Mapping zinc-responsive elements in *Schizosaccharomyces pombe*.

THESIS

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By

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Abstract

Zinc is an essential trace mineral that is an important co-factor for over 300 enzymes involved in the catalysis of many biochemical reactions. Due to the wide range of roles zinc has in the body, a deficiency can lead to severe health issues. These include problems associated with the gastrointestinal tract, central nervous system, the immune system, skeletal system, and the epidermis. In humans, the expression of many genes is regulated in response to zinc deficiency. However, the factor(s) that mediate these responses are unknown. Therefore, it is important to understand how zinc deficiency is “sensed” at the cellular level. Currently, there is no known sensor of zinc deficiency in higher eukaryotes. To identify factors necessary for zinc sensing, we used the fission yeast model system Schizosaccharomyces pombe. Many genes, including adh4 (alcohol dehydrogenase 4) and vel1 are regulated by zinc in S. pombe. The objective of the study was to determine if adh4 and vel1 are transcriptionally regulated in response to zinc limitation. In order to test this hypothesis, a series of promoter truncations were created to map the site of the zinc-responsive element in adh4 and vel1. We have found that both adh4 and vel1 contain sequences necessary for zinc responsive regulation and are regulated at the transcriptional level.
Dedication

Dedicated with love to my parents, who constantly supported me throughout my education and instilled in me the values of hard work and determination. This thesis is also dedicated to my fiancé, Jordan, for his constant encouragement, support, and motivation.
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First and foremost, I would like to thank Dr. Amanda Bird for her guidance, expertise, and instruction during the course of my studies and thesis work. Her insight, patience, and knowledge of zinc regulation in *S. pombe* helped me to further develop my skills and education while at The Ohio State University. I am grateful for her participation as my advisor.

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CHAPTER 1

Introduction

Zinc is an essential trace mineral that is an important co-factor for over 300 enzymes involved in the catalysis of many biochemical reactions. Zinc can also have a structural role in some proteins. In these cases, zinc typically helps to stabilize small domains, such as the $C_2H_2$ zinc finger domain. The $C_2H_2$ zinc finger is the most common DNA binding motif found within the human genome (1). Zinc fingers are comprised of one alpha-helix structure and two beta-strands. Once a single zinc atom binds to the conserved cysteine and histine residues, the zinc finger forms into a compact domain, which easily fits into the major groove of DNA (1,2). Because of this, zinc has an important role in gene expression, as the binding allows for the initiation of transcription and ultimately gene expression.

Due to the role of zinc in a wide variety of cellular functions, a deficiency can have catastrophic effects on an individual. Affected systems in the human body include the gastrointestinal tract, central nervous system, the immune system, skeletal system, and the epidermis (2). Because of the severe ramifications of zinc deficiency,
it is important to recognize and understand the mechanisms by which fluctuations in zinc levels are “sensed” at the cellular level.

In many organisms, including humans, many genes are regulated at a transcriptional level in response to zinc deficiency; however, the mechanism(s) behind these transcriptional changes are largely unknown. The only known eukaryotic factor that senses cellular zinc deficiency is Zap1, from the budding yeast Saccharomyces cerevisiae (3). Zap1 is a transcription factor that mediates the cellular response to zinc deficiency through binding to zinc-responsive elements in the promoter regions of target genes during periods of low zinc (4). It induces the expression of genes necessary for zinc uptake, as well as repressing genes encoding proteins that require zinc as a cofactor.

In the fission yeast Schizosaccharomyces pombe, many genes that are regulated by zinc deficiency in the budding yeast are also regulated by zinc. These include alcohol dehydrogenase 4 (adh4), which encodes a mitochondrial zinc-dependent alcohol dehydrogenase (5). Although there is a significant overlap between the target genes regulated by zinc deficiency in S. pombe, and S. cerevisiae, there is no Zap1 homolog in the S. pombe genome. Due to the strong regulation of gene expression by zinc, the absence of a Zap1 homolog, and the robust genetics of the fission yeast model system, we opted to use S. pombe as a tool to identify genes that are necessary for the regulation of gene expression in response to zinc.
1.1 Research Objectives

We hypothesize that *adh4*, and other zinc-regulated genes in *S. pombe*, are transcriptionally regulated in response to zinc limitation. In order to test this hypothesis, we propose the following objectives:

Objective #1: Locate the zinc-responsive element in the *adh4* promoter using promoter mapping

Objective #2: Perform site-directed mutagenesis to enable fine resolution of the zinc responsive element (ZRE) in the *adh4* promoter

Objective #3: Determine whether similar elements are found in the promoters of other zinc-regulated genes

The identification of the ZRE(s) in multiple zinc-regulated genes will provide us with a means to directly determine which genes are regulated in response to zinc deficiency and a method of identifying the factors necessary for zinc-dependent regulation of gene expression.
Chapter 2

Literature Review

2.1 Functions of zinc within the human body

Zinc, an essential trace mineral, is necessary for the normal growth and of the human body. Next to iron, zinc is the most abundant trace mineral found in the human body (6). The element has many roles, which can be summed into three categories: catalytic, regulatory, and structural (7). For example, zinc ions are necessary for the catalytic activity of RNA polymerases, which help initiate RNA synthesis (7). The structural role of zinc can be observed in zinc-fingers, which help transcription factors bind to specific sequences in the promoter regions of DNA. Because of this, zinc helps to regulate transcription. Other examples of zinc-dependent enzymes include carboxypeptidases, superoxide dismutase, and alkaline phosphatase (8). Carboxypeptidases assist in the digestion of peptides during digestions. Superoxide dismutase assists in the body's antioxidant defense while alkaline phosphatase has an important role in bone metabolism (8). Because zinc
contributes to a number of different biochemical functions, it is crucial to the overall health of the body.

2.1.1 Dietary recommendations and regulatory mechanisms

Currently, the Recommended Daily Allowance (RDA) for zinc intake is 11mg/day for men and 8mg/day for women, with the tolerable upper limit set at 40mg/day (9). Zinc needs are slightly increased during pregnancy and childhood development (10). While zinc toxicity is a concern, homeostatic mechanisms enable the control of zinc levels within the cell. One point of this regulation occurs in the small intestine at the point of absorption. When dietary levels of zinc are high, absorption across the epithelial cells decreases; alternatively, when dietary zinc levels are low, absorption increases (11). Another point of regulation occurs once zinc has entered the epithelial cells of the small intestine. When zinc concentrations increase, the metal will bind to thionein, forming the active protein metallothionein (12). This mechanism protects the cell from toxic levels of zinc. When zinc levels drop, zinc will be released from metallothionein, either for cellular use or transportation to the blood for distribution to other tissues. Zinc bound to these proteins within the epithelial cells of the small intestine may also be lost and excreted in the feces after cell turnover occurs.
Zinc status also helps to regulate the amount of metallothionein present within cells at any given time. Studies have indicated that an increase in metallothionein mRNA occurs when dietary zinc increases after a period of low status (10). The promoter for the metallothionein gene contains a metal-response-element (MRE), to which MTF-1 (metal-responsive-element binding transcription factor) binds to activate transcription (13). Zinc, unlike other metals, activates the binding of MTF-1 to the MRE, as MTF-1 contains several zinc finger proteins (14). Therefore, as intracellular zinc levels increase, metallothionein levels do as well.

2.1.2 Zinc Deficiency in Humans

On the other end of the spectrum, zinc deficiency, common in the elderly and childhood populations, causes a variety of complications (15). Sign and symptoms of zinc deficiency include growth retardation, hypogonadism in males, loss of appetite, and impaired immune function (16). Additionally, hair loss may occur. Dermatitis, present especially around the mouth and eyes, increases in severity as the deficiency increases (17).

Cases of zinc deficiency were first discovered in Middle Eastern boys, ages 12-20, suffering from dwarfism and hypogonadism (18). It was noted that following zinc administration, sexual maturation and growth increased in a few months (19).
Unleavened bread, commonly consumed in the villages of these countries, contains high amounts of phytic acid (20). This phytic acid can form a complex with minerals, including zinc, leading to poor absorption in the gastrointestinal tract (20). In leavened breads, the fermentation by yeast helps to activate phytase found in wheat, which breaks down phytic acid complexes and allows for the release of zinc and other nutrients for digestion (21,22). Yeast itself also contains phytase, adding to the breakdown of phytic acid complexes (22).

Zinc deficiency may also occur in those with gastrointestinal diseases that decrease the absorption of zinc across the small intestine. Acrodermatitis enteropathica, a rare autosomal recessive disorder, causes a mutation to occur in Zip4, an intestinal zinc transporter (23). This mutation affects intestinal zinc absorption and is characterized by dry, scaly skin, especially around the mouth, alopecia, and diarrhea in infants and children (23). Vegetarians also are prone to zinc deficiency, as the bioavailability of zinc from plant sources is not as high as that of animal, particularly red meats and seafood (24). Foods such as legumes and grains, which may contain zinc, also contain phytic acid, which binds zinc, inhibiting its absorption across the small intestine (24). Because of the impact of zinc deficiency on these populations, it is important to understand the mechanisms responsible for the “sensing” of cellular zinc levels in higher eukaryotes.
2.2 "Sensing" zinc deficiency

2.2.1 Mechanisms for "sensing" zinc deficiency in *S. cerevisiae*

The only known eukaryotic factor that senses cellular zinc deficiency is the Zap1 protein from *S. cerevisiae*, which specifically activates target gene expression under zinc-limiting conditions (3). In response to zinc deficiency, Zap1 induces transcription of *ZRT1* and *ZRT2*, which encode zinc uptake transporters. The *ZAP1* gene itself is also regulated in response to zinc; when zinc replete cells become zinc limited, Zap1 is produced, enabling an increase in target gene expression (3). While some of this Zap1 zinc-dependent regulation occurs at a transcriptional level, the majority of the regulation occurs at the post-translational level (25). Zap1 also controls the expression of *FET4*, which encodes for a transporter of iron, copper, and zinc.

Zap1 activates gene expression by binding in a site-specific manner to zinc-responsive elements (ZREs) that are located in the promoters of these genes (7). The protein is able to do so through the binding of five C2H2 zinc-fingers (ZnF3-7) that are located in its DNA binding domain (DBD) (26). ZnF1 and ZnF2 are not required for DNA binding but have a different function (25,26).
Zap1 contains two activation domains, known as AD1 and AD2 (25). AD2 has been mapped to the region containing ZnF2; no activation function has been found in ZnF1 (27). However, both ZnF1 and ZnF2 are required for the repression of AD2 function. ZnF1 and ZnF2 are important in zinc responsiveness when Zap1 is bound to DNA. In zinc-replete conditions, zinc binds to both zinc fingers and allows for them to interact, therefore inducing a conformational change in AD2 (27). This change renders AD2 unable to activate target gene expression (27). The other activation domain, AD1, is thought to act like a zinc (II)-binding sensor domain. When intracellular zinc is low, no zinc is bound to the region, allowing for AD1 to activate transcription. However, when intracellular zinc levels rise, zinc binds to the ZRD, enabling a conformational change which in-turn masks AD1, repression its function (28).

*ZRT1*, one of the Zap1 target genes, encodes a high affinity zinc transporter. This gene is required for growth in zinc-limited conditions and therefore helps to increase the amount of zinc available to a cell when zinc conditions are low (29).
When cells are zinc limited, there is a 10-fold increase in ZRT1 mRNA levels when compared to zinc-replete cells. ZRT2, on the other hand, encodes the low affinity zinc transporter, which enables entry of zinc into cells during zinc-replete conditions (30).

The number and arrangement of the ZREs can directly influence how a target gene is regulated by zinc. ZRT1 contains three ZREs in its promoter that act in an additive manner (4). When a single ZRE is deleted from the ZRT1 promoter, gene expression is still able to occur; however, deletion of two ZREs greatly decreases activity. When all three ZREs are deleted, gene expression disappears (4). Only a single ZRE is necessary for activation. However, stronger activation can be obtained when multiple ZREs are present. ZRT2, like ZRT1, contains three ZREs in its promoter region. Because ZRT2 encodes a low affinity zinc-transporter, higher levels of zinc are required for repression. When cells are zinc limited, ZRT2 is still transcribed; however, as zinc limitation increases, ZRT2 transcription is repressed and ZRT1 transcription increases in order to better bring zinc into the cell (4). The three ZREs in the promoter for ZRT2 allow for this repression to occur. Zap1 is known to bind to ZRE1 and ZRE2 in the promoter of ZRT2 and promote activation of transcription. However, when zinc-limiting conditions increase, Zap1 acts as a repressor, binding to ZRE3, which is located downstream of the TATA box and upstream of the transcriptional start site on the ZRT2 promoter (31). Through this binding, Zap1 inhibits the initiation of transcription.
Figure 2: Activation and repression of ZRT2 by Zap1 during high and low zinc conditions

Zap1 also helps to control intracellular zinc levels by regulating the levels of genes encoding a number of alcohol dehydrogenases. ADH1 and ADH3 are repressed during periods of low zinc, while Zap1 induces ADH4. ADH1 encodes the major cytoplasmic alcohol dehydrogenase in S. cerevisiae. When cellular zinc levels are low, Zap1 induces the expression of Zinc Regulated RNA1 (ZRR1), which causes transcriptional interference (32). ZRR1 allows for the transient displacement of Rap1 and Gcr1, two proteins necessary for activation of ADH1. It is hypothesized
that ADH1/3 bind two molecules of zinc per monomer while ADH4 binds only one, therefore allowing for the cell to conserve zinc for other uses (33).

2.2.2 Use of promoter mapping to identify zinc responsive elements in S. pombe

While Zap1 displays no known homologs in S. pombe, many of its target genes are conserved in, and are regulated similarly by zinc in S. pombe. So far, the majority of the research has focused on the regulation of adh4 by zinc. Adh4 is a mitochondrial alcohol dehydrogenase whose expression levels are tightly regulated by zinc (34). In wild-type cells, adh4 is not expressed when cellular zinc levels are high (5). Because adh4 is highly regulated by zinc, it acts as a useful tool to identify if the gene is under transcriptional control and identify zinc-responsive promoter elements that are necessary for the transcriptional regulation. One method to identify binding sites for transcription factors is to create promoter reporter fusions. In this method, the promoter region of a targeted gene is fused to a reporter gene, which commonly include luciferase, lacZ (which encodes beta-galactosidase), and chloramphenicol acetyl-transferase (35). In previous work, promoter mapping was used to map the ZRE in the adh4 promoter in S. pombe. In this study, the authors generated a series of fusions of the adh4 promoter to the lacZ reporter gene and assayed for beta-galactosidase activity in wild-type cells grown under zinc-limited and zinc-replete conditions (Figure 1). From these reporter assays, it was concluded that ‘various repressors and activators control the expression of adh4 (5).
Figure 3: Previously published adh4-lacZ truncation data (5).

The reporters constructed by the Whitehall group utilized the predicted start codon in the adh4 promoter from amino acids -1380 to 115, which was determined using the S. pombe gene database (5). In our lab, we used a 5’ RACE (Rapid Amplification of cDNA Ends) to map the transcriptional start site of the adh4 transcript. We have found that the annotation of the gene in the S. pombe data base genome was incorrect and that the genuine adh4 transcript contained an additional upstream untranslated exon and intron. As a consequence, this results in a different adh4 translational start site than what is annotated in the genome database. In light of this new information, in this research thesis, we generated a new series of constructs to map the location of the ZRE in the adh4 promoter.
CHAPTER 3

Methods

3.1 Cloning

In order to construct the reporter strains necessary for mapping the adh4-promoter, a cloning strategy was developed (Figure 4).

Figure 4: Cloning strategy for construction of reporter plasmids
### 3.1.1 Primer construction and Polymerase Chain Reaction

Primer sets were constructed and ordered for the construction of the reporter plasmids needed to map the *adh4* promoter. Each primer set corresponded to a specific truncation length of the *adh4* promoter. The same was done for the *vel1* promoter (Table 1).

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Primers Used</th>
<th>Primer Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>adh4</em>-759-lacZ</td>
<td>Adh4-1</td>
<td>CTGGGATCATGGCTTTCGAAGAGGTG</td>
</tr>
<tr>
<td></td>
<td>Adh4-759</td>
<td>GATCTGCGGCGGCGCCATGTAATTAT’TCCCAAGT</td>
</tr>
<tr>
<td><em>adh4</em>-F500-lacZ</td>
<td>Adh4-1</td>
<td>CTGGGATCATGGCTTTCGAAGAGGTG</td>
</tr>
<tr>
<td></td>
<td>Adh4F500</td>
<td>GATCTGCGGCGGACGGCTTTCGAGAATGCTTCTC</td>
</tr>
<tr>
<td><em>adh4</em>-449-lacZ</td>
<td>Adh4-1</td>
<td>CTGGGATCATGGCTTTCGAAGAGGTG</td>
</tr>
<tr>
<td></td>
<td>Adh4-459</td>
<td>GATCTGCGGCGGCTTTCGAGAAGAAGGAGATG</td>
</tr>
<tr>
<td><em>adh4</em>-420-lacZ</td>
<td>Adh4-1</td>
<td>CTGGGATCATGGCTTTCGAAGAGGTG</td>
</tr>
<tr>
<td></td>
<td>Adh4-420</td>
<td>GATCTGCGGCGGATAATGCGATTGCTTTCGACC</td>
</tr>
<tr>
<td><em>adh4</em>-352-lacZ</td>
<td>Adh4-1</td>
<td>CTGGGATCATGGCTTTCGAAGAGGTG</td>
</tr>
<tr>
<td></td>
<td>Adh4-352</td>
<td>GATCTGCGGCGGAATAGTTTCTATCTCTAG</td>
</tr>
<tr>
<td><em>adh4</em>-206-lacZ</td>
<td>Adh4-1</td>
<td>CTGGGATCATGGCTTTCGAAGAGGTG</td>
</tr>
<tr>
<td></td>
<td>Adh4-206</td>
<td>GATCTGCGGCGGCAAAATCAGTTTGCAG</td>
</tr>
<tr>
<td><em>adh4</em>-93-lacZ</td>
<td>Adh4-1</td>
<td>CTGGGATCATGGCTTTCGAAGAGGTG</td>
</tr>
<tr>
<td></td>
<td>Adh4-93</td>
<td>GATCTGCGGCGGCTATTGAGAATGTCATGACACC</td>
</tr>
<tr>
<td><em>adh4</em>-35-lacZ</td>
<td>Adh4-1</td>
<td>CTGGGATCATGGCTTTCGAAGAGGTG</td>
</tr>
<tr>
<td></td>
<td>Adh4-35</td>
<td>GATCTGCGGCGGCTATTGAGAATGTCATGACACC</td>
</tr>
<tr>
<td><em>vel1</em>-600-lacZ</td>
<td>Vel1-1</td>
<td>CTGGGATCCATCGTATGGAATGTCCCTCTG</td>
</tr>
<tr>
<td></td>
<td>Vel1-600</td>
<td>GATCTGCGGCGGAATGATAATCCAGTAGGACATAC</td>
</tr>
<tr>
<td><em>vel1</em>-385-lacZ</td>
<td>Vel1-1</td>
<td>CTGGGATCCATCGTATGGAATGTCCCTCTG</td>
</tr>
<tr>
<td></td>
<td>Vel1-385</td>
<td>GATCTGCGGCGGAGAGGAGATCTACACAC</td>
</tr>
<tr>
<td><em>vel1</em>-252-lacZ</td>
<td>Vel1-1</td>
<td>CTGGGATCCATCGTATGGAATGTCCCTCTG</td>
</tr>
<tr>
<td></td>
<td>Vel1-252</td>
<td>GATCTGCGGCGGCTATTGAGAATGTCATGACAC</td>
</tr>
<tr>
<td><em>vel1</em>-148-lacZ</td>
<td>Vel1-1</td>
<td>CTGGGATCCATCGTATGGAATGTCCCTCTG</td>
</tr>
<tr>
<td></td>
<td>Vel1-148</td>
<td>GATCTGCGGCGGACGATCATGATCTACACG</td>
</tr>
</tbody>
</table>

Table 1: Primers constructed and used for reporter strain generation
Polymerase chain reaction (PCR) was used in order to amplify each truncation using the full-length *adh4* or *vel1* promoter as a template. A typical PCR reaction is displayed in Table 2.

| PCR Reaction                  |  
|-------------------------------|---
| 0.5 μl template DNA          | 5 μl 10x thermopol buffer |
| 1 μl primer #1               | 0.5 μl T`aq` polymerase    |
| 1 μl primer #2               | 41 μl dH₂O                  |
| 1 μl dNTPs                    |                            |

Table 2: Sample PCR reaction

A master stock was created for each PCR reaction in order to perform a total of six identical reactions. Once separated into 50 μl aliquots, the tubes were placed in the PCR machine. Denaturation occurred at 94° C for a total of 4 minutes. The reaction was then subjected again to 94° C for a period of 30 seconds. Annealing of the primers to the template DNA occurred at 60° C for a period of 30 seconds. Extension of the primers occurred at 72°C for 1 minute and 15 seconds via DNA Polymerase. Beginning with step two, this reaction cycled for a total of 33 cycles to allow for amplification of the desired sequence. A final step in the PCR reaction occurred for a period of 5 minutes at 72°C.
3.1.2 Vector and insert digestion, purification, and ligation

The PCR product was pooled and purified using the Qiagen PCR Purification Kit. Following purification, both the PCR product (insert) and the JK148 vector were digested with *Bam* HI and *Eag* I for a period of 2 hours at 37° C. Reaction measurements can be found in Table 3.

<table>
<thead>
<tr>
<th>Insert Digest</th>
<th>Vector Digest</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 μl purified PCR product</td>
<td>20 μl vector</td>
</tr>
<tr>
<td>10 μl 10x BSA</td>
<td>10 μl 10x BSA</td>
</tr>
<tr>
<td>10 μl NEB 3</td>
<td>10 μl NEB 3</td>
</tr>
<tr>
<td>1.5 μl <em>Eag</em> I</td>
<td>1.5 μl <em>Eag</em> I</td>
</tr>
<tr>
<td>1.5 μl <em>Bam</em> HI</td>
<td>1.5 μl <em>Bam</em> HI</td>
</tr>
<tr>
<td>27 μl dH₂O</td>
<td>57 μl dH₂O</td>
</tr>
</tbody>
</table>

Table 3: Digest reactions for both the insert and vector

Following digestion, both the vector and insert were gel purified. 20 μl of loading dye was added to each digest prior to administration to a 1% agarose gel. After separation via gel electrophoresis, the insert and vector were cut out and placed into separate 1.5 ml tubes for purification using the Qiagen Gel Extraction Kit. Once purified, a ligation reaction was completed in order to construct the plasmid. Concentrations for the insert and vector were determined based on band brightness. The vector + insert were incubated together at 65°C for a period of 5 minutes, and then placed on ice for 2 minutes. 1.5 μl of 10x ligation buffer and 1.0 μl of T4 ligase were added to the ligation. The remaining amount of reaction was
brought to a total volume of 15 μl with dH₂O. This reaction was incubated at room temperature for 2 hours.

3.2 Transformation and integration

3.2.1 E. coli transformation

Following completion of the ligation reaction, the plasmids were cloned into E. coli cells. A vial of E. coli was thawed on ice; 2 μl of the ligation reaction was added and mixed gently. The E. coli + ligation mixture was incubated on ice for 30 minutes and then heat shocked in a 42°C water bath for 30 seconds. The vial was then placed on ice for 2 minutes. 250 μl of SOC media was added to the reaction and placed in a 37°C incubator for 1 hour. Following incubation, the reaction was centrifuged (3 minutes/3500 rpm) and the supernatant was removed. The pellet was resuspended in 50 μl of new SOC media and the cell mixture was spread onto a pre-warmed plate supplemented with 100 μg/ml of the antibiotic ampicillin (LB AMP), and was incubated at 37°C overnight. Clones that grew on the LB AMP plates were streaked onto new LB AMP plates and were inoculated into 5 ml LB AMP stocks for overnight growth. Samples were mini-prepped using the Promega DNA Extraction kit to obtain the plasmid. Correct clones were confirmed by restriction digest and sequencing.
3.2.2 Integration into S. pombe

Correct reporter plasmids were linearized with \textit{Nru} I for integration into the wild-type \textit{S. pombe} genome. For the digest reaction, 10 \( \mu l \) of the plasmid, 5 \( \mu l \) of NEB 3, 5 \( \mu l \) 10x BSA, 1 \( \mu l \) \textit{Nru} I and 29 \( \mu l \) dH\(_2\)O were combined in a 1.5 ml tube and incubated at 37\° C for 2 hours. Wild-type cells (JW81) were grown in 5 ml YES media overnight at 31\° C. Cells were then grown to a stationary phase for an additional 4 hours at 31\° C in 50 ml fresh YES media. Following growth, the cell culture was centrifuged (3000 rpm/5 minutes) and the supernatant was discarded. The pellet was washed with 10 ml 1x lithium acetate solution (0.1 M LiAc, 10 mM TRIS, 1 mM EDTA), centrifuged, and the supernatant discarded. The remaining pellet was resuspended in any residual lithium acetate. 60 \( \mu l \) of the cell suspension was combined with 7 \( \mu l \) of the linearized reporter plasmid and incubated at 31\° C for 15 minutes. Concurrently, salmon sperm DNA (10 mg/ml) (ssDNA) was boiled in a water bath for 10 minutes and placed on ice for 2 minutes. 7 \( \mu l \) ssDNA and 320 \( \mu l \) of 40\% PEG/LiAc solution were added to the cell solution and incubated again at 31\° C for 15 minutes. Following incubation, the solution was heat shocked at 42\° C for 15 minutes and centrifuged (3500 rpm/2 minutes). The supernatant was discarded and cells were resuspended in 800 \( \mu l \) 0.5x YES media. Cells were grown with shaking for 45 minutes at 31\° C. After growth, the cells were again centrifuged (3500 rpm/2 minutes) and resuspended in 150 \( \mu l \) 1x TE (10mM TRIS, 1 mM EDTA). This resuspension was then plated on EMM-leucine plates and grown in a 31\° C incubator.
Because the JK148 plasmid contains a leucine nutritional marker, correct transformants were screened based on their ability to grow on EMM-leucine plates. These transformants were additionally screened for beta-galactosidase activity under low-zinc growth conditions.

3.3 Beta-galactosidase assays

Reporter assay strains were grown in 5 ml cultures of YES media for ~16 hours at 31°C. After growth, 500 μl of the cell culture was used to inoculate 5 ml of new YES media for 6 hours to reach and maintain exponential growth. The cultures were centrifuged (3500 rpm/5 minutes) and the supernatant discarded. Cell cultures were washed in 5 ml zinc-limiting EMM (ZL-EMM), vortexed, and centrifuged (3500 rpm/5 minutes). The supernatant was again discarded, and the cells were washed for a second time in ZL-EMM. 500 μl of the cell solution was added to both the low (ZL-EMM) and high (ZL-EMM+ 200 uM Zn) zinc growth conditions (5 ml). These cultures were grown in a 31°C incubator for ~16-18 hours.

The cells were then centrifuged (3500 rpm/5 minutes) and the supernatant was discarded. Cells were washed in 5 ml of cold lacZ buffer (16.08 g Na₂HPO₄*7H₂O, 5.5 g NaH₂PO₄*H₂O, 10 ml 1.0 M KCl stock, 1 ml 1.0 M MgCl₂ stock). Cells were again centrifuged (3500/5 minutes), the supernatant discarded, and were resuspended in 5 ml cold lacZ buffer.
In order to measure cell density, 250 μl of cells were added to 750 μl dH₂O in a cuvette, and the density was measured at a wavelength of 600 OD using a spectrophotometer. Following measurement, 2 ml of cells were transferred to a pre-chilled 15 ml tube and placed on ice. 50 μl of 0.1% SDS and 50 μl of chloroform were added to each tube, which were then vortexed for 10 seconds to mix.

Once mixed, 1500 μl of each cell solution was divided evenly into 3-1.5 ml tubes. At time 0, 100 μl of chilled 4 mg/ml ortho-Nitrophenyl-β-galactoside (ONPG) was added to the first tube and vortexed. ONPG was then added to the remaining tubes at 10-second intervals. Once all the tubes received ONPG and a yellow color was observed, each reaction was stopped by the addition of 250 μl of 1M Na₂CO₃. This was carried out in the same 10-second interval fashion. Tubes were centrifuged (12000 rpm/1 minute). To analyze the beta-galactosidase activity, 600 μl from each tube was added to a cuvette for measurement at an OD of 420.

### 3.4 Site-directed Mutagenesis

Site directed mutagenesis uses constructed synthetic oligonucleotide primers in order to construct a deletion or mutation at a specific point in a DNA sequence. A PCR reaction was set up to begin site directed mutagenesis of the adh4 promoter.
This reaction included 5 μl reaction buffer, 2 μl dsDNA plasmid template, 1 μl 10 μM primer #1, 1 μl μM primer #2, 1 μl dNTPs, and 39.5 μl ddH₂O. 1 μl of PfuTurbo DNA polymerase was added after mixing. Each reaction was placed in the PCR machine. The first cycle lasted 30 seconds at 95°C for denaturation. The second part of the cycling included three different time and temperature settings. The first part included a 30 second cycle at 95°C, followed by one minute at 55°C, and 68°C for 12 minutes. These three time and temperature settings were cycled a total of 18 times. Following the PCR reaction, each tube was treated with 1 μl of the restriction enzyme Dpn I and was gently mixed. This restriction enzyme is used to digest the parenteral (non-mutated) DNA generated in the PCR reaction step. The digestion reaction was incubated at 37°C for 1 hour. After the digestion occurred, transformation into E. coli was carried out in a similar fashion as with the reporter plasmid construction. 3 μl of the digestion was added to a vial of E. coli cells and incubated on ice for 30 minutes. The solution was then heat shocked at 42°C for 30 seconds and placed on ice for 2 minutes. 250 μl of SOC media was added and the reaction was incubated at 37°C for 1 hour. Following incubation, the reaction was centrifuged (3 minutes/3500 rpm) and the supernatant discarded. 50 μl of new SOC media was used to resuspend the pellet, which was spread onto a LB AMP plate and incubated at 37°C overnight. Resulting clones were screened via sequence analysis for correctness and later integrated into wild-type S. pombe cells.
Chapter 4

Results

4.1 Transcriptional Regulation

Both the adh4-lacZ and vel1-lacZ reporter plasmids were integrated into a wildtype strain (JW81) and a strain containing the loz1-1 mutation to test for transcriptional regulation. The loz1-1 allele is a recessive mutation that leads to loss of zinc sensing and constitutive expression of all zinc-regulated genes. In normal wildtype cells, adh4 and vel1 are expressed in zinc-limiting conditions and are repressed during zinc-replete conditions. As a result of the loz1-1 mutation, adh4 and vel1 are expressed in both zinc-limiting and zinc-replete conditions.

In wildtype cells, it was observed that the adh4-lacZ and vel1-lacZ reporter strains displayed β-galactosidase activity in zinc-limited conditions. In the loz1-1 background, the zinc-responsive regulation of the adh4-1400-lacZ and vel1-lacZ reporters was lost (Figure 5). As controls, the levels of JK148 and sod1-lacZ were examined.
JK148 is the empty vector and served as a negative control. *Sod1-lacZ* served as the positive control, as it is not regulated by zinc levels and is therefore constitutively expressed in both conditions.

Figure 5: *LacZ* analysis of various constructs under high (+) and low (-) zinc conditions in both wildtype and *loz1-1* mutant strains.

The *adh4*-promoter fusion that was generated contained both the *adh4* promoter and 5’ untranslated region (5’ UTR). Because 5’ UTRs can contain regulatory elements (36), a new construct was generated in which the *adh4* 5’ UTR was
replaced with the \( nmt1 \) 5’ UTR (\( nmt1{-}\text{adh4-lacZ} \)). When the activity of the fusion was compared to the \( \text{adh4-1400-lacZ} \) construct in a wild type strain, it was noted that the fusion displayed a similar type of regulation as the \( \text{adh4-1400-lacZ} \) construct (Figure 6).

![Graph showing LacZ analysis of \( \text{adh4-1400-lacZ} \) and \( \text{adh4-nmt1-lacZ} \) fusions to determine if regulation occurred at the promoter or the 5’ UTR.]

Figure 6: LacZ analysis of \( \text{adh4-1400-lacZ} \) and \( \text{adh4-nmt1-lacZ} \) fusions to determine if regulation occurred at the promoter or the 5’ UTR.
4.2 Promoter Mapping

4.2.1 Mapping of the adh4 promoter

To map the precise location of the ZRE in the adh4 promoter, a series of truncated reporters were generated and introduced into a wild-type strain. Figure 7 displays the complete adh4-lacZ data for the truncated reporter fusions that were generated. It was noted that the adh4-500-lacZ and the adh4-1400-lacz construct both displayed β-galactosidase activity under zinc-limited conditions but not under zinc-replete conditions. Further truncations were created in order to find sites where this regulation was lost. The adh4-759-lacZ construct lost regulation. However, regulation was regained in the adh4-500-lacZ, adh4-449-lacZ, and adh4-432-lacZ constructs. This regulation was again lost with further deletions (adh4-420-lacZ, adh4-352-lacZ, and adh4-206-lacZ) and restored in shorter deletions (adh4-93-lacZ and adh4-35-lacZ). JK148 and sod1-lacZ served as negative and positive controls, respectively.
4.2.2 Mapping of the vel1-promoter

*Vel1*, another gene known to be under zinc dependent regulation within *S. pombe*, was also mapped in the same promoter-truncation method used to locate the ZRE in *adh4*. Truncated promoter plasmids were integrated into a wildtype (JW81) yeast strains.
strain and assessed via beta-galactosidase assays. JK148 once again served as a negative control with no beta-galactosidase activity and sod1-lacZ served as the positive control, as it is constitutively expressed regardless of zinc levels. The full-length vel1-lacZ promoter reporter construct produced beta-galactosidase during periods of zinc deficiency (Figure 8). However, this production severely decreased in the presence of zinc. Additionally, vel1-600-lacZ, vel1-385-lacZ, and vel1-252-lacZ produced similar results, with higher beta-galactosidase activity during periods of zinc deficiency. The vel1-148-lacZ construct produced minimal beta-galactosidase regardless of cellular zinc status. However, this production did not match the levels produced in both conditions for the sod1-lacZ positive control.

Figure 8: Mapping data for the vel1 promoter in a wildtype S. pombe strain
CHAPTER 5

Discussion

5.1 Conclusions

Currently, there is no known sensor of zinc deficiency in higher eukaryotes. The only known sensor, Zap1, is found in the budding yeast \textit{S. cerevisiae}. This transcription factor is responsible for the regulation of zinc-responsive genes during periods of zinc deficiency. Zap1 carries out this function by binding to zinc responsive elements found in the promoter regions of these genes. The fission yeast \textit{S. pombe} displays similarity to \textit{S. cerevisiae} in the genes regulated by zinc; however, there is no Zap1 homolog present. Therefore, \textit{S. pombe} was utilized as a model organism to identify how certain are genes regulated during periods of zinc deficiency.

We hypothesized that \textit{adh4}, a gene that encodes a mitochondrial alcohol dehydrogenase, was transcriptionally regulated during periods of zinc deficiency. If \textit{adh4} was under transcriptional control, there should be production of mRNA during
periods of zinc deficiency and none during periods of zinc repletion. However, if
*adh4* were post-transcriptionally regulated, mRNA would be produced regardless of
cellular zinc status. The mRNA would then be degraded in times of zinc repletion
but kept intact during zinc deficiency. Translating this to the β-galactosidase assays,
if the reporter constructs produced β-galactosidase during periods of zinc deficiency
but not during periods of zinc repletion, it could be concluded that the specific gene
of interest was transcriptionally regulated. However, if β-galactosidase was
produced under both conditions, there would be evidence to support the role of
post-transcriptional regulation.

When the *adh4* and *vel1* reporter constructs were integrated into a wildtype yeast
strain, β-galactosidase was produced in zinc-limiting conditions only. However,
when these reporter constructs were integrated in the *loz1-1* mutant strain, β-
galactosidase was constitutively expressed in both conditions. The data therefore
suggests that both the *adh4* and *vel1* promoter contain sequences necessary for
zinc-responsive regulation of gene expression when expressed in the wildtype
strain. This zinc responsive regulation is lost in the *loz1-1* mutant strain, further
supporting the hypothesis that that *adh4* and *vel1* are both regulated at the
transcriptional level in response to zinc.
To further test this hypothesis, a new construct containing the \textit{adh4} promoter and \textit{nmt1}-5' UTR was constructed. The new construct was compared to the original \textit{adh4} reporter construct containing the \textit{adh4}-5'UTR. This was done in order to distinguish between transcriptional regulation occurring at the promoter itself or at the 5' UTR of \textit{adh4}. Both reporter constructs displayed a similar amount of zinc-dependent \(
\beta\)-galactosidase activity, indicating that the regulation maps to the promoter region of \textit{adh4} and is independent of the 5' UTR. This further helps to support the role of zinc-dependent transcriptional regulation.

In order to map the location of the zinc-responsive element in the \textit{adh4} promoter, a series of promoter truncations were constructed and analyzed through \(
\beta\)-galactosidase assays. Zinc-dependent regulation was observed in a number of constructs, including the full-length \textit{adh4}-1400-lacZ, \textit{adh4}-500-lacZ, \textit{adh4}-449-lacZ, \textit{adh4}-432-lacZ, \textit{adh4}-93-lacZ, and \textit{adh4}-35-lacZ constructs. However, a loss of regulation is observed in the \textit{adh4}-759-lacZ, \textit{adh4}-440-lacZ, \textit{adh4}-352-lacZ, and \textit{adh4}-206-lacZ constructs. While the data shows the presence of transcriptional regulation along the \textit{adh4} promoter, loss of regulation occurs after nucleotide 432 and is regained at nucleotide 93.

Our lab has established that there is a repressor present to facilitate this regulation; however, the mapping data seems to suggest that a zinc-regulated activator is also present. We have hypothesized several models to explain the behavior of the \textit{adh4} promoter.
The first hypothesis states that a repressor is bound somewhere along the \textit{adh4} promoter regardless of cellular zinc status (Figure 9). This could explain the repression of the \textit{adh4} gene during periods of zinc-repletion. When cellular zinc becomes deficient, a zinc-responsive activator binds to promoter to facilitate activation of \textit{adh4} transcription, overriding the bound repressor. With subsequent deletions, the activator-binding site is removed, causing the loss of zinc-responsive regulation in periods of low zinc. The problem with this model is that the deletion of the repressor leads to constitutive expression – if there is a zinc responsive activator, zinc-dependent regulation should still occur.

![Diagram](image)

**Figure 9:** Theory 1-Zinc dependent activator binds to promoter during zinc deficiency to activate transcription
The second theory suggests that the repressor does not itself bind to the DNA but to the activator in times of zinc repletion, inhibiting its activity, which represses the transcription of \textit{adh4}. However, in times of zinc deficiency, the activator is not bound by the repressor and is able to bind to the promoter to promote transcription of the \textit{adh4} gene (Figure 10). The promoter truncations therefore would have deleted the binding site for the activator, explaining the loss of regulation in the shorter truncations.

\begin{center}
\includegraphics[width=\textwidth]{figure10.png}
\end{center}

\textbf{Figure 10: Theory 2- Repressor binds to the activator in times of zinc repletion}
The last theory suggests that the repressor and activator binding sites overlap on the \textit{adh4} promoter. This would explain the regulation occurring with the \textit{adh4-432-lacZ} construct and the abrupt loss of regulation at the \textit{adh4-420-lacZ} construct. By deleting the overlapping activation site, the zinc-dependent transcriptional regulation is lost, allowing the repressor to inhibit transcription of \textit{adh4} (Figure 11).

![Diagram of activator (A) and repressor (R) binding sites on the \textit{adh4} promoter with and without zinc (Zn) ions]

Figure 11: Theory 3- Activator and repressor binding sites overlap

Promoter truncations were also conducted for \textit{vel1}, another gene under zinc-dependent regulation. As stated previously, data suggested that \textit{vel1} is under transcriptional zinc-dependent regulation in a wildtype strain. Mapping data for the \textit{vel1} promoter is much less complex than that of \textit{adh4}. Zinc-dependent
regulation is maintained through the vel1-252-lacZ construct, and is then lost at the vel1-148-lacZ construct. This leads us to believe the ZRE in the vel1 promoter lies between nucleotides 252 and 148. This also suggests that vel1 is under the same type of zinc dependent transcriptional control as adh4, another zinc regulated gene.

5.2 Limitations

To provide a fine resolution of the zinc-responsive element in the adh4-promoter, we intended to utilize site-directed mutagenesis to generate deletions in the 449-420 nucleotide region. Three sets of primers for the deletions were constructed, including 449-440, 438-420, and 429-420. However, attempts to transform the deletions into E. coli were unsuccessful, and therefore we were not able to construct deletion strains in S. pombe. Because this was unsuccessful, we were unable to develop a fine resolution of the zinc-responsive element. Therefore, we continued to map the designated area with another reporter construct, adh4-432-lacZ.
5.3 Future Studies

Results from our experiments show that *adh4*, as well as *vel1*, are under zinc-dependent transcriptional control. While a repressor has been established to function in this regulation, the identity of the factor necessary for gene activation is unknown. The data from this study however supports the existence of a transcriptional activator. Future studies to map a consensus will aid in the identification of additional target genes and further our understanding of how Loz1 regulates gene expression in response to zinc.
References


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