MULTIPLE ROLES FOR THE ZEBRAFISH TRANSCRIPTION ACTIVATOR SBF/STAF

A Dissertation

by

KARI MICHELE HALBIG

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2008

Major Subject: Biochemistry

MULTIPLE ROLES FOR THE ZEBRAFISH TRANSCRIPTION ACTIVATOR SBF/STAF

A Dissertation

by

KARI MICHELE HALBIG

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Approved by:

Chair of Committee,	Gary R. Kunkel
Committee Members,	Dorothy E. Shippen
	Donald W. Pettigrew
	Julian L. Leibowitz
	Arne C. Lekven
Head of Department,	Gregory Reinhart

May 2008

Major Subject: Biochemistry

ABSTRACT

Multiple Roles for the Zebrafish Transcriptional Activator Protein SBF/Staf.

(May 2008)

Kari Michele Halbig, B.S., University of Illinois-Urbana

Chair of Advisory Committee: Dr. Gary R. Kunkel

Eukaryotic transcriptional activators stimulate transcription of genes otherwise expressed at low levels. The typical activator operates by binding to specific sites on DNA with its activating region contacting the multiprotein machinery that directs transcription. SBF/Staf is a transcriptional activator that binds to the SPH element found in the promoters of genes for snRNAs and genes that code for mRNAs. SBF/Staf binds to SPH through a reiterated zinc finger DNA binding domain and also contains two distinct activation domains, one for snRNA genes and one for mRNA genes. To test the role of SBF/Staf *in vivo*, morpholino antisense oligos were used to knock down SBF/Staf expression in zebrafish. A high percentage of developing zebrafish embryos exhibited abnormalities. Co-injection of a synthetic mRNA construct rescued the morpholino-induced knockdown. Furthermore, both the mRNA and snRNA activation domains have significant roles in the function of SBF/Staf because when each domain was removed separately, partial rescue of the knockdown phenotype was obtained. When both domains were removed, no rescue of the phenotype was observed. Unexpectedly, knockdown of SBF/Staf expression in zebrafish embryos caused an increase in steady-

state levels of all endogenous mRNAs tested, as well as transcripts produced from co-injected U6 maxigenes. However, quantitative RT-PCR analysis showed a relatively smaller increase in the steady-state levels of several mRNAs from genes that contain a SPH element in their promoters. In zebrafish U6 genes, the SPH element is in the unique location of being next to the TATA box, instead of ~220 bp upstream of the start site as in mammals. To determine the significance of the proximally-located SPH element for transcription of the zebrafish U6 snRNA gene, the SPH element was mutated. Transcription of a zebrafish U6 maxigene was reduced to 20.6% in transfected ZF4 cells and 26.8% in injected embryos, compared to that of the U6 maxigene with a normal promoter. This work indicates a more global role of SBF/Staf in mRNA gene transcription, instead of only activating the transcription of snRNA and a few mRNA genes, leading to an increased importance of the role of SBF/Staf in transcriptional control.

DEDICATION

I would like to dedicate this work to my parents, Karl and Jackie Halbig. Without their support, love, and telling me my whole life that I can be and do whatever I put my mind to, I would not be what I am today. I thank my grandmothers, who both loved me without question and gave me the friendly and tenacious attitude that I am complimented on today. Finally to James whose daily support and love through the toughest of time of my "Ph. D. process" made it possible to write my Ph. D. dissertation.

ACKNOWLEDGEMENTS

First, I would like to thank Dr. Gary Kunkel for taking a chance on me and taking me into his laboratory. He has been the best advisor a graduate student could have, allowing me to blossom into a better scientist through constant guidance and support. I would also like to thank my committee members Dorothy Shippen, Donald Pettigrew, Arne Lekven, and Julian Leibowitz for their patience and support throughout my academic career and for teaching me how think a problem through. Additionally, I would like to thank Ry Young for instructing me in how to be a better teacher through passion for the subject, and to Jim Hu for being there for advice during the tough times.

The first thing that one needs to know about graduate school is that you will not make it through without your friends. They have become my family away from home and we have shared in the good times when we laughed, and the hard times when we cried. We helped each other grow as scientists and as people. They were always there to listen and give a hug when needed. I would like to give a special thanks to Adrienne Zweifel, Libby Badgett, Michelle Heacock, Cathy Kelton, Gwen Knapp, and Matt Watson. To be successful graduate students need great lab members and I would like to thank the Lekven Lab for adopting me, teaching me all they know about zebrafish, and tolerating all my questions. Though they don't always get the credit they deserve I would like to thank my "Aunties" Tillie, Daisy and Juanita; they were always there with help, advice, and an attentive ear.

Finally, I enjoyed my time as an officer of the Biochemistry Graduate Student Association and the leadership skills that I learned will be carried over into the next chapter of my life.

TABLE OF CONTENTS

CHAPTER Page

LIST OF FIGURES

CHAPTER I

INTRODUCTION

SCOPE

The research in this dissertation was performed to determine the biological effect of SBF (SPH-binding factor)/Staf (selenocysteine tRNA gene transcription activating factor) in zebrafish development and the significance of the proximally-located SPH element for transcription of zebrafish U6 snRNA genes. This introduction provides the background information on the different classes of promoters transcribed by eukaryotic RNA polymerases and activator proteins of transcription. The first part of the introduction focuses on eukaryotic RNA polymerases gene promoters, the activation domain of transcription activation proteins, the enhancer elements that these activators bind to, and the U6 snRNA gene which is the focus of our laboratory. The second part focuses on the endpoints of the developmental zebrafish pathways involved in transcriptional control.

SIGNIFICANCE

There are many small, stable RNAs that serve essential functions in eukaryotic cells, which are not translated but operate in RNA-protein complexes. Some examples

---

This dissertation follows the style of Molecular and Cellular Biology.

of non-translated RNAs are ribosomal RNAs, snoRNAs for pre-ribosomal RNA processing, the RNA template of telomerase, and the pre-mRNA splicing cofactors U1, U2, U4, U5, and U6 snRNAs. In our lab we are studying the snRNA promoters, specifically the human U6-1 gene promoter as a representative of a highly efficient promoter that provides a simple model system to study different mechanisms of eukaryotic transcription (38). In vertebrates, spliceosomal snRNA genes are transcribed very efficiently, producing one transcript every 2-4 seconds in cultured human cells (21). The human U6 snRNA gene promoter contains two regions, the proximal region and the distal region. The proximal region, or basal promoter, contains a proximal sequence element (PSE) that binds the SNAP complex and a TATA box that binds a TBP (TATA –binding protein)-containing complex. Together these two complexes, along with other, less-characterized proteins, recruit RNA polymerase III to the human U6 snRNA gene promoter. A distal region contains the Oct element and the SPH element, and is located approximately 200-250 bp upstream from the transcriptional start site. These two elements are bound by the Oct-1 protein and the SPH binding factor (SBF/Staf), respectively. It is this distal, enhancer-like region that allows for the U6 snRNA gene to have a transcription level 8-100 fold higher than basal levels. Our laboratory has focused on the role of SBF/Staf in transcriptional activation. Unexpectedly, SBF/Staf also stimulates transcription for a large number of protein-coding genes through a different activation domain (91). In order to further study the different roles of the two activation domains of SBF/Staf in a whole animal, my work employed the model vertebrate, *Danio rerio* (zebrafish). Zebrafish is a good model system to approach this

question because of the following: 1) SBF/Staf is conserved in vertebrates, including humans, mouse, zebrafish, *Fugu*, and *Xenopus*, but is absent in nonvertebrates (62); 2) protocols are well established for the micro-injection of anti-sense morpholino oligonucleotides; 3) synthetic mRNA allows for the study of gene knockdown, rescue, or gain of function phenotypes; and 4) zebrafish embryos are optically clear allowing for the phenotypic outcome of the microinjection to be visualized easily. In the beginning of our investigation it was discovered that the zebrafish U6 snRNA gene promoters have an unusual layout in comparison to the human U6 snRNA promoter. In zebrafish U6 genes, the SPH element is in the unique location of being next to the TATA box, instead of ~220 bp upstream of the start site as in the human U6 snRNA genes and in the other zebrafish snRNAs. This unusual location may facilitate the investigation of the mechanism of SBF/Staf activation at U6 snRNA promoters.

EUKARYOTIC RNA POLYMERASES TRANSCRIBE DIFFERENT CLASSES OF PROMOTERS

Eukaryotic transcription has an increased complexity over prokaryotic transcription in that eukaryotic transcription uses three different RNA polymerases, whereas prokaryotes use only one (79). Also, instead of binding directly to the promoter sequence, eukaryotic polymerases need to interact with a variety of additional proteins to specifically initiate transcription. Eukaryotic RNA polymerase I is responsible for the transcription of the genes that encode for the 45S ribosomal RNA precursor that is then processed to yield the 28S, 18S and 5.8S rRNA. RNA polymerase II transcribes the

genes that encode for mRNAs and U1, U2, U4, and U5 snRNAs. The genes that encode for tRNAs, 5S rRNA and the U6 snRNA are transcribed by RNA polymerase III (79).

All genes contain a promoter that has a core sequence element that is recognized by a factor that binds to DNA specifically and provides a site for the formation of a DNA-protein complex. The DNA-protein complex is sufficient to allow for the RNA polymerase to load onto the DNA. With all three polymerases a second protein component is needed to connect the nucleating factor to the polymerase to form the preinitiation complex (110).

The genes transcribed by RNA polymerase I contain a promoter located 150 bp upstream from the transcriptional start site. The promoter is recognized by two transcription factors, the upstream binding factor (UBF) and the selectivity factor 1 (SL1). SL1 is made up of four protein subunits and one of the units is the TATA-binding protein (TBP). These two factors together recruit RNA polymerase I. Since the rRNA promoter does not contain a TATA-box, TBP is associated with rRNA genes by binding through other proteins in the SL1 complex (93). This is similar to the way that TBP binds with the Initiator (Inr) sequences of RNA polymerase II genes that lack a TATA-box.

Some RNA polymerase II transcribed genes contain a basal promoter with a TATA-box located 25-30 bp upstream from the transcription start site (108). The basal transcription factor that binds to the TATA-box is TFIID, which contains TBP, and TBP associated factors (TAFs). TFIIB binds to TBP and acts as a bridge to the RNA polymerase II-TFIIF complex. TFIIH and TFIIE need to bind to initiate transcription.

TFIIH is a multi-subunit factor that is made up of two helicases and a protein kinase that phosphorylates the repeated sequence in the C-terminus of the RNA polymerase which releases the RNA polymerase from the initiation complex (79). RNA polymerase II gene promoters may also have an Inr sequence that spans the start site. Even promoters lacking a TATA-box still need TFIID to start transcription but the TAFs bind the Inr sequence instead of TBP.

The genes transcribed by RNA polymerase III can be divided into three groups based on the promoter structures and transcription factor requirements. The first group is responsible for the transcription of tRNAs and contains two promoter elements, an A box and B box, that lie within the transcribed gene sequence. Transcription factor TFIIIC binds directly to the B box and the A box orients TFIIIC to the transcriptional start site. Transcription factor TFIIIB binds and recruits the RNA polymerase. The second group of promoters is represented by the 5S rRNA gene (79). The promoter elements for the second group are also located within the transcribed sequence and contain an A box and a C box. Transcription factor TFIIIA initiates transcription by binding to the C box and allows for the assembly of TFIIIC, TFIIIB, and lastly, RNA polymerase III (110).

The final group does not rely on internal promoter elements and instead has proximal and distal promoter elements located upstream of the transcribed gene. The proximal promoter is made up of a TATA-box and a proximal sequence element (PSE). The distal promoter contains an OCT element, that binds the Oct-1 protein and, in many cases, an SPH element bound by the SBF protein. The distal region has been shown to

be an enhancer element (79). One member of this group is the set of vertebrate U6 snRNA genes whose gene product is needed to remove introns from pre-mRNA.

U6 snRNA AND GENES

U6 snRNA is a non-translated RNA that has a main role in mRNA splicing. During splicing the 5' splice and branch sites are recognized partially by a base-pairing interaction with U1 and U2 snRNAs respectively. Then U6 snRNA replaces U1 at the 5' splice site and forms a base-pairing reaction with U2 snRNA that juxtaposes the 5' splice site and branch site, the reactants of the first of the two transesterification reactions of splicing. Finally the two exons are bound and kept in alignment partially by interactions in a conserved loop in the U5 snRNA (15, 66, 100). U6 snRNA mutagenesis data have defined two catalytically crucial domains that are evolutionary conserved: the ACAGAGA box and the AGC triad. Mutagenesis of either of these two domains leads to a block in the first and second steps of splicing (100).

The U6 snRNA gene is a good model for the study of the regulation of eukaryotic transcription not only because is it highly conserved, but also because it has a high level of transcription. In the human genome there are several copies of the U6 snRNA genes dispersed throughout the genome, unlike the U1 and U2 snRNA genes that are tightly clustered. In our lab, chromatin immunoprecipitation and transfection experiments were used to determine if the U6 snRNA genes identified via the human genome project were transcriptionally active. Domitrovich and Kunkel (2003) determined that five of the nine U6 snRNA genes identified were transcriptionally

active. The human U6 snRNA gene promoter contains two regions, the proximal region and the distal region. The proximal region, or basal promoter, contains a proximal sequence element (PSE) that binds the SNAP complex and a TATA box that binds the TFIIIB complex and specifies the selection of RNA pol III. Together these two complexes recruit RNA polymerase III to the human U6 snRNA gene promoter. A distal region contains the Oct element and the SPH element and is located approximately 200-250 bp upstream from the transcriptional start site. These two elements are bound by the Oct-1 protein and the SPH binding factor (SBF/Staf), respectively (Figure 1).

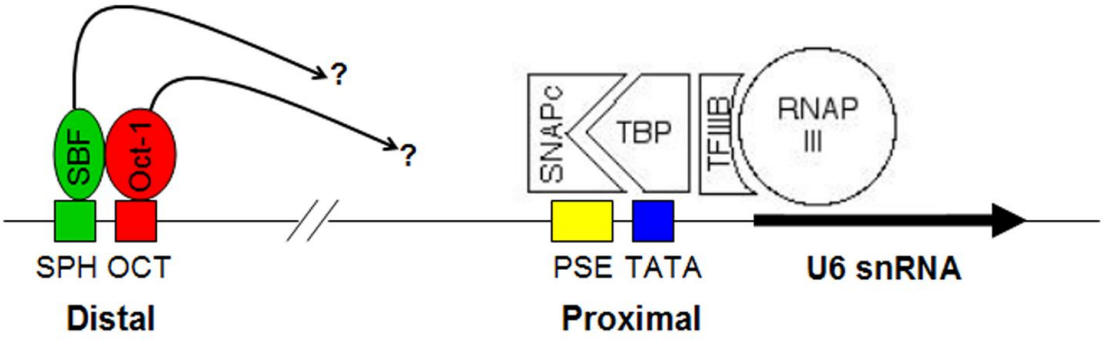

Figure 1. Model for Transcription Complex on Human U6 snRNA Promoter.

It is this distal, enhancer-like region that allows for the U6 snRNA gene to have a transcriptional activity 8-100 fold higher than basal levels. In the beginning of the investigation into zebrafish as a model system we discovered that zebrafish U6 snRNA gene promoters have an unusual organization in comparison to the human U6 snRNA promoter. Zebrafish U6 genes contrast the human U6 genes in that the SPH element is next to the TATA box, instead of ~220 bp upstream of the start site. This unique

location might facilitate the investigation into the mechanism of SBF/Staf activation at U6 snRNA promoters because one could study the protein-protein interactions between SBF/Staf and TBP. Due to the fact that the SPH element and TATA box are located next to one another in the promoter of the zebrafish U6 snRNA gene the likelihood of SBF/Staf and TBP being physically next to each other is very likely. This would increase the chance of detecting protein-protein interactions between the two proteins.

TRANSCRIPTIONAL REGULATION IN EUKARYOTES

Eukaryotic gene transcription is an intricate biochemical process that is tightly regulated at many levels. Biochemical and genetic analysis of various model organisms has identified a great number of protein factors responsible for transcriptional control. While the large assortment of gene-specific DNA-binding regulators was anticipated, the complexity of the general machinery in relation to prokaryotes was not. Corresponding with this increased complexity in cis-control elements, 5-10% of the total coding capacity of metazoans is dedicated to proteins that regulate transcription (52). These proteins fall into three major classes: sequence-specific DNA binding proteins that control gene-selective transcriptional activation or repression; general, but diverse, components of large multi-protein RNA polymerase machines required for promoter recognition; and RNA synthesis, and chromatin remodeling and modification complexes.

The *S. cerevisiae* genome encodes a total of ~ 300 transcription factors, which includes both sequence-specific DNA-binding proteins and subunits of general transcription complexes (105). In contrast, the genome sequences of *C. elegans* and

Drosophilia reveal at least 1,000 transcription factors each and there may be as many as 3,000 transcription factors in the human genome (3, 81). Thus, the complexity of an organism correlates with an increase in both the ratio and absolute number of transcription factors per genome. Yeast contains an average of one transcription factor per 20 genes, while humans appear to contain one factor for every ten genes (52). Given that transcription factors can work in combination, this two-fold increase in the number of factors could produce an expansion in the regulatory complexity.

Metazoan genes contain highly structured regulatory DNAs that direct complex patterns of expression in many different cell types during development (52). The basal promoter is compact and composed of ~60 bp straddling the transcription start site (See Figure 2).

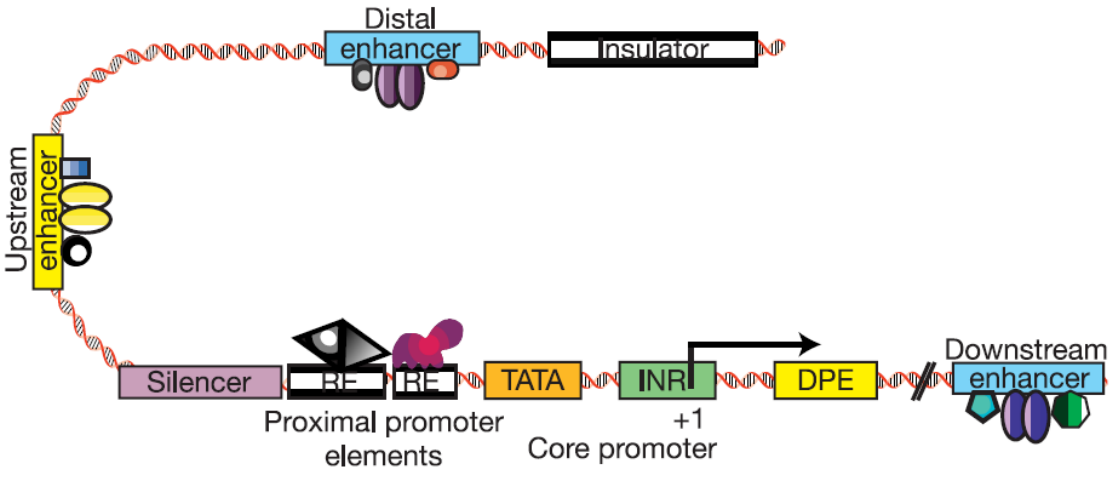

Figure 2. Complex Metazoan Transcriptional Control Modules. A complex arrangement of multiple clustered enhancer modules interspersed with silencer and insulator elements which can be located 10-50 kb either upstream or downstream of a composite core promoter containing TATA box (TATA), Initiator sequence (INR), and downstream promoter element (DPE) (Modified from 53).

There are at least three different sequence elements that can recruit the TBP containing TFIID initiation complex: TATA-box, Inr, and the downstream promoter element (DPE) (94). Many genes contain binding sites for proximal regulatory factors just upstream of the basal promoter. These factors do not always function as classical activators or repressors; instead they may serve as "tethering elements" that recruit distal enhancers to the basal promoter (18, 95). Insulator DNAs prevent enhancers associated with one gene from inappropriately regulating a neighboring gene (16). These regulatory DNAs are scattered over distances of roughly 10 kb in fruit flies and 100 kb in mammals. It is this elaborate organization of the regulatory DNA elements that allows for the tight control of gene expression and provides an explanation for the use of multiple enhancers, silencers, and promoters to control the expression of an individual metazoan gene.

There are three major methods for regulating the binding and function of the RNA pol II complex at the basal promoter. The first is divergent TFIID complexes that bind specific sequence elements within the basal promoter and recruit RNA pol II. It is now clear that metazoans have evolved multiple related TFIID complexes that can function at distinct promoters through the use of tissue-specific TAFs and TBP related factors (TRFs) that are not found in yeast (Figure 3A). One example of a tissue-specific TAF is $TAF_{II}105$, related to the ubiquitously expressed human $TAF_{II}130$, which operates as part of a unique TFIID complex in follicle cells of the ovary to permit the selective activation of a small subset of genes (32). The diverse TFIID complexes have also evolved through the duplication of TBP. There is only one TBP gene and no TRFs

in yeast but there are four TRFs (TRF1-4) in addition to TBP in *Drosophila* (39, 43, 75, 101). These findings suggest that metazoans have evolved TFIID-related transcription complexes responsible for recognizing distinct basal promoters with specific regulatory activities.

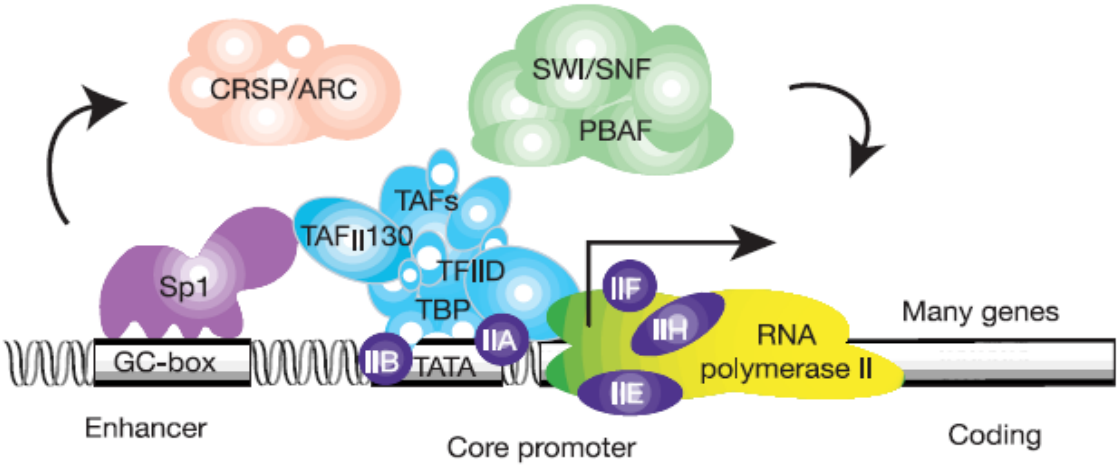

Figure 3. The Multi-Subunit General Transcription Apparatus. The eukaryotic transcriptional apparatus can be subdivided into three broad classes of multi-subunit assembles that include the RNA pol II core complex and associated general transcription factors (TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH), multi-subunit cofactors (mediator, CRSP, TRAP, ARC/DRIP, etc) and various chromatin modifying or remodeling complexes (SWI/SNF, PBAF, ACF, NURF, and RSF). (Modified from 27)

A second mechanism to facilitate the binding and function of RNA pol II is the multi-subunit transcription complexes that are related to the yeast mediator complex. While yeast has one mediator complex, metazoans have several related complexes: TRAP, CRSP, ARC/DRIP, SMCC, and hMed. These complexes are recruited to the DNA through interactions with a variety of sequence-specific transcriptional activators, including nuclear receptors such as the vitamin D receptor and the thyroid hormone receptor (2, 31, 37, 76). Like TFIID, these cofactor complexes may serve as bridges between activators in the distal region on the basal promoter. However, they may not

function solely through the simple recruitment of RNA pol II, but can also be induced to undergo conformational changes that may be essential for activating transcription (96). The cofactor complexes represent some of the most dramatic examples of the diversification of general transcription complexes in evolution (41, 64, 82, 96). Unlike the other components of the general transcription machinery, most of the protein subunits that comprise the yeast mediator and metazoan cofactor complexes are not highly conserved. Thus, in contrast to the strong conservation of TFIID and RNA pol II subunits, cofactor complexes have diversified greatly between eukaryotes, and have expanded among metazoans (34, 57).

Finally, there are enzymatic complexes that remodel or modify chromatin. The enzymes that either remodel nucleosomes (Swi/Snf, Baf/Brm, Acf, and Nurf) or covalently modify histones via acetylation or methylation represent another potential source of regulatory diversification during metazoan evolution (29, 35, 45, 98, 103). Although such complexes are found in yeast, there is only limited conservation of similar subunits in mammals. There is also emerging evidence that remodeling complexes such as the BAFs (Brahma-related gene (BRG1)/Brahma (BRM)-associated factor) have diversified in mammals along with the acquisition of specialized cell types. One example is the neuron-specific BAF complex in mammals, which has no apparent counterpart in fruit flies or nematodes (70). Also, some of the remodeling complexes mediate transcriptional repression rather than activation, such as the MBD2 protein that controls repression on methylated DNA templates by recruiting NuRD, a complex that contains both a Swi/Snf activity and histone deacetylase activity (29).

ENHANCER ELEMENTS OF TRANSCRIPTION

Since their identification in the early 1980s, transcriptional enhancers have been the subject of numerous studies because of their ubiquitous roles in higher eukaryotic gene regulation. Enhancers are classically defined as cis-acting independent of their position and orientation with respect to the transcriptional initiation site (7). This stimulatory role distinguishes enhancers from basal promoter elements which bind the basal transcription machinery and determine the site of transcriptional initiation (94). Enhancer-associated proteins can bind in sequence-specific or sequence-non-specific manners, as well as indirectly through protein-protein interactions. Typical enhancers span 200-1,000 bp, and bind to dozens of sequence-specific proteins (4). Enhancer elements are commonly found in clusters. Single factor binding sites are in general insufficient to drive gene expression, a mechanistic feature that appears to prevent unwanted activation by randomly occurring binding sites. Instead, multiple, clustered sites are a hallmark of enhancers, and presumably reflect the synergy required for important, but weak, protein-protein interactions to occur (28).

There are two manners in which enhancers are suggested to affect gene expression: either in a binary, "stochastic" manner, or a continuous, "rheostatic" manner. In the first case the transcriptional expression level of a gene has either an "on" or "off" state and the activity of the enhancer is to turn the gene to the "on" state (13). The second, rheostatic model, suggests that enhancers might also quantitatively regulate transcription rates through a continuous spectrum, depending on the amount and nature of the bound factors. This model is supported by direct manipulation of transcription

factor concentrations on synthetic enhancers (10, 80) and by sequence changes that affect individual factor binding sites within an enhancer that also quantitatively affect the strength of the activation (46).

ACTIVATION DOMAINS OF TRANSCRIPTION ACTIVATION PROTEINS

Eukaryotic transcriptional activators stimulate transcription of genes otherwise expressed at low levels. The typical activator operates by binding to specific sites on DNA, contacting its activating region to the multiprotein machinery that directs transcription (73). The activation domain of a transcription activator is the region of the protein that recruits the basal transcriptional machinery or mediator proteins through protein-protein interactions to the gene promoter. Transcriptional activators can also recruit chromatin remodeling factors to a specific gene promoter allowing the transcriptional machinery access to the promoter. This allows for an increased level of transcription of a specific gene. Activation domains do not have a well defined organization and have no obvious sequence similarity but may structurally have an alpha helix and are sometimes rich in proline, glutamine, acidic amino acids, or hydroxylated amino acids.

For many activators and genes, the specificity of activation is determined solely by the DNA binding address of the activator (73). For example, the activator Gal4 ordinarily activates genes required for galactose metabolism in yeast, but when any of a wide array of genes is modified to contain a Gal4 binding site nearby, Gal4 will also activate that gene. Gal4 contains two functional domains. One is the DNA binding

domain (DBD) that binds to Gal4 UAS (upstream activation sequence) but does not activate transcription. The Activation domain (AD), which is inactive on its own, works when fused to a heterologous DNA-binding domain to activate a reporter gene (72). Thus, Gal4 is a modular protein with the DBD and AD functions carried on separable domains. When Gal4 was artificially expressed in a wide array of eukaryotes, it was found in each case to activate a reporter gene bearing Gal4 binding sites (UAS) nearby. Thus, the mechanism of activation is widely conserved (74).

The recruitment model postulates that transcriptional activators, through the use of protein-protein interactions, recruit the transcriptional machinery to the DNA. Activating regions that are not tethered to DNA do not activate; they must be brought to the DNA for recruitment to move forward. The activator can either lie adjacent to the gene or further upstream, where the DNA loops out to allow the protein-protein interactions. Activation of transcription by recruitment is more complicated in eukaryotes than in bacteria in that an activator must recruit to the gene a more elaborate transcriptional machinery (57, 59). Whereas recruitment of RNA polymerase itself suffices in bacteria, at least 50 proteins must be assembled in eukaryotes. Some of these proteins are found in complexes and some must be independently recruited to the gene (14, 19). Some of the recruited complexes modify histones to increase the affinity for other complexes and make the further recruiting task of an activator easier. Recruited complexes interact with and attract polymerase and promote elongation of transcription. Therefore, the activator, by simple binding or recruiting reactions, triggers a cascade of events that results in expression of a specific gene (1, 42, 50).

THE SBF/STAF PROTEIN

SBF/Staf is an activator protein that binds to the SPH element and activates/enhances the transcription of the downstream gene. SBF/Staf was first demonstrated to be able to activate RNA polymerase III transcription *in vivo* by the Carbon lab (92). SBF/Staf binds to the SPH element through a seven zinc finger DNA binding domain and contains two distinct activation domains, one for snRNA genes and one for mRNA genes that can stimulate snRNA promoters and synthetic mRNA promoters as determined by Schuster *et al*.1998 (91) (Figure 4).

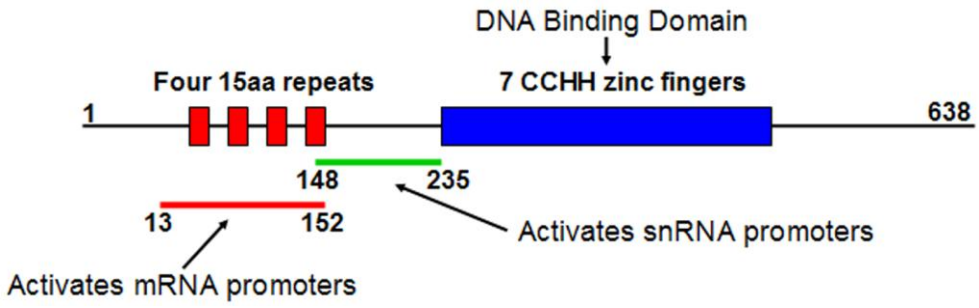

Figure 4. Primary Structure of the Human SBF/Staf Protein.

The central part of SBF/Staf contains seven contiguous zinc fingers of the C_2-H_2 type (92). Not all of the seven zinc fingers are required for binding to SPH sites. Zinc finger 1 exhibits a flexible requirement, since it contacts the DNA of *X.laevis* $tRNA^{Sec}$, but not at the human U6-1 snRNA SPH element (86). This flexibility allows for the maximum transcription activation from the $xtRNA^{Sec}$ and human U6-1 snRNA promoters (87). The non-utilization of the first zinc finger at the human U6-1 snRNA promoter enables

the simultaneous binding of SBF/Staf and Oct-1 to their cognate DNA motifs (87). The two physically separate and functionally distinct activation domains are located in the N-terminus. The activation domain covering residues 207 to 224 of *Xenopus* SBF/Staf functions solely in the transactivation of RNA polymerase II and RNA polymerase III snRNA-type promoters. The other activation domain, residues 84-176 of the *Xenopus* protein, is restricted to transcriptional activation from mRNA promoters (91).

Until recently, only eight protein-coding genes had been described to be regulated by SBF/Staf: 1) mouse cytosolic chaperonin containing t-complex polypeptide 1 (TCP1) (47); 2) human interferon regulatory factor (IRF3) (55); 3) human neuronal nitric oxide synthase (NOS1) (85); 4) human transaldolase (TALDO1) (36); 5) mouse aldehyde reductase (AKR1A1) (8); 6) human mitochondrial ribosomal protein S11 (MRPS11) (40); 7) human synaptobrevin-like 1 (SYBL1) (24); 8) human budding uninhibited benzimidazole receptor 1 (BubR1) (61). Vertebrates also contain two proteins, ZNF143 and ZNF76, which are related to SBF/Staf. ZNF143 constitutes a human ortholog of SBF/Staf and ZNF76 a paralog of ZNF143 (63). ZNF76 and ZNF143 are thought to play the same role. However, recent results suggest that ZNF76 functions as a transcriptional repressor through interactions with TBP and that sumoylation modulates its transcriptional properties (111). A recent study by Myslinski, *et. al.* 2006 (60) identified 1175 SBF/Staf binding sites (SPH elements) in 938 promoters of four different mammalian genomes. Thus, SBF/Staf may be one of the most prevalent activators for mammalian genes. The group also demonstrated that the presence of the SPH elements alone were sufficient to direct the expression of a

luciferase reporter gene, suggesting that SBF/Staf can recruit the RNA pol II transcription machinery.

ENDPOINTS OF PATHWAYS IN ZEBRAFISH DEVELOPMENT INVOLVE TRANSCRIPTIONAL CONTROL

In early development higher eukaryotic cells are totipotent. The control of cell differentiation is controlled by selective gene expression. Differential gene expression during metazoan embryogenesis typically is regulated in response to several signaling transduction pathways including the Wnt, FGF (Fibroblast Growth Factor), TGF-β (Transforming Growth Factor Beta) super family, and Hedgehog pathways. Loss of function of any member of any of these pathways will lead to disruption of embryo development. The major signal transduction pathways follow similar schematic outlines. Each receptor spans the cell membrane and has an extra cellular region, a transmembrane region, and a cytoplasmic region. When a ligand binds to its receptor it causes a conformational change in the receptor's cytoplasmic domain. The conformational change gives the cytoplasmic domain an enzymatic activity, which is usually a kinase activity. The activated cytoplasmic domain phosphorylates a downstream target protein which becomes activated in response. Eventually, the signal

cascade of phosphorylation will activate a dormant transcription factor. The main function of these pathways is to use intercellular signaling to regulate cell fate decisions by altering the transcriptional program of the target cells in an instructive manner. For the purpose of this study, knowledge of these pathways may help in the interpretation of the phenotypes seen after morpholino injection to knock down SBF/Staf levels. An overall description of these signaling pathways and mutant phenotypes found after mis-expression of members of the pathways in zebrafish follows.

One important signalling pathway is the Wnt pathway (33). Wnt binds to the Frizzled protein receptor activating the Disheveled protein. Activated Disheveled then inhibits the activity of glycogen synthase kinase-3 (GSK3). With GSK3 inhibited, β-catenin dissociates from the APC protein and enters the nucleus. Once in the nucleus β-catenin forms a heterodimer with either LEF or TCF DNA-binding proteins to become a transcription factor (Figure 5) (9, 17).

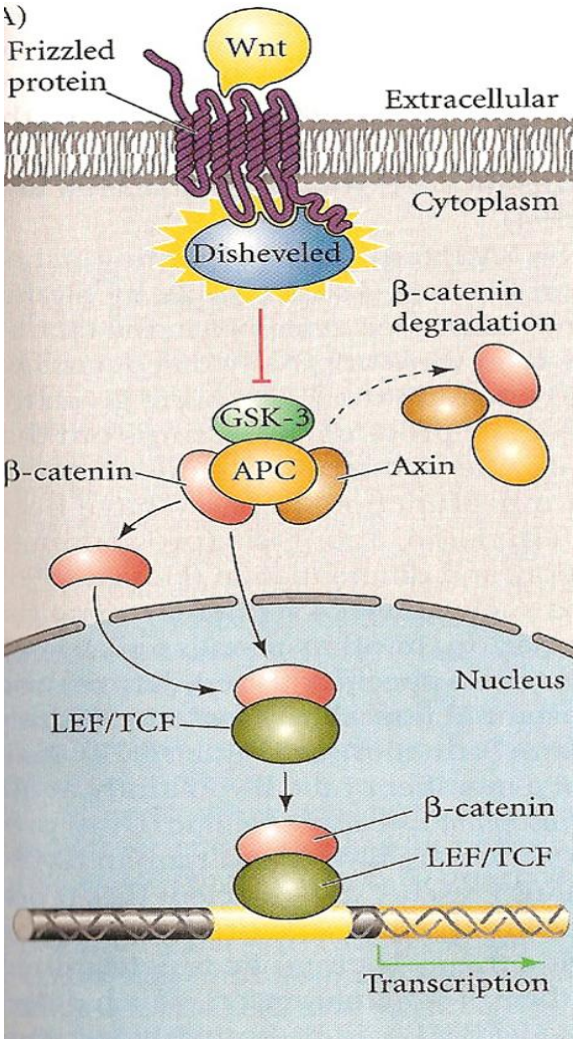

Figure 5. The Wnt Signal Transduction Pathway. The Wnt protein binds to its receptor Frizzled activating Disheveled allowing it to become an inhibitor of GSK3. β-catenin is then free to associate with LEF or TCF and become an active transcription factor. (Modified from 33)

Two genes in the human Wnt signaling pathway contain SPH sites in their promoters, Frizzled-4 and GSK3-α (60). These sites were confirmed by visual inspection to be in analogous positions in the corresponding zebrafish genes. Hence, there is a chance that SBF/Staf may be involved in the Wnt signaling pathway. Three transcription factor genes found in zebrafish to be targets of Wnt signaling are cdx4 (23), MITF/nacre (27,

84, 107), and bozozok (83). Based on analysis of these genes, mutation or knockdown of Wnt and members of the Wnt pathway has been shown to lead to phenotypes such as lack of somites and tailbud (Wnt-3A, mouse) (97) and mis-patterened mesoderm (Wnt-8A, zebrafish) (51). It is known that loss of bozozok (transcription factor that is regulated by β-catenin) and TCF3 (transcriptional repressor of Wnt target genes) leads to a variable loss of dorsal mesoderm and forebrain, and loss of forebrain and midbrain, respectively, in zebrafish (89). Target genes of the Wnt pathway in other model organisms can be found at the web site www.stanford.edu/~rnusse/wntwindow.htm.

The FGF (fibroblast growth factor) pathway is a pathway important for limb development and lens induction (102). The FGF ligand activates the FGF receptor (FGFR) which is a tyrosine kinase. When the ligand binds, the receptor autophosphorylates the cytoplasmic domain of the receptor and the adaptor protein then serves as a bridge to link the phosphorylated receptor to the G protein Ras. Once GAP (GTPase-activating protein) activates Ras, the Ras protein associates with Raf kinase. Raf kinase activates the MEK (Mitogen-Activated Protein Kinase Kinase) by phosphorylation, MEK then phosphorylates ERK (Extracellular Signal-Regulated Kinase), and then ERK enters the nucleus and phosphorylates a specific transcription factor (Figure 6).

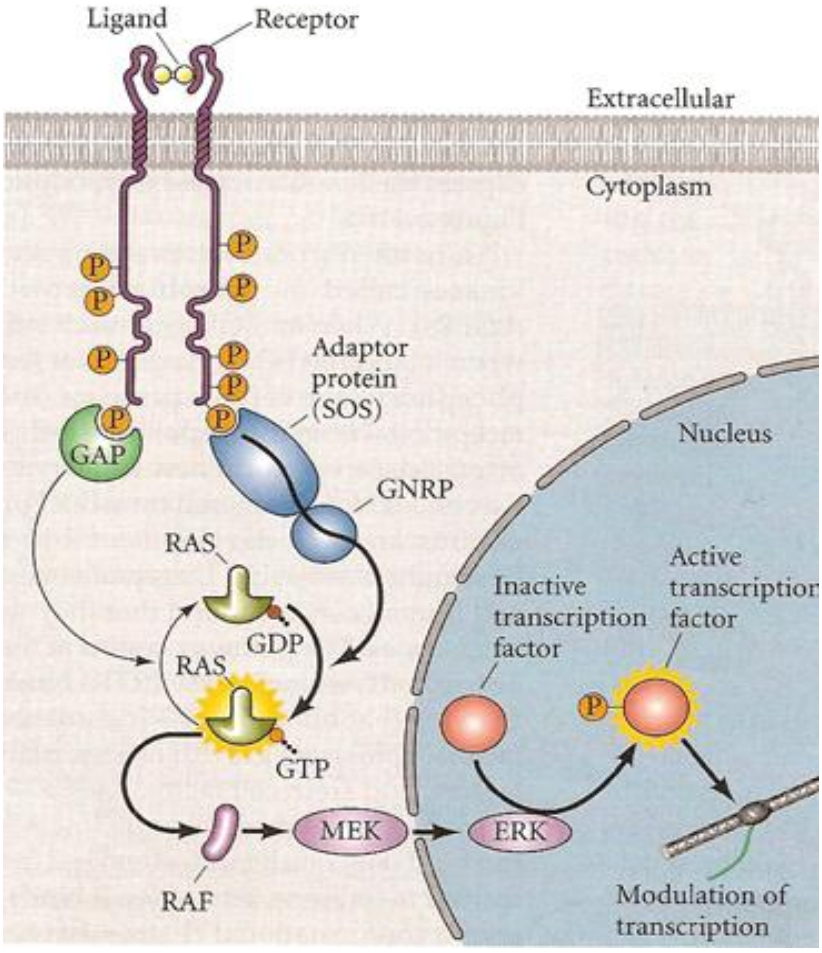

Figure 6. General FGF Pathway. (Modified from 33)

One example of a transcription factor that is activated by the FGF pathway is Mitf that binds to the p300/CBP histone acetyltransferase protein that enables it to activate the transcription of genes encoding tyrosinase and other proteins of the melanin-formation pathway (71). The FGFR1 gene contains an SPH element in the promoter of the human gene (60) and also has an SPH element in the zebrafish gene promoter as confirmed by visual inspection. Therefore, there is also a chance that SBf/Staf may be

involved in the FGF signaling pathway. Several zebrafish mutants have been generated by mutagenesis with ENU (ethylnitrosourea) for FGF genes. Mutants in FGF8 lack a cerebellum and the midbrain-hindbrain boundary organizer (77). Embryos mutant for FGF24 lack pectoral fin buds (30). Mutation of the FGF10 gene leads to embryos that have no pectoral fin buds and a severely dysmorphic heatopancreatic ductal system (26, 68). Knockdown of FGF16, FGF19, and FGF21 by morpholino injection generated zebrafish embryos that lacked fin buds, impaired development of the forebrain, and haematopoiesis (58, 67, 106).

Members of the TGF-β (transforming growth factor-beta) super family signaling pathway dimerize in the C-terminal domain to make both homodimers and heterodimers with other TGF-β family members and then are secreted from the cell. One member of the TGF-β super family is the BMP (bone morphogenetic proteins) family that induce bone formation, regulate cell division, apoptosis, cell migration, and differentiation. BMPs are thought to work through diffusion and the range of the diffusion is determined by the N-terminal amino acids (69). Another member of the TGF-β super family is Nodal that signals through the activin receptor, and affects mesoderm and endoderm formation(90). It is encoded by the *cyclops* and *squint* genes in zebrafish, and mutants display cyclopia or cyclopia and dorsal mesoderm defects respectively (89, 90). TGF-β binds to a type II TGF-β receptor and then the type II receptor binds to a type I TGF-β receptor to phosphorylate either the serine or threonine on the type I TGF-β receptor. The type I receptor activates Smad 1 and 5 in the case of BMP, Smad 2 and 3 for Nodal. These phosphorylated Smads then bind to Smad 4 to form a transcription complex that

enters the nucleus (Figure 7). Nodal also uses the FoxH1 co-activator in the transcription of Nodal controlled genes. Zebrafish that are mutant in the Smad 5, BMP2b, and BMP7 genes are severely dorsalized (89).

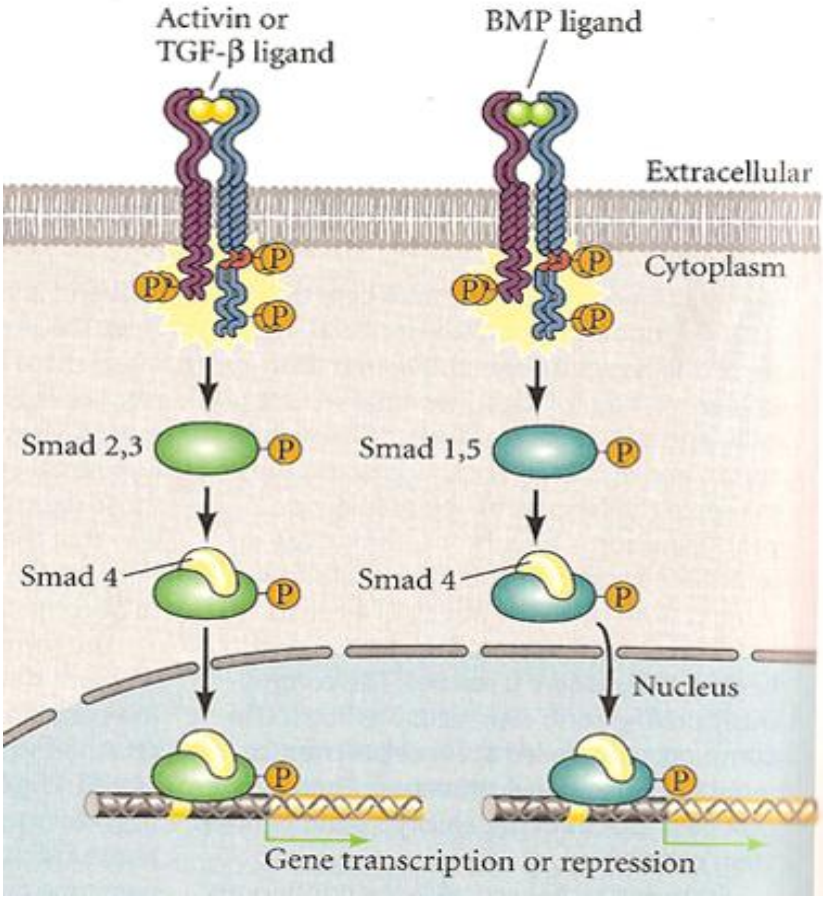

Figure 7. The Smad Pathway Activated by TGF-β Super Family Ligands. (modified from 33)

A fourth developmental pathway is the Hedgehog pathway named after the hedgehog signaling protein in *Drosophila*. *Drosophila* hedgehog works in concert with other molecules to lay down the basic framework of the embryo, determining anterior-posterior relationships in developing structures(6). There are three homologues of the

Drosophila Hedgehog found in vertebrates, sonic hedgehog (*shh*), desert hedgehog (dhh), and indian hedgehog (ihh). Desert hedgehog is expressed in the testes, and mice mutant in *dhh* are defective in spermatogenesis (11). Indian hedgehog is expressed in the gut and helps catalyze bone growth (12). Sonic hedgehog has the greatest number of functions of the three hedgehog genes and is responsible for vertebrate limb formation and neural differentiation by directing motor neurons to only come from the ventral portion of the neural tube (56). Hedgehog proteins bind the Patched receptor, and the signal is transduced by the interaction of Patched and Smoothened. Patched is a negative regulator of Smoothened and in the absence of a Hedgehog ligand Smoothened is inactive and the Cubitus interuptus (Ci) protein tethered to the microtubules is cleaved. When Ci is cleaved the free portion can enter the nucleus and act as a transcriptional repressor. When a Hedgehog ligand binds to Patched it causes a conformational change in Patched that releases it from Smoothened. Smoothened then releases Ci from the microtubules, and the intact Ci protein enters the nucleus and acts as a transcriptional activator (5, 54). Loss of zebrafish Hedgehog signaling leads to ventral spinal cord defects, deficiencies in ventral forebrain specification, absence of an optic chiasm due to retinal axon guidance defects, absence of slow muscle fiber types, malformations of the dorsal aorta, ventral curvature of the body and defects in pectoral fin development (44).

The Hedgehog signaling pathway controls the expression of the Wnt gene family, TGF-β/BMP family, and the Patched protein(6) (Figure 8).

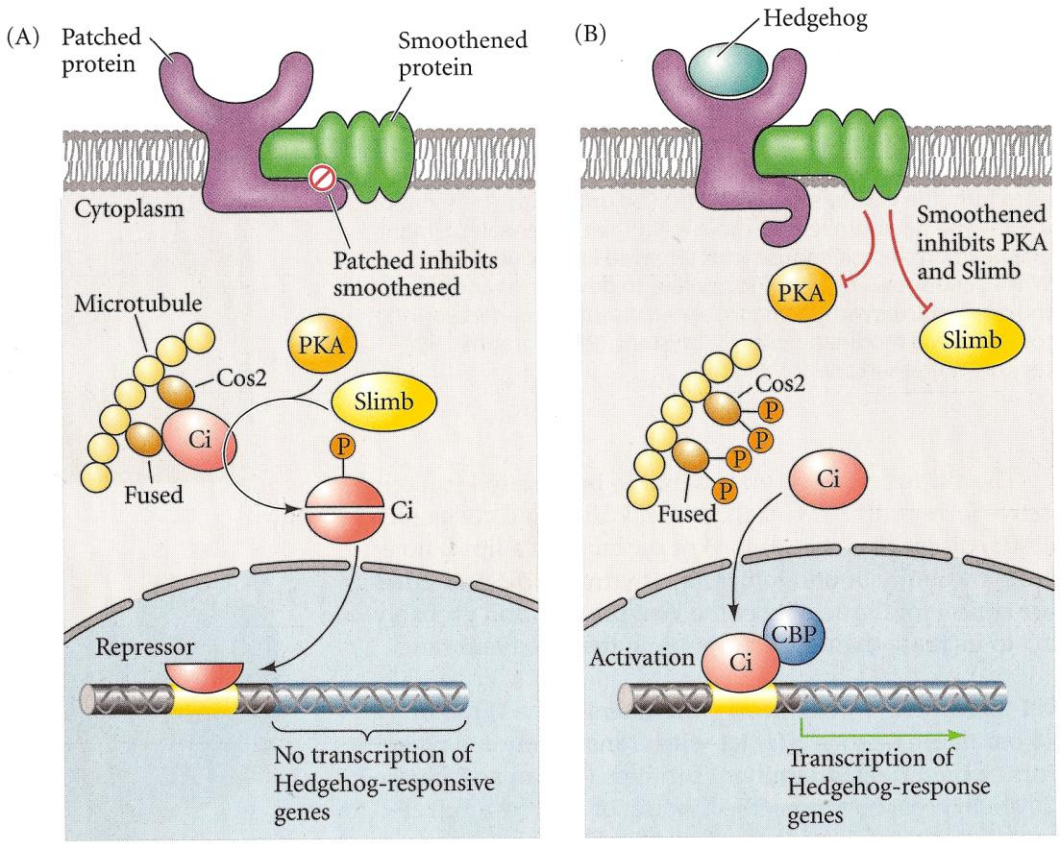

Figure 8. The Hedgehog Signal Transduction Pathway. A). In the absence of Hedgehog binding to Patched, the Ci protein is tethered to the microtubules by the Cos2 and Fused proteins. This binding allows for PKA and Slimb proteins to cleave Ci into a transcriptional repressor. B). When Hedgehog binds to Patched its conformation is changed and is released from the inhibition of Smoothened. Smoothened then releases Ci from the microtubules and inactivates the cleavage protein PKA and Slimb. The Ci protein enters the nucleus, binds a CBP protein and acts as a transcriptional activator. (Modified from 33)

In summary, two of the major developmental signaling pathways have components that contain SPH sites in the promoter. In the case of the Wnt signaling pathway the transcription factors bozozok, cdx4, and MITF/nacre, whose expression is regulated by the Wnt pathway, lead to the loss of dorsal mesoderm and forebrain (bozozok), defects in haematopoiesis (cdx4), and loss of pigment cell differentiation

(MITF/nacre). In the second signaling pathway, the FGF pathway, the FGF receptor 1 (FGFR1) gene contains an SPH element in the promoter. Loss of FGF signaling leads to a loss of dorsal ventral patterning. The phenotypes described in Chapter III of morpholino injected zebrafish embryos such as a change in the cell fate of dorsal and ventral cells or circular system stress may be due to a loss in these developmental signaling pathways.

GOAL

The research in this dissertation focuses on characterizing the role of SBF/Staf in both zebrafish development and transcriptional control. The overall goal of this research is to provide insight into the role of SBF/Staf in general embryonic development as well as to determine the significance of the proximally-located SPH element for transcriptional control of the zebrafish U6 snRNA genes. First I set out to determine the phenotype of a SBF/Staf morphant embryo and whether the phenotype could be rescued by in vitro transcribed mRNA. In addition, I tested whether mutant SBF/Staf proteins, defective in either mRNA or snRNA activation domains, could rescue the phenotype. Furthermore, I investigated the enhancer function of the proximally-located SPH element in the zebrafish U6 snRNA genes.

CHAPTER II

MATERIALS AND METHODS

RAPID RNA ISOLATION FROM ZEBRAFISH EMBRYOS

Embryos were collected at the indicated time-point and homogenized in GHCl buffer (7.5 M guanidinium hydrochloride, 0.025 M NaOAc pH 7.0, 5 mM DTT, 0.5% N-laurylsarcosinate, 0.5% DEPC) with a dounce homogenizer using 0.5 ml of GHCl buffer per 100 embryos. The homogenate was then extracted once with an equal volume of acid phenol-$CHCl_3$, and the upper phase was extracted with an equal volume of $CHCl_3$. The RNA was then ethanol precipitated by transferring the upper phase into a new tube with 50 µl of ethanol and 2 µl of 1M acetic acid for each 100 µl of GHCl solution. The sample was incubated at -20 ˚C overnight and the RNA was pelleted by centrifuging the sample at 14k RPM for 30 min at 4 ˚C. The pellet was then resuspended in one half the starting volume of GHCl buffer. The RNA was then reprecipitated and stated above, the pellet was washed in 100% ethanol, and dried briefly in a Speed Vac (Savant). Once dried the RNA was resuspended in 20-30 µl of H_2O-DEPC and stored at -80 ˚C.

PLASMID CONSTRUCTIONS

Zebrafish small RNA genes

Plasmid DNAs containing zebrafish U6 and U4 snRNA genes were constructed by G. Kunkel as described previously (22). Briefly, zebrafish snRNA genes were

amplified by PCR from zebrafish genomic DNA (provided by A. Lekven), and DNA fragments were ligated into pGEM-T (Promega) according to the manufacturer's protocol. Primer sequences are given under "Oligos Used." To make the gene a "maxigene" a XhoI restriction site was inserted into the 3' end of the zebrafish U6 snRNA gene. Maxigene versions of both zU6-1 and zU4-1 genes were constructed according to the QuickChange protocol (Stratagene) using primers ZU61MAXITOP/ZU61MAXIBOT and ZU4MAXITOP/ZU4MAXIBOT, respectively. Mutations within the SPH and TATA elements in the zebrafish U6-1 gene promoter were added using the QuickChange protocol with the primer sets, ZU61SPHMUTTOP/ZU61SPHMUTBOT and ZU61TATAMUTTOP/ZU61TATAMUTBOT, respectively. All mutations, as well as the fidelity of the entire plasmid insert, were verified by sequencing of plasmid DNAs. Large-scale amounts of plasmid DNA were prepared using a Qiagen plasmid maxi kit.

Zebrafish SBF/Staf plasmid DNAs used as templates for rescue mRNA synthesis

Plasmid DNAs containing the zebrafish SBF/Staf ORF were constructed by G. Kunkel. A cDNA containing the zebrafish SBF/Staf gene was obtained from Open Biosystems. We named this plasmid pME18S-FL3/zZNF143. The ORF contained within this insert was lacking a full-length gene and contained a reading frame error in the coding region near the amino-terminus of the encoded protein. Hence, in order to construct a full-length ORF ligated behind a T7 promoter, three DNA fragments were ligated as follows. The "correct" amino-terminal region fragment was prepared by RT-

PCR using total RNA from zebrafish ZF4 cells and ligated into a pGEM-T vector (Promega). This fragment was excised using KpnI and PvuII and purified by agarose gel electrophoresis. The main body of the SBF/Staf ORF was excised from pME18S-FL3/zZNF143 using PvuII and XhoI and purified by agarose gel electrophoresis. The third DNA fragment was the pBlueScript SK (Stratagene) vector opened at KpnI and XhoI sites, and purified by chromatography on Sepharose CL-4B. The three-fragment ligation reaction was used to transform E. coli XL1-Blue competent cells. The insert of the subsequent plasmid, named pBS/FLZSBF, was sequenced entirely to verify its successful construction. A single myc tag was inserted at the amino-terminus of the encoded SBF/Staf protein using the QuickChange protocol and the primer set ZSBFMYCTOP/ZSBFMYCBOT. All subsequent deletions within the zSBF/Staf ORF started with the parent plasmid pBS/mycZSBF and were constructed using the QuickChange protocol in which the oligonucleotides base-paired across the deletion endpoints and looped out the template DNA. The DNA templates used in the *in vitro* transcription reaction were made using the QuickChange (Stratagene) protocol using oligos that looped out the DNA encoding for the amino acids to be deleted. For each PCR-based reaction 5 µl of 10X Pfu Ultra HF Buffer, 1 µl 10 mM dNTPs mix, 50 ng plasmid template, 1.25 oligo mix (50 ng/µl each), 1 Pfu Ultra HF polymerase, and H_2O up to 50 µl. The PCR program used in the reaction was as follows: 95˚C for 3 min to denature the template; 95˚C for 30 sec, 50˚C for 1 min, 68˚C for 10 min, cycled 20 times; then cooled to 4˚C. The reaction was then treated with DpnI for 2 hr at 37˚C. Ten microliters of the reaction was used to transform chemically competent E. coli XL1-Blue

cells. The plasmid DNA was isolated using the Qiagen mini-prep kit and inserts of all plasmids were sequenced completely to verify deletions and ensure that no other mutations were added to the SBF/Staf ORF. Plasmid minipreps were prepared using a Qiagen miniprep kit, and such preparations were suitable for mRNA synthesis *in vitro*.

TRANSFECTIONS

Zebrafish ZF4 cells (ATCC #CRL-2058) were transfected in 6-well plates with 500 ng of the appropriate U6 maxigene reporter plasmid plus 500ng of a U4 maxigene control plasmid for RNA recovery using 1:3 ratio (1µg DNA:3 µl Lipofectamine) of Lipofectamine 2000 (Invitrogen). Total RNA was isolated 48 hr post-transfection and analyzed by RT-PCR. For RT-PCR 105 ng of total RNA were treated with TURBO DNase (Ambion) per the manufacturer's instructions. The treated RNA was reversed transcribed using random hexamer primers (Roche) and Superscript III (Invitrogen) per the manufacturer's instructions. The resulting cDNA was then amplified by PCR using 25 µl Go Green Master mix (Promega), 3 µl of 10mM forward primer, 3 µl of 10mM reverse primer, 2 µl (3.5ng) of cDNA, and H_2O to a final volume of 50 µl. Primer sets used were designed to amplify the expression of the U6 maxigene, U4 maxigene, and the 5.8S rRNA (See Oligos Used). The PCR program used for amplification was 95˚C for 1 min, 57˚C for 2 min, and 72˚C for 2 min. and was repeated 35 times. The products were separated by electrophoresis on 18% polyacrylamide gels, stained with EtBr and the relative band intensities were quantitated using the Bio-Rad Gel Doc XR and Bio-Rad Quantity One software.

MICROINJECTION OF MORPHOLINOS INTO ZEBRAFISH EMBRYOS

The wild-type fish used were the AB strain (Westerfield) and were maintained as described previously (104). The morpholinos (MOs) used in the microinjections were designed to block the translation of SBF/Staf. The sequence of the MOs and location of their target sequence is illustrated in Figure 9.

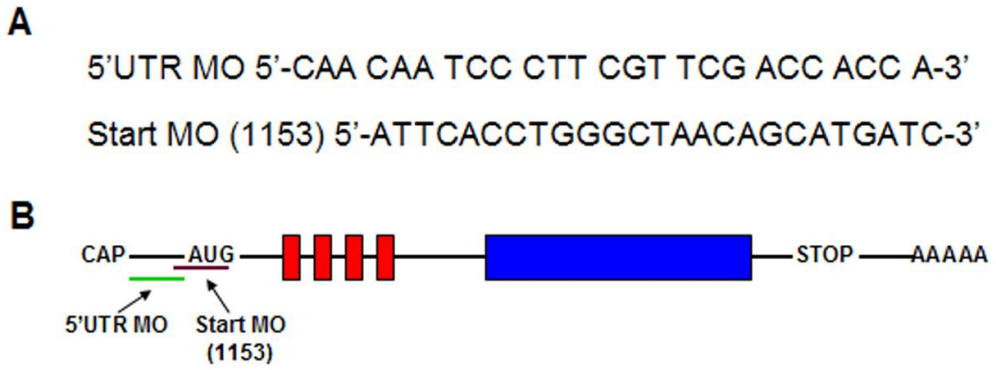

Figure 9. Morpholino Sequence and Location of Binding. A. Sequence of the two MOs used for embryo injections. B. Location on the SBF/Staf mRNA in which the MO will bind and block translation.

The two translation blocking MOs (5 ng/nl each) were simultaneously injected and the phenotypes resulting from the injection were classified at 48 hpf. To determine the specificity of the MO knockdown, a capped rescue wild-type SBF/Staf mRNA that contained a myc tag on the 5' end and did not contain the binding sites for the MOs was transcribed *in vitro* using the mMESSAGE mMACHINE T7 Ultra kit (Ambion) per the manufacturer's instructions. Rescue mRNAs of SBF/Staf were prepared that either lacked the mRNA activation domain and snRNA activation domain simultaneously or

independently. These rescue mRNAs were co-injected with both MOs and the phenotypes were classified at 48 hpf.

REAL TIME PCR (qPCR)

For each reaction the following was mixed: 1.5 µl of primer mix (30 pmol/µl of each), 10 µl 2X SYBR Green qPCR master mix (Applied Biosystems), cDNA sample, and H_2O to 20 µl final volume. The samples were cycled in an iCycler machine (Bio-Rad) using the following program: 95˚C for 15 min; 95˚C for 30 sec, 58˚C for 30 sec, and 72˚C for 30 sec, cycled 40 times with data collected at this time; 95˚C for 1 min; 55˚C for 1 min; and 55˚C for 10 sec, cycled 80 times with an increase in setpoint temperature by 0.5˚C which allowed for data for a melting curve to be collected and analyzed. The iCycleriQ software (Bio-Rad) was used to analyze the qPCR data collected.

OLIGOS USED

<u>PLASMID CONSTRUCTION</u>

ZU61-305: 5'-AGG ACA CCTCAA CAA AAG CTC CTC-3'
CZU61+290: 5'-GCT CTA ATG CGC GGC TGG CAG TGC-3'
ZU41-300: 5'-GTG CGT TTG TAT GTT AGG AAA TAC G-3'
CZU4+132: 5'-GCC AGC AAC GCG GCT CGC CTT CAT C-3'
ZU61MAXITOP: 5'-ATG ACA CGC AAA TTC CCT CGA GGC GTG AAG CGT TCC ATC-3'
ZU61MAXIBOT: 5'-GAT GGA ACG CTT CAC GCC TCG AGG GAA TTT GCG TGT CAT-3'
ZU4MAXITOP: 5'-TTG AAA ACT TTA CCC GAC TCG AGG AAA TAC CCC GCC GTG A-3'
ZU4MAXIBOT: 5'-TCA CGG CGG GGT ATT TCC TCG AGT CGG GTA AAG TTT TCA A-3'

ZU61SPHMUTTOP: 5’-CAC ATG AAA CAC ATA GTT CGA AGT CAC TGG TAT A-3’
ZU61SPHMUTBOT: 5’-TAT ACC AGT GAC TTC GAA CTA TGT GTT TCA TGT G-3’
ZU61TATAMUTTOP: 5’-CAG AAG TCA CTG GTA GCG CTA GCC GTC CTC CAG A-3’
ZU61TATAMUTBOT: 5’-TCT GGA GGA CGG CTA GCG CTA CCA GTG ACT TCT G-3’
ZSBFMYCTOP: 5’-GGG ATT GTA CCA TGG AGC AAA AAT TGA TTT CCG AGG AGG ACT TGA TGC TGT TAG CCC AGG-3’
ZSBFMYCBOT: 5’-CCT GGG CTA ACA GCA TCA AGT CCT CCT CGG AAA TCA ATT TTT GCT CCA TGG TAC AAT CCC-3’
F-KpnI zZNF143: 5’-AGC TTG GTA CCA TGC TGT TAG CCC AGG TGA ATC GG-3’
R-zZNF 143 279-300: 5’-ATC CAT CCT CCA GCT GAA TCA C-3’

RESCUE CONSTRUCTS

MYCZSBFDEL2TO149TOP: 5’-TCC GAG GAG GAC TTG ATG CCT CAG TCC AAC ACC ATC-3’
MYCZSBFDEL2TO149BOT: 5’-GAT GGT GTT GGA CTG AGG CAT CAA GTC CTC CTC GGA-3’
MYCZSBFDEL151TO225TOP: 5’-GCA TAT ATT CAA CAT ATG CCT GGA GAA AAG GCC TTC CG-3’
MYCZSBFDEL151TO225BOT: 5’-CGG AAG GCC TTT TCT CCA GGC ATA TGT TGA ATA TAT GC-3’
MYCZSBFDEL2TO225TOP: 5’-TCC GAG GAG GAC TTG ATG GGA GAA AAG GCC TTC CG-3’
MYCZSBFDEL2TO225BOT: 5’-CGG AAG GCC TTT TCT CCC ATC AAG TCC TCC TCG GA-3’

RT-PCR

F-zU61-17: 5’-GTG CTT GCT TCG GCA GC-3’
MAXI2U6: 5’-TTC ACG CCT CGA GGG AAT-3’
F-zU4 4-25: 5’-TTT GCG CAG TGG CAG TAT CGT-3’
R-zU4maxi: 5’-GGT ATT TCC TCG AGT CGG GTA AAG TTT TC-3’
F-5.8S2-25: 5’-AAC TCT TAG CGG TAC ACT CGG-3’
R-5.8S91-114: 5’-GCA AAG TGC GTT CGA AGT GTC GAT-3’
F-zU61-23: 5’-GCC GTC CTC CAG ACT CCC AGC TCG-3’
R-ZU61+193: 5’-CTG CGT ACT GAA CGC TTA AAC TCC-3’

REAL-TIME PCR

ZTFIIB510: 5’-TGC TAT TGC TTC AGC CTG CCT CTA-3’
CZTFIIB625: 5’-TGA AGC AGC GAC CAA TCT CCT TCT-3’
ZPLK1579: 5’-GTT GTG GGC TTT CAC GGG TTC TTT-3’
CZPLK1731” 5’-ACC CTG AAT AGT CTG GCG CAT GAA-3’
ZMDH1672: 5’-ATG CTG TGA ATG ACG AA GCT GGC-3’
CZMDH1791: 5’-AGA TGG CTT TGG CAG CAG ACA TTG-3’
ZSNAPC3555: 5’-TTT CAG ACT CTG CAT GTG TTG GGC-3’
CZSNAPC3671: 5’-TGG TAC CAT GTC TGG AGT GTT GCT-3’

CHAPTER III

THE BIOLOGICAL EFFECT OF SBF/STAF IN ZEBRAFISH DEVELOPMENT

INTRODUCTION

As stated in Chapter I, Myslinski, *et. al.* (2006) identified 1175 SBF/Staf binding sites (SPH elements) in 938 promoters of four different mammalian genomes. The group also demonstrated that the presence of the SPH elements alone was sufficient to direct the expression of a luciferase reporter gene, suggesting that SBF/Staf can recruit the RNA pol II transcription machinery. This raises the question of whether SBF/Staf is able to activate the expression of both mRNA and snRNA encoding genes. In order to further study the different roles of the two activation domains of SBF/Staf, the Kunkel lab attempted a knock-down of the SBF/Staf activity in cultured human cells using RNAi strategies. While the SBF/Staf RNA was successfully targeted with this approach, the endogenous protein levels could never be reduced by more than approximately 50%. Because this level of knockdown does not allow an accurate evaluation of SBF/Staf function, an alternate experimental system was required. The zebrafish (*Danio rerio*) appeared to be a good model system to approach this question because of the following: SBF/Staf is conserved in vertebrates, but is absent in invertebrates (62); protocols are well established for the micro-injection of anti-sense morpholino oligos and synthetic mRNA to study gene knockdown, rescue, or gain of function phenotypes; zebrafish embryos develop rapidly outside the body of the mother; and embryos are optically clear allowing for the phenotypic outcome of the microinjection to be visualized easily. Very little is known about the role of SBF/Staf in vivo. According to an in situ hybridization

experiment submitted to the Zebrafish Information (ZFIN) website, SBF/Staf, referred to as *znf143*, does not show a specific localization (99). This result is not surprising because the known role for SBF/Staf was in activation of the spliceosomal snRNA gene and a few encoding mRNAs and would be needed in all tissues. However, we hypothesized that the downstream expression of other genes controlled by SBF/Staf may result in distinct phenotypes that may be uncovered by these experiments.

Expression of a specific protein can be suppressed throughout a developing embryo, producing a "knockdown" by the use of morpholino (MO) antisense oligodeoxynucleotides (65). Morpholinos are made up of a six member morpholine ring instead of the ribose ring and a nonionic phosphorodiamidate backbone in place of the anionic phosphodiester backbone. It is these two features that keep the morpholino from being detected by the cell's nucleases and being degraded. The bases of a morpholino oligo are the same as for a DNA oligo. Morpholinos can be designed to act on a particular mRNA and cause a "knockdown" by either blocking the ribosome from initiating translation or the mRNA splicing machinery from binding to the splice site. Using the MOs against SBF/Staf will allow for the characterization of the role of SBF/Staf in zebrafish embryonic development.

RESULTS

Morpholino injection into zebrafish embryos to knock down SBF/Staf protein levels

For the MO injection two MOs were synthesized, one complementary to the 5'UTR of SBF/Staf mRNA (5'UTR MO) and another that is complementary to a region

including the 5'UTR and includes the SBF/Staf translation start site (1153 MO) (See Figure 9). First I determined which MO would give the greatest proportion of embryos displaying a phenotype, and then I varied the concentration of each MO that was injected to determine whether the response was dose-dependent. Different stock concentrations of each MO (1, 2, and 4 µg/µl) were injected into embryos resulting in an estimated final concentration in the embryo of 2.5, 5, and 10 µM respectively. The MOs were injected as a 1:1 mixture with the stock final concentrations varying between 1, 2, and 4 µg/µl. When the 5'UTR and 1153 MOs were injected individually, the percentages of embryos that displayed a phenotype were as follows: for the 5'UTR at 48 hours post fertilization (hpf) 8, 5.5, and 11%; at 72 hrs 11, 8.5, and 11%; for the 1153 MO at 48 hpf 7.7, 16.7, and 17.7%; at 72 hpf 11.3, 24, and 31%. When both MOs were injected together at 1, 2, or 4 µg/µl the percentages of embryos displaying a phenotype at 48 hpf were 3, 19, and 95% and at 72 hpf the percentage of embryos were 6, 23, and 100% (See Figure 10). All MO injections were repeated at least three times. From these data it was determined that the most efficient knockdown was achieved with a combination of both MOs at a final concentration of 4 µg/µl.

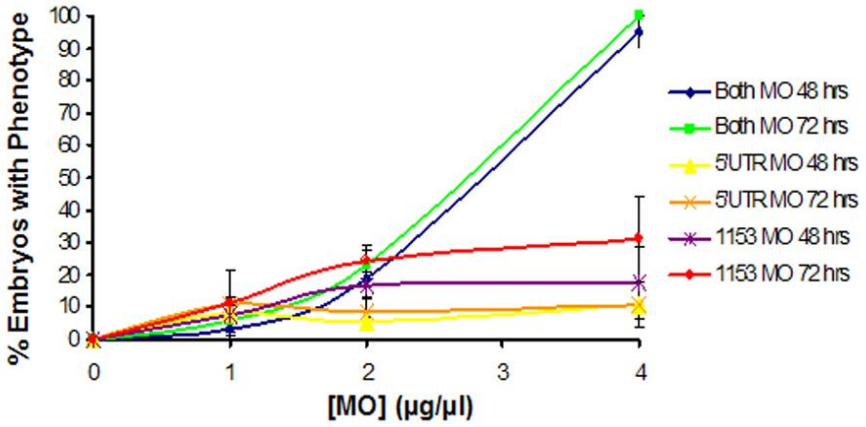

Figure 10. Morpholino Knockdown of SBF/Staf. Embryos were injected with different amounts of two different morpholinos, either separately or together. Embryos were scored at 48 and 72 hpf. Data represent three independent injections. The error bars show one standard deviation above and below the mean value.

When the combination of MOs was injected into the embryos, a varying degree of phenotypes was seen in the morphants (MO injected embryos). Figure 11 illustrates the general classes of the morphant phenotypes seen 48 hours after injection. The phenotypes range in severity of the morphant phenotypes is a usual result from MO injection. Class 1 (kinked tail) and class 2 (curved body) morphants lack the ventral tail fin. The kink in the tail (C1) and the general curved body (C2) maybe due to a change in the cell fates between the dorsal and ventral cells in the tail or a change in the growth rate of those cells (Figure 11B, C). In the class 3 morphants, the somite chevron shape is absent. There are opaque regions by the eye and the ear. This is indicative of cell death which can either be specific to a loss of the protein needed in the development of those structures or it may be a non-specific cell death caused by the MO injection triggering a p53 response. There is not a yolk extension and there is edema around the heart with the

heart extended away from the body of the fish. This heart phenotype may be due to stress on the circulatory system (Figure 11D). In the phenotypes seen in the class 4 and class 5 morphants the shape of the somites and the yolk extension is lost as well as the tail bud length. These morphants also have the extended heart and edema around the heart characteristic of circulatory system stress (Figure 11E, F). In the class 6 morphant the phenotype is that the embryo has no eyes, ears, or cell differentiation except for the heart structures (Figure 11G). The same range of phenotypes, in smaller proportions, was found when 5'UTR or 1153 MOs were injected individually.

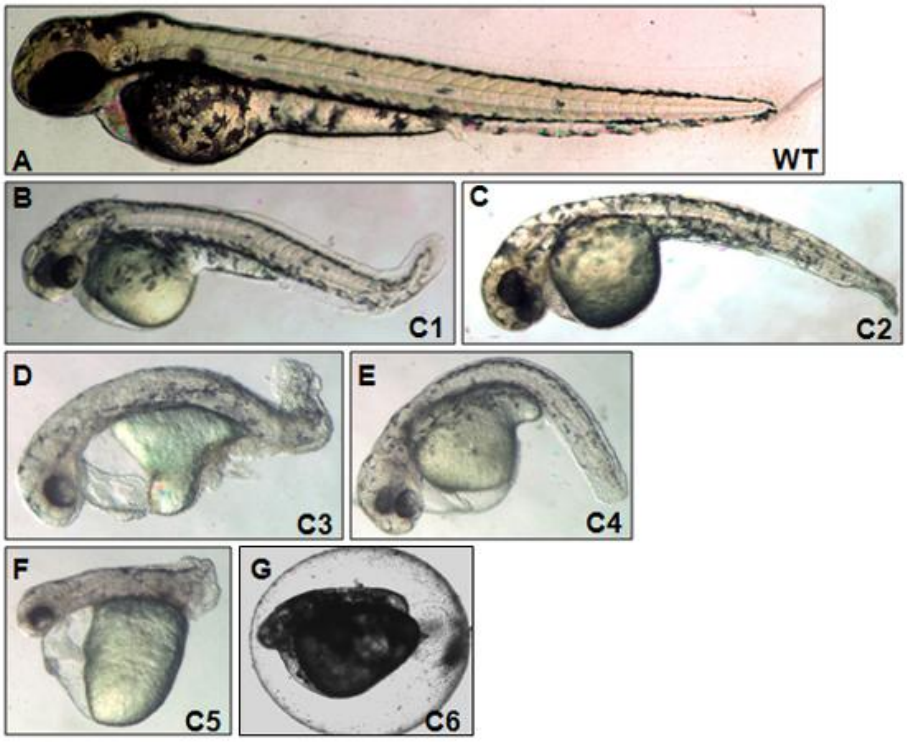

Figure 11. Phenotypes of Embryos Injected with MOs. A: Wild type 48 hpf embryo; B-G: Morphant phenotypic classes of 48 hpf embryos

Rescue of morphant phenotypes with different mRNA constructs of SBF/Staf

To confirm that the phenotypes seen in the injected embryos were due to the specific knock down of SBF/Staf and not a general toxic effect of the morpholino, a myc-tagged version of SBF/Staf was constructed so the protein expressed from the injected mRNA could be differentiated from the endogenous form. Transcription of this gene by T7 RNA polymerase produced a mRNA that did not contain the complete binding sites for the MOs, and thus injection of this transcript is expected to rescue the mutants induced by MO addition. First I determined the amount of mRNA to be injected that would not induce an overexpression phenotype. Different amounts of the rescue SBF/Staf mRNA (0-300 ng/µl) were injected into wildtype embryos and then scored after 48 hrs for morphological abnormalities. Figure 12A it is shows that between 15 and 30 ng/ul (0.48 nM_{Final} and 0.95 nM_{Final} in the embryo) of the rescue SBF/Staf mRNA alone does not induce an abnormal phenotype. It should be noted that with the higher concentrations the SBF/Staf rescue mRNA does produce phenotypes similar to MOs knockdown. This result was surprising but may be due to the fact that SBF/Staf has a global role in gene expression as seen in Carbon 2006 (60) and is further addressed in the Discussion section.

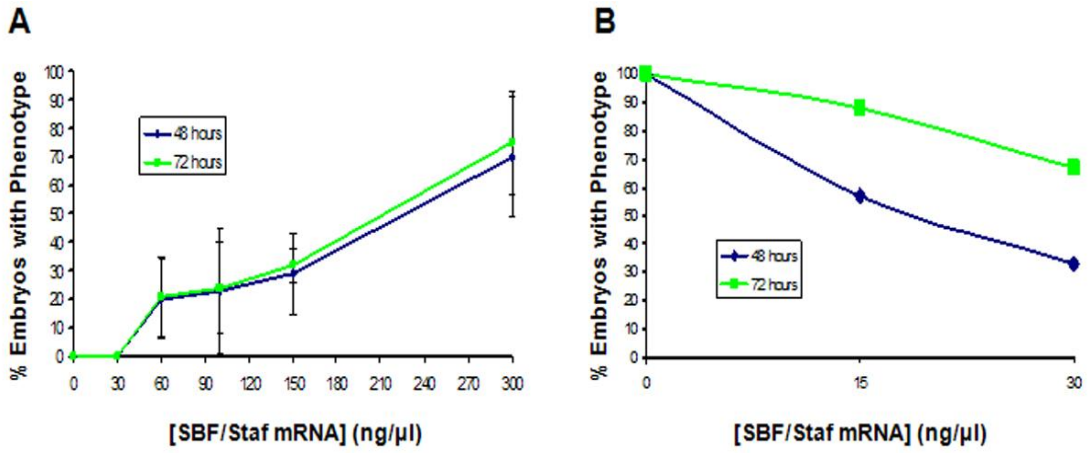

Figure 12. Gain of Function and Rescue of Phenotypes. A: Embryos were injected with increasing amounts of SBF/Staf mRNA and were scored at 48 and 72 hpf. B: Embryos were scored at 48 and 72 hpf after being injected with MOs and either 0,15, or 30 ng/µl rescue SBF/Staf mRNA. In panel A, the error bars show one standard deviation above and below the mean value.

The rescue SBF/Staf mRNA (30 ng/ul) was injected simultaneously with the MOs (4 µg/µl) and the morphants were scored 48 and 72 hrs after the injection. With the injection of the rescue SBF/Staf mRNA (30 ng/µl) and MOs the morphant phenotypes were reduced from 100% to only 33% of injected embryos illustrating that the knockdown by the MOs is specific for the knockdown of SBF/Staf (Figure 12B).

We next wanted to determine which of the two activation domains of SBF/Staf was responsible for the morphant phenotypes seen with the MO injection. We also wanted to determine if we could delineate the morphant phenotypes to those that were in response to losing the mRNA gene activation and those that could be due to a general loss in mRNA spicing (loss of the snRNA activation domain). Three deletion mutants of SBF/Staf genes were constructed. Rescue mRNAs were synthesized that lacked the mRNA activation domain (Δ2-149), the snRNA activation domain (Δ151-225), or both

(Δ2-225). The Δ2-225 rescue mRNA was constructed to make sure that the phenotypes seen were not due to the simple loss of the DNA binding domain since this construct only contains the DNA binding domain and the C-terminal region of uncharacterized function. Each of these rescue mRNAs were injected with the MOs and the phenotypes were scored 48 hrs after injection. When the rescue Δ2-225 mRNA was injected it was unable to rescue the morphant phenotype, but both the Δ2-149 and Δ151-225 mRNAs were able to rescue approximately 50% of the embryos to a wildtype phenotype (Figure 13). These results indicate that both the mRNA and the snRNA activation domain are responsible for the morphant phenotypes seen in the injected embryos. Luciferase transfection assays performed by Dr. Gary Kunkel demonstrated that when the deletion SBF/Staf mRNA constructs were expressed in ZF4 cells the Δ151-225 construct was able to activate the expression of the luciferase mRNA reporter gene, whereas the Δ2-149 and Δ2-225 constructs were not (results not shown). Therefore the deletion construct proteins function as expected in the zebrafish embryonic cell line.

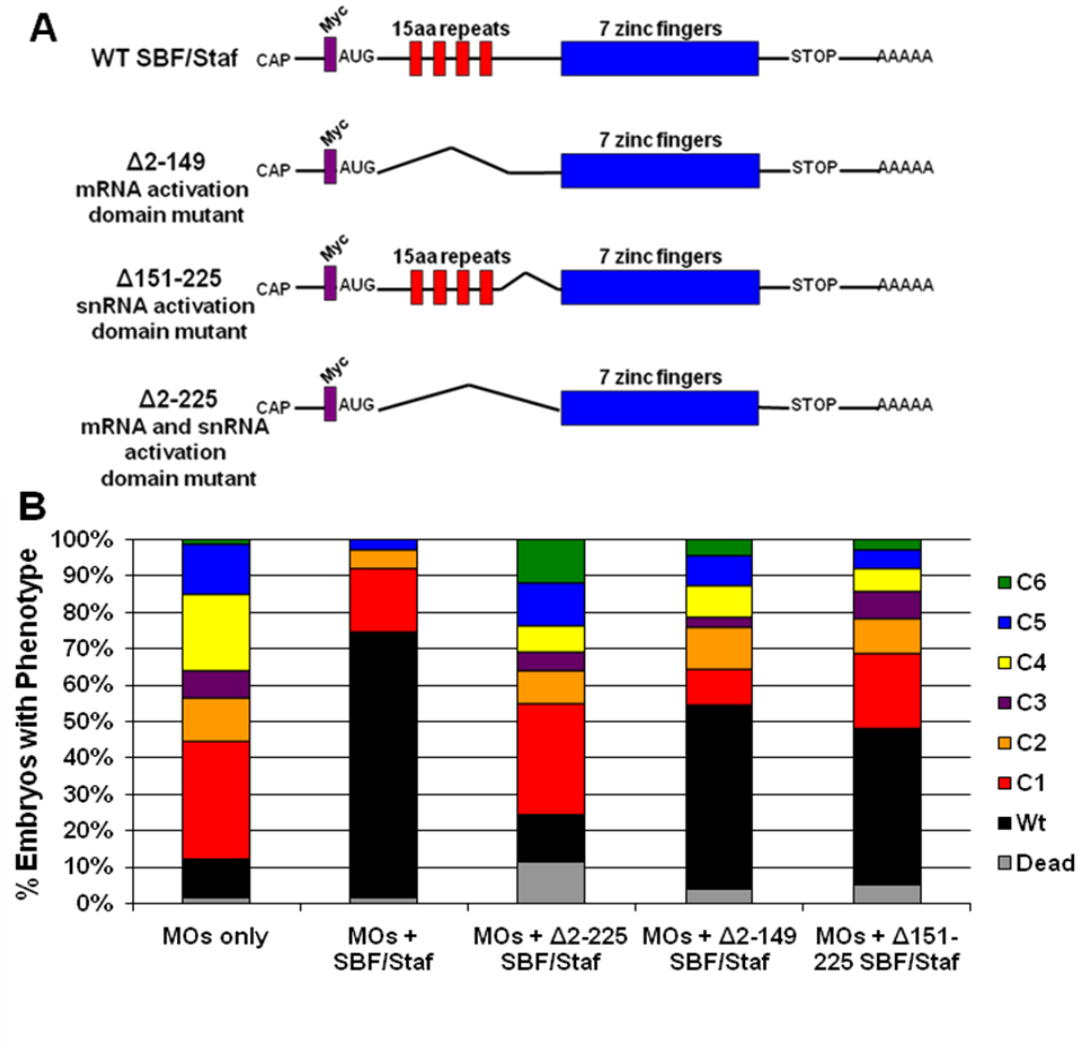

Figure 13. Rescue of Morphant Phenotypes with Different mRNA Constructs of SBF/Staf. A: Diagrams of deletion rescue SBF/Staf mRNAs. B: Rescue data for injection of the different deletion rescue SBF/Staf mRNAs by morphant phenotypic class. Each set of data represents the average of three independent injections.

Injection of embryos with morpholinos affects expression of a U6 snRNA maxigene and various mRNA levels

Next we wanted to determine the effect of the knockdown of SBF/Staf on U6 snRNA expression in the developing embryos. U6 snRNA is expected to have a long

half-life, so I examined transient expression from an injected plasmid. In order to differentiate from the endogenous U6 snRNA gene, embryos were injected with a U6 maxigene construct (zU6Maxi) with and without MOs. Total RNA was collected at the 16 somite stage and processed for RT-PCR. As a negative control, when embryos were injected with an unrelated MO that produces no phenotype the U6 maxi RNA level was unaffected (results not shown). The expression of the U6 maxi snRNA gene was then quantified by densitometry of RT-PCR using oligos for the U6 maxi snRNA and normalized to 5.8S rRNA levels. When SBF/Staf expression is knocked down by the MOs the expression of the U6 snRNA maxigene was increased approximately ten-fold (Figure 14).

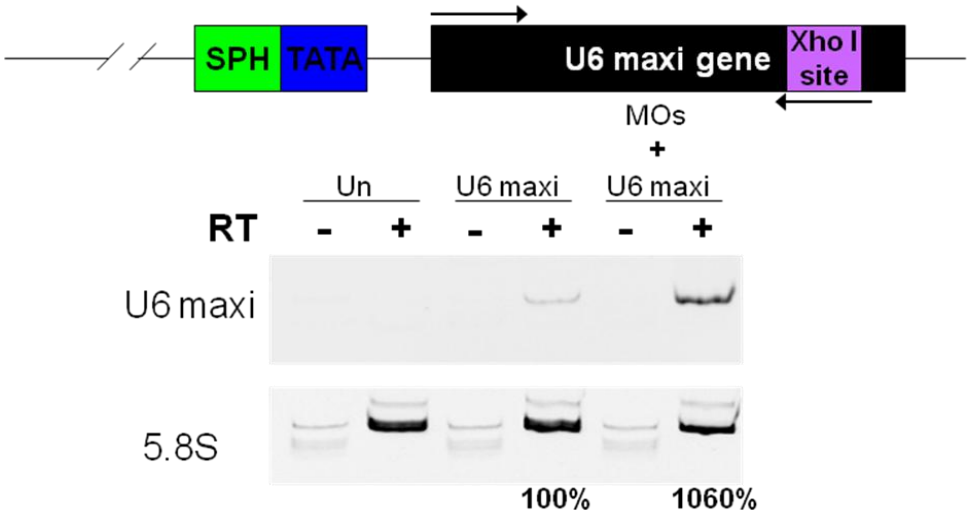

Figure 14. RT-PCR of Total RNA From Embryos Injected With MOs and a U6 Maxigene Promoter Construct. (Top) Maxigene construct with arrows indicating the location of the primers used in PCR. (Bottom) PAGE gels of the RT-PCR reactions.

This result was surprising because a simple expectation was that reduction of SBF/Staf, an activator for human U6 gene transcription, would result in decreased zebrafish U6

maxi expression. Therefore, we investigated the effect of SBF/Staf knockdown on the expression of several mRNAs expressed during zebrafish development.

qRT-PCR to determine the expression of different mRNAs in morpholino-injected embryos

We wanted to determine the effect of SBF/Staf knockdown on mRNA levels from genes that either contain an SPH-element in the promoter or that lack identifiable SPH sites. Total RNA was collected from both uninjected and MOs-injected zebrafish embryos at the 16 somite stage, treated with DNase I, and processed to make cDNA. Oligos were used in qRT-PCR to detect the expression levels of 5.8S rRNA, TFIIB (Transcription Factor IIB), MDH (malate dehydrogenase), PLK (polo-like kinase), and SNAPc3 (a subunit of the small nuclear RNA activating complex). 5.8S rRNA levels were used as an RNA loading control in the qRT-PCR and TFIIB was used as a normalization control because its promoter does not contain an SPH-element. MDH, PLK, and SNAPc3 genes all have SPH-elements in their promoters, and expression may be affected by the decrease in SBF/Staf protein with the injection of the MOs. Similar to U6 maxi RNA levels, steady-state levels of these mRNAs were increased after injection of the MOs (Figure 15). We do not understand the mechanism of this overall increase in both snRNA (U6) and mRNA levels upon MO-mediated knockdown of SBF/Staf, but we speculate about various possibilities in the discussion section. Note that 5.8S rRNA levels are unaffected.

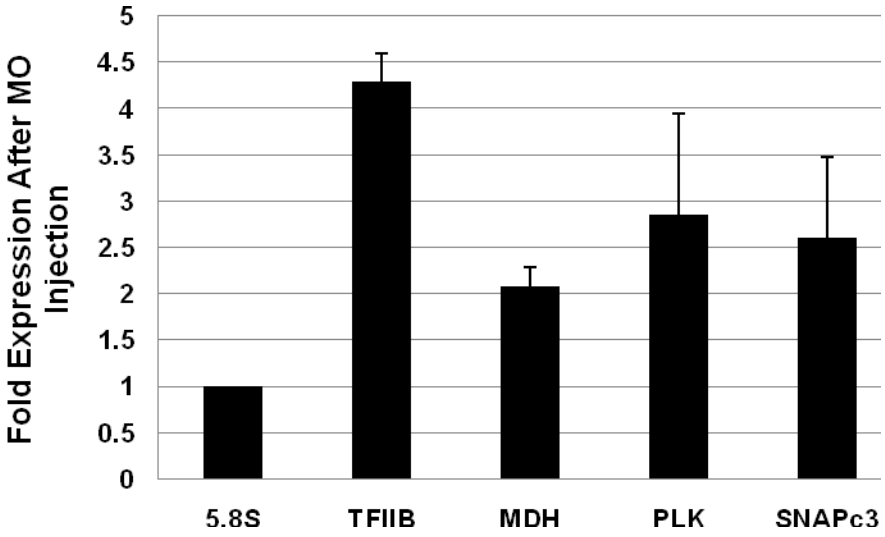

Figure 15. Quantitative RT-PCR of Total RNA from Embryos Injected with MOs Normalized to 5.8S rRNA. All data was normalized to 5.8S rRNA levels using the following equation: $\Delta C_{T,target} = (C_{T,target} - C_{T,\ 5.8S})_{MO} - (C_{T,target} - C_{T,5.8S})_{WT}$. The error bars show one standard deviation above the mean value.

A more careful analysis of the qRT-PCR data demonstrated a relatively modest decrease of mRNAs for genes containing SPH sites when compared to expression from the TFIIB gene which lacks an SPH site. To determine the expression levels of MDH, PLK, and SNAPc3 relative to TFIIB, the $2^{-\Delta\Delta CT}$ method to compare the C_T values was used as described in Livak and Schmittgen (53). The expression levels of MDH, PLK, and SNAPc3 were decreased by approximately one-half after MO-injection, when compared to TFIIB (Figure 16).

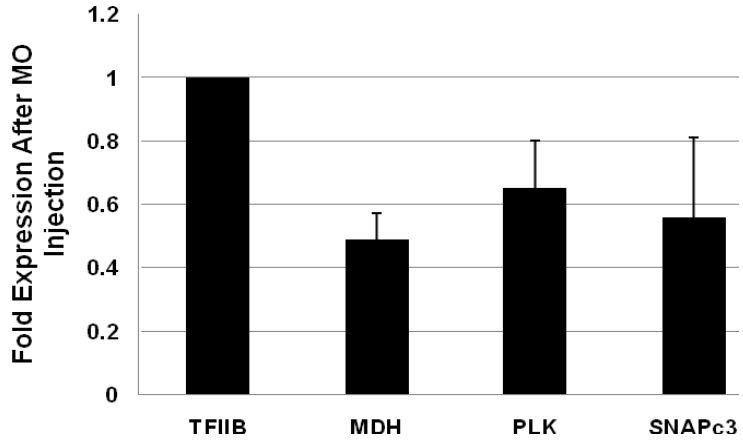

Figure 16. Quantitative RT-PCR of Total RNA from Embryos Injected with MOs. All data was normalized to TFIIB using the following equation: $\Delta C_{T,target} = (C_{T,target} - C_{T,\ 5.8S})_{MO} - (C_{T,target} - C_{T,5.8S})_{WT}$; $\Delta\Delta C_{T,target} = (\Delta C_{T,target} - \Delta C_{T,TFIIB})$. Error bars show one standard deviation above the mean value.

DISCUSSION

From the MO injections we see that SBF/Staf has a global role in the development of zebrafish embryos. This is supported by the general, overall phenotypes that were seen upon the injection of the MOs together and that the same morphant phenotypes were seen when the MOs were injected separately. When MOs are co-injected, there is a synergistic effect in the percentage of embryos that display a morphant phenotype. The global role in development is also supported by a study by the Carbon lab which used a combined *in silico* and biochemical approach to identify 1175 conserved SPH elements, the binding site for SBF/Staf, distributed in 938 mRNA gene promoters of four mammalian genomes (60). The SPH element shows a significant positional preference and occurs mostly within 200 bp upstream of the transcription start site. Chromatin immunoprecipitation assays with 295 of the promoters established that

90% contain *bona fide* SPH elements. The co-injection of the rescue SBF/Staf mRNA with the MOs supports the conclusion that the morphant phenotypes result from a MO specific knockdown of the SBF/Staf protein because a majority of the morphant phenotypes are rescued. The co-injection of SBF/Staf rescue mRNAs encoding deletions illustrated that both the mRNA activation domain and the snRNA activation domain are important to the zebrafish embryo development, because when each activation domain was deleted independently only half of the embryos were rescued. It was only when both activation domains were deleted that the synthetic SBF/Staf mRNA could not rescue the morphant phenotype. The data also shows that when the SBF/Staf rescue mRNA was injected at higher concentrations, the phenotypes seen in the morphants were the same as those seen with the knockdown of SBF/Staf. Since SBF/Staf had been shown to have a role in the expression of many mRNAs by the Carbon lab (60) when SBF/Staf is overexpressed in the embryo there may also be an overexpression of many other mRNAs leading to a gain-of-function phenotype that is similar to the loss-of-function phenotype. From the MO injections we also see an increase in the steady-state levels of mRNAs so the morphant phenotypes seen may be due to an increase of mRNA levels.

When the U6 maxigene plasmid was co-injected into embryos with the MOs, the expression level of the U6 maxigene was higher than when the U6 maxigene was injected alone (Figure 14). This result was unexpected because SBF/Staf is known to be an activator of snRNA gene promoters (88). However, this increase may be a general effect, because several mRNA levels were increased upon SBF/Staf MOs injection

(Figure 15). The increase in the U6 maxigene plasmid expression may be due to the MO injection stabilizing the plasmid DNA but this is unlikely because there was also an increase seen in the endogenous mRNAs. One scenario may explain the increase in RNA levels upon SBF/Staf knockdown. Possibly, overall RNA stabilization is a result of decreased expression of a RNA turnover protein that has an SPH element in the promoter of its gene. Therefore, there is a decrease in the expression of a gene encoding for a RNA destabilization protein due to the knockdown of SBF/Staf.

CHAPTER IV

THE SIGNIFICANCE OF THE PROXIMALLY-LOCATED SPH ELEMENT FOR TRANSCRIPTION OF ZEBRAFISH U6 SNRNA GENES

INTRODUCTION

The snRNA SPH element was first recognized in chicken U1, U2, and U4 distal regions, but it is now clear that the SPH element is present in many vertebrate snRNA-type promoters (55, 60, 78, 88, 109). The SPH element was first discovered to be an enhancer of the chicken U1 snRNA expression by the Stumph lab and while there was a basal level of transcription without the SPH element, transcription was greatly enhanced by the presence of the SPH element (78). The Kunkel lab determined that full activation of the human U6 promoter *in vivo* depends on there being both the Oct-1 and SPH element in the promoter and that the binding of SBF/Staf to the SPH element is dependent on Mg^{+2}(22, 48). Experiments using Sarkosyl to limit transcription to a single-round demonstrated that promoters containing either an Oct-1 or an SPH element support an increased number of pre-initiation complexes *in vitro* (49). In humans it has been found that enhancers of snRNA genes and mRNA genes are not completely interchangeable, suggesting the mechanism of snRNA gene activation may be distinct (20).

The zebrafish U6 snRNA gene promoters have an unusual organization in comparison to the human U6 snRNA promoter (Figure 17). In the zebrafish U6 snRNA gene, the SPH element is located next to the TATA element in the proximal region of the

promoter. Also, there is no distinguishable PSE (proximal sequence element) site and current studies in the Kunkel lab are being performed to determine if there is a bona-fide PSE site. All other zebrafish snRNA genes have the standard promoter structure with an SPH element located ~230 bp upstream from the start of the snRNA gene. With this location one could expect a different role for the SPH element and SBF/Staf in the expression of the zebrafish U6 snRNA gene. Due to the unique location of the SPH element being next to the TATA element, there could be a potential protein-protein interaction between SBF/Staf and TBP (or another component of the RNA polymerase III transcription apparatus). We set out to determine if this proximally located SPH element was a functional transcriptional element.

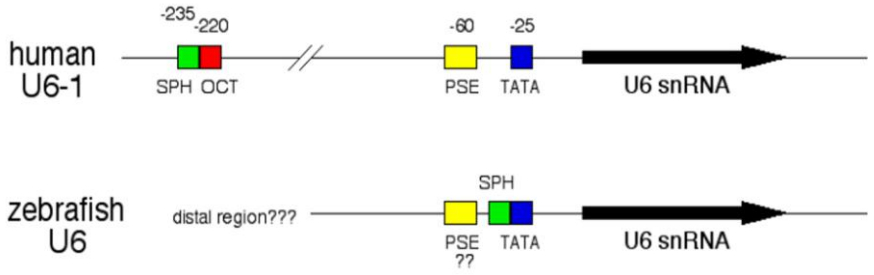

Figure 17. SPH Element Is Located in a Novel Position in Zebrafish U6 snRNA Promoters.

RESULTS

Proximally-located SPH element plays a functional role in U6 snRNA maxigene expression

In the zebrafish U6 snRNA gene the SPH element is located in an unusual position (See Figure 17). Previous work in the lab by G. Kunkel used DNase 1

footprinting with an end-labeled zebrafish U6 snRNA promoter and the DNA binding domain of SBF/Staf to determine that the SPH element is a binding site for SBF/Staf (Figure 18). This binding was shown to be specific by using an electrophoretic mobility shift assay in which unlabeled SPH DNA sequence was able to compete with the binding of the SBF/Staf protein to labeled zebrafish U6 snRNA promoter sequence, and a non-specific DNA sequence was not able to compete (Figure 18). It should be noted that the human SBF/Staf was used in these experiments and that the human and zebrafish SBF/Staf proteins are 71% identical overall, about 50% identical in the snRNA activation domain, and almost 100% identical in the mRNA activation domain and DNA binding domain.

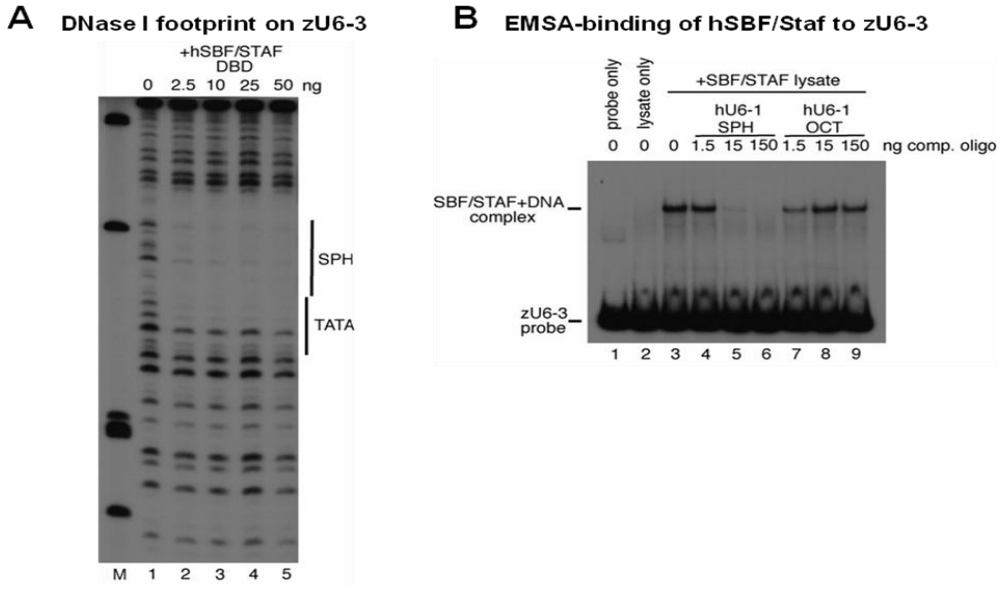

Figure 18. DNase I Footprint on zU6 Promoter and EMSA-Binding of hSBF/Staf to zU6 Gene. (A) DNase I footprinting assay with increasing amounts of the DNA binding domain of hSBF/Staf against a end-labeled zU6 promoter. (B) EMSA-binding assay of hSBF-Staf to zU6 promoter DNA. Increasing amounts of hSPH sequence was used to compete the interaction and hU6 Oct-1 DNA was used as a non-specific competitor.

Next, zebrafish U6 snRNA maxigene constructs were made in which an XhoI site was inserted in the 3' end of the gene allowing for the distinction of the maxigene transcripts from the endogenous U6 snRNA. The promoter that included 300 bp of 5' flanking sequence including the SPH element and TATA box was cloned upstream of the U6 snRNA maxigene in the pGEM parental plasmid. Plasmids were also constructed with the SPH and TATA box mutated independently by changing several bp within each element. These plasmids were transiently transfected in the zebrafish ZF4 cell line (ATCC #CRL-2058) using Lipofectamine 2000 (Invitrogen). Total RNA was isolated from transfected cells, treated with DNase, and specific maxigene transcription was detected by RT-PCR analysis. The expression of the U6 snRNA maxi gene was quantified from each of the different promoter constructs. When the TATA-element was mutated the expression of the U6 snRNA maxigene was decreased to 22.7% of the expression from the wildtype promoter. When the SPH element was mutated, expression was decreased to 20.6% of wildtype (average of three independent transfection experiments). From the data in Figure 19 it can be determined that the proximally-located SPH element is important for the transcription of the zebrafish U6 snRNA gene despite its unusual location.

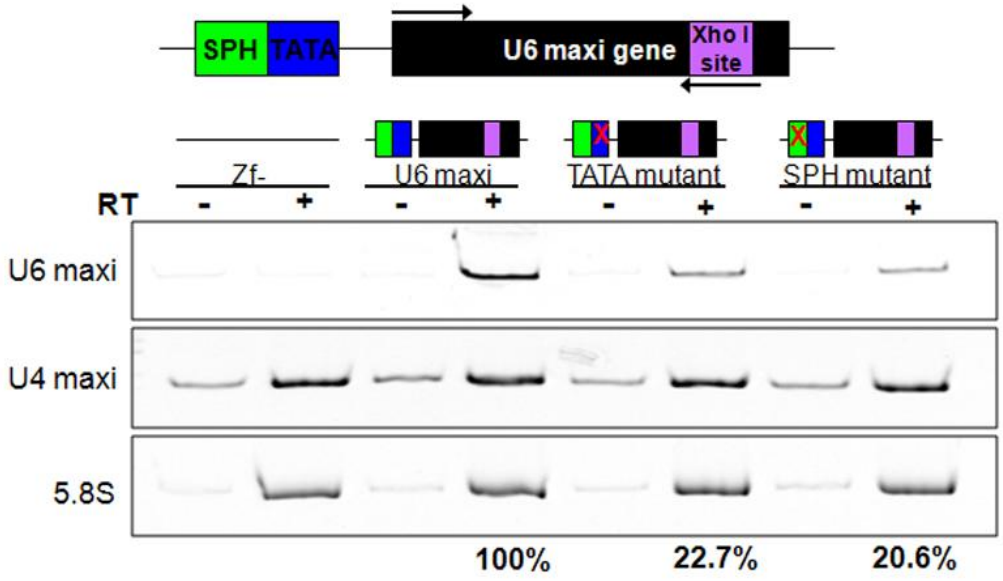

Figure 19. RT-PCR of Total RNA Recovered from Transiently Transfected ZF4 Cells. (Top) Maxigene construct with arrows indicating the location of the primers used in PCR. (Bottom) PAGE gels of the RT-PCR reactions. Diagram above each set of lanes indicates the location of the promoter mutation with a red X.

Injection of embryos with morpholinos affects expression of a U6 snRNA maxigene and U6 snRNA maxigene mutants

Since in previous experiments the injection of MOs into embryos increased the steady-state levels of U6 maxigene transcript, I decided to inject embryos with the different SPH and TATA mutant plasmids under these conditions. RNA was collected at the 16 somite stage and processed for RT-PCR. The expression of the U6 maxi snRNA gene was then quantified using oligos for the U6 maxi snRNA and normalized to 5.8S rRNA levels.

When the TATAMUT construct was injected with MOs the expression of the U6 maxigene was decreased to 1.6% of the expression of the U6 maxigene construct with

the wildtype promoter. These data support previous studies that both the TATA element and SBF/Staf are needed to obtain the highest levels of human U6 snRNA gene transcription. These data where further supported with the SPHMUT construct being injected with MOs and the expression of the U6 maxigene decreasing only to 26.8% of the expression of the U6 maxigene with a wildtype promoter (Figure 20).

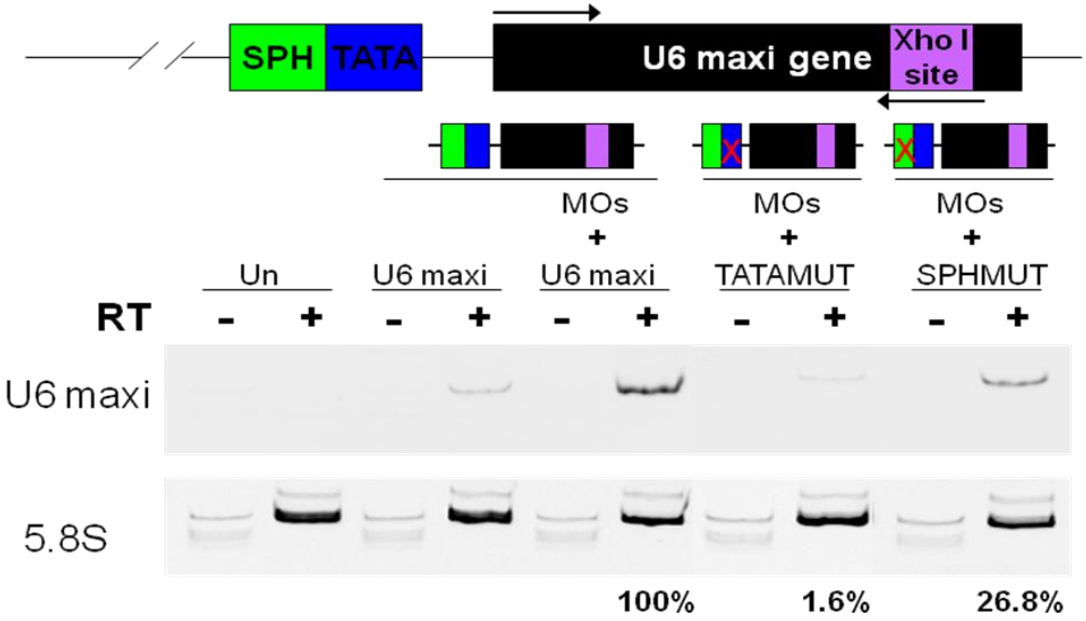

Figure 20. RT-PCR of Total RNA from Embryos Injected With MOs and a U6 Maxigene Mutant Promoter Constructs. (Top) Maxigene construct with arrows indicating the location of the primers used in PCR. (Bottom) PAGE gels of the RT-PCR reactions. Note that the first six lanes are that same as in Figure 13.

DISCUSSION

For human RNA polymerase III snRNA gene promoters, such as U6 snRNA, the SPH-element is located approximately 230 bp upstream from the transcription start site. In the case of the zebrafish U6 snRNA gene the SPH-element is located in the proximal

region of the promoter, next to the TATA box. We set out to determine if this SPH-element played a role in the expression of the zebrafish U6 snRNA gene. First we confirmed that SBF/Staf could bind at the SPH-element using DNase I footprinting and EMSA. When the U6 snRNA maxigene expression in transfected ZF4 cells was quantified using RT-PCR it was shown that the proximally-located SPH-element is not only bound by SBF/Staf, but also SBF/Staf has a role in the expression of the U6 snRNA gene. Unlike mammalian U6 snRNA promoters the expression of the U6 maxigene is not completely abolished with the loss of the TATA element. Possibly the SPH-element alone can function as a basal promoter. Alternatively, other unidentified proximal elements may play a role(s). For example the zU6 snRNA promoter may contain a PSE, typically found in snRNA genes. It is to the advantage of the organism to have multiple basal promoter elements for an essential gene in mRNA maturation, because without the splicing machinery no functional proteins could be produced by the cell. This unusual location of the SPH element is also seen in mRNA gene promoters, where it is sometimes present at the approximately -30 position in place of the TATA-element (60). It should be noted that for other zebrafish snRNA promoters the SPH element is located in the normal location; only the U6 snRNA gene is different. In the zebrafish embryo the TATAMUT and SPHMUT plasmid constructs illustrated that the proximally-located SPH element does have a role in the transcription of the U6 maxigene by the decrease in the level of expression of the U6 maxigene when both SBF/Staf is knocked down and the SPH-element is mutated.

There are a couple possibilities for why the decrease of the expression of the U6 maxigene seen with the TATAMUT and SPHMUT constructs was different in the transfected ZF4 embryonic cell line versus the whole embryo. There could be a different chromatin structure of the plasmids in the ZF4 cells versus the embryo. Alternatively, the higher level of expression seen in the ZF4 cells transfected with the TATAMUT versus the embryo injected with the TATAMUT could be due to non-specific transcription in the ZF4 cells.

CHAPTER V

SUMMARY

The level of eukaryotic gene expression is in most cases controlled by the gene's basal promoter as well as enhancer elements that direct binding of activator proteins. The U6 snRNA promoter provides a simple model system to study the mechanism of eukaryotic transcription. SBF/Staf is one important activator protein that binds the SPH element in U6 snRNA enhancers. However, this protein has the ability to stimulate transcription of both snRNA and protein-coding genes through distinct activation domains. Using zebrafish as a model I first looked at the role that the two activation domains of SBF/Staf play in zebrafish development. I also investigated the role of the unusual proximal location of the SPH element in the transcription of zebrafish U6 snRNA genes.

Work described in Chapter III illustrates the phenotypic effects of a knockdown of the SBF/Staf protein on a developing zebrafish embryo. Two translation blocking MOs that were designed against SBF/Staf were injected either independently or together into zebrafish embryos. When the morphant phenotypes were scored, the phenotypes seen varied from a simple tail tip malformation to an almost complete loss of cell differentiation. To confirm that the morphant phenotypes were the result of the MO knockdown of SBF/Staf, a capped rescue mRNA that did not contain the binding sites for the MOs was synthesized. When the rescue mRNA was injected with the MOs into the zebrafish embryos most of the embryos displayed a wildtype phenotype. Using this rescue strategy I then attempted to delineate the morphant phenotypes that were due to a

lack of the SBF/Staf mRNA activation domain and those due to a lack of the snRNA activation domain. Rescue mRNAs were synthesized lacking either or both the activation domains and were co-injected with the MOs. The rescue mRNAs that had either the mRNA activation domain only (Δ2-149) or the snRNA activation domain only (Δ151-225) were both able to rescue the morphant phenotype to a wildtype phenotype in about 50% of the embryos. Remaining mutant embryos were not distinguishable between the two different rescue mutants. The rescue mRNA that lacked both activation domains (Δ2-225) was not able to rescue the morphant phenotypes. There appeared to be an increase in the severity of the phenotypes that remained after the injection of this mRNA and the MOs. The phenotypes described in Chapter III of morpholino injected zebrafish embryos, such as a change in the cell fate of dorsal and ventral cells or circular system stress, may be due to a loss in the Wnt and FGF developmental signaling pathways.

Next I wanted to study the effect on U6 snRNA transcription and several mRNA gene targets by the loss of the SBF/Staf protein. Using the U6 maxigene constructs to inject with the MOs, an increase in the steady-state levels of the U6 snRNA maxigene expression was found. This increase was also seen when I looked at the mRNA levels for several genes. Upon a more careful analysis, I found that the increased levels of expression of mRNAs for genes containing SPH sites were less than for a control gene lacking an SPH site in its promoter. A possible reason for the apparent global increase in the steady-state levels of both the snRNA and the mRNAs could be due to the loss of a RNA destabilization or nuclease protein that is responsible for RNA turnover and has

an SPH element in its promoter. Further studies could use a MO designed to knock down a protein with these functions. One could determine whether U6 snRNA maxigene and mRNA expression is affected. In addition, injection of a synthetic mRNA encoding the putative RNA destabilization protein would be predicted to rescue the elevated RNA levels caused by SBF/Staf MOs. Myslinski *et. al.* detected a number of mammalian SBF/Staf target gene promoters that direct transcription of genes important in RNA metabolism(60).

In Chapter IV I chose to investigate the role of the proximally-located SPH element in the zebrafish U6 snRNA gene. When we first decided to use the zebrafish model system it was discovered that while all the rest of the snRNA gene promoters have architectures similar to the human snRNA promoters, the zebrafish U6 snRNA genes did not. The SPH element is located next to the TATA element and this raised the question of whether or not this element was functional. Previous DNase I footprinting and EMSA binding assays using the zebrafish U6 snRNA promoter DNA sequence showed that SBF/Staf binds to this proximally-located SPH element in a specific manner. To study whether the proximally-located SPH element was functional, U6 maxigene constructs were made with a mutant SPH element (SPHMUT) or a TATA element (TATAMUT). Using transfected zebrafish embryonic cells I found that the SPH element was functional because there was a decrease in the expression of the U6 maxigene when the SPH element was mutated. The TATAMUT and SPHMUT constructs were also injected into zebrafish embryos with the MOs to investigate the functionality of the proximally-located SPH element *in vivo*. As expected, when the

TATA element was mutated the expression of the U6 maxigene dropped dramatically. When the SPH element was mutated the decrease in the expression of the U6 maxigene was similar to that seen in the transfected cells. These experiments illustrated that the SPH element plays an important role at its proximal location. Further studies need to be done to determine if this unusual location next to the TATA element facilitates protein-protein interaction between SBF/Staf and components of the transcription machinery, for example, TATA-binding protein (TBP) or TBP-associated proteins.

The results presented in this dissertation have increased the knowledge of an enhancer element and activator protein that has recently been shown to have a more global role in gene transcription than previously thought. Both activation domains of SBF/Staf have been shown to be important in zebrafish development, and the proximally-located SPH element has been shown to be functional regardless of its unusual location for a snRNA gene. Future studies will illuminate specific developmental targets of SBF/Staf activation, as well as biochemical mechanisms by which the protein works at snRNA and mRNA gene promoters.

REFERENCES

1. **Agalioti, T., G. Chen, and D. Thanos.** 2002. Deciphering the transcriptional histone acetylation code for a human gene. Cell **111:**381-92.

2. **Akoulitchev, S., S. Chuikov, and D. Reinberg.** 2000. TFIIH is negatively regulated by cdk8-containing mediator complexes. Nature **407:**102-6.

3. **Aoyagi, N., and D. A. Wassarman.** 2000. Genes encoding Drosophila melanogaster RNA polymerase II general transcription factors: diversity in TFIIA and TFIID components contributes to gene-specific transcriptional regulation. J Cell Biol **150:**F45-50.

4. **Arnosti, D. N., and M. M. Kulkarni.** 2005. Transcriptional enhancers: intelligent enhanceosomes or flexible billboards? J Cell Biochem **94:**890-8.

5. **Aza-Blanc, P., F. A. Ramirez-Weber, M. P. Laget, C. Schwartz, and T. B. Kornberg.** 1997. Proteolysis that is inhibited by hedgehog targets Cubitus interruptus protein to the nucleus and converts it to a repressor. Cell **89:**1043-53.

6. **Bale, A. E.** 2002. Hedgehog signaling and human disease. Annu Rev Genomics Hum Genet **3:**47-65.

7. **Banerji, J., S. Rusconi, and W. Schaffner.** 1981. Expression of a beta-globin gene is enhanced by remote SV40 DNA sequences. Cell **27:**299-308.

8. **Barski, O. A., V. Z. Papusha, G. R. Kunkel, and K. H. Gabbay.** 2004. Regulation of aldehyde reductase expression by STAF and CHOP. Genomics **83:**119-29.

9. **Behrens, J., J. P. von Kries, M. Kuhl, L. Bruhn, D. Wedlich, R. Grosschedl, and W. Birchmeier.** 1996. Functional interaction of beta-catenin with the transcription factor LEF-1. Nature **382:**638-42.

10. **Biggar, S. R., and G. R. Crabtree.** 2001. Cell signaling can direct either binary or graded transcriptional responses. Embo J **20:**3167-76.

11. **Bitgood, M. J., and A. P. McMahon.** 1995. Hedgehog and Bmp genes are coexpressed at many diverse sites of cell-cell interaction in the mouse embryo. Dev Biol **172:**126-38.

12. **Bitgood, M. J., L. Shen, and A. P. McMahon.** 1996. Sertoli cell signaling by desert hedgehog regulates the male germline. Curr Biol **6:**298-304.

13. **Blackwood, E. M., and J. T. Kadonaga.** 1998. Going the distance: a current view of enhancer action. Science **281:**60-3.

14. **Bryant, G. O., and M. Ptashne.** 2003. Independent recruitment in vivo by Gal4 of two complexes required for transcription. Mol Cell **11:**1301-9.

15. **Burge, C. B., Tuschl, T., Sharp, P.A.** 1999. Splicing of precursors to mRNAs by the spliceosomes, p. 525-560. *In* R. F. Gesteland, Cech, T.R., Atkins, J.F. (eds.), The RNA World. Cold Spring Harbor Laboratory Press, Woodbury, NY.

16. **Burgess-Beusse, B., C. Farrell, M. Gaszner, M. Litt, V. Mutskov, F. Recillas-Targa, M. Simpson, A. West, and G. Felsenfeld.** 2002. The insulation of genes from external enhancers and silencing chromatin. Proc Natl Acad Sci USA **99 Suppl 4:**16433-7.

17. **Cadigan, K. M., and R. Nusse.** 1997. Wnt signaling: a common theme in animal development. Genes Dev **11:**3286-305.

18. **Calhoun, V. C., A. Stathopoulos, and M. Levine.** 2002. Promoter-proximal tethering elements regulate enhancer-promoter specificity in the Drosophila Antennapedia complex. Proc Natl Acad Sci **99:**9243-7.

19. **Cosma, M. P., T. Tanaka, and K. Nasmyth.** 1999. Ordered recruitment of transcription and chromatin remodeling factors to a cell cycle- and developmentally regulated promoter. Cell **97:**299-311.

20. **Dahlberg, J. E., and E. T. Schenborn.** 1988. The human U1 snRNA promoter and enhancer do not direct synthesis of messenger RNA. Nucleic Acids Res **16:**5827-40.

21. **Dahlberg, J. E. a. L., E.** 1988. Structure and Function of Major and Minor Small Nuclear Ribonucleoprotein Particles. Springer-Verlag, Berlin.

22. **Danzeiser, D. A., O. Urso, and G. R. Kunkel.** 1993. Functional characterization of elements in a human U6 small nuclear RNA gene distal control region. Mol Cell Biol **13:**4670-8.

23. **Davidson, A. J., P. Ernst, Y. Wang, M. P. Dekens, P. D. Kingsley, J. Palis, S. J. Korsmeyer, G. Q. Daley, and L. I. Zon.** 2003. cdx4 mutants fail to specify blood progenitors and can be rescued by multiple hox genes. Nature **425:**300-6.

24. **Di Leva, F., M. I. Ferrante, F. Demarchi, A. Caravelli, M. R. Matarazzo, M. Giacca, M. D'Urso, M. D'Esposito, and A. Franze.** 2004. Human synaptobrevin-like 1 gene basal transcription is regulated through the interaction

of selenocysteine tRNA gene transcription activating factor-zinc finger 143 factors with evolutionary conserved cis-elements. J Biol Chem **279:**7734-9.

25. **Domitrovich, A. M., and G. R. Kunkel.** 2003. Multiple, dispersed human U6 small nuclear RNA genes with varied transcriptional efficiencies. Nucleic Acids Res **31:**2344-52.

26. **Dong, P. D., C. A. Munson, W. Norton, C. Crosnier, X. Pan, Z. Gong, C. J. Neumann, and D. Y. Stainier.** 2007. Fgf10 regulates hepatopancreatic ductal system patterning and differentiation. Nat Genet **39:**397-402.

27. **Dorsky, R. I., D. W. Raible, and R. T. Moon.** 2000. Direct regulation of nacre, a zebrafish MITF homolog required for pigment cell formation, by the Wnt pathway. Genes Dev **14:**158-62.

28. **Droge, P., and B. Muller-Hill.** 2001. High local protein concentrations at promoters: strategies in prokaryotic and eukaryotic cells. Bioessays **23:**179-83.

29. **Feng, Q., and Y. Zhang.** 2003. The NuRD complex: linking histone modification to nucleosome remodeling. Curr Top Microbiol Immunol **274:**269-90.

30. **Fischer, S., B. W. Draper, and C. J. Neumann.** 2003. The zebrafish fgf24 mutant identifies an additional level of Fgf signaling involved in vertebrate forelimb initiation. Development **130:**3515-24.

31. **Fondell, J. D., H. Ge, and R. G. Roeder.** 1996. Ligand induction of a transcriptionally active thyroid hormone receptor coactivator complex. Proc Natl Acad Sci **93:**8329-33.

32. **Freiman, R. N., S. R. Albright, S. Zheng, W. C. Sha, R. E. Hammer, and R. Tjian.** 2001. Requirement of tissue-selective TBP-associated factor TAFII105 in ovarian development. Science **293:**2084-7.

33. **Gilbert, S. F.** 2006. Developmental Biology, 8th ed. Sinauer Associates, Inc., Sudderland, MA.

34. **Glass, C. K., and M. G. Rosenfeld.** 2000. The coregulator exchange in transcriptional functions of nuclear receptors. Genes Dev **14:**121-41.

35. **Goodman, R. H., and S. Smolik.** 2000. CBP/p300 in cell growth, transformation, and development. Genes Dev **14:**1553-77.

36. **Grossman, C. E., Y. Qian, K. Banki, and A. Perl.** 2004. ZNF143 mediates basal and tissue-specific expression of human transaldolase. J Biol Chem **279:**12190-205.

37. **Gu, W., S. Malik, M. Ito, C. X. Yuan, J. D. Fondell, X. Zhang, E. Martinez, J. Qin, and R. G. Roeder.** 1999. A novel human SRB/MED-containing cofactor complex, SMCC, involved in transcription regulation. Mol Cell **3:**97-108.

38. **Hernandez, N.** 2001. Small nuclear RNA genes: a model system to study fundamental mechanisms of transcription. J Biol Chem **276:**26733-6.

39. **Holmes, M. C., and R. Tjian.** 2000. Promoter-selective properties of the TBP-related factor TRF1. Science **288:**867-70.

40. **Ishiguchi, H., H. Izumi, T. Torigoe, Y. Yoshida, H. Kubota, S. Tsuji, and K. Kohno.** 2004. ZNF143 activates gene expression in response to DNA damage and binds to cisplatin-modified DNA. Int J Cancer **111:**900-9.

41. **Ito, M., and R. G. Roeder.** 2001. The TRAP/SMCC/Mediator complex and thyroid hormone receptor function. Trends Endocrinol Metab **12:**127-34.

42. **Jenuwein, T., and C. D. Allis.** 2001. Translating the histone code. Science **293:**1074-80.

43. **Kaltenbach, L., M. A. Horner, J. H. Rothman, and S. E. Mango.** 2000. The TBP-like factor CeTLF is required to activate RNA polymerase II transcription during C. elegans embryogenesis. Mol Cell **6:**705-13.

44. **Karlstrom, R. O., O. V. Tyurina, A. Kawakami, N. Nishioka, W. S. Talbot, H. Sasaki, and A. F. Schier.** 2003. Genetic analysis of zebrafish gli1 and gli2 reveals divergent requirements for gli genes in vertebrate development. Development **130:**1549-64.

45. **Khavari, P. A., C. L. Peterson, J. W. Tamkun, D. B. Mendel, and G. R. Crabtree.** 1993. BRG1 contains a conserved domain of the SWI2/SNF2 family necessary for normal mitotic growth and transcription. Nature **366:**170-4.

46. **Knight, J. C.** 2004. Allele-specific gene expression uncovered. Trends Genet **20:**113-6.

47. **Kubota, H., S. Yokota, H. Yanagi, and T. Yura.** 2000. Transcriptional regulation of the mouse cytosolic chaperonin subunit gene Ccta/t-complex polypeptide 1 by selenocysteine tRNA gene transcription activating factor family zinc finger proteins. J Biol Chem **275:**28641-8.

48. **Kunkel, G. R., T. C. Cheung, J. H. Miyake, O. Urso, K. J. McNamara-Schroeder, and W. E. Stumph.** 1996. Identification of a SPH element in the distal region of a human U6 small nuclear RNA gene promoter and characterization of the SPH binding factor in HeLa cell extracts. Gene Expr **6:**59-72.

49. **Kunkel, G. R., and J. D. Hixson.** 1998. The distal elements, OCT and SPH, stimulate the formation of preinitiation complexes on a human U6 snRNA gene promoter in vitro. Nucleic Acids Res **26:**1536-43.

50. **Kurdistani, S. K., and M. Grunstein.** 2003. Histone acetylation and deacetylation in yeast. Nat Rev Mol Cell Biol **4:**276-84.

51. **Lekven, A. C., C. J. Thorpe, J. S. Waxman, and R. T. Moon.** 2001. Zebrafish wnt8 encodes two wnt8 proteins on a bicistronic transcript and is required for mesoderm and neurectoderm patterning. Dev Cell **1:**103-14.

52. **Levine, M., and R. Tjian.** 2003. Transcription regulation and animal diversity. Nature **424:**147-51.

53. **Livak, K. J., and T. D. Schmittgen.** 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods **25:**402-8.

54. **Lum, L., and P. A. Beachy.** 2004. The Hedgehog response network: sensors, switches, and routers. Science **304:**1755-9.

55. **Mach, C. M., B. W. Hargrove, and G. R. Kunkel.** 2002. The Small RNA gene activator protein, SphI postoctamer homology-binding factor/selenocysteine tRNA gene transcription activating factor, stimulates transcription of the human interferon regulatory factor-3 gene. J Biol Chem **277:**4853-8.

56. **Machold, R., S. Hayashi, M. Rutlin, M. D. Muzumdar, S. Nery, J. G. Corbin, A. Gritli-Linde, T. Dellovade, J. A. Porter, L. L. Rubin, H. Dudek, A. P. McMahon, and G. Fishell.** 2003. Sonic hedgehog is required for progenitor cell maintenance in telencephalic stem cell niches. Neuron **39:**937-50.

57. **Malik, S., and R. G. Roeder.** 2000. Transcriptional regulation through Mediator-like coactivators in yeast and metazoan cells. Trends Biochem Sci **25:**277-83.

58. **Miyake, A., Y. Nakayama, M. Konishi, and N. Itoh.** 2005. Fgf19 regulated by Hh signaling is required for zebrafish forebrain development. Dev Biol **288:**259-75.

59. **Myers, L. C., and R. D. Kornberg.** 2000. Mediator of transcriptional regulation. Annu Rev Biochem **69:**729-49.

60. **Myslinski, E., M. A. Gerard, A. Krol, and P. Carbon.** 2006. A genome scale location analysis of human Staf/ZNF143-binding sites suggests a widespread role for human Staf/ZNF143 in mammalian promoters. J Biol Chem **281:**39953-62.

61. **Myslinski, E., M. A. Gerard, A. Krol, and P. Carbon.** 2007. Transcription of the human cell cycle regulated BUB1B gene requires hStaf/ZNF143. Nucleic Acids Res **35:**3453-64.

62. **Myslinski, E., A. Krol, and P. Carbon.** 2004. Characterization of snRNA and snRNA-type genes in the pufferfish Fugu rubripes. Gene **330:**149-58.

63. **Myslinski, E., A. Krol, and P. Carbon.** 1998. ZNF76 and ZNF143 are two human homologs of the transcriptional activator Staf. J Biol Chem **273:**21998-2006.

64. **Naar, A. M., P. A. Beaurang, S. Zhou, S. Abraham, W. Solomon, and R. Tjian.** 1999. Composite co-activator ARC mediates chromatin-directed transcriptional activation. Nature **398:**828-32.

65. **Nasevicius, A., and S. C. Ekker.** 2000. Effective targeted gene 'knockdown' in zebrafish. Nat Genet **26:**216-20.

66. **Nilsen, T.** 1998. RNA-RNA interactions in nuclear pre-mRNA splicing, p. 279-307. *In* R. Simons, Grunberg-Manago, M. (eds.), RNA Structure and Function. Cold Spring Harbor Laboratory Press, Woodbury, NY.

67. **Nomura, R., E. Kamei, Y. Hotta, M. Konishi, A. Miyake, and N. Itoh.** 2006. Fgf16 is essential for pectoral fin bud formation in zebrafish. Biochem Biophys Res Commun **347:**340-6.

68. **Norton, W. H., J. Ledin, H. Grandel, and C. J. Neumann.** 2005. HSPG synthesis by zebrafish Ext2 and Extl3 is required for Fgf10 signalling during limb development. Development **132:**4963-73.

69. **Ohkawara, B., S. Iemura, P. ten Dijke, and N. Ueno.** 2002. Action range of BMP is defined by its N-terminal basic amino acid core. Curr Biol **12:**205-9.

70. **Olave, I. A., S. L. Reck-Peterson, and G. R. Crabtree.** 2002. Nuclear actin and actin-related proteins in chromatin remodeling. Annu Rev Biochem **71:**755-81.

71. **Price, E. R., H. F. Ding, T. Badalian, S. Bhattacharya, C. Takemoto, T. P. Yao, T. J. Hemesath, and D. E. Fisher.** 1998. Lineage-specific signaling in melanocytes. C-kit stimulation recruits p300/CBP to microphthalmia. J Biol Chem **273:**17983-6.

72. **Ptashne, M.** 2005. Regulation of transcription: from lambda to eukaryotes. Trends Biochem Sci **30:**275-9.

73. **Ptashne, M., and A. Gann.** 1997. Transcriptional activation by recruitment. Nature **386:**569-77.

74. **Ptashne, M. and A. Gann.** 2002. Genes and Signals. Cold Spring Harbor Laboratory Press, Woodbury, NY.

75. **Rabenstein, M. D., S. Zhou, J. T. Lis, and R. Tjian.** 1999. TATA box-binding protein (TBP)-related factor 2 (TRF2), a third member of the TBP family. Proc Natl Acad Sci **96:**4791-6.

76. **Rachez, C., B. D. Lemon, Z. Suldan, V. Bromleigh, M. Gamble, A. M. Naar, H. Erdjument-Bromage, P. Tempst, and L. P. Freedman.** 1999. Ligand-dependent transcription activation by nuclear receptors requires the DRIP complex. Nature **398:**824-8.

77. **Reifers, F., H. Bohli, E. C. Walsh, P. H. Crossley, D. Y. Stainier, and M. Brand.** 1998. Fgf8 is mutated in zebrafish acerebellar (ace) mutants and is required for maintenance of midbrain-hindbrain boundary development and somitogenesis. Development **125:**2381-95.

78. **Roebuck, K. A., R. J. Walker, and W. E. Stumph.** 1987. Multiple functional motifs in the chicken U1 RNA gene enhancer. Mol Cell Biol **7:**4185-93.

79. **Roeder, R. G.** 1996. Nuclear RNA polymerases: role of general initiation factors and cofactors in eukaryotic transcription. Methods Enzymol **273:**165-71.

80. **Rossi, F. M., A. M. Kringstein, A. Spicher, O. M. Guicherit, and H. M. Blau.** 2000. Transcriptional control: rheostat converted to on/off switch. Mol Cell **6:**723-8.

81. **Ruvkun, G., and O. Hobert.** 1998. The taxonomy of developmental control in Caenorhabditis elegans. Science **282:**2033-41.

82. **Ryu, S., S. Zhou, A. G. Ladurner, and R. Tjian.** 1999. The transcriptional cofactor complex CRSP is required for activity of the enhancer-binding protein Sp1. Nature **397:**446-50.

83. **Ryu, S. L., R. Fujii, Y. Yamanaka, T. Shimizu, T. Yabe, T. Hirata, M. Hibi, and T. Hirano.** 2001. Regulation of dharma/bozozok by the Wnt pathway. Dev Biol **231:**397-409.

84. **Sarto, I., T. Klausberger, N. Ehya, B. Mayer, K. Fuchs, and W. Sieghart.** 2002. A novel site on gamma 3 subunits important for assembly of GABA(A) receptors. J Biol Chem **277:**30656-64.

85. **Saur, D., B. Seidler, H. Paehge, V. Schusdziarra, and H. D. Allescher.** 2002. Complex regulation of human neuronal nitric-oxide synthase exon 1c gene transcription. Essential role of Sp and ZNF family members of transcription factors. J Biol Chem **277:**25798-814.

86. **Schaub, M., A. Krol, and P. Carbon.** 1999. Flexible zinc finger requirement for binding of the transcriptional activator staf to U6 small nuclear RNA and tRNA(Sec) promoters. J Biol Chem **274:**24241-9.

87. **Schaub, M., E. Myslinski, A. Krol, and P. Carbon.** 1999. Maximization of selenocysteine tRNA and U6 small nuclear RNA transcriptional activation achieved by flexible utilization of a Staf zinc finger. J Biol Chem **274:**25042-50.

88. **Schaub, M., E. Myslinski, C. Schuster, A. Krol, and P. Carbon.** 1997. Staf, a promiscuous activator for enhanced transcription by RNA polymerases II and III. Embo J **16:**173-81.

89. **Schier, A. F.** 2001. Axis formation and patterning in zebrafish. Curr Opin Genet Dev **11:**393-404.

90. **Schier, A. F.** 2003. Nodal signaling in vertebrate development. Annu Rev Cell Dev Biol **19:**589-621.

91. **Schuster, C., A. Krol, and P. Carbon.** 1998. Two distinct domains in Staf to selectively activate small nuclear RNA-type and mRNA promoters. Mol Cell Biol **18:**2650-8.

92. **Schuster, C., E. Myslinski, A. Krol, and P. Carbon.** 1995. Staf, a novel zinc finger protein that activates the RNA polymerase III promoter of the selenocysteine tRNA gene. Embo J **14:**3777-87.

93. **Sharp, P. A.** 1992. TATA-binding protein is a classless factor. Cell **68:**819-21.

94. **Smale, S. T., and J. T. Kadonaga.** 2003. The RNA polymerase II core promoter. Annu Rev Biochem **72:**449-79.

95. **Su, W., S. Jackson, R. Tjian, and H. Echols.** 1991. DNA looping between sites for transcriptional activation: self-association of DNA-bound Sp1. Genes Dev **5:**820-6.

96. **Taatjes, D. J., A. M. Naar, F. Andel, 3rd, E. Nogales, and R. Tjian.** 2002. Structure, function, and activator-induced conformations of the CRSP coactivator. Science **295:**1058-62.

97. **Takada, S., K. L. Stark, M. J. Shea, G. Vassileva, J. A. McMahon, and A. P. McMahon.** 1994. Wnt-3a regulates somite and tailbud formation in the mouse embryo. Genes Dev **8:**174-89.

98. **Tamkun, J. W., R. Deuring, M. P. Scott, M. Kissinger, A. M. Pattatucci, T. C. Kaufman, and J. A. Kennison.** 1992. Brahma: a regulator of Drosophila homeotic genes structurally related to the yeast transcriptional activator SNF2/SWI2. Cell **68:**561-72.

99. **Thisse, B. and Thisse, C.** 2004, posting date. Fast Release Clones: A High Throughput Expression Analysis, http://zfin.org.

100. **Valadkhan, S.** 2005. snRNAs as the catalysts of pre-mRNA splicing. Curr Opin Chem Biol **9:**603-8.

101. **Veenstra, G. J., D. L. Weeks, and A. P. Wolffe.** 2000. Distinct roles for TBP and TBP-like factor in early embryonic gene transcription in Xenopus. Science **290:**2312-5.

102. **Vogel-Hopker, A., T. Momose, H. Rohrer, K. Yasuda, L. Ishihara, and D. H. Rapaport.** 2000. Multiple functions of fibroblast growth factor-8 (FGF-8) in chick eye development. Mech Dev **94:**25-36.

103. **Wang, W., Y. Xue, S. Zhou, A. Kuo, B. R. Cairns, and G. R. Crabtree.** 1996. Diversity and specialization of mammalian SWI/SNF complexes. Genes Dev **10:**2117-30.

104. **Westerfield, M.** 2000. The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish (Danio rerio), 4th ed. University of Oregon Press, Eugene.

105. **Wyrick, J. J., and R. A. Young.** 2002. Deciphering gene expression regulatory networks. Curr Opin Genet Dev **12:**130-6.

106. **Yamauchi, H., Y. Hotta, M. Konishi, A. Miyake, A. Kawahara, and N. Itoh.** 2006. Fgf21 is essential for haematopoiesis in zebrafish. EMBO Rep **7:**649-54.

107. **Yasumoto, K., K. Takeda, H. Saito, K. Watanabe, K. Takahashi, and S. Shibahara.** 2002. Microphthalmia-associated transcription factor interacts with LEF-1, a mediator of Wnt signaling. Embo J **21:**2703-14.

108. **Young, R. A.** 1991. RNA polymerase II. Annu Rev Biochem **60:**689-715.

109. **Zamrod, Z., and W. E. Stumph.** 1990. U4B snRNA gene enhancer activity requires functional octamer and SPH motifs. Nucleic Acids Res **18:**7323-30.

110. **Zawel, L., and D. Reinberg.** 1995. Common themes in assembly and function of eukaryotic transcription complexes. Annu Rev Biochem **64:**533-61.

111. **Zheng, G., and Y. C. Yang.** 2004. ZNF76, a novel transcriptional repressor targeting TATA-binding protein, is modulated by sumoylation. J Biol Chem **279:**42410-21.

APPENDIX

PAPERS AUTHORED BY KARI MICHELE HALBIG ON PRE-EDITED mRNA BINDING PROTEINS FROM *Trypanosoma brucei*

The following is a list of papers published, as well as my contribution to those papers, from the Cruz-Reyes lab:

Halbig, K., Sacharidou, A., Cifuentes-Rojas, C., De Nova-Ocampo, M., Cruz-Reyes, J. 2006. Preferential interaction of a 25 kDa protein with an A6 pre-mRNA substrate for RNA editing in Trypanosoma brucei. *International Journal of Parasitology*. 2006 Oct;36(12):1295-304.

The purpose of this study was to search for protein interactions with a functional editing substrate by using a site-specific radiolabeled RNA single-site labeled mRNA and "zero-distance" photocrosslinking with mitochondrial lysate fractions that were partially purified by ion-exchange chromatography. A 25 kDa crosslink was observed that was protein-dependent and was shown to be specific through competition experiments with both homologous and heterologous RNA competitors. Mapping and substrate truncation analyses suggest that the crosslinking activity primarily targeted a predicted stem-loop region containing the first editing sites. Pre-annealing with guide RNA abolished crosslinking suggesting that pre-mRNA structure may be required. Interestingly, this preferential protein interaction with the A6 substrate seemed to require ATP, but not hydrolysis. As in other biological systems, fine regulation in vivo may be brought about by transient networks of relatively low-specificity interactions in which additional factors bind to mRNAs and/or editing complexes in unique higher-order assemblies.

For this study I was responsible for the production of all RNA substrates, growth of the PF trypanosomes, the preparation of the mitochondrial extracts, and the enrichment of the editing complex by the use of chromatography. I also performed the crosslinking assays including all competition assays, the adenylation assays, the nucleotide requirement assays, and the mRNA structural requirement assays. Finally I also performed the isopycnic sedimentation assay of the 25 kDa crosslink in both BF and PF extracts. I presented this work at the 2005 Gordon Research Conference in RNA Editing in Ventura, CA.

Miller, MM., **Halbig, K.**, Cruz-Reyes, J., Read, LK. (2006). RBP16 stimulates trypanosome RNA editing in vitro at an early step in the editing reaction. *RNA*. 2006 Jul;12(7):1292-303

This work was done in collaboration with Dr. Laurie Read's laboratory and all the experiments were done by Melissa Miller. I contributed the purified editing complex used in the work.

Sacharidou, A., **Halbig, K**., Cifuentes-Rojas, C., Hernandez, A., Dangott, L., De Nova-Ocampo, M.,Cruz-Reyes, J. (2006). RNA editing complex interactions with a site for full-round U deletion in Trypanosoma brucei. *RNA.* 2006 Jul;12(7):1219-28

The basic enzymatic activities and protein composition of these RNA editing complexes had been under intense study by several labs, but the specific protein interactions with functional pre-mRNA/gRNA substrates had not been studied. Editing complexes purified through extensive ion-exchange chromatography and immunoprecipitation make specific cross-linking interactions with A6 pre-mRNA

containing a single 32P and photoreactive 4-thioU at the scissile bond of a functional site. For full-round U deletion at least four direct protein-RNA contacts are detected at this site by cross-linking. All four interactions (p40, p50, p60, and p100) are stimulated by unpaired residues just 5' of the pre-mRNA/gRNA anchor duplex, but strongly inhibited by pairing of the editing site region. All four of the interactions also co-purified with known members of the editing complex. Furthermore, competition analysis with homologous and heterologous transcripts suggests preferential contacts of the editing complex with the mRNA/gRNA duplex substrate. This apparent structural selectivity suggests that the RNA-protein interactions observed may be involved in recognition of editing sites and/or catalysis in assembled complexes.

For this study I was responsible for the preparation of the mitochondrial extracts and the enrichment of the editing complex via chromatography. I also performed the crosslinking assays of the A6 mRNA and gRNA with the editing complexes with different levels of purified editing complex as well as the culturing of the PF trypanosomes.

Cifuentes-Rojas, C., **Halbig, K**., Sacharidou, A., De Nova-Ocampo, M.,Cruz-Reyes, J. (2005). Minimal pre-mRNA substrates with natural and converted sites for full-round U insertion and U deletion RNA editing in trypanosomes. *Nucleic Acids Res*. Nov 23**;33(20):**6610-20.

The structural and functional composition of editing complexes is intensively studied, but the molecular interactions in and around editing sites was not completely

understood, which was the focus of this study. In this study, a systematic analysis of distal RNA requirements for full-round insertion and deletion by purified editing complex was performed. Minimal substrates were defined for efficient editing of A6 and CYb model transcripts, and established a new substrate, RPS12. Important differences were observed in the composition of substrates for insertion and deletion. It was also shown for the first time that natural sites can be artificially converted in both directions: from deletion to insertion or from insertion to deletion. The site conversions enabled a direct comparison of the two editing modes at common sites during substrate minimization and demonstrate that all basic determinants directing the editosome to carry out full-round insertion or deletion reside within each editing site. Surprisingly, I was able to engineer a deletion site into CYb, which exclusively undergoes insertion in nature.

For this study I was responsible for the preparation of the CYb mRNA and gRNA constructs for both insertion and deletion editing as well as the preparation of the mitochondrial extracts and the enrichment of the editing complex. I also performed the editing assays for both CYb deletion and insertion.

Halbig, K., De Nova-Ocampo, M., Cruz-Reyes, J. (2004) Complete cycles of bloodstream trypanosome RNA editing in vitro.
RNA. Jun;**10(6)**:914-20.

Previous work on the requirements for RNA editing in trypanosomes had only been studied in the procyclic or insect form of the parasite because it was the only form in which an *in vitro* assay had been established. During the life cycle of *Trypanosome*

brucei it also is found in the bloodstream form when it has infected a mammal host. The establishment of an *in vitro* assay that recreates efficient RNA editing in bloodstream *T. brucei* would be a valuable tool for mechanistic studies of regulation. In this work Dr. Monico De Nova-Ocampo and I were able to establish a robust in vitro system that reconstitutes full cycles of both U insertion and U deletion in bloodstream trypanosomes, and allowed for the first direct comparisons of the in vitro systems for strains of mammalian and insect stages.

For this study I infected the rats with the BF trypanosomes, prepared the mitochondrial extracts and the enrichment of the BF editing complex via chromatography. I also ran isopycnic sedimentation on both PF and BF mitochondrial extracts as well as assayed for the location of the editing complex by adenylation. Finally I developed all the figures for the paper as well as performed the quantitation of the editing assays. I presented this work at the Molecular Parasitology Meeting XIV at Marine Biological Laboratory in Woods Hole, MA in 2003.

VITA

NAME: Kari Michele Halbig

ADDRESS: Department of Biochemistry and Biophysics
TAMU 2128
College Station, TX 77843
kmhalbig@tamu.edu

EDUCATION: B.S., Biochemistry (2001)
University of Illinois
Urbana, IL

Ph.D., Biochemistry (2008)
Texas A&M University
College Station, TX

HONORS AND AWARDS:

2007	2nd Place in the Biochemistry Graduate Student Research Competition
2005	President, Biochemistry Graduate Association
2004	Graduate Recruiting and Admissions Committee Representative

SELECTED PUBLICATIONS: (from eight total)

Halbig, K. and Kunkel, G.R. "Zebrafish U6 Small Nuclear RNA Gene Promoters Contain a SPH Element in an Unusual Proximal Location" Manuscript in preparation.

Halbig, K., Lekven, A.C., Kunkel G.R. "The Role of the Multifunctional Transcriptional Activator SBF/Staf/ZNF143 in Zebrafish Development" Manuscript in preparation

SELECTED PRESENTATION: (from five total)

Halbig, K., and Kunkel, G.R. "Multiple Roles for the Zebrafish Transcriptional Activator Protein SBF/Staf" The Fourth Biennial Texas and Southern Plains Zebrafish Conference, *Texas A&M University*, College Station, TX (2007)