7-10 contains increasing masses of 208-1 DNA in the absence of Sir3p, serving as an internal control.
Although exact affinities have not been determined, Sir3p affinity for 208-1 can be defined as the lowest, in relation to the p29/p58 and PX199 fragments. We propose this, since in direct competition with the other fragments, it required higher $\gamma_{\text{Sir3}}$ values to deplete the free 208-1 DNA than those observed for the p29/p58 and PX199 fragments and it requires higher $\gamma_{\text{Sir3}}$ to induce distinct or well-defined Sir3p/208-1 complexes than those observed for the p29/p58 and PX199 sequences. Even in direct competition the PX199 and 208-1 fragments did not out-compete p29-p58 for Sir3p binding. According to these results, the order of Sir3p affinity is p29/p58, PX199, then 208-1, listing in order of decreasing affinity.

The competition studies also emphasized the effect of increasing molarity on Sir3p-DNA binding. As the molarity increases, the Sir3p-DNA interaction, binding and complex formation also increases. This adds weight to the concept that Sir3p-DNA binding occurs in a cooperative manner.

Sir3p molarity appears to play a critical role in the formation of distinct complex. In the previous experiments performed using individual DNA templates, the Sir3p molarity in each reaction mix was lower than those used in these set of experiments, and while we saw Sir3p-DNA interaction and binding and also the formation of indistinct or undefined complexes (indicated by smears in the gels), we did not see the formation of Sir3p-DNA complexes. In these experiments, Sir3p molarity per reaction mix has increased, and now we observe the formation of high molecular weight distinct Sir3p-DNA complexes with all three fragments and very little to no smearing in the gels.
Chapter Four

Discussion

Transcriptional silencing is critical for gene regulation in eukaryotes. In vivo studies have shown that the mutation or deletion of the silencing proteins yield partial or complete derepression of the mating type genes at the HMR and HML loci and telomeric regions in yeast (Kyrion et al., 1993; Moretti et al., 1994). The loss of the function of these proteins subsequently results in the loss of telomeric silencing and defects in the maintenance of the silent loci (Kyrion et al., 1993; Moretti et al., 1994; Moretti and Shore, 2001). Genetic and biochemical evidence indicate that in yeast, Sir3p appears to be the pivotal silencing component, primarily for the spread of the silent state. Knockout of the Sir3 gene results in abnormalities in the propagation of the silent state throughout the locus (Lustig et al, 1996; et al., 1997; Gardner and Fox, 2001). While Sir3p interactions with its silencing partners (Sir4, Rap1, ORC, etc) have been characterized, very little emphasis has been placed on the DNA binding properties of Sir3 protein. In fact, no established role has been assigned to the DNA component in the silencing process that occurs at the HMR and HML loci or telomeric regions in yeast.

In this work we confirm that Sir3p is a DNA binding protein and characterize its affinity for DNA fragments with differences in bendability and curvature that mimic conformations and structure of DNA typically found associated with the nucleosome core particle and linker DNA. The DNA within the nucleosome takes on non-uniform curvature and conformations conferred by the DNA-sequence dependent flexibility and histone-induced bendability, since the DNA must accommodate protein interactions such as
those formed between the negatively charged DNA phosphate backbone and the highly basic histone tails of the core histones (Luger et al., 1997; Beutel and Gold, 1992; Cloutier and Widom, 2004). In this study, the p29-p58 fragment, a relatively linear DNA template was used to mimic the linker DNA found in chromatin, the PX199 and the 208-1 fragments were used as substitutes for the DNA found at various locations around the histone octamer. The 4WJ was used as a mimic of the entry/exit site of nucleosomal DNA.

We report several observations: (1) Sir3p seems to bind with a higher affinity for straight DNA when compared to sharply bent, curved DNA or four-way junction DNA, (2) Sir3p induces the formation of high molecular DNA–protein structures with straight, sharply bent and curved DNA fragments at high Sir3p molar ratios and, (3) increasing Sir3p molarity increases Sir3p ability to form more defined or distinct DNA-protein complexes.

The first finding is surprising, since we hypothesized that Sir3p would display a higher affinity for the bent PX199 fragment or the curved 208-1 fragment (nucleosomal DNA substitutes) than for the p29-p58 straight DNA (linker DNA substitute). We based our hypothesis on the EcoR1 digestion assay (Georgel et al., 2001) that showed that Sir3p did not bind to, or was not sufficient to protect the linker DNA, which is relatively straight in comparison to DNA around and between the nucleosome core particle. However, our data (based on triplicate experiments) suggest the opposite is true, that in our in vitro system, Sir3p has a higher affinity for straight DNA than it does for bent or curved DNA, since we observed Sir3p/p29-p58 binding, depletion and complex formation at lower molar ratios than those observed for PX199 and 208-1.
Sir3p also bound and depleted the four-way junction DNA, but it required higher molar ratios to detect Sir3p-4WJ binding than the molar ratios at which we saw Sir3p binding with the other DNA fragments. None of the Sir3p/DNA ratios used in this study completely depleted the 4WJ and while increase in Sir3p molarity resulted in the formation of Sir3p-DNA complexes with the other fragments (p29/p58, PX199 and 208-1) Sir3p did not form complexes with the 4WJ under the Sir3p concentrations used in this study. The four-way junction is a transient feature of nucleic acid molecules and a number of structural and architectural proteins, specifically linker histones and the SWI/SNF chromatin remodelers have been shown to have an affinity for four-way junction DNA conformations (Peterson et al., 1994; Zlatanova and Van Holde, 1998). However, in our in vitro system, under the conditions used in the Sir3p-4WJ binding experiments (see experimental procedures) Sir3p did not bind to the 4WJ conformation with appreciable affinity. The order of Sir3p affinity (not exact affinities) for the DNA fragments used in this study, that we have observed, is p29/p58, PX199, 208-1 and then the 4WJ. Therefore we propose that if a Sir3p-DNA component is involved in Sir3p-dependent silencing at the silent chromosomal regions in yeast, Sir3p is likely to bind to nucleosomal DNA regions mimicked by the PX199 and 208-1 sequence than to those that may resemble the 4WJ.

Our data also supports reports from other studies that show that Sir3p binds DNA with little to no sequence specificity (Guarenete, 1999; Gasser and Cockell, 2001; Georgel et al., 2001). This conclusion is supported by the fact that previous studies have established that Sir3p does not have a specific DNA binding motif. While Sir3p may not need a specific DNA binding motif to interact with DNA, its binding may depend on the presence of specific amino acid residues, such as basic lysine and arginine residues.
These residues would confer the ability for Sir3p to bind to and stably interact with the negatively charged DNA backbone of any DNA sequence. Some proteins contain ‘basic patches’ or groups of conserved basic amino acids that can serve as an interaction site for DNA and other negatively charged binding partners (Gong et al., 2000; and Kulish et al., 2000) One or several basic patches could be one of the mechanisms used by Sir3p protein to bind to DNA with differences in curvature and inherent bendability such as the p29/p58, PX199, and 208-1 sequences. However, the primary amino acid structure of Sir3p (Figure 27) does not contain uninterrupted stretches of lysine or arginine residues larger than four, nor any clusters, that would indicate the presence of a basic patch. However, the secondary structure of Sir3p is not known, therefore folding i.e. secondary protein interactions between amino acids that are distant from each (in the primary amino acid sequence) may still result in formation of basic patches that may act as cognate sites for DNA binding.

Future Sir3p studies may include the disruption of Sir3p-DNA interactions by the mutation or deletion of key amino acid residues. Key residues needed for Sir3p DNA binding can be determined by serial deletions of the amino acid sequence of Sir3p, and the use of these mutant sequences in Sir3p-DNA binding studies. Sir3p mutants, where specific amino acid residues have been deleted can also be used in Sir3p-histone interaction studies, to compare and contrast the amino acid residues needed for Sir3p binding to DNA and those needed for Sir3p interaction with DNA. Determining key residues need for both Sir3p-DNA and Sir3p-Histone interactions will give us insight into whether or not DNA and histones compete for Sir3p binding sites or residues, in an in vivo environment.
The primary structure contains a number of basic lysine and arginine residues that may serve as key amino acid residues for DNA interaction. However, no clusters or un-interrupted stretches (above 4) of basic residues that could serve as a basic patch for DNA interaction have been determined. While the primary sequence of Sir3p has been elucidated, the secondary and tertiary structures, that would show Sir3p folding and interactions between amino acids within the sequence have not been determined. The primary structure contains a number of basic lysine and arginine residues that may serve as key amino acid residues for DNA interaction. However, no clusters or un-interrupted stretches (above 4) of basic residues that could serve as a basic patch for DNA interaction is observed in the primary amino acid sequence. Green: K and R, Yellow: S and T, Red: Y. Sir3p: Theoretical pI/Mw: 6.02 / 111359.98
Another noteworthy observation made in this work, was the fact that Sir3p molarity was critical for the formation of distinct DNA-Sir3p complexes. At lower Sir3p molarity we observed Sir3p binding and depletion of all four DNA fragments (p29-p58, PX199, 208-1, and 4WJ) and the formation of indistinct DNA-Sir3p complexes as was indicated by smears on the gel. However, as Sir3p molarity increased, so did the Sir3p-DNA binding or interaction with the nucleosomal and linker DNA substitutes (PX199 and 208-1) and we observe the formation of clearly defined, distinct, low electrophoretic mobility complexes (no smearing or indistinct complexes at high sir3p molarity). Sir3p-dependent silencing in vivo is characterized by the organization of chromosomal regions into highly condensed, transcriptionally repressed tertiary structures. Therefore, it is not too farfetched to propose an in vivo model in which Sir3p binds to DNA within the nucleosome core particle, at high local Sir3p molarity and perhaps this Sir3p-DNA interaction may play a role play in spreading and enhancing the formation of highly condensed repressed chromosomal regions that are typical of the silenced regions at the budding yeast mating type loci and telomeric regions.

However, the fact that Sir3p bound and depleted, to varying degrees, all the fragments used in this study, does not entirely elucidate a role for a DNA component in silencing in vivo at the mating type loci or telomeric regions of Saccharomyces cerevisiae. Sir3p interactions with the tails of H3 and H4, and with its other binding partners such as Sir4, Rap1 and ORC, have to be taken into consideration. In an in vitro system, such as the experiments performed in this study, in the absence of histone and other proteins, then Sir3p is free to interact with DNA. However, a number of studies have found that when Sir3p is placed in the context of chromatin as was done using the nucleosomal DNA in the EcoR1 linker accessibility assay then Sir3p does not seem to
bind to seemingly accessible DNA (Gartenburg, 2000; Gardener and Fox, 2001 and Georgel et al, 2001). The presence of the histones (and accompanying highly basic tails) may act to target Sir3p to the histones and nucleosomal DNA, since Sir3p has such a high affinity or preference for the basic residues in the histone tails.

A number of our propositions or conclusions have been based on the EcoR1 linker accessibility assay done by Georgel et al, where Sir3p did not bind to or was not sufficient to protect the linker DNA. In another study done by Ghedelli et al. in 2001, recombinant Sir3 protein protected the linker DNA in a dinucleosome from cleavage which supports a role for Sir3p in binding of linker DNA in chromatin. However, there is only one linker histone and less nucleosomal DNA in a dinucleosome system than in a nucleosomal array. Therefore in a dinucleosome system Sir3p may be forced to bind to the linker DNA, while in the nucleosomal array there are other binding sites that Sir3p can associate with. However, the concept that Sir3p can interact with DNA in chromatin, and possibly play a role in enhancing the silent state of specific chromosomal regions within yeast is a plausible concept. Even if Sir3p does not bind linker DNA, this does not altogether eliminate a DNA component in Sir3p-dependent silencing. DNA in close contact with the histone core, intuitively or molecularly speaking, does not seem as accessible to protein binding as does the linker. DNA’s negatively charged phosphate backbone make connections with the histone proteins, through the histone tails and the histone-fold motif, which diminishes access to other protein factors (Luger et al., 1997). However, the DNA in the nucleosome core particle is not uniformly distributed around the histone proteins (Luger et al., 1997). There are a number of morphological distortions in nucleosomal DNA that include but are not limited to sharply bent, curved and looped DNA. (Luger et al., 1997; Lowary and Widom, 1997; Zlatanova et al., 1998; Richmond
and Davey, 2003). In distorted regions the DNA is not as tightly associated with the histone octamer, and this would allow access to chromatin architectural proteins such as remodeling complexes and in this case the silencing protein Sir3p. If there is a DNA component in Sir3p-dependent silencing, these non-uniform regions could provide interaction sites for Sir3p binding and spreading.

The order of Sir3p affinity that we have observed, (p29-p58, then PX199, then 208-1 then 4WJ) is unexpected, since a number of studies have suggested that DNA fragments with inherent bending due to their sequence tend to accommodate binding of protein and other cellular components (Beutel and Gold, 1992; Lowary and Widom, 1998; Cloutier and Widom, 2004). Furthermore, much of the genomic DNA of eukaryotic organisms (70-80%) is sharply bent by structural proteins especially by the activity of the core histones that package DNA (Cloutier and Widom, 2004; Schwarz and Hansen, 1994). There are DNA sequences, that have specific structural conformations that act to bias their own packaging, meaning histones would preferentially bind to these sequences to form stable nucleosomes. These DNA sequences are referred to as “nucleosome positioning sequences” and they rarely have a straight configuration. Instead, many nucleosome positioning sequences have been shown to have curved, slightly bent or distorted morphologies (Lowary and Widom, 1998; Cloutier and Widom, 2004; Cloutier and Widom, 2005). In fact, the 208 tandemly repeated 5S rDNA sequence from *Lytechinus variegatus* (Sea urchin) has been shown to have the properties of a nucleosome positioning sequence. Based on these observations, one can reason that DNA with inherent bendability or DNA that can be induced to curve or bend by protein binding would improve protein-DNA dynamics in comparison to straight or linear DNA molecules. With this reasoning in mind, it seemed that in line with a number of other DNA
binding proteins, that Sir3p would have a preference or display a higher affinity for the curved or sharply bent DNA. However it is only fair to mention that there are a number of DNA binding proteins that do have an affinity for linear DNA configurations like Sir3p displayed in this study. Furthermore, it is important to keep in mind that this is an *in vitro* study and that Sir3p has been taken out of its native context, in an attempt at an initial characterization of the DNA binding properties of the Sir3 protein. Further, investigations must be done, to possibly pinpoint key residues in the Sir3p amino acid sequence that may bias its binding to one DNA conformation over another. Long term Sir3p investigations should include manipulation of Sir3p *in vivo*, in a system that mimics its native environment, to gain an accurate picture of Sir3p DNA binding abilities. For now, our findings establish Sir3p as DNA partner and with a preference for straight DNA configurations. We also show that Sir3p can organize DNA into various complexes showing an increasing hierarchical level of organization and the formation of these complexes depends on Sir3p molarity (Figure 28). In our EMSAs of Sir3p-DNA binding, at the lowest level of organization we observe free or unbound DNA, suggesting very little Sir3p-DNA binding (Figure 28A). At the intermediate level of organization, we notice complete depletion of free DNA, an indication of a significant increase in DNA-Sir3p interaction. The rapid depletion of DNA at this level (higher Sir3p/DNA ratios) implies a highly cooperative mode of Sir3p DNA binding, where the binding of one or two molecules of Sir3p enhances Sir3p-DNA interactions, allowing more Sir3p molecules to bind to quickly deplete the free DNA (Figure 28B). Finally, at the highest level of organization we observe the assembly of DNA and protein into high molecular DNA-protein complexes (Figure 28C). Here we propose that these structures are induced when several molecules of Sir3p are bound to the DNA fragment, and since we know that
Sir3p homodimerizes, we also suggest some protein-protein or protein-DNA-DNA-protein (stacked layers of DNA and protein) interaction possibly in the arrangement depicted in figure 28C.

**Figure 28: Effect of Sir3p molarity on Sir3p-DNA hierarchical organization.**

Sir3p binds DNA in a molar ratio and molarity dependent manner. At the low Sir3p/DNA ratios Sir3p/DNA binding was imperceptible, while at higher ratios, DNA-Sir3p interaction increases and free DNA is completely depleted by Sir3p binding. At low Sir3p molarities we see Sir3p-DNA binding or depletion of free DNA but no complexes formed. As the molarity increases we observe the formation of Sir3p-DNA complexes. **A,** There is very little DNA-Sir3p interaction. (Lane 1 contains 1 Kb+ DNA size markers that do not show up in the southern blot.) **B,** Increasing Sir3p molar ratios results in tangible DNA-Sir3p association, seen as smears; suggesting the binding of 1 or possibly 2 Sir3p molecules per DNA fragment. **C,** The final Sir3p-DNA hierarchy that results from increased Sir3p molar ratio is the formation of distinct complexes, possibly as a result of more than 2 Sir3p molecules per DNA template or more than likely DNA-Sir3p-Sir3p-DNA or Sir3p-DNA-DNA-Sir3p interactions (depicted in illustration on right).
In the context of Sir3p-dependent silencing at the yeast HMR and HML loci, and telomeric regions, we propose the model depicted in figure 29. This scenario involves a DNA component in the establishment of a silent state in *Saccharomyces cerevisiae* where Sir3p is recruited to the loci to play a role in the nucleation of silencing events (Figure 29A). The histone tails, specifically H3 and H4, serve as the high affinity binding sites for Sir3p and are critical for the recruitment of Sir3p to the nucleosome core particle. Sir3p binding would serve as a stabilizing influence at the silent loci and in a cooperative manner, allow the binding of additional Sir3p to enhance the level of organization and promote silencing nucleation events (Figure 29B). Previous studies have suggested that Sir3p is the key factor that allows the spreading of the silent state to distant chromosomal regions. This model suggest that once Sir3p is recruited by its interactions with the histone tails and other silencing factors, then as Sir3p molarity increases and high affinity binding sites are filled, then a DNA component is involved, and Sir3p may associate with DNA, using it as a “track” to move along the length of the chromosome to propagate the silent state throughout the locus resulting in the repressed heterochromatin-like regions found in *Saccharomyces cerevisiae* (Figure 29C).
Figure 29: Proposed model of role of Sir3p-DNA interaction in silencing.

Cumulatively our data suggest the involvement of a DNA component in Sir3p dependent silencing. Model we propose involve A, Sir3p initially associates with its high affinity targets; the histone N-termini of histones H3 and H4. These interactions would take place at low local Sir3p concentration and would help to nucleate silencing events. B, When the high affinity sites are filled, and Sir3p concentration increases, then a DNA component is added, with Sir3p using the DNA as a “track to spread the silent state to distal regions of the chromosome to form the highly condensed and compact, transcriptionally repressed chromosomal regions found in yeast. C, We suggest that at high local Sir3p concentration, Sir3-DNA and Sir3-Sir3 associations help in the formation of stable heterochromatin-like structures and assist in the maintenance of the silent state.
This study lays out a very simple and concise picture of Sir3p-DNA binding interaction, with Sir3p having the highest affinity for the straight DNA fragment (p29-p58), intermediate binding affinity for the sharply bent DNA (PX199) and a lower affinity for the slightly curved fragment (208-1). This seems to apply well in a chromatin context, in that the linker DNA, the relatively straight DNA, would seem to be the most accessible to Sir3p activity, then next in line for Sir3p binding would be bent or distorted regions of DNA around the octamer core that is loosely associated with the histone proteins. The final region in line for Sir3p binding interaction would be the slightly curved regions, which are in closer contact with the histones than the bent or considerably distorted regions. In my limited experience however, research is never simple or concise, therefore the picture or scenario proposed above, may at best, be part of the Sir3p-DNA binding story and at worse, contain no actual descriptions about the way Sir3p interacts with DNA in its native environment at silent chromosomal domains.
Conclusion and Perspectives

Although more in depth studies must be done to fully characterize Sir3-DNA interactions and their role in Sir3p dependent silencing in a number of systems, in terms of chromatin, Sir3 protein can no longer be defined as only a histone binding protein. The DNA component must be added or at least investigated before it is absolutely ruled out as a factor in the formation of heterochromatin-like conformations and silencing at the yeast mating type loci and the telomeres of *Saccharomyces cerevisiae*. This study demonstrated that Sir3p is promiscuous in its DNA binding, associating with a number of DNA fragments, from different organisms, with differences in sequence, curvature, bending ability and native conformations. However, we observe that Sir3p does seem to have a higher affinity for some DNA conformations than others as can be seen if we compare Sir3p binding to the p29-p58 straight fragment (highest affinity in this study) with its binding to the 4WJ (lowest affinity in this study). Furthermore, Sir3p is able to bind DNA and organize high molecular weight DNA-protein structures at high Sir3p concentration. This evidence begs that we develop experimental systems to determine whether DNA plays a role in the initiation, establishment or maintenance of silent states.

As regards this study, efforts are being made to further characterize DNA-Sir3p interaction by calculating exact affinities for each of the fragments used in this work, and based on those numbers, a precedent can be set for the type of DNA that should be used in future investigations of DNA-Sir3p binding studies. Even though Sir3 protein shows little affinity for four-way junction DNA within the confines of the Sir3p titration used in this study, this DNA conformation can be further manipulated using conditions with and
without Mg\(^{2+}\) (magnesium changes or flattens 4WJ conformation) to further characterize Sir3p-DNA binding activity. Furthermore we are examining Sir3p-DNA relationship with longer DNA fragments such as the 208-7 and the 208-12 tandemly repeated 5S rDNA fragments from *Lytechinus variegatus*. Comparisons can be made between Sir3p silencing in yeast and silencing in humans, perhaps using the MeCP2 methylation-dependent silencing protein, to compare silencing mechanisms used by each protein. Cumulatively, the results from this study and from future studies will hopefully give us a clearer picture of Sir3p silencing at the HMR and HML silent loci and telomeric regions in yeast and these mechanisms may have some application to how chromatin associated proteins act to establish heterochromatin in higher eukaryotes.
Appendix

Intensity Tests Analysis of DNA Sir3p binding and Depletion of Free DNA

Sir3p and DNA samples were treated as described under experimental procedures. Samples were electrophoresed and nucleic acids were transferred using Southern Blotting procedure. Blots were imaged using X-ray film (Kodak BioMax™). Images were scanned using an EPSON PERFECTION 1650™ scanner. The intensity (integrated area of peaks) of free DNA used as an internal control and the free DNA remaining in the lanes with low Sir3p molar ratios ($r_{\text{Sir3}}$) were gained using AlphaEaseFC™ computer software. This program selects each free DNA band within the scanned image of the Gel and from integrated peak areas generates intensity values for each band (Figure 30 A and B).

Figure 30: Intensity Test and Values.

A, The intensity software selects bands corresponding to free DNA and based on the integrated surface area of each band, B, an intensity value is assigned to each band. The intensity values and the known DNA masses of the controls (Lanes 5-8) are used to determine the masses of the free DNA remaining in the sample lanes (Lanes 1-4).
The intensity value (minus a background value) produced for each free DNA band of the internal control or calibration is plotted against the corresponding known mass of each free DNA band of the control. The graph produced is linear in nature and produces a \( y=mx \) formula that can be used to extrapolate the mass of remaining free DNA within the sample lanes with low Sir3 molar ratios (Figure 31). The \( y \)-value represents the integrated surface area of each DNA band, \( m \) is the slope of the line and the \( x \)-value represents the mass of remaining free DNA.

**Figure 31: Graph of Intensity Values against Masses of Control DNA.**

The internal DNA calibration was a titration of DNA, consisting of four increments 50, 37.5, 25 and 12.5 (ng). Intensity values (23, 17, 11 and 6) were assigned to each DNA band within the control calibration and these values were plotted against the known DNA masses (50, 37.5, 25 and 12.5) and the result was a linear graph with a \( y = mx \) formula (\( y = 0.456X \)) that was used to calculate the unknown DNA masses within the sample lanes.
Figure 32: Calculating unknown DNA masses within sample lanes.

The \( y = mx \) formula is gained from the linear graph, where the intensity values assigned to the controls are plotted against the known masses of the controls. The \( y = mx \) formula is used to determine the masses of free DNA remaining in the sample lanes. The DNA Depletion value per sample lane is determined by subtracting the final mass of free DNA in the sample lane from the initial DNA mass (50 ng) that was incubated with increasing ratios of Sir3 protein.

Free DNA is depleted by the binding of Sir3p at higher molar ratios. The molar ratio of Sir3p needed to deplete half (25 ng) of the initial DNA per sample (50 ng) was determined by plotting the calculated \( x \)-values (mass of free DNA remaining in each sample lane) against the corresponding mass of Sir3p used in that sample (Figure 33). The Sir3p mass needed for depletion of half of the initial mass of free DNA was gained from the plot and used to calculate the molar ratio needed for half-depletion of initial mass of free DNA. The Sir3p molar ratio \( (r^{Sir3}) \) needed for depletion of half of the initial mass of free p29/p58 linear DNA was compared to the \( r^{Sir3} \) values needed to deplete half of the initial PX199 bent DNA, the 208-1 curved DNA and the 4-way junction DNA.
These values will be used to determine the affinity constant of Sir3p for each DNA fragment. The rationale behind this method was that the fragment with the highest affinity for Sir3p would require lower $r^{Sir3}$ values for binding and depletion. Furthermore, the Sir3 molar ratios needed for half depletion and complete depletion of free DNA and for the formation of DNA-protein complexes would be lower for the fragment with the highest affinity for Sir3p.

![Figure 33: Half-depletion Values.](image)

The initial DNA mass used per sample was 50 ng. Six samples of DNA were incubated with increasing Sir3p molar ratios. The DNA depletion at each Sir3p increment was calculated and plotted as shown above and the mass of Sir3p need for half-depletion (25 ng) was determined using graph. In the graph above, it required 0.6 μg of Sir3p to half-deplete the free DNA. This corresponds to an $r^{Sir3}$ value of 12.
References


