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INVESTIGATION OF THE DNA-BINDING PROPERTIES OF *DROSOPHILA* ADULT ENHANCER FACTOR-1 ZINC FINGER PROTEIN

A Major Qualifying Project Report

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ABSTRACT

Adult enhancer factor-1 (Aef-1) is a 4 finger DNA-binding zinc finger protein which functions as an adult alcohol dehydrogenase gene (*Adh*) repressor in *Drosophila*. During larvae development it also plays a role in decreasing expression of the *Adh* gene. This project used mutagenesis of the Aef-1 gene and the bacterial one-hybrid system to examine DNA binding specificity and recognition of the Aef-1 zinc fingers. The intent of this project was to investigate the importance of each zinc finger, and binding specificity of a 3 finger Aef-1 construct.

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BACKGROUND

Structure and Function of Cys_2His_2 Zinc Finger Proteins

Cys_2His_2 zinc finger proteins are a family that contain multiple cysteine and/or histidine residues that interact with a zinc cation. The CysHis residues are stabilized by a single zinc ion, which causes the finger protein domain to fold around it (Berg et al., 1997). Proteins that contain this domain are very common in eukaryotic organisms because of their ability to bind to DNA, RNA, and other proteins (Mackay et al., 1998). The focus of this MQP is to investigate the DNA-binding activities of various mutants of the *D. melanogaster* zinc finger protein adult enhancer factor-1 (Aef1). The study of zinc fingers is an important step in understanding how genes can be regulated. The ability to select and design fingers with specific binding sites may prove to be a useful tool for the study of gene regulation and possibly for gene therapy.

Transcription Factor-IIIA (TFIIIA) is a Cys_2His_2 zinc finger domain protein that contains nine tandem repeats of an approximately 30 amino acid long motif (Wolfe et al., 2000). The consensus sequence for this type of zinc finger was discovered to be (Phe/Tyr)-X-Cys-X_{2-5}-Cys-X_3-(Phe/Tyr)-X_5-ψ-X_2-His-X_{3-5}-His, where X represents any amino acid, and ψ is a hydrophobic residue (Wolfe et al., 2000). The two cysteines and two histidines (underlined in the above sequence) interact with zinc to cause the protein to fold into a ββα domain (Figure 1) (Wolfe et al., 2000). Each finger binds a single zinc ion that is held between the two anti parallel β-sheets and the α-helix. The zinc ion

(shown as a sphere in the figure) is positioned between two cysteines at one end of the β-sheets and two histidines in the C-terminal portion of the α-helix.

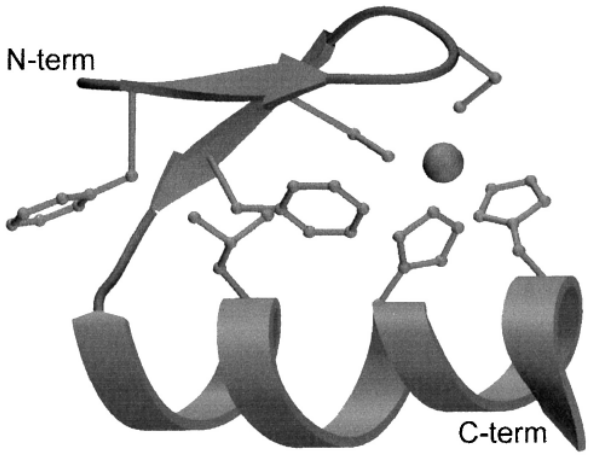

Figure 1: Diagram of the ββα domain motif from finger 2 of the Zif268 (Wolfe et al., 2000).

Again Cys_2His_2 zinc finger proteins are very common in eukaryotes. They are found in animals, plants, and fungi. The number of fingers and length of the linker between the fingers differ between the kingdoms (Wolfe et al., 2000). The affinity and the specificity can also vary greatly making these proteins very useful for gene regulation. Several human zinc finger proteins have been isolated. The human genome may encode hundreds of zinc finger binding proteins (Hueber et al., 1991). Interestingly, the Cys_2His_2 zinc finger protein motif is not naturally found in the genome of *Escherichia coli*, but potential zinc finger-like proteins have been identified in other bacteria (Claudia et al., 2002). This suggests that even though the motif is not commonly found in bacteria it is not completely foreign to prokaryotes.

Protein/DNA Binding and Recognition

The Cys_2His_2 zinc finger motif typically requires 2 to 4 tandemly arranged zinc fingers, and when only one finger is present additional secondary structures are found that assist in the binding of DNA (Wolfe et al., 2000). Zinc fingers can cooperatively work with other DNA binding proteins, or can bind DNA with sufficient specificity and affinity to function independently. The affinity for DNA increases as the number of zinc fingers increases, but tends to drop after three fingers. This may be due to conformational changes in the DNA caused by the zinc fingers. In humans, zinc fingers may play a role in DNA and/or RNA binding (Hueber et al., 1991).

Zif268 is a well characterized 3 zinc finger protein used as a model for DNA binding and recognition. It is a mammalian transcription factor originally discovered in mice, and has a distinct pattern of expression in the brain. Its induction has been shown to be associated with neuronal transcriptional activity (Knapska et al., 2004). Zif268 binds DNA by way of the α-helix of each finger fitting into the major groove of DNA, and the binding of successive fingers causes it to wrap around DNA (Wolfe et al., 2000). Proteins that contain three tandem fingers, each making two or more base contacts, tend to dock in a manner similar to Zif268 (Wolfe et al., 2000). Most zinc fingers studied have a similar binding arrangement to Zif268 even though there are other known binding patterns.

Each finger of Zif268 typically interacts with a 3 base pair segment of the primary strand of DNA (Figure 2). Finger 1 of Zif268 binds at the 3' end (lower right of the figure) and finger 3 binds at the 5' end (upper right of the diagram). Each finger binds DNA with a similar docking pattern. The amino acids at positions -1, 3, and 6 of the

helix in finger 3 (R, E, R) are positioned so that they make contact with the primary strand of the DNA. The amino acid at position 2 (D) interacts with a base on the complementary strand and one base downstream of the 3 base pair site that the finger sits on (Figure 2). This pattern of the amino acid at the 2nd position contacting one base pair upstream and on the complimentary strand can be seen in each finger. Each of the fingers contains an Aspartic Acid and an Arginine at positions 2 and -1 respectively (Wolfe et al., 2000).

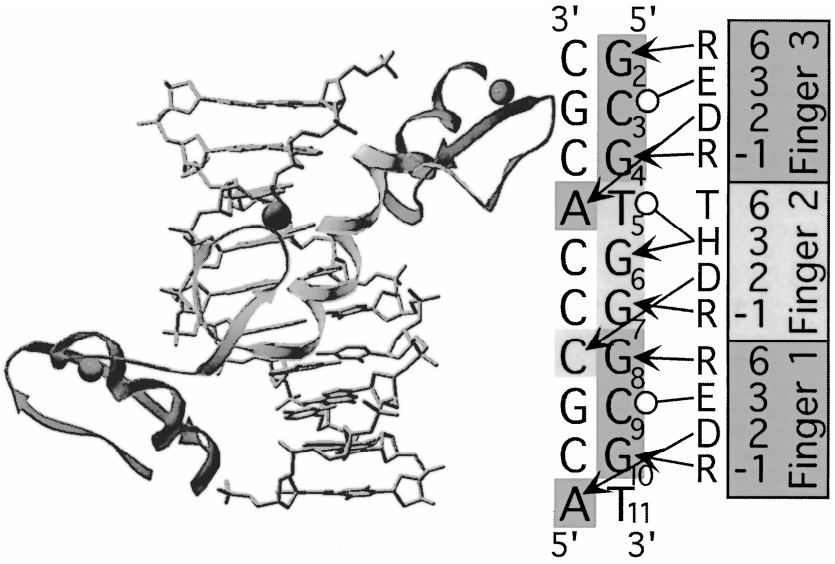

Figure 2: Zif268 DNA binding motif and the base contacts of the 3 fingers (Wolfe et al., 2000).

The fingers in a Cys_2His_2 zinc finger protein are connected by a linker region which controls the spacing between each finger. A common linker arrangement has five residues between the final histidine of one finger and the first conserved aromatic of the next finger (Wolfe et al., 2000). The length of the linkers plays a role in the binding affinity of the fingers, and the linker can be changed to manipulate the binding affinity of the fingers.

Selection and Prediction of Zinc Finger Binding Sites

The ability to select and design fingers with specific binding sites may prove to be a useful tool in the future of biology and medical research. Research shows that taking fingers from different proteins and/or rearranging them can change the binding affinity and specificity of the fingers. Several methods have been developed that allow for selection of zinc fingers. Many zinc finger proteins are designed by phage display which I a sequential selection protocol that successively selects one finger at a time to create a new zinc fingers.

Predicting zinc finger DNA binding specificity is as important to the study and development of zinc finger technology as designing and selecting fingers. Being able to accumulate the data generated from selecting and examining different zinc fingers will allow for quicker design and investigation of zinc fingers through the study of patterns and motifs. Predicting the binding specificity zinc fingers is based on the fact that certain residues in the recognition helix tend to contact specific bases on DNA. Through structural studies, a recognition code has been developed that can be used to predict what a zinc finger might bind to in context. However the code can only help predict what bases the fingers will contact because of side chain-side chain interactions, water-mediated contacts, and changes in the docking arrangement (Wolfe et al., 2000).

Bacterial One-Hybrid System

The bacterial one-hybrid (B1H) system is a system for determining the DNA-binding specificity of transcription factors. This system is similar to the yeast one-hybrid system which can determine DNA-binding specificity. The B1H system provides an

advantage over the yeast one-hybrid system in that it can obtain a higher transformation efficiency, which allows libraries containing more than 100-fold greater complexity to be searched (Meng et al., 2005). The B1H system contains three components: the transcription factor expression vector, a library of randomized binding sites in the reporter system, and the bacterial strain. In the original bacterial one-hybrid system, developed by the Wolfe lab, the DNA-binding domain of the transcription factor is expressed as a fusion with the α-subunit of RNA polymerase (Figure 4). The α-subunit is required for *E. coli* to survive, but for this project an ω-subunit fusion was used. The ω-subunit fusion was developed and provided by Wolfe lab. It has been shown that this fusion yields less of a background of breakthrough cells and is a stronger promoter. This may be because the ω-subunit is not required for the cells to survive.

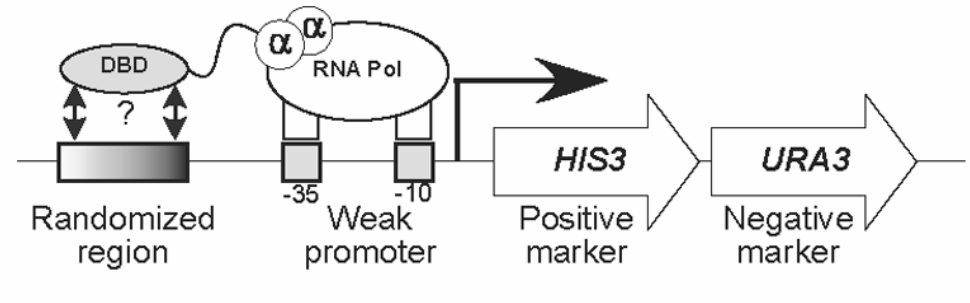

Figure 4: A schematic of the bacterial one-hybrid system with an α-subunit fusion. In this project an ω-subunit fusion was used (Meng et al., 2005). Binding of the zinc finger protein (fused to α-pol to DNA increases expression of His3 positive marker, and URA3 selection marker.

The reporter vector contains a 28 base pair RNA pol binding site upstream of the promoter of the two reporters. The reporters are the yeast HIS3 and URA3 genes. The reporter genes allow for positive and negative selection respectively. If a DNA-binding domain, "the bait," recognizes a target site, "the prey," in the reporter vector, RNA polymerase will be recruited to the promoter and the reporter genes will be activated.

When the cells are grown on minimal medium containing 3-amino-triazole (3-AT), a competitive inhibitor of HIS3, selection for an active promoter is observed. Selection with medium using 5-flouro-orotic acid (5-FOA) that is converted into a toxic compound by uracil provides selection against an active promoter (Meng et al., 2005). This negative selection step is useful when not using an already purified library; however a purified library provided by the Wolfe lab was used for this project. Reporter vectors identified in a library screen as positive for a zinc finger DNA-binding site can be isolated by selecting for increasing levels of HIS3 expression (Figure 5). From this screen, a series of positives can be picked and sequenced so a "sequence logo" can be developed. The logo shows the preferred binding domain for a particular protein. However, the sequence logo is a base off of a cumulative analysis of many clones. The more colonies sequenced the more accurate the logo becomes.

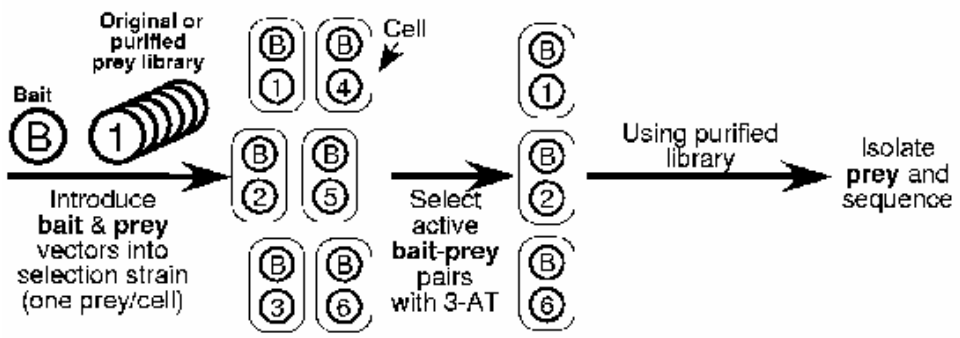

Figure 5: The B1H selection system using a purified library (Meng et al., 2005).

Adult Enhancer Factor-1

Aef-1 is a zinc finger transcriptional repressor that plays a role in the stage specific regulation of the alcohol dehydrogenase (*Adh*) gene. The *Adh* gene helps to control alcohol levels in flies during larvae and adult stages. The gene has two tandem

promoters. The distal promoter is active in late third instar larvae and adults, while the proximal promoter is active during embryogenesis and early larval development (Ren et al., 1998). The promoters are turned on by the *Adh* adult enhancer (AAE) and the *Adh* larval enhancer (ALE) respectively. *Drosophila's* natural environment especially during development consists of fermenting plant materials. *Drosophila melanogaster* often encounters high levels of ethanol in its environment, and is well equipped to deal with the toxic effects of ethanol. Ethanol becomes used both as an energy source and for lipid biosynthesis (Guanier et al., 2003).

A possible function of Aef-1 in adult flies is to decrease but not shut off the activity of the AAE (Ren et al., 1998). The level of Aef-1 mRNA is highest in adult flies but low in the late embryo and larval stages (Ren et al., 1998). This may be because fly larvae can be found in fruits which have a high level of alcohol and once it develops into an adult it isn't exposed to as much alcohol. The Aef-1-binding site in the AAE and in two other *Drosophila* fat body enhancers overlaps a sequence recognized by the mammalian transcription factor CCAAT/enhancer-binding protein (Falb et al., 1992). These two proteins also bind to overlapping sites in a liver specific regulatory element of the human *Adh* gene (Falb et al., 1992). Thus, the role of the Aef-1 DNA element in regulating enhancer activity is highly conserved in evolution.

DNA Binding Activity of Aef-1

Aef-1 contains four zinc finger repeats that each recognizes nucleotides ACA. The recognition helix of each finger contains a glutamine, serine, threonine, and asparagine at the -1, 2, 3, and 6 position respectively (Figure 6).

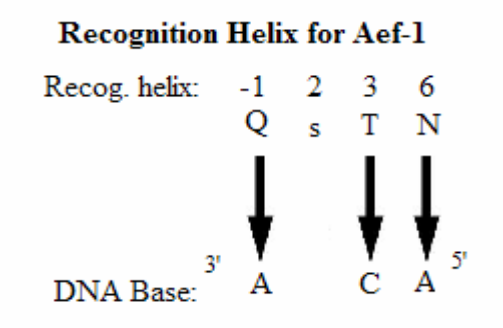

Figure 6: The recognition helix for each of the four zinc fingers of Aef-1.

The glutamine interacts with an adenine on the 3' end, and the asparagine interacts with an adenine on the 5' end. Threonine interacts with the cytosine in between. Thus the preferred binding domain for Aef-1 is ACAACAACAACA with a cytosine one base downstream of the actual site (Figure 7).

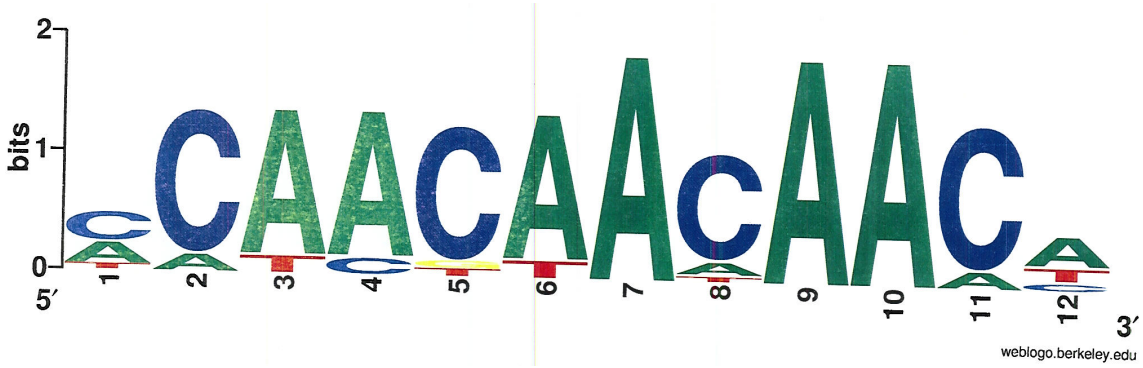

Figure 7: Sequence logo of the Aef-1 DNA binding protein (http://weblogo.berkeley.edu/)

Zinc fingers play an important role in gene function and regulation. Much has been learned through biochemical studies about the structure and function on them in biology. Efforts have been made to design fingers using information about known interactions. Many of the methods use the Zif268 DNA binding motif as a model for

designing proteins with DNA-binding specificity. It is important to develop a database of fingers that recognize specific sites in context. The Aef-1 protein is a good model to study because it is highly conserved in nature. The 3 finger constructs may also prove to be a very good 3 finger ACA binding proteins and give valuable information about how Aef-1 binds in context.

PROJECT PURPOSE

In an attempt to investigate the DNA binding properties of Aef-1, this project used mutagenesis to create various 3 finger proteins from the 4 finger Aef-1. In one construct, finger 4 of Aef-1 was removed so that only fingers 1-3 remain. In a second construct, finger 1 was removed so that only fingers 2-4 remain. Two constructs, Aef-1 gateway and Aef-1(1-4), which both contain all 4 fingers, were used as positive controls. They should both bind effectively to the Aef-1 binding site and generate a strong His-3 selection on 3-AT plates. Original "odd" is a zinc finger protein that contains a frame shift mutation in the first finger that doesn't allow it to bind DNA. It was used as a negative control in the experiments. The constructs were built and provided by the Wolfe lab. These fingers were then analyzed using an activity assay with different binding sites to directly analyze binding activity, and the bacterial one-hybrid system to identify the preferred DNA binding sites that the 3 finger constructs prefer.

The activity assay looked at 4 different DNA binding sites: ACAACAACAA, ACAACAACAC, ACAACAACAACA, and a negative control multiple cloning site (MCS). The ACAACAACAA binding site has adenine at the 3' end while the ACAACAACAC binding site has a cytosine at the 3' end. Since finger 1 docks on the 3' end of the binding site changing the base on this end allows for analysis of which base it may prefer one base downstream of the binding site. The ACAACAACAACA binding site gives insight as to what each of the 3finger constructs bind in the natural Aef-1 binding site. There is a cytosine one base downstream in the ACAACAACAACA binding site. This is important because the ACAACAACAA and ACAACAACAC site

will show which base one base downstream of the binding site is more efficient for gene activation. The MCS site is a control sight that should show no binding. The data showed that finger 1 may prefer an adenine at the 3' end when the 4th finger isn't present. When the 4th finger was present it seemed that a cytosine is preferred at the 3' end as was the case for the Aef-1 constructs containing fingers 1-4 or fingers 2-4.

Using the bacterial one-hybrid system further analysis was done that showed the specific binding of the constructs in a sequence logo. This will allow for analysis of how the protein might bind in context and what base finger 1 most likely prefers. The logos showed finger one selecting an adenine but further colonies would need to be sequence in order to get better resolution. This data shows the importance of each zinc finger, and binding specificity of the 3 finger Aef-1 constructs.

METHODS

Medium

NM medium was composed of 50 ml of M9 salts supplemented with 0.50 ml of 10 μM ZnSO4, 100 μM $CaCl_2$, 1 μM $MgSO_4$, 10 mg/ml thiamine, 5 ml of 20 mM adenine, 5 ml of 20 mM Uracil (left out in the selection media), and 5 ml of an amino acid mixture (containing all amino acids except cysteine, methionine, and histidine). For the recovery 1.75 ml of the NM medium was add to 8.25 ml of sterile H_20 in order to dilute it along with 100 μl of 10% Histidine and 50 μl of a 10 mg/ml stock solution of tetracycline. Medium containing just the minimal components, no antibiotics, histidine or IPTG was used for titering. For the plates, 87 ml of the medium was mixed with 400 ml of bacto-agar. and 1 to 15 mM 3-AT. 100 μl of a 25 mg/ml stock solution of kanamycin (the selection marker for the binding site plasmids), 100 μl of a 100 mg/ml stock solution of carbencillin (the selection marker for the Aef-1 plasmids), and 50 μl of 100 μM IPTG were also added to the plates.

Single Site Activity Assay

Single colonies were picked from previously grown *E. coli* double transformed with the Aef-1 plasmid constructs and the binding site plasmids clones containing the HIS3 URA3 reporters. Overnight cultures were set up in 2xYT medium containing both 100 μl of 25 mg/ml kanamycin and 100 μl of 100 mg/ml carbencillin along with 50 μl of 10 mg/ml tetracycline and 100 μM IPTG. 50 μl subcultures were put in 5 ml of the liquid medium, and grown for 1.5 hours at 37ºC.

The cells were pelleted in a table top centrifuge at speed is 18,000xg and then recovered in enriched NM liquid medium. After the NM recovery, the cells were transferred to 1.5mL eppendorf tubes and spun down for 2 minutes on maximum speed. The cells were washed four times with sterile water by spinning them down at 18,000xg and the media was siphoned off. After the wash, the cells were pelleted and resuspended in 150 µl of unenriched NM medium. The resuspended cells were titered on the NM plates in eight 5 µl drops at 10 fold dilution steps, beginning with a 10 fold dilution at the first drop. The constructs were plated in duplicate so that there were two rows of each. The cells were titered on plates that contained increasing amount of 3-AT.

Binding Site Selections

Electro-competent cells transformed with each plasmid construct (bait) and the purified (prey) library were grown in SOC medium for one hour at 37ºC. The cells were pelleted in a table top centrifuge at speed is 18,000xg and then recovered in enriched NM liquid medium. The cells were washed four times with sterile water and once with NM medium. They were then resuspended in NM medium and plated on positive selection plates containing 5 and 10 mM 3-AT. Approximately 4 x 10^7 cells were plated for each construct. The cells were allowed to grow for 48 hours at 37ºC or until well-defined colonies were visible on the plates. Colonies were isolated and PCR was used with the library primers to amplify the 28bp library. The DNA was purified by QIAGEN QIAquick PCR purification columns and sent for sequencing.

The sequences were analyzed using Ape –A Plasmid Editor. Overrepresented binding site sequence motifs within the randomized region of the isolated prey were

identified using an MEME algorithm (http://meme.sdsc.edu/meme/website/meme.html). Sequence logos were generated from the aligned sequences representing each of the overrepresented motifs using WebLogo server (http://weblogo.berkeley.edu/).

RESULTS

Single Site Activity Assay

The positive control plates (Figure 8) for the single site activity assay showed each construct growing since there is no selective pressure from 3-AT in these cultures. Each of these plates contained histidine which also allowed the cells to grow despite whether the constructs activate the reporter or not (Figure 8). This allows you to get a rough estimate of how many cells were plated which can be compared with how many cell survived at the higher stringencies of 3-AT.

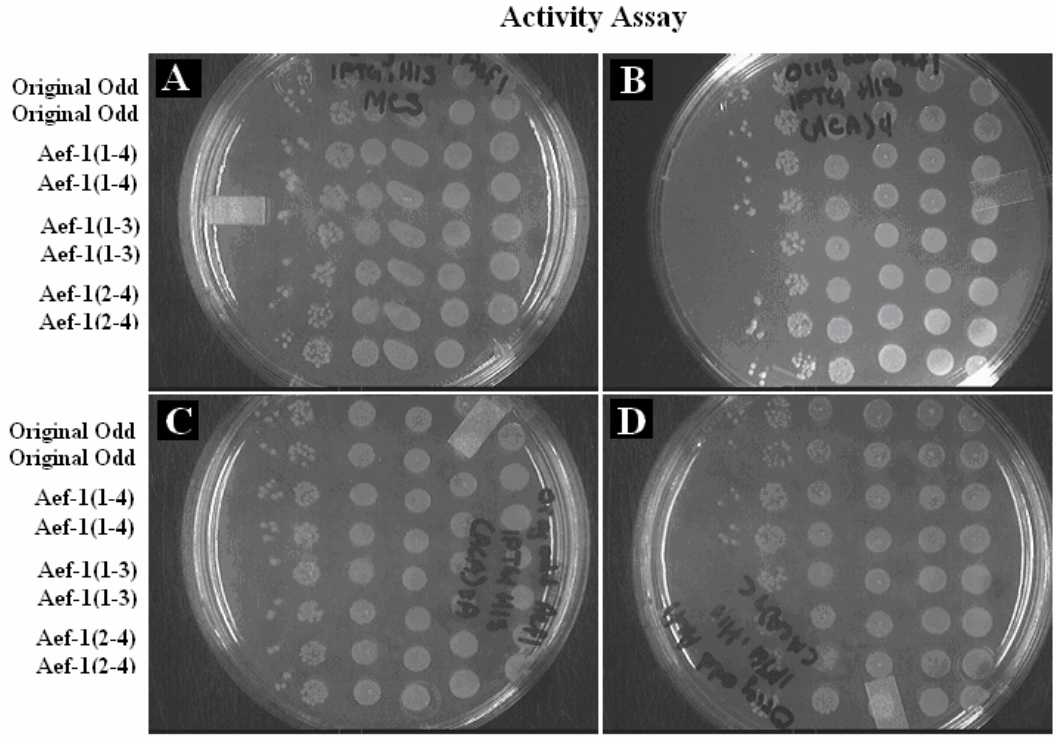

Figure 8: The HIS Positive Control Plates. The cells should grow on each binding site despite activation. A) This is the negative control binding site. B) This is the ACAACAACAACA known Aef-1 binding site. C) This is the ACAACAACAA binding site. D) This is the ACAACAACAC binding site.

The cells were titered on plates that contained increasing amounts of 3-AT to show how strong the constructs bound the sites (Figure 9). A digression of survival should be seen on the plates as the 3-AT concentration increases. The multiple cloning site (MCS) as a "binding site" was used as a negative control; none of the proteins showed activation for this site. The ACAACAACAACA binding site (Panel B) is the known binding site for Aef-1. Aef-1(1-4) and Aef-1(2-4) both showed similar activation on this site while Aef-1(1-3) showed poor survival with increasing amounts of 3-AT. This pattern was consistent for each of the binding sites. For the ACAACAACAA binding site (Panel C) Aef-1(1-3) increased in survival. All of the constructs seemed to have the lowest affinity for the ACAACAACAC binding site (Panel D). For each binding site, "original odd" a negative control protein, did not show any activation (Figure 9).

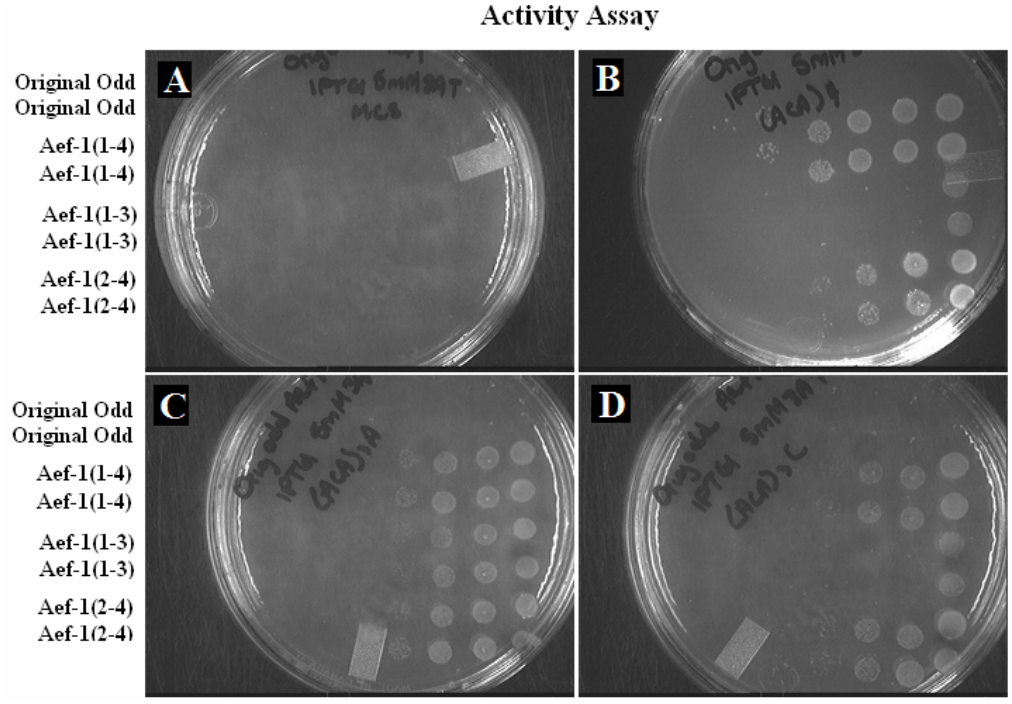

Figure 9: This shows the 5mM 3-AT plates. A) This is the MCS negative control binding site. B) This is the ACAACAACAACA known Aef-1 binding site. C) This is the ACAACAACAA binding site. D) This is the ACAACAACAC binding site.

Binding Site Selections

For the binding site selection plates (Figure 10), approximately 4 x 10^7 cells were plated on 10 mM 3-AT selection plates. Very few colonies grew on the negative control "original odd" plate (Panel A). The colonies that did grow on this plate were small and isolated. Approximately 3800 colonies grew on the positive Aef-1(1-4) control plates (Panel B). Approximately 1760 colonies grew on the Aef-1(1-3) plates (Panel C), and 2352 colonies grew on the Aef-1(2-4) plates (Panel D). These were both over the background.

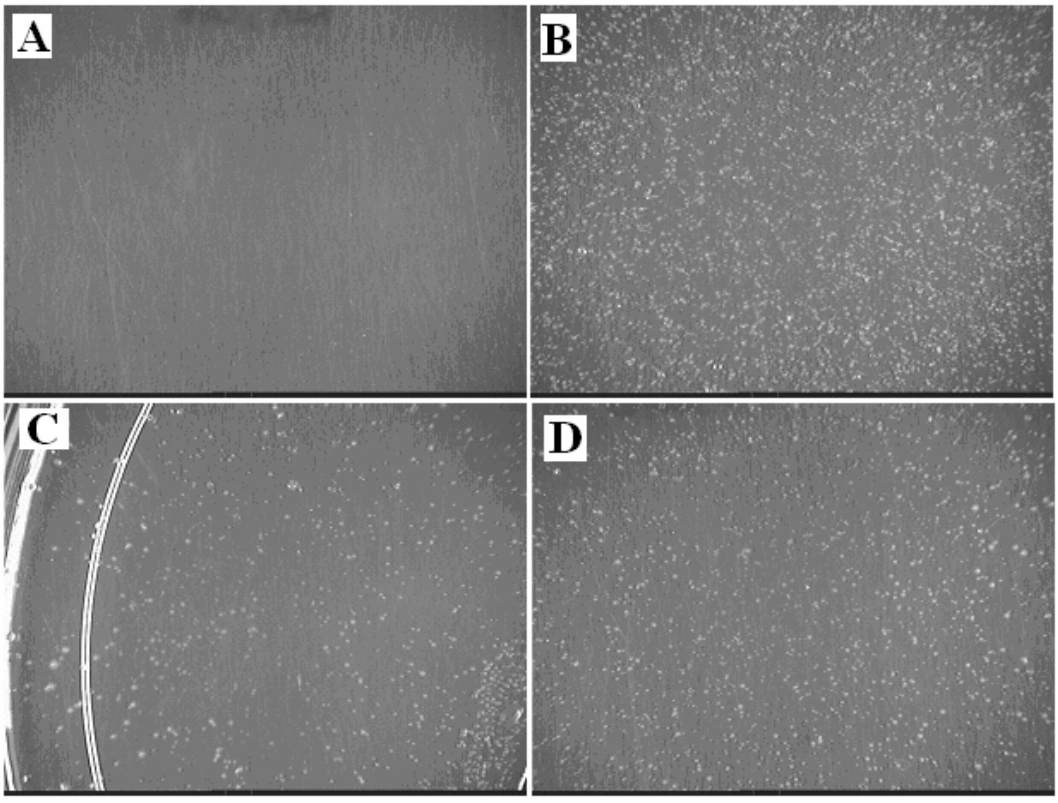

Figure 10: This shows the selection plates for each construct transformed with the 28bp library at 10 mM 3-AT. A) This is the original "odd" negative control plate. Very few colonies grew on this plate and they were small. B) This is the Aef-1(1-4) construct. C) This is the Aef-1(1-3) construct. D) This is the Aef-1(2-4) construct. Each construct grew over background.

The sequence logo developed from the sequenced library for Aef-1(1-3) showed that finger 1 preferred an adenine at the 3' end. 11 out of 12 sequenced colonies selected an adenine (Figure 11).

Figure 11: Sequence logo of the Aef-1(1-3) DNA binding protein (http://weblogo.berkeley.edu/)

The sequence logo developed from the sequenced library for Aef-1 (2-4) (Figure 12) showed that finger 2 may prefer a cytosine at the 3' end. 7 out of 9 sequenced colonies selected a cytosine (Figure 12). Unfortunately more sequences would need to be done in order for the data to provide more resolution of the binding site.

Figure 12: Sequence logo of the Aef-1(2-4) DNA binding protein (http://weblogo.berkeley.edu/)

DISCUSSION

The generation of novel DNA binding zinc finger domains of defined specificity is important to the future of this technology. The characterization of zinc finger domains developed for the recognition of ACA DNA binding sites is important for increasing understanding of the recognition codes and how they apply to the many naturally occurring zinc finger proteins of unknown function. Attempts to design zinc fingers that recognize this ACA binding site have shown in many cases that the residues not expected to make base-specific contacts actually had effects on specificity. The three helical positions −1, 3, and 6 of a zinc finger domain are insufficient to allow for the fine specificity of the DNA binding domain to be predicted. Finger position also seems to be as important as a recognition code for residues.

The data from this project shows that when the 4^{th} finger is present, Aef-1 repressor protein is specifying for a cytosine at the 3' end of the binding site. However when that 4^{th} finger is removed, the Aef-1 specifies for an adenine at the 3' end. This may be the reason for the variability of the base at this end. So while many of the amino acids found in a designed protein at the key contact positions −1, 3, and 6 are those that are consistent with a simple code of recognition, the optimal specific recognition is sensitive to the context in which these residues are presented. Also the position of the finger in context plays a significant role as to what the protein will bind. The Aef-1 protein is a good model for how ACA binding proteins bind in context.

The activity assay showed the Aef-1(1-4) and the Aef-1(1-3) displayed similar survival on each binding site. At the 3' end of the ACAACAACAACA binding there is a

cytosine, and it makes sense that Aef-1(2-4) shows similar survival for these binding sites since the logo shows the construct specifying for a cytosine at this end. The data showed that finger 1 may prefer an adenine at the 3' end when the 4th finger is not present. The activity assay showed that Aef-1(1-3) survived better on the ACAACAACAA site, while it did not survive well on either of the other two sites. This may indicate that the 4th finger plays a significant role in binding affinity. The variability in specificity of finger 1 may be what allows Aef-1 to be used as a transcriptional element in more than one *Drosophila* enhancer.

Many transcription factors like Aef-1 and other regulatory proteins that interact with DNA contain zinc fingers. The application of transcription factors is wide spread in the genome. It is important to develop a database of how transcription factors have been shown to bind DNA to aid our understanding of a recognition code. The study of zinc fingers is an important step in understanding how genes can be regulated. The ability to select and design fingers with specific binding sites may prove to be a useful tool for the study of gene regulation.

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