HETERODIMERIC TRANSCRIPTION FACTORS IN A YEAST ONE-HYBRID ASSAY

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ABSTRACT

The study of spatiotemporal gene expression, especially in the context of gene regulatory networks, is greatly important for the complete understanding of the role gene regulation has in organism development and function. Transcription factors (TFs), key regulators of spatiotemporal gene expression, bind to specific *cis*-regulatory elements (CREs) and facilitate or repress the transcription of their associated gene. Some TFs can bind with other TFs to form heterodimers, resulting in a change in their target binding site. Heterodimer TFs have been implicated in a number of important gene regulatory networks and the study of them represents an area vital to a complete understanding of gene regulatory network dynamics. The yeast one-hybrid (Y1H) system is an important and powerful assay for the detection of protein-DNA interactions (PDIs) in a cell-based assay. However, the Y1H system has not yet been able to detect heterodimer PDIs. To achieve this, a modified Y1H system was developed using novel plasmid constructs to coexpress Gal4 activation domain (AD)-fusion *C. elegans* TF heterodimer pairs in existing and newly constructed bait reporter yeast strains. This project for the first time demonstrates robust Y1H reporter activation by heterodimeric TFs. The clones and strategies developed by this project provide a foundation to effectively screen dimerizing TFs for sequence-specific interactions as well as further optimize the assay for reliable results.

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INTRODUCTION

In the study of biology, there is a constant push to achieve a deeper understanding of how organisms develop and function. A key process to examine in this pursuit is the differential regulation of gene expression. Since complete genome sequences have become available to the scientific community there has been a concerted effort to determine exactly when and where genes are expressed. Such spatiotemporal expression is seen as integral to organism development, cell specificity, and responses to environmental stimuli. Indeed it is the ability to selectively activate genes that allows a multi-cellular organism to vary cell phenotype while maintaining the same genetic information. Eukaryotic systems in particular have a wide variety of methods to regulate gene expression. Chromatin remodeling (Li et al, 2007), epigenetics (Ahmed & Brickner, 2007), mRNA degradation (Garneau et al, 2007), and the regulation of protein synthesis (Sonenerg & Hinebusch, 2009) all impact the overall expression of a gene. While these methods of gene regulation have been shown to be vital to organism growth and function, the most studied and understood method of regulation of genes in eukaryotes involves transcription factors.

TRANSCRIPTION FACTORS

Transcription factors (TFs) are a diverse family of DNA-binding proteins that influence the transcription of genes into mRNA in eukaryotic cells. When one is discussing TFs, it is important to specify whether one is referring to general or site-specific TFs. General transcription factors

(GTFs) are required for RNA polymerase II recruitment to a gene and include such nomenclature designations as TFIIE and TFIIF. These TFs are recruited to a transcriptional start site by TATA-binding protein (TBP) that associates with the 5'-TATAAA-3' DNA sequence upstream of the start site. Site-specific TFs, rather than relying on the universal TATA sequence, recognize and bind to the specific *cis*-regulatory element of a gene. Once the TF is bound to its target region, it can recruit coactivators and GTFs to initiate transcription or repressing agents to prevent transcriptional machinery from binding. While there is a substantial amount of structural and functional variety in what constitutes an activating TF, two features remain constant: an activation domain (AD) and DNA-binding domain (DBD). The AD of a TF is what allows for RNA polymerase II, whether directly or through coactivators and mediators, to be recruited for transcription of its associated gene. An example of this is the widely used and well-characterized Gal4 TF, a protein important to the regulation of galactose metabolism through the glycolytic pathway (Traven et al, 2006). The crystal structure of Gal4 bound to DNA in Figure 1 shows the DBD anchored into the double helix of the DNA and the freely available AD.

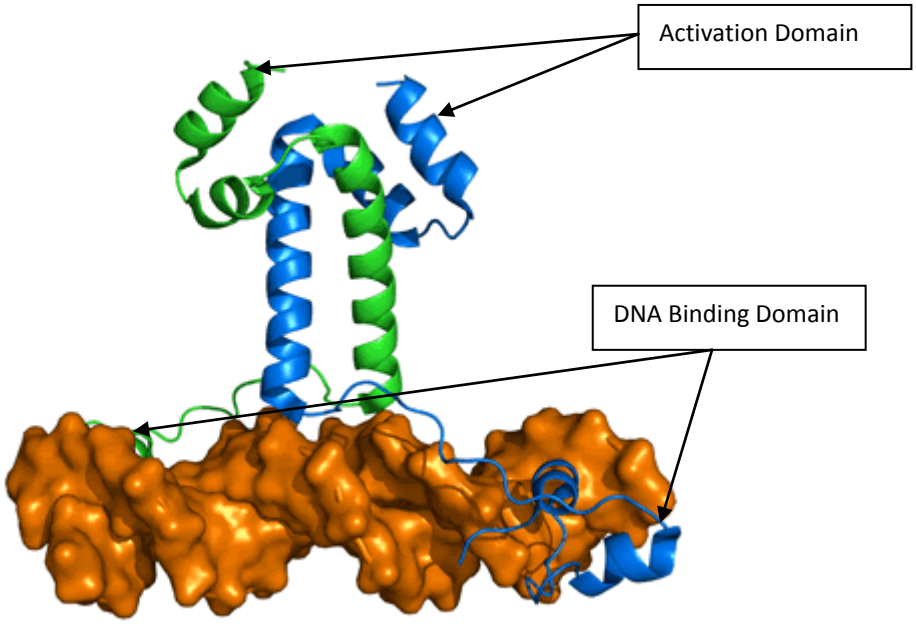

Figure 1. Gal4 TF Bound to DNA (Hong et al, 2008)

Gal4, under galactose inducing conditions, binds to a number of other proteins including mediator and TBP. This results in the recruitment of general TFs and subsequently RNA polymerase II to transcribe galactose metabolism genes. The DBD allows a particular TF to interact with a specific sequence of DNA based on the characteristics of the TF. These include a shape that allows them to have a significant surface area contact with their target DNA, and a DNA binding domain with specific-surface chemistry that allows the TF to interact both with the negatively charged phosphate backbone of DNA and makes sequence specific bonds with the DNA's bases.

In addition to being able to bind to DNA and activate transcription as a monomer, TFs have been shown to form homodimers (*i.e.* Gal4) and heterodimers. Heterodimers are complexes made of two unlike proteins bound together, usually through hydrophobic interactions. Research into heterodimeric transcription factors has produced a significant amount of

information about their characteristics as complex regulators of gene expression. Heterodimeric TFs have been shown to be able to bind to different DBDs than that of their monomer components (Hai & Curran, 1991). Additionally, they can form out of not only functional monomeric TFs, but also TFs which could not otherwise bind DNA except as part of a dimer pair. The *C. elegans* TF HLH-2, for instance, does not have a strong DNA binding affinity alone. When it is coupled with another TF such as HLH-10 however, its affinity DNA greatly increases (Grove et al, 2009). Heterodimerizing monomers also do not necessarily just form one heterodimer complex. A single monomer can interact with a number of other TFs to form heterodimers that bind to completely different DNA sequences and activate different genes. All of this adds levels of complexity to TF mediated gene regulatory networks. If one considers the most recently established number of TFs in *C. elegans*, 934 (Reece-Hoyes et al, 2005) and theorizes the possibility of each TF dimerizing with every other TF and then subtracts out the homodimers, one can see that there is an immediate increase from 934 to $(934)^2$-933 = 871,423 possible unique TF complexes through heterodimerization. Of course not every TF dimerizes, but it gives an idea of the multiple orders of magnitude potential increase in the complexity of TF-mediated gene regulation heterodimerization lends to.

Heterodimeric TF regulation has been implicated in a range of biological phenomena including viral infection (Cassetti & Moss, 1996), cell proliferation (Helin et al, 1993), and homeostasis (Wang et al, 1995). Among the families of TFs that have been shown to dimerize, the main families grouped by DNA binding domain are the basic region leucine zipper (bZIP), nuclear hormone receptor (NHR), homeodomains (HD), and basic region helix-loop-helix (bHLH). These families provide a basis to begin characterizing heterodimers. To do so, one

needs a method to study the interaction between the heterodimer and its DNA binding site - a method such as the yeast one-hybrid system.

THE YEAST ONE-HYBRID SYSTEM

In eukaryotic gene regulation, the protein-DNA interactions between transcription factors and their specific binding sites are highly important. A number of different methods have been developed to study them. These include gel-shift (Taylor et al, 2000), DNAse I footprinting (Leblanc & Moss, 2000), and chromatin immunoprecipitation (Kuo & Allis, 1999) assays. While these assays all allow for protein-DNA interaction detection, they do not have the benefits of cell-based assays that are increasingly important in understanding the implications of these interactions on a holistic level. To do this, scientists had to use a different live-cell based assaying method such as the yeast one-hybrid system.

The origins of the yeast one-hybrid (Y1H) system stems from its predecessor: the yeast two-hybrid system (Y2H). The two-hybrid system was first developed to detect protein-protein interactions by assaying for the merger of AD and DB-fusion proteins and their ability to activate a reporter gene (Fields & Song, 1989). At the heart of this assay lies the Gal4 TF. What makes Gal4 useful in this regard is that it consists of distinct and separable DB and AD domains. In order to test if two proteins interact, the proteins are translated with the Gal4 AD or DB attached to the N-terminus and are expressed in yeast containing the Gal4 binding site upstream of a reporter gene. A reporter gene in this context is simply a gene whose expression can be assayed for and will be addressed in greater detail later in this paper. Once expressed in

the yeast, the DB-fusion will bind to its Gal4 target sequence but not trigger transcription of the reporter gene due to the lack of the activation domain. Similarly, the AD-fusion protein could initiate transcription of the reporter gene but has no means by which to bind to an upstream sequence. If there is an interaction between the fusion proteins, the Gal4 AD and DB will reconstitute a functional Gal4 transcription factor and allow for transcription of the reporter gene.

The yeast one-hybrid system uses similar concepts to the yeast-two hybrid system, but is used to examine protein-DNA interactions rather than protein-protein interactions. It was first developed as a strategy to identify the gene that encodes the DNA-interacting subunit of the yeast origin recognition complex (Joachim & Herskowitz, 1993) and later adapted for higher throughput studies (Deplancke et al, 2004). Instead of expressing AD and DB-fusion proteins , a single AD- fusion protein is made and expressed in yeast containing a specific DNA 'bait' sequence cloned upstream of the reporter gene. If the AD-fusion protein interacts with a particular DNA bait of interest, then transcription of the reporter will be activated via the attached AD. This model is illustrated by Figure 2.

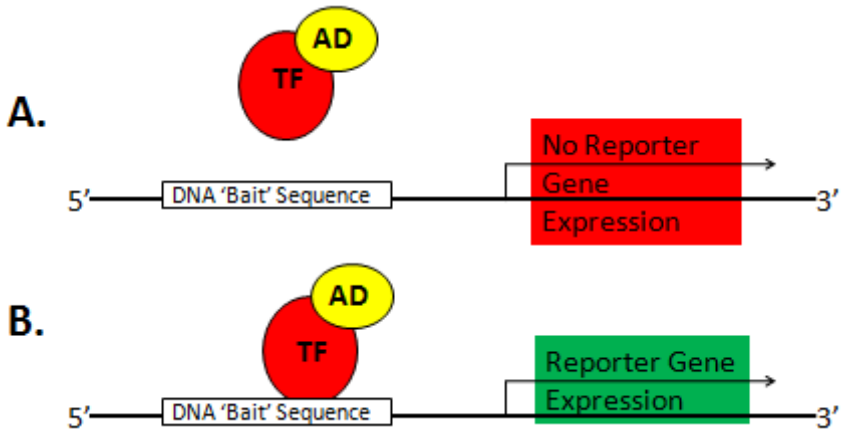

Figure 2. Yeast One-Hybrid System Schematic **A.** If there is no interaction between the bait sequence and the TF there will be no activation of the reporter gene. **B.** When the bait sequence and AD-fusion TF interact, the reporter gene is activated.

Similarly to the Y2H system, reporter genes are necessary in a yeast one-hybrid system to indicate the presence or absence of an interaction between the AD-fusion TF and the DNA bait sequence. This can be accomplished through a number of different reporter strategies including auxotrophic and color producing markers. A prominent color producing reporter gene used is the *E. coli* derived lacZ gene (Serebriiskii & Golemis, 2000). LacZ is part of the well characterized lactose operon that facilitates the expression of genes that allow for lactose metabolism in *E.coli*. The gene lacZ encodes for β-galactosidase (β-gal), an enzyme whose catalytic activity cleaves beta-glycosidic bonds. In order to assay for lacZ activation, one must in turn test for presence of β-gal. In order to avoid the complications that would arise by treating this as an auxotrophic marker and forcing the yeast to utilize lactose as a carbon source, a different strategy was developed. The chemical bromo-chloro-indolyl-galactopyranoside, or X-gal, functions as assayable substrate for β-gal (Serebriiskii & Golemis, 2000). Once digested, it is

cleaved into a galactose and a form of indole that once oxidized forms a dark blue precipitate. The consequence of this is that when X-gal is introduced to an environment containing β-gal, a blue color is produced. The presence or absence of blue in yeast cells introduced to X-gal can therefore be correlated to the expression of the lacZ reporter gene.

An example of an auxotrophic reporter is the HIS3 gene. The HIS3 reporter gene encodes for imdazoleglycerol-phosphate dehydratase, which facilitates the sixth step of the histidine biosynthesis pathway in yeast (Alifano et al, 1996). By using a yeast strain deficient in this vital enzyme, yeast will grow on media lacking histidine only if a supplemental copy of the gene is present and active in the yeast. When an AD-bound transcription factor binds upstream of HIS3 in the bait strain yeast, it activates production of the His3 protein. The production of the His3 protein allows the yeast to synthesize its own histidine once again. Simply assaying for the ability of the yeast to grow on histidine lacking media, however, does not accurately report AD-fusion upstream binding. There are inherent levels of self-activation for HIS3 within the yeast stain due to the presence of a minimal promoter upstream of the HIS3 reporter gene itself. Both HIS3 and lacZ have upstream minimal promoter, as yeast ADs require them to.

This is actually to the benefit of the assay for a number of reasons. First, it allows for HIS3 to serve the role as an auxotrophic selector for an intact reporter gene. Secondly, it allows yeast that do not have HIS3 activation via AD-fusion binding to still grow and be assayed for lacZ activity. In order to determine if there is AD-fusion mediated HIS3 activation, one must assay for the relative amount of the His3 protein it produces. One way to do this is by measuring the competitive antagonism of His3 with 3-amino-1,2,4-triazol (3AT). 3AT is an

organic compound that competitively inhibits His3 by occupying its active site. This is the same active site which catalyzes the sixth step in histidine synthesis. For yeast to exhibit growth on media containing 3AT, they must therefore produce a significantly greater concentration of His3 protein to produce enough histidine. This allows one to correlate the ability to grow well on 3AT-inclusive media to an activated HIS3 reporter gene. Using two reporter systems such as lacZ and HIS3 allows one to minimize the uncertainty of the results of one particular assay and serves to decrease background 'noise' when performing Y1H assays.

PROJECT CONTEXT

In the study of gene regulation in the nematode *C. elegans*, 934 transcription factors have been predicted and listed in wTF2.0 (Reece-Hoyes et al 2005). When combined with the creation of the *C. elegans* promoterome (Dupuy et al, 2004), this resource provides an unprecedented opportunity to completely map the TF-promoter interactions in *C. elegans*. The lab of Dr. Marian Walhout at UMass Medical School has undertaken that challenge by utilizing custom developed high throughput yeast one-hybrid techniques to individually screen each transcription factor against each promoter in *C. elegans*. A major issue associated with the project however is how to handle predicted TFs that do not show binding to any member of the promoterome. While there are a number of technical reasons why a certain TF may turn up as a false-negative in the assay, a possibility that is especially important to the TF interaction mapping is that some of the predicted TFs only bind to DNA as a heterodimer. This explains why Y1H assays that only test one monomeric TF against a DNA bait produces a negative result.

In order to test this, it has been proposed that those TFs that do not show up in the initial Y1H screening be tested for interaction with other non-binding TFs through Y2H assays. This gives a possible candidate pool for heterodimers that consist of non-binding monomers. These could then be tested as heterodimers against the promoterome again to map their binding. The only issue with this approach is that heterodimers have not been shown to be detectable in a Y1H assay before. This project aims to demonstrate through the utilization of well characterized heterodimeric TFs that the Y1H assay can detect heterodimeric TF site specific binding. Previous work in the lab has established a number of potential *C. elegans* heterodimeric TFs and their binding sites for possible use in study (Grove et al, 2009). Of these, the HLH-2/HLH-10 and MXL-1/MDL-1 heterodimers were chosen due to the availability of their clones, previously created compatible reporter bait strains, established data on their characteristics as heterodimers, and the availability of the heterodimer DNA binding sequence. The goal was to establish heterodimeric TF detection in both the lacZ and HIS3 reporters used by the lab in the Walhout Lab's Y1H studies and to troubleshoot possible issues that may arise in the transition from monomer/homodimers to heterodimer Y1H assaying.

MATERIALS AND METHODS

MXL-1/MDL-1 1X AND 3X BAIT STRAIN CREATION

To create the MXL-1/MDL-1 1XBS and 3XBS bait strains, entry clones containing a 1X and 3X repeat of the CCACGTGC binding sequence were created by annealing primers containing the target sequence flanked by the HindII or BamHI cut sites and utilizing those restriction sites

for ligation into the entry backbone pMW#4. This was transformed into DH5α *E.coli* cells and from that transformation three colonies were picked, cultured, plasmid prepped, and plasmids sequenced to verify the presence and integrity of the inserted binding site. Once the plasmids were verified for having the correct insert, the binding site was move from the pMW#4 entry vector into the HIS3 and lacZ Y1H reporter destination vectors via a Gateway (Hartley et al, 2000) LR reaction. These LR products were then transformed once again into DH5α, cultured, and plasmid prepped to yield MXL-1/MDL-1 1X and 3X Y1H reporter destination vector stocks. In order to create the final bait strains, the reporter bait strain plasmids were linearized through NcoI digestion for the lacZ vector and XhoI for the HIS3 vector and then transformed into the lab yeast strain designated 611. All yeast strains used in this project contain endogenous GAL4 and GAL80 deletions as well as mutations in URA3, HIS3, TRP1, and LEU2. The transformed yeast was grown on –UH media to select integrant colonies possessing both the URA3-containing lacZ reporter and HIS3 reporter construct, after which they were streaked for single colonies and tested for both lacZ and HIS3 self-activation (*i.e.* expression of reporters in the absence of a TF). The colony that demonstrated the lowest amount of self-activity for both reporters was then used as the MXL-1/MDL-1 1X or 3X bait strain in heterodimer Y1H testing.

Gap Repair Synthesis of pDEST-AD-LEU2 2μ

Gap repair synthesis of the pDEST-AD-LEU2 2μ vector first required the creation of a LEU2 PCR product with flanking sequences homologous to those flanking the TRP1 gene in the 2μ vector. LEU2 gap repair (GR) primers were created (see Appendix) and were used in PCR on

the pDEST-AD-LEU2 ARS/CEN vector. A gradient of annealing temperatures from 55°C to 72°C was used in the PCR, with those producing the most LEU2 GR product pooled for later use. In parallel to this, pDEST-AD-TRP1 2µ was digested with Bsu361 at vendor specifications and left to incubate overnight at 37°C overnight. Once both LEU2 GR product and digested pDEST-AD-TRP1 2µ were available, they were transformed via the via high efficiency transformation into lab yeast strain mAV-103 and mAV-203, plated on –L media, and allowed to grow for several days at 30°C. The transformed yeast was then streaked for single colonies, spotted onto –L plates, and then replica plated onto –L as well as –T plates. Those colonies that grew on –L but not –T were then subject to a yeast plasmid prep to extract the newly created pDEST-AD-LEU2 2µ vector. After verifying that the death cassette and LEU2 gene was intact in the plasmid through *E.coli* and yeast transformations, respectively, the vector was cultured and extracted from death cassette resistant DB3.1 *E.coli.* This yielded a stock of the final pDEST-AD-LEU2 2µ vector.

YEAST TRANSFORMATIONS

For Y1H transformations where one plasmid was transformed into the bait strain at a time, a low efficiency transformation method was used. To do this, the bait strain was first streaked out onto YEPD media and allowed to grow overnight in a 30°C incubator. A small amount (about one toothpick end full per three transformations) was washed in 1mL sterile dH_2O and then 1mL 10mM Tris-EDTA/ 0.1M Lithium (TE/LiAc) solution. After resuspending in 50µL TE/LiAc and 5µL 10mg/mL boiled salmon sperm DNA per transformation was added to the yeast and the solution was separated into 50µL aliquots. From here 100-200ng of the

transforming DNA and 300µL of TE/LiAc in 40% (w/v) PEG was combined with each aliquot and mixed thoroughly. Following a 30 minute 30°C air and 20 minute 42°C water bath incubation, the transformations were spun down briefly and their supernatant removed, allowing for resuspension in 50µL sterile dH_2O and plating on the appropriate auxotrophic yeast media. These plates were then allowed to incubate at 30°C for up to three days until distinct colonies had formed. If it was necessary to transform a second plasmid, the previously mentioned process was repeated using the transformed yeast as the bait strain. When the required plasmid combinations were transformed, up to 20 of these colonies were pooled and replated for Y1H reporter assaying.

In the event that the two AD-fusion plasmids were to be transformed in at the same time, as was done at the beginning of the project, a high efficiency transformation method had to be used. As with the low efficiency method, the yeast was first freshly streaked out and allowed to grow on the appropriately selecting media plate overnight. The bait yeast was then grown in a rich media YEPD sterile liquid culture, shaking at 30°C with a starting OD_{600} of 0.2 ± 0.02. After reaching an OD_{600} of 0.4-0.6, the culture was spun down and resuspended in sterile dH_2O at $1/5^{th}$ the original culture volume. At this point the rest of the transformation procedure mimics that of the low efficiency transformation with a few notable exceptions. First, the final TE/LiAc resuspension volume before adding salmon sperm DNA is determined by the following equation in order to maintain a consistent amount of yeast used per transformation with this method.

$$\frac{V_{Resuspension} = V_{Original\ Culture} \times OD_{600}}{100}$$

Additionally, both AD-fusion plasmids are added together before the TE/LiAC/PEG solution at 100-200ng each.

HIS3 AND LACZ ASSAYING

LacZ reporter assaying was conducted by first replica plating the Y1H transformed yeast onto a membrane filter placed over a rich media plate. After allowing to grow overnight at 30°C, the membrane was removed and immersed in liquid nitrogen to lyse the yeast and fix them to the membrane. The frozen membrane was then placed over X-gal containing solution soaked paper filters and incubated at 37°C until noticeable color was produced. HIS3 assaying was conducted by replica plating the Y1H transformed yeast onto 5mM, 10mM, 20mM, and 40mM 3AT plates and recording growth after 5 days of 30°C incubation.

RESULTS

Preliminary assaying for the plausibility of detecting heterodimers TF activation through the Y1H system was carried out using existing plasmid and bait strains created through previous projects in the Walhout lab. The *C. elegans* HLH-2/HLH-10 and MXL-1/MDL-1 heterodimers were chosen for this study because previous work showed that they do not bind strongly to DNA as monomers or homodimers, their binding site was already determined by PBM data, and the ready availability of clones and bait strains to conduct initial tests. Of particular importance in trying to prove that heterodimeric TF PDIs can be detected in a Y1H assay was finding a positive control for each binding site tested. This requires that one have a secondary TF that binds to the same sequence as the heterodimer. It was determined that the proteins CES-1 and HLH-30 were available and bound as monomers or homodimers to the HLH-2/HLH-10 and MXL-

1/MDL-1 binding sites respectively. For a negative control TF, the FLH-1 was used as its binding sequence is significantly different from that of either heterodimer. This allows one to determine if the TFs activate all the baits non-specifically, such as by binding the reporter vector backbone.

The HLH-2 and HLH-10 TF ORFs, AD-vectors and CES-1 1XBS bait strain were already available in the lab, so the HLH-2/HLH-10 heterodimer Y1H transformations were set up according to Table 1 for the preliminary Y1H pilot. In order to select for both monomer expressing vectors in the yeast, a Gateway-compatible 2µ AD-fusion destination vector with TRP1 auxotrophic selection (pDEST-AD-TRP1 2µ) and a Gateway compatible ARS/CEN AD-fusion destination vector with LEU2 auxotrophic selection (pDEST-AD-LEU2 ARS/CEN) were used. With selection for lacZ and HIS3 reporters driven by URA3 and HIS3 auxotrophic selection respectively, the heterodimer Y1H transformations were maintained on –UHLT media for the duration of their growth and assaying.

	Prey			**Prey**	
Bait	*TF*	*Vector*	**Bait**	*TF*	*Vector*
CES-1 1XBS	HLH-2	pDEST-AD-LEU2 ARS/CEN	FLH-1 1XBS	HLH-2	pDEST-AD-LEU2 ARS/CEN
	None	pDEST-AD-TRP1 2µ		None	pDEST-AD-TRP1 2µ
CES-1 1XBS	None	pDEST-AD-LEU2 ARS/CEN	FLH-1 1XBS	None	pDEST-AD-LEU2 ARS/CEN
	HLH-10	pDEST-AD-TRP1 2µ		HLH-10	pDEST-AD-TRP1 2µ
CES-1 1XBS	HLH-2	pDEST-AD-LEU2 ARS/CEN	FLH-1 1XBS	HLH-2	pDEST-AD-LEU2 ARS/CEN
	HLH-10	pDEST-AD-TRP1 2µ		HLH-10	pDEST-AD-TRP1 2µ
CES-1 1XBS	CES-1	pDEST-AD-TRP1 2µ	FLH-1 1XBS	CES-1	pDEST-AD-TRP1 2µ
	None	pDEST-AD-LEU2 ARS/CEN		None	pDEST-AD-LEU2 ARS/CEN
CES-1 1XBS	FLH-1	pDEST-AD-TRP1 2µ	FLH-1 1XBS	FLH-1	pDEST-AD-TRP1 2µ
	None	pDEST-AD-LEU2 ARS/CEN		None	pDEST-AD-LEU2 ARS/CEN
CES-1 1XBS	None	pDEST-AD-TRP1 2µ	FLH-1 1XBS	None	pDEST-AD-TRP1 2µ
	None	pDEST-AD-LEU2 ARS/CEN		None	pDEST-AD-LEU2 ARS/CEN

Table 1. Preliminary HLH-2/HLH-10 Heterodimer Y1H Experiment Design

The lacZ and HIS3 results from that experiment, as seen in Figure 3, showed that only the positive control (compare the negative controls in column 6 to column 4 for CES-1 1XBS and column 5 for FLH-1 1XBS) yielded significant reporter gene activation(*i.e.* blue color in the lacZ assay and growth in the HIS3 assay). As it had been previously shown via Y2H that HLH-2 and HLH-10 dimerize and through PBM data that the heterodimer they form binds to the specific bait sequence used in the CES-1 1XBS, coexpressing them should have resulted in activation (see column 3).

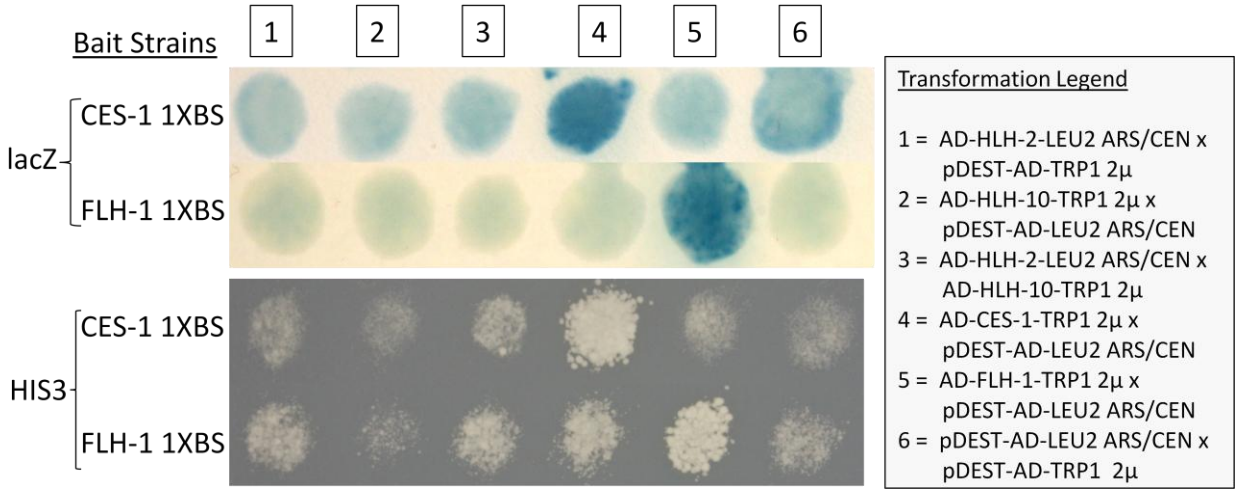

Figure 3. HIS3 and lacZ Assay Results from Initial Pilot Heterodimer Y1H Assay

After repeating the experiment with both high and low efficiency transformation techniques and varying amounts of plasmid initially transformed into the yeast, the results were the same. In order to explain the lack of activation of either reporter gene from the heterodimer, it was suggested that perhaps using vectors with significantly different copy numbers was affecting the outcome of the assay. The AD-TRP1 vector used in the experiment has a 2µ origin of replication, while the AD-LEU2 vector has an ARS/CEN origin of replication.

ARS/CEN vectors maintain only about 1-2 copies of itself in the yeast cell at any given time, resulting in any encoded protein having a much lower overall expression than those encoded by a plasmid that contains a much higher copy number within the cell. 2µ plasmids are bacterially-sourced high copy number plasmids that maintain 50-100 copies per cell (Veit & Fangman, 1988). By expressing both member proteins inside 2µ vectors, it was reasoned that similarly high amounts of plasmid would lead to equivalent amounts of protein produced. While it was not practical at the time to compare the relative amount of each protein being produced in the yeast to test this, it was decided that an AD-LEU2 2µ vector had to be created.

In order to create the AD-LEU2 2µ vector, the LEU2 gene from the existing ARS/CEN vector was swapped in for the TRP1 gene in the 2µ vector backbone. Although the most straightforward way to do this would have been to utilize restriction sites on either side of each gene, the vectors were not compatible in this respect. Instead, the LEU2 and TRP1 swap was facilitated by utilizing the homologous repair mechanisms within the yeast through a process known as gap repair. A PCR product consisting of the LEU2 gene from the ARS/CEN vector flanked by ends that are homologous to either side of the TRP1 gene in the 2µ vector was created. The 2µ vector was digested with Bsu361, resulting in a single cut within the TRP1 region. The digested TRP1 vector and LEU2 PCR product were then cotransformed into yeast. Homologus repair machinery within the yeast, recruited by the double strand break in TRP1, recognize the matching regions around the LEU2 and TRP1 genes, replacing the damaged TRP1 with the intact LEU2. This then allows the yeast to be selected for on leucine deficient media and the new pDEST-AD-LEU2 2µ to be extracted for use. A diagram summarizing the process can be found in Figure 4. The specific gap repair primers can be found in the Appendix.

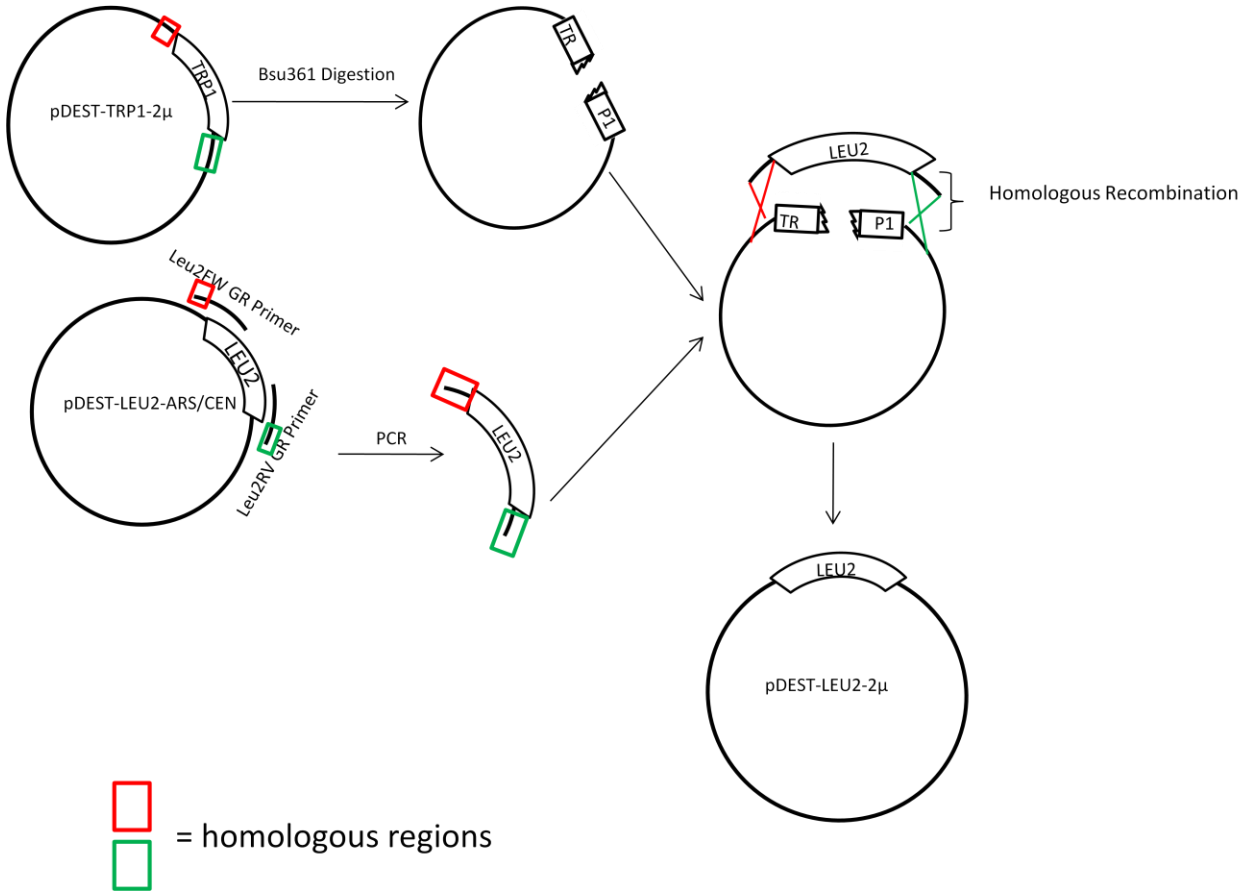

Figure 4. Gap Repair Construction of pDEST-AD-LEU2 2µ

After the completed pDEST-AD-LEU2 2µ vector was obtained, the heterodimer Y1H assay was repeated. This time the MXL-1/MDL-1 heterodimer was also included in the experiments as MXL-1/MDL-1 1X and 3XBS bait strains had been recently created. Additional promoter based reporter strains containing the heterodimer binding site were also added into the assay, with PB0507.1 containing the HLH-2/HLH-10 binding site and R13F6.5 containing the MXL-1/MDL-1 binding site. The setup of these heterodimer transformations can be found in Tables 2 and 3. Both high efficiency double transformations of both plasmids and two sequential low efficiency transformations of the monomer containing AD-vector were performed, but did not show different results. The final results, shown in Figures 5 and 6, are a

product of two sequential low efficiency transformations. This method was used consistently towards the end of the project due to its relatively easy and reliable procedure.

	Prey			Prey	
Bait	*TF*	*Vector*	**Bait**	*TF*	*Vector*
CES-1 1XBS	HLH-2	pDEST-AD-LEU2 2µ	PB0507.1	HLH-2	pDEST-AD-LEU2 2µ
	None	pDEST-AD-TRP1 2µ		None	pDEST-AD-TRP1 2µ
CES-1 1XBS	None	pDEST-AD-LEU2 2µ	PB0507.1	None	pDEST-AD-LEU2 2µ
	HLH-10	pDEST-AD-TRP1 2µ		HLH-10	pDEST-AD-TRP1 2µ
CES-1 1XBS	HLH-2	pDEST-AD-LEU2 2µ	PB0507.1	HLH-2	pDEST-AD-LEU2 2µ
	HLH-10	pDEST-AD-TRP1 2µ		HLH-10	pDEST-AD-TRP1 2µ
CES-1 1XBS	CES-1	pDEST-AD-TRP1 2µ	PB0507.1	CES-1	pDEST-AD-TRP1 2µ
	None	pDEST-AD-LEU2 2µ		None	pDEST-AD-LEU2 2µ
CES-1 1XBS	FLH-1	pDEST-AD-TRP1 2µ	PB0507.1	FLH-1	pDEST-AD-TRP1 2µ
	None	pDEST-AD-LEU2 2µ		None	pDEST-AD-LEU2 2µ
CES-1 1XBS	None	pDEST-AD-TRP1 2µ	PB0507.1	None	pDEST-AD-TRP1 2µ
	None	pDEST-AD-LEU2 2µ		None	pDEST-AD-LEU2 2µ
FLH-1 1XBS	HLH-2	pDEST-AD-LEU2 2µ			
	None	pDEST-AD-TRP1 2µ			
FLH-1 1XBS	None	pDEST-AD-LEU2 2µ			
	HLH-10	pDEST-AD-TRP1 2µ			
FLH-1 1XBS	HLH-2	pDEST-AD-LEU2 2µ			
	HLH-10	pDEST-AD-TRP1 2µ			
FLH-1 1XBS	CES-1	pDEST-AD-TRP1 2µ			
	None	pDEST-AD-LEU2 2µ			
FLH-1 1XBS	FLH-1	pDEST-AD-TRP1 2µ			
	None	pDEST-AD-LEU2 2µ			
FLH-1 1XBS	None	pDEST-AD-TRP1 2µ			
	None	pDEST-AD-LEU2 2µ			

Table 2. HLH-2/HLH-10 Heterodimer Y1H Experiment Design

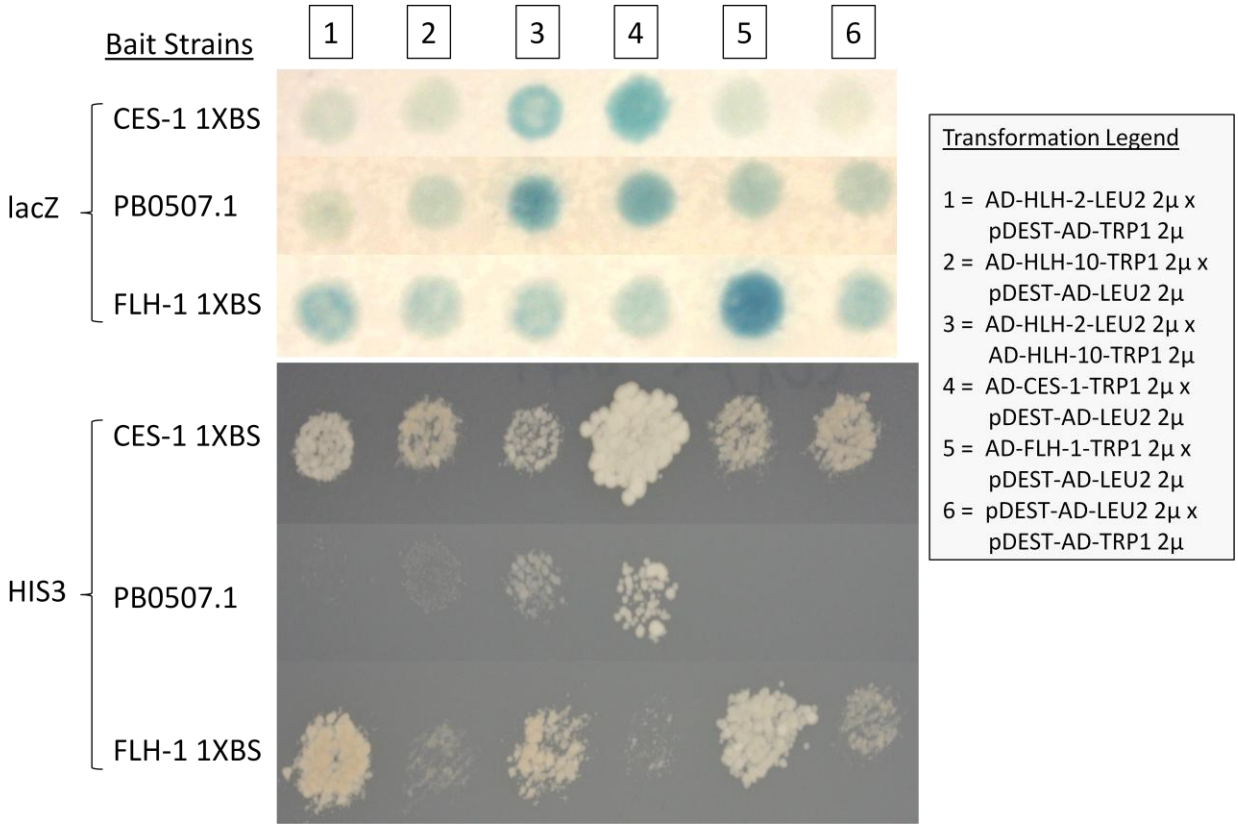

Figure 5. HIS3 and lacZ Assay Results for the HLH-2/HLH-10 Heterodimer Y1H Assay

As Figure 5 shows, in addition to the positive controls working as in Figure 3, HLH-2/HLH-10 clearly activates the lacZ reporter in both of its target bait strains when both proteins are expressed in 2µ vectors (compare column 3 to negative control column 6). The HIS3 reporter was only mildly activated in the PB0507.1 bait strain and no noticeable activation in could be detected in the CES-1 1XBS bait strain. The lack of FLH-1 1XBS lacZ activation by the heterodimer demonstrates that HLH-2/HLH-10 is interacting with the reporter in a bait sequence-specific manner. The FLH-1 1XBS HIS3 results suggest that HLH-2 binds as a monomer/homodimer (column 1 vs. column 6) and also that the HLH-2/HLH-10 heterodimer can bind. However, HLH-2 is known to be unable to bind to DNA alone (Grove et al, 2009) and

the predicted site for the HLH-2/HLH-10 heterodimer does not exist in the FLH-1 1XBS bait strain. While the apparently HIS3 activation by HLH-2 in FLH-1 1XBS is not readily explainable, FLH-1 1XBS is not a true negative control strain as HLH-2 was not specifically tested against it to verify a lack of binding. The fact that the lacZ assay does not agree with these HIS3 results suggests that they are technical false positives.

	Prey			**Prey**	
Bait	*TF*	*Vector*	**Bait**	*TF*	*Vector*
MXL-1/MDL-1 1XBS	MXL-1	pDEST-AD-LEU2 2µ	R13F6.5	MXL-1	pDEST-AD-LEU2 2µ
	None	pDEST-AD-TRP1 2µ		None	pDEST-AD-TRP1 2µ
MXL-1/MDL-1 1XBS	None	pDEST-AD-LEU2 2µ	R13F6.5	None	pDEST-AD-LEU2 2µ
	MDL-1	pDEST-AD-TRP1 2µ		MDL-1	pDEST-AD-TRP1 2µ
MXL-1/MDL-1 1XBS	MXL-1	pDEST-AD-LEU2 2µ	R13F6.5	MXL-1	pDEST-AD-LEU2 2µ
	MDL-1	pDEST-AD-TRP1 2µ		MDL-1	pDEST-AD-TRP1 2µ
MXL-1/MDL-1 1XBS	HLH-30	pDEST-AD-TRP1 2µ	R13F6.5	HLH-30	pDEST-AD-TRP1 2µ
	None	pDEST-AD-LEU2 2µ		None	pDEST-AD-LEU2 2µ
MXL-1/MDL-1 1XBS	FLH-1	pDEST-AD-TRP1 2µ	R13F6.5	FLH-1	pDEST-AD-TRP1 2µ
	None	pDEST-AD-LEU2 2µ		None	pDEST-AD-LEU2 2µ
MXL-1/MDL-1 1XBS	None	pDEST-AD-LEU2 2µ	R13F6.5	None	pDEST-AD-LEU2 2µ
	None	pDEST-AD-TRP1 2µ		None	pDEST-AD-TRP1 2µ
MXL-1/MDL-1 3XBS	MXL-1	pDEST-AD-LEU2 2µ	FLH-1 1XBS	MXL-1	pDEST-AD-LEU2 2µ
	None	pDEST-AD-TRP1 2µ		None	pDEST-AD-TRP1 2µ
MXL-1/MDL-1 3XBS	None	pDEST-AD-LEU2 2µ	FLH-1 1XBS	None	pDEST-AD-LEU2 2µ
	MDL-1	pDEST-AD-TRP1 2µ		MDL-1	pDEST-AD-TRP1 2µ
MXL-1/MDL-1 3XBS	MXL-1	pDEST-AD-TRP1 2µ	FLH-1 1XBS	MXL-1	pDEST-AD-TRP1 2µ
	MDL-1	pDEST-AD-LEU2 2µ		MDL-1	pDEST-AD-LEU2 2µ
MXL-1/MDL-1 3XBS	HLH-30	pDEST-AD-TRP1 2µ	FLH-1 1XBS	HLH-30	pDEST-AD-TRP1 2µ
	None	pDEST-AD-LEU2 2µ		None	pDEST-AD-LEU2 2µ
MXL-1/MDL-1 3XBS	FLH-1	pDEST-AD-TRP1 2µ	FLH-1 1XBS	FLH-1	pDEST-AD-TRP1 2µ
	None	pDEST-AD-LEU2 2µ		None	pDEST-AD-LEU2 2µ
MXL-1/MDL-1 3XBS	None	pDEST-AD-TRP1 2µ	FLH-1 1XBS	None	pDEST-AD-TRP1 2µ
	None	pDEST-AD-LEU2 2µ		None	pDEST-AD-LEU2 2µ

Table 3. MXL-1/MDL-1 Heterodimer Y1H Experiment Design

The MXL-1/MDL-1 results, shown in Figure 6, demonstrate strong heterodimer lacZ and HIS3 reporter activation in the 3XBS bait strain (compare column 3 to column 6). The 1XBS and PR13F6.5 bait strains however did not produce collaborative results. HLH-30 was intended to be a positive control for these experiments based on its ability to bind as a homodimer to the sites that occur within MXL-1/MDL-1 1XBS, 3XBS, and R13F6.5 baits. However, the results apparently disprove this prediction. The R13F6.5 bait strain's high self activity unfortunately did now allow for any reliable data to be collected from it. As for the 1XBS bait strain, the heterodimer did not activate but instead seemed to repress the lacZ minimal promoter. The inability of the FLH-1 TF to activate the FLH-1 1XBS bait strain suggests that the binding site may have sustained mutations in the course of this assay.

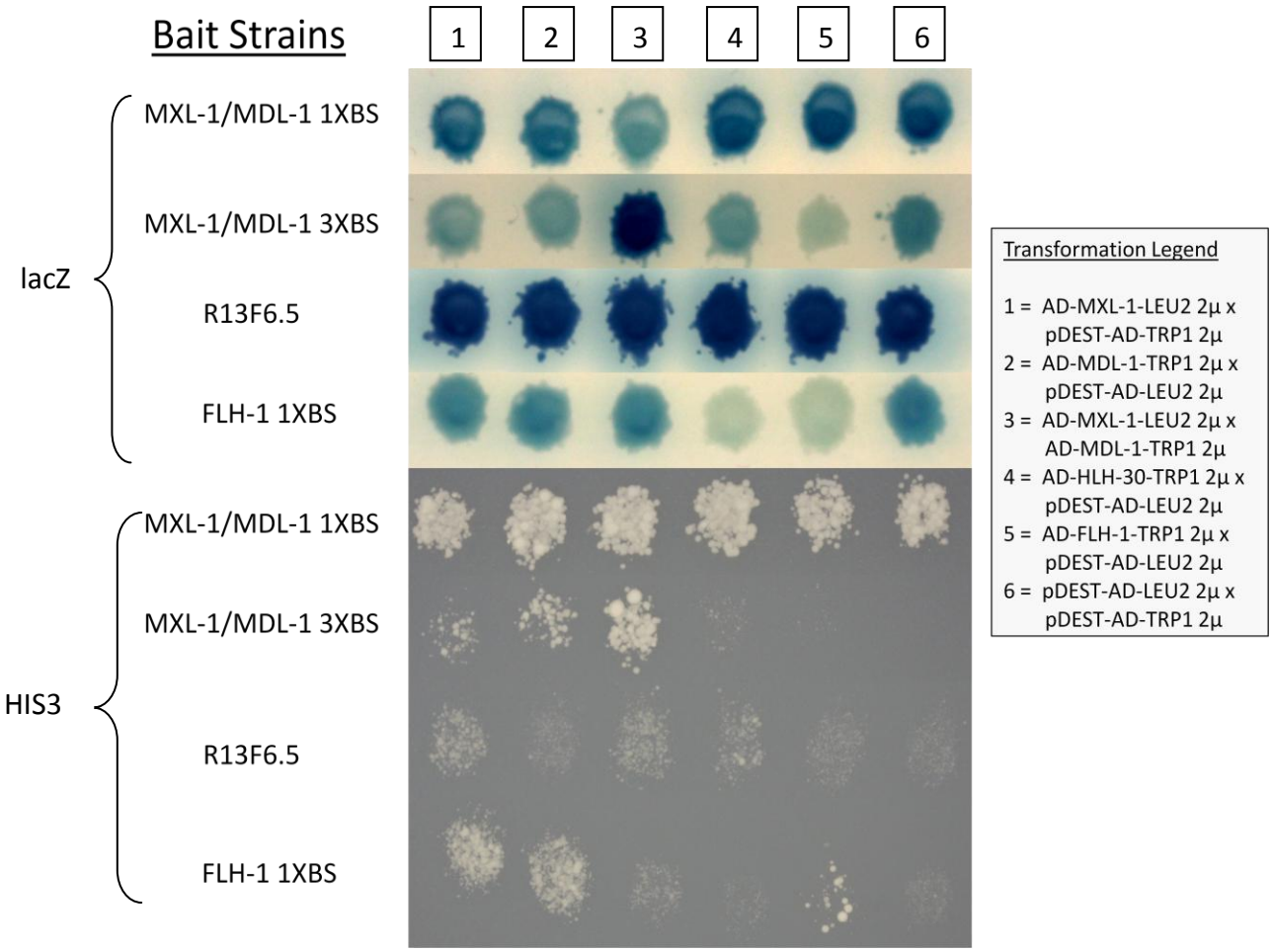

Figure 6. HIS3 and lacZ Results for the MXL-1/MDL-1 Heterodimer Y1H Assay

The HIS3 activation caused by both monomers and heterodimer caused in the MXL-1/MDL-1 3XBS bait strain could result from cross contamination between the MXL-1 and MDL-1 plasmid stocks. To test this, the plasmids were constructed from freshly prepared pDEST-AD vectors from bacterial glycerol stock and verified wild-type clonal entry vectors for the proteins that had been synthesized by an earlier project in the lab. Upon trying to transform the AD-MXL-1-LEU2 2µ vector into the yeast bait strains, they were now not able to grow on –L media. This is particularly puzzling as the other AD-vector clones created from the same destination vector stock did confer the LEU2 positive phenotype. Tests were done to verify that the pDEST-

AD-TRP1 2µ was not accidently used instead of pDEST-AD-LEU2 2µ by use of the Bsu361 digestion site (data not shown). Unfortunately there was not enough time in the project to fully investigate the cause of this plasmid lethality in the yeast.

DISCUSSION

This paper presents evidence for the successful use of a modified Y1H system to report on the interactions between heterodimeric TFs and their DNA binding domains. The success of the assay results presented demonstrates that heterodimers can robustly activate the lacZ reporter gene and mildly activate the his3 reporter in a binding site specific manner. This was accomplished by transforming both dimer members into the reporter yeast bait stains in separate AD-fusion 2µ vectors.

Comparing the results from the initial pilot and later heterodimer Y1H testing produces interesting questions regarding various characteristics of the assay. First, why would the two 2µ AD-plasmids allow heterodimeric reporter activation whereas the combination of the 2µ and ARS/CEN plasmid did not? First of all, the fact that the reporter yeast cotransformed with pDEST-AD-LEU2 ARS/CEN and pDEST-AD-TRP1 2µ were able to grow on –UHLT media demonstrates that both plasmids as well as the reporter constructs were present and functional in the yeast. Not only are the genes present, but they are creating functioning proteins. Most of the auxotrophic systems work by synthesizing an enzyme that completes a step in a certain amino acid's synthesis pathway missing in the original strain. This suggests that something about the vectors themselves not related to their expression is affecting the assay results. It is possible that 2µ and ARS/CEN vectors are incompatible, a theory which has some supporting

data from a publication on the affect of 2µ plasmids on yeast life span. It suggested that the presence of ARS containing plasmids can affect 2µ plasmid DNA levels (Falcon et al, 2005). If having both 2µ and ARS/CEN plasmids in the same yeast leads to 2µ loss, it could exacerbate the low levels of ARS/CEN based TF production and result in a net loss in heterodimer concentration. It is unlikely that the 2µ vector levels could be so reduced that they would fall below the 1-2 copy ARS/CEN levels. If there was native competition for the 2µ expressed protein, however, the overall lowering of protein available to form the heterodimeric TF may still occur.

With the different results from using a 2µ LEU2 vector instead of a LEU2 ARS/CEN not satisfactorily explainable by incompatibility between the two plasmid types, one needs to look into other dynamics of the system. If an assumption is made that that there is no insert-specific protein-DNA interaction in the yeast which would affect the AD-fusion protein product in either vector, then the factors which could affect the expression of each protein on a per-plasmid basis should remain constant. Both pDEST-AD-LEU2 2µ and pDEST-AD-LEU2 ARS/CEN vectors share identical promoter regions, gateway sites, auxotrophic selection, and bacterial replication origins. Indeed the only practical difference between the two is their yeast replication origin and correlatively, their copy number in the yeast. The increased number of plasmids in the yeast can create a higher intracellular concentration of the protein. By this reasoning, one can say that the 2µ vector in heterodimer Y1H system produced significantly more AD-fusion protein than the ARS/CEN. Having a lower concentration of TFs means that there would be fewer instances where they would interact with their binding site and in turn activate the reporter genes in the bait strains.

This problem is intensified by the need for both dimer halves to find and bind to each other before binding to DNA, resulting in the need for more TFs to be present to bind to their bait with the same frequency as a homodimer or non-dimzerizing TF. The protein produced by the low copy number restricts heterodimer formation due to its limited availability relative to its most robustly produced partner. Although the amount of AD-fusion protein present in the yeast was at no point quantified during this project, the inferred difference of numbers in the dimerizing protein population provides an explanation for the lack of reporter signal seen in the initial heterodimer Y1H experiments. If the AD-fusion protein level was low enough, the amount of reporter gene generated from it could have been eclipsed by the latent self-activation of the bait strains via the reporters' minimal promoters. Although it would have been prudent to assay for the AD-fusion protein levels in the yeast to verify this, time restrictions prevented this from being performed.

The other major challenge that arose during the project was trying to explain the difference between the HIS3 and lacZ reporter data. There should have been a direct correlation to positives in the two reporter genes. However, as comparing the HIS3 and lacZ results demonstrates, the connection was not so simple. Indeed, throughout the project the HIS3 reporter showed far less robust results than lacZ assays conducted on the same yeast. In comparing the CES-1 1XBS lacZ and HIS3 results in Figure 5, one can see that while the HLH-2/HLH-10 demonstrated a strong positive for the lacZ, it showed no such tendency in the HIS3. The most likely reason for this has to do with the way the reporter enzymes themselves work. The lacZ reporter is generally regarded as more sensitive than the HIS3 as even a small amount of β-gal, if given enough substrate and time, can produce a lot of blue pigment. HIS3, on the

other hand, requires a much higher concentration in order to see growth by overcoming 3AT antagonism.

Going forward, it would be prudent to explore the use of other reporter genes to replace HIS3 and lacZ with something more quantifiable and consistent. A possibility for this is to express red (RFP) or green fluorescent protein (GFP) rather than an enzyme that must then react with a substrate to report on its expression. Fluorescent protein functions by emitting certain colors when excited by a certain wavelength of light. The intensity of the color produced is proportional to the amount of fluorescent protein present, allowing one to quantify the amount of protein by measuring the luminescence. If the production of fluorescent protein were tied to reporter expression then one could measure the direct amount of the reporter and not the product of its enzymatic action. Both RFP (Rodrigues et al, 2001) and GFP (Cormack et al, 1997) have been shown to be functional reporters in yeast. Furthermore, yeast enhanced green fluorescent protein (yEGFP) has already been optimized for use in a high-throughput Y2H assay with expression measurements of flow cytometry (Chen et al, 2007). The inclusion of flow cytometry into a GFP/RFP based Y1H would allow for a highly reliable and quantifiable measurement of reporter activation not based on the eye's determination of color differences. Additionally one would save both the reagent and time investment required to perform current lacZ and HIS3 reporter assaying. The pursuit of RFP and GFP as Y1H reporters, particularly heterodimer Y1H assaying, would thus be highly desirable.

If the use of GFP and RFP does not result in clearer and more reliable data for the heterodimer Y1H assay, one would need to go back and reconsider a more fundamental

element of the assay. The Gal4 AD, which is attached to each dimer half in the current Y1H system, is central to the success of the assay. However, no work as been performed on the affect of two activation domains on heterodimeric TF DNA binding. It could be that the dual ADs create steric interference that limits its accessibility to coactivators and general transcription factors that allow them to recruit RNA polymerase II. The obvious course to test this would be to remove the AD domain from one of the two expression vectors, clone one of the heterodimer halves into the bait strain with its AD inclusive partner, and then run the assay again. While this seems relatively straightforward, there are issues with the approach. By not including the AD in on one of the two proteins, one cannot show through the assay that the non-AD TF is not binding to the bait region. If the non-AD TF were binding by itself, it would not be able to activate the reporter gene. A possible way around this would be run two iterations of each assay. In this approach, dimer piece A would be without the AD in one assay and dimer piece B would be without the AD in the other. This would allow one to directly show that a positive assay result for the heterodimer is a result of the complex and not one of its member proteins.

All in all the results of this project are encouraging for the use of the detection of heterodimer protein-DNA interactions using the Y1H system. The inclusion of heterodimers in Y1H screens represents an important improvement in the thoroughness and impact of those screens. Through additional troubleshooting and the pursuit of different reporter strategies, the screening of heterodimers through the Y1H system has the potential to yield important data to further our understanding of how genes are regulated.

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APPENDIX

GAP REPAIR PRIMERS

1. 40bp in the 5'->3' direction upstream of the TRP1 ORF:

GTGAGTATACGTGATTAAGCACACAAAGGCAGCTTGGAGT

2. First 20bp of LEU2 ORF in 5'->3' direction:

ATGTCTGCCCCTAAGAAGATCGTC

3. Reverse Complement of the 40bp immediately downstream of the TRP1 ORF 5'->3':

TGCACAAACAATACTTAAATAAATACTACTCAGTAATAAC

4. Reverse Complement of the last 20bp (5'->3') of the LEU2 ORF:

TTA AGCAAGGATTTTCTTAACTTCTTCGG

Leu2->Trp1 Forward Gap Repair Primer:

5'-GTGAGTATACGTGATTAAGCACACAAAGGCAGCTTGGAGTATGTCTGCC CCTAAGAAG ATCGTC -3'

Leu2->Trp1 Reverse Gap Repair Primer:

5' -TGCACAAACAATACTTAAATAAATACTACTCAGTAATAACTTAAGCAAG GATTTTCTTAACTTCTTC GG -3'