Protein-protein interaction experiment

Introduction

Saccharomyces cerevisiae is a unicellular eukaryotic fungus (Clark 2010). Investigating protein-protein interactions involved in the activation of GAL4 genes in yeast that regulate the metabolic breakdown of carbon energy sources is the purpose of this project.

In the yeast two hybrid system the binding domain is supposed to bind to the UAS of the GAL4 gene in the yeast (BIOL 466). The bait is bound to the UAS and the prey is bound to the binding domain; the activation domain is attached to the prey. If the bait and the prey interact it corresponds to an interaction between the respective attached domains that will allow for the activation domain to recruit the transcriptional machinery required to transcribe the reporter gene which is HIS3 (BIOL 466). This process provides insight into why the relatively small number of eukaryotic genes of yeast, and similarly humans, can have so many functional protein manifestations (BIOL 466). This, in part, is due to protein-protein interactions resulting in the variation of function upon the interaction and modification of proteins. Interacting proteins can serve many purposes in cellular function diversity (Rapely 2008). The way products interact can be based on how they are modified after translation, which proteins interactions are taking place at a given time, and the environments and where these processes are taking place (Rapely 2008). The purpose of this experiment is to use protein-protein interactions to monitor how Gal4p domains responsible for activation and binding interact in yeast by manipulating the GAL4 gene to provide a signal that can easily be monitored.

Protein-protein interactions can serve as a way of signalling fitting proteins and to help dictate their function. This can be used in molecular biology to determine whether an aunt two parts of the gene need to interact in order for the expression of a gene to take place. In the Yeast Two Hybrid (Y2H) experiment protein-protein interactions are used to determine the functionality of the GAL4 gene responsible for transcription of the protein Gal4p (BIOL 466). Yeast two hybrid (Y2H) can be done in a forward fashion or a reverse fashion. The forward fashion investigates whether protein-protein interactions take place, resulting in a reporter gene signal, while reverse investigates disrupting protein-protein interactions resulting in a toxic reporter gene signal (BIOL 466). The process involves GAL4 that is required for galactose gene expression in the presence of glucose (Clark 2010). It is a transcription factor with two domains that are involved in either DNA binding or transcription activation (Clark 2010). It also finds to a region on the Yeast genome called the UAS. These two domains can be split from each other and still function independently, similar to the physical separation of the Alpha and omega fragments in E. coli Alpha-complementation (BIOL 466). A reporter gene for places the GAL4 wild type gene to monitor the interaction of the bait and prey (Rapley 2008). In the case of this project, the reporter gene is wild type HIS3 (BIOL 466 lab. Man.).

RT-PCR is an in vitro process that presents a solution to the problem of introns in eukaryotic DNA by converting mRNA products into cDNA (complementary DNA) which consists only of exons that commonly code for the genes of interest in this project (Rapley 2008; Clark 2010). Reverse transcriptase derived from retroviruses often have the ability to degrade

RNA-DNA double stranded hybrids which is not a desirable function for the RT-PCR and is often eliminated n the reverse transcriptase enzyme used (Rapley 2008). Similar to PCR, RT-PCR requires the addition of dNTPs, primers for the amplified region of interest and buffers with the appropriate salt concentrations and temperature for the enzyme to function. The primers used in the project will be designed to amplify the mRNA of interest as opposed to other methods that use random primers with no selectivity or dT oligonucleotide primers designed to ensure amplification of mRNA over rRNA in RT-PCR (Rapley 2008). The RNA is denatured first in the process in the presence of primers. Then enzyme, buffer, dNTPs, and salts are added with the inclusion of RNase-denaturing solution like DEPC H20 (Rapley 2008). The temperature is set for ideal enzyme function for the conversion of the mRNA fragment of interest until the solution is heated to inactivate the extension cycle (Rapley 2008).

The yeast two hybrid (Y2H) process involves the isolation of yeast RNA and its conversion into a cDNA templates. Through the use of PCR the cDNA is amplified after which the prey will be ligated into the vector (Rapley, 2008). The purpose of this experiment is to monitor the interaction of the bait and prey and compare the results to four plasmid preparations on an agarose gel.

The bait and prey system is used in the Y2H system to determine Protein-protein interaction (Rapley, 2008). By connecting the bait and prey system to genes in the yeast and introducing a measurable signal that corresponds to protein-protein interaction, the experiment can be monitored (Clark 2010).

The yeast two-hybrid takes advantage of proteins that bind to other proteins (Clark 2010). By using a protein with two domains, such as a binding and activation domain, these domains can be manipulated to provide a signal that corresponds to leather not they are interacting with each other (Clark 2010). For this project, the binding domain of Gal4p and also its activation domain are manipulated to use the GAL4 gene of yeast as a system to monitor Protein-Protein interactions. The protein's binding domain customarily a binds to the UAS domain upstream from the GAL4 and the activation domain usually binds RNA polymerase for transcription (BIOL 466). In the two-hybrid system, the two domains of the protein are split apart because they can still function if they are not attached to each other; these pieces can be attached to other proteins that have a known affinity for each other which are called the " bait" and the " prey" (Clark 2010). By taking advantage of MAP kinase activity and the fact that the MAPSP1 protein readily binds very tightly to p14, an endosomal protein (BIOL 466).

The key to monitoring the activation of the gene and interest depends on the bait and gray five each other and interacting a line for the transcription of a gene that provide some sort of signal that the interaction between the activation and by the domains through the bait and prey system has occurred (Clark 2010). For those projects that the prey is MP1, a kinase, and the bait is p14, respectively (Biol 466 lab). The bait is attached to the binding domain and the parade is attached to the activation domain.

The purpose of this experiment is to explore protein-protein interactions by manipulating the molecular machinery of the yeast using the bait and prey

system and screening for the signal using the Y2H parameters set up using molecular biology applications.

Methods

As per BIOL466 Lab Manual 2011 except waited only 45 minutes instead of 60 minutes for incubation in week 7; also waited 35 minutes instead of 45 minutes when incubating the tubes in Week 8; in week 9 for tube 3 used more than 0. 5 ï ¡g of C because the volume of plasmid required was below 2 ï ¡L.

Results

For this experiment, in order to amplified the desired protein product, the RT-PCR process is used to produce RNA fragments from DNA that are specific to regions of the yeast genome that contain GAL elements. The specific primers used are included below (See below). The negatively charged nucleic acid migrates to the positively charged end of the agarose gel network in the presence of the buffers and other solutions (BIOL466 Oligos, 2010)

Following the insertion of the vectors into the cells, the yeast two-hybrid screening on various plates with different medium content were used to confirm the bait and prey interaction within the cell that should result in a positive signal from the reporter gene HIS3 when no histidine is present in the medium.

The expected results for the plates containing leucine, tryptophan and histidine were that only the successful ligation and interaction of the bait and prey system will give a positive signal (Figure 2B). Positive signal

corresponds to the plate with abundant amount of Colonies (plate C). The other plates-A, B, and D–have no growth.

The expected results for the plates containing leucine, tryptophan and no histidine were that only the successful insert of His3 or his3 auxotrophs as well as cells with the interaction of the bait and prey system will give a positive signal (Figure 2A). Positive signal corresponds to the plate with abundant amount of Colonies (plate B, C, and). The other plate-plate A,–has no significant growth.

Discussion

The oligonucleotides for the bait and prey were designed to hybridize to upper part of the 5′ end of the DNA fragment of interest as well as the 3′ end of the DNA fragment of interest (BIOL 466). The fragments of interest were transformed into DH5ï ¡ E. coli for fast replication of the fragments to have a large sample to purify and extract in a relatively short period of time.

In this project the restriction enzyme digestion is performed simultaneously because the enzymes thrive well in similar buffer conditions (NEB double digest). The restriction enzymes recognize different sequences in the DNA and create 5′ overhangs that do not have compatibility with the other enzyme’s overhang. Therefore, relegation of the fragments with itself upon the addition of ligase will not occur and the probability of the recombinant plasmid forming increases significantly; this aspect of the primer design also requires consideration of the possibility that the restriction enzymes of choice do not cut the insert within the sequence and are only found in the multiple cloning site (MCS) of the plasmid used (Rapley 2008). RNase

removal with DEPC helps minimize loss of RNA product to these enzymes that have disulfide bonds that contribute to their heat resistance (Rapley 2008).

This project was carried out by first isolating yeast RNA from cell culture. The RNA was extracted and purified in several steps resulting in a purified sample. The sample purification was confirmed via spectrophotometric analysis of the RNA sample and measuring the A260/A280 Ratio (Table 2). Following the purification steps of the RNA, the RNA was converted to cDNA through the using of reverse transcriptase polymerase chain reaction (RT-PCR). The conversion of RNA to the C cDNA template using a reverse transcriptase enzyme derived from viral functions was amplified to yield the portions of DNA containing the bait and prey sequences (Oligos, 2010) (Sequence 1 and 2). The primers used for amplification are derived from oligonucleotides from the yeast genome. These oligonucleotides for the prey (MP1) are ligated into the vector (pGADT7).

The RNA gel of the yeast contains some smears which may be the result of proteins or denatured nucleic acids contaminating the sample and the purity of the sample can be monitored by calculating A260/A280 (Lui 2009). The spectrophotometric measurements of concentration of nucleic acids were used to assess purity as well as normalized the values of nucleic acids inserted into the wells. This was done to insure that each lane show they represent the value for the samples used.

While conducting the many steps for this project, the first steps involved PCR of the yeast genome region of interest and cloning those fragments into two

different vectors where one vector contained the prey protein which was attached to the activation domain. The other vector contained the bait which was attached to the binding domain (Clark 2010). The vectors were selected so that the gene of interest would be in the correct open reading frame (ORF) and the protein of interest would be properly transcribed. After the recombination process the yeast have both vectors the reporter gene will be expressed (Clark 2010). For this project the reporter gene is HIS3 therefore expression will allow auxotrophic histidine variants to grow in mediums that don’t have histidine (Clark 2010). The protein-protein interaction is detected using the reporter gene and plating these colonies on medium that will confirm or deny the presence of the Protein-Protein interaction within the cells.

By mating yeasts containing one vector with one kind of domain, a binding domain, with yeast containing another vector with the other kind of domain, and activating domain, the resulting hybrid likely have the bait and prey interaction which will allow for the activation of the reporter gene. The reporter gene will give a visual signal when plated on histidine because the reporter gene used is HIS3. There are possibilities of false positives in this experiment so the medium for plating was checked twice: one medium contained histidine, tryptophan, and leucine and another media contained only tryptophan and leucine (Figure 3). In the agarose gel Lanes one and two corresponds to the positive and negative controls, respectively (Figure 2). Lane one contains a band at about 450 bp and lane two has no band present, both as expected. Lane three contains the ligation with the band at the same size as lane one but the band is much darker suggesting that there

is more ligation product than the control plasmid found in lane one. Also, the products of lane four suggests the insert is found based on its higher location of jell which corresponds to larger sized plasmid containing an insert.

The sample found in the A sample and A plate can be confirmed based on the relatively low presence of the plasmid that has the smallest molecular weight which corresponds to the smaller sized restriction map of the four choices. This conclusion is also supported by the presence of no colonies on the A plates suggesting the lack of Histidine production capabilities for the yeast plated on the triple drop out as well as the presence of a leucine auxotrophy on the double drop out plate indicating the presence of the Mp1 plasmid and nothing to offset the auxotrophy in either medium.

The lighter band on the agarose gel for the A sample suggests this sample was difficult to synthesize or extract though the process did take place based on evidence of the cDNA presence on the gel (Figure 1A and B). The results indicating the B sample was the Human insert of p14 is initially not clear based on the results of the plates alone which had the same results as the result for plate D in both the double and triple drop out. The results are distinguished, however, upon the analysis of the restriction maps and the agarose gels. The difference between the sedlin and p14 recombinant is based on the size of the insert in each respective sample inserted into the pGBKT7 vector (Sequence 3 and 4). The sedlin insert is much larger and so has a larger bp that corresponds to a band that would be comparatively higher than that of the p14 recombinant plasmid (Vectors 3 and 4, Figure 1A and B). From the overall evaluation of the data pertaining to samples B and D, the identity of B is the mouse sedlin recombinant. The recombinant gives

a red color on the double drop out because of the mutation of the ade2 gene producing a red by product (BIOL 466).

Comparing these findings to the restriction maps generated by NEB cutter, the differences between lane four and lane three suggesting one has a larger insert than the other and noting the sedlin insert in the pGBKT7 is larger than the p14 in the same plasmid and the location of the cut sites for each leads to the conclusion that sedlin must be from lane D and the plate D is the sedlin recombinant yeast sample.

In conclusion, the use of GAL4 in the yeast and manipulating its transcription factor by separating it from its activation domain provides a way to monitor Protein-Protein interactions. These interactions are monitored using the two-hybrid system involving a bait and prey attached to each separate domain on two separate vectors which are then brought together after the yeast mate. The success of the insertion of the respective bait-attached gene into a vector, or the prey protein in the same respect, into the vector is monitored by agarose gel electrophoresis and compared to various controls. Furthermore, the Protein-Protein interactions were monitored through the activation of a reporter gene of HIS3 when plated on medium containing histidine and not containing histidine to confirm the presence of colonies where the bait and preyed are interacting and the activation by the domains of Gal4p interacts to recruit RNA polymerase to make histidine. Reporter gene activation is a screening process to identify the protein interaction of interest and provides insight into the way that Protein-Protein interactions within a cell dictate how the protein functions. The yeast two-hybrid method can be applied to the human genomic studies for monitoring genetic

pathways and complex protein interactions stemming from the fact that humans have 30, 000 genes that behave as though there are 150, 000 genes (Rapely 2008; BIOL 466).

Possible sources of error include the limits of using this bait system includes the requirement that the proteins need to interact with the nucleus (Clark 2010). This limitation can be avoided by using membrane systems that can interact in the cytoplasm or other variations of the two-hybrid system (Clark 2010). Also, the presence of a beta carotene other than the one uses project interacting with the prey may occur (Rapley 2008). Perhaps using to proteins in a purified setting and monitoring their interaction as a control for this part of the experiment can provide insight in to what extent there may be a rogue bait protein (Rapley 2008).

Other sources of error may include mRNA degradation resulting in low yield of intact, complete product. Also large fragments greater than 200 bp are not very easily converted and may require a different amplification strategy like gateway cloning (Rapley 2008; BIOL 466).