

ONE-HYBRID SCREEN (EJ Stockinger)

I. INTRODUCTION

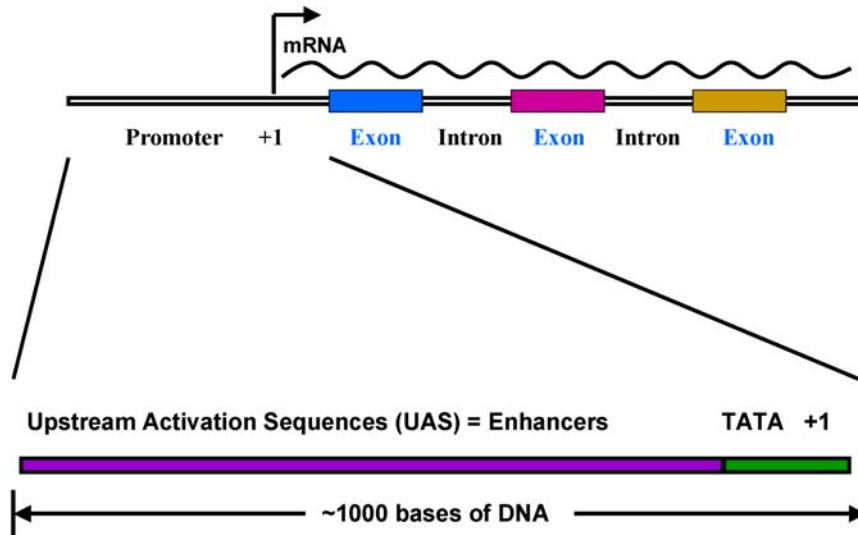
The yeast *Saccharomyces cerevisiae* is a single-celled lower eukaryote. Because it is a eukaryote it has been used as a model organism to help understand how higher eukaryotes (i.e., people and plants) function. Many of the biochemical pathways we now know and understand were worked out using yeast. Its genome was the first eukaryote to be completely sequenced. Yet humans have been manipulating yeast for thousands of years. It is yeast that produces CO₂, which causes bread to rise. It is yeast that ferments the sugars in wort and must into alcohols, creating beer and wine. In fact one of the reasons that yeast is so well studied in modern biology is because it plays such a central role in so many processes near and dear to us humans. Understanding a little about how to work with yeast can also be quite rewarding because the basic principles such as good sterile technique apply equally to the kitchen (for all you homebrewers) as to the lab.

The yeast one hybrid and the yeast two hybrid screens are methods that arose because researchers were working to understanding the basic principles of how transcription factor proteins bind to DNA to turn on genes. We will use the yeast one hybrid to demonstrate the basics of gene activation and to become familiar with methodology novel to working with yeast. The procedure is also written in the format such that the yeast-one hybrid screen could be set up as part of one's own research project if so desired. The first lab will consist of transforming two different yeasts with the same recombinant DNA molecule. The recombinant molecule encodes a protein that will turn on a gene in one of the transformed yeast strains but not in the other yeast strain. This is because the second strain lacks the specific DNA binding site recognized by the encoded protein (but it still has the coding region of that same gene). The second lab will consist of processing those transformed yeast so that we can actually observe this visually. In the third lab we will isolate protein from yeast cells and in the final lab we will measure the activity of one particular protein, β -galactosidase, which is the gene product encoded by the gene under control of the recombinant molecule we transformed into yeast. To understand how the yeast one (and two)-hybrid systems work however we must first review a few basics about gene structure and transcription factors. At the end of the procedure are several questions designed to test your understanding of the concepts presented in the basics described below.

A classical gene consists of multiple structural units. In its simplest form one portion is the protein-encoding portion of the DNA. This is the region of DNA transcribed into mRNA, which in turn, is translated by the ribosome to yield the functional protein. The other structural unit of the gene relevant to this discussion is the regulatory region of the gene. This is generally referred to as the **promoter** and usually this is **5-prime** (5'), or upstream of the transcribed portion of the gene. Promoters have at least two known functions. One function is to insure that transcription initiates at the correct position, which is the +1 site. The other function usually ascribed to a promoter is to direct expression of a gene spatially and/or temporally and/or in response to external signals. Spatially means what cells or tissues; temporally means what stage or time in the life cycle of the organism. External signals can be from another cell or tissue within the same organism; e.g., hormonal signals (in plants this could be auxins, cytokinins, gibberellins, ethylene, abscissic acid) or from the environment (drought, cold, heat, etc). Regardless, determinants of spatial, temporal and external-signal control over gene expression are usually contained in the particular combinations of DNA sequences of the promoter. These are called **cis-acting elements** because they are molecularly linked to the transcribed portion of the gene. One frequently found *cis*-acting promoter element is the TATA box, a stretch of 8 bases consisting entirely of A·T base pairs. For most eukaryotic genes the TATA box is located about 25 bases upstream; i.e., -25 of the mRNA startpoint (which is +1). (In prokaryotes the TATA is at about -10.) As it turns out most eukaryotic genes do not have a TATA box, rather it seems to be the case that only the most highly expressed genes possess the

TATA box. The TATA box also helps to direct correct positional initiation of the mRNA transcript, which explains why it is almost always position at about -25 relative to the start site of transcript initiation.

Gene promoters control the regulation of mRNA production



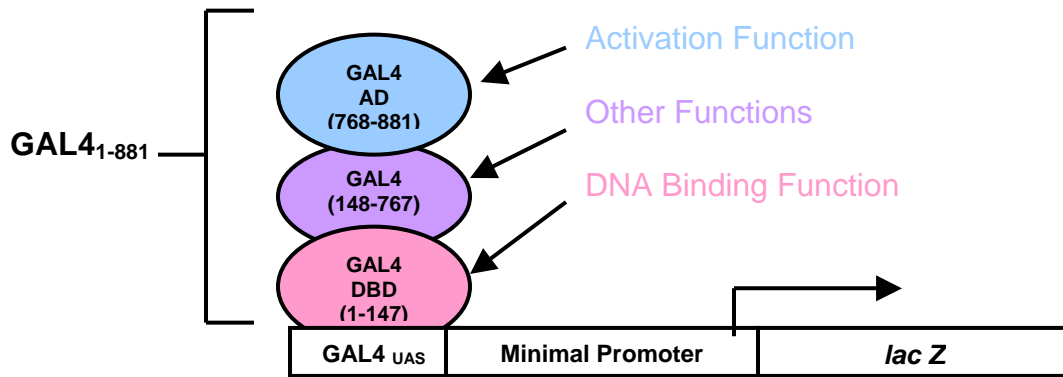
Whereas the *cis*-acting TATA box element is present in most genes, the *cis*-acting elements conferring the unique expression pattern characteristic of any given gene are necessarily by definition, gene specific. At this point in time, determining exactly what nucleotides constitute any given *cis*-acting regulatory element requires a case-by-case approach through empirical determination (explained in more detail below under “*Definition of the cis-acting regulatory element*”). However as more and more genes are examined for their expression patterns, the number of known *cis*-acting DNA regulatory elements will only increase. In a large part this is due to the use of DNA microarrays and synthetic oligonucleotide “chips.” These tools will be covered in greater detail in week nine by Dr. J.C. Jang. Conceptually these techniques allow for every gene in the genome to be analyzed for its expression pattern. In addition, more and more genomes are being fully sequenced. As a result numerous analytical tools have been developed to analyze promoter sequences. Some of these online databases contain every single known *cis*-acting DNA regulatory element. All one needs to do is simply copy and paste your favorite promoter sequence as a query into one of these programs. If there is a known *cis*-acting DNA regulatory element present in your promoter, then it should show up in the output analyses of you promoter. For example go to The Arabidopsis Information Resource (TAIR) web site at <http://www.arabidopsis.org/>. Under analysis tools go through the **Motif Analysis** link to <http://www.arabidopsis.org/tools/bulk/motiffinder/index.html>. At this site there are two databases (<http://oberon.rug.ac.be:8080/PlantCARE/index.html> and <http://www.dna.affrc.go.jp/htdocs/PLACE/>) that allow one to query a promoter sequence against all known or published *cis*-acting DNA elements. In addition there are programs that allow one to identify over-represented motifs in the upstream region of a set of co-regulated genes. These are Motif Sampler (<http://www.esat.kuleuven.ac.be/~thijs/Work/MotifSampler.html>) and AceAlign (<http://atlas.med.harvard.edu/>). Links to these analyses tools can also be found on the TAIR web site.

Just as there are *cis*-acting DNA regulatory elements there are *trans*-acting regulatory factors. *Trans* means that they are not physically connected as part of the same molecule; i.e., gene being transcribed. These are proteins encoded elsewhere in the genome, which then make their way to the site of action. For

example the *trans*-acting factor that binds to the TATA box is the TATA Binding Protein or TBP. The gene encoding TBP is transcribed in the nucleus, mRNA is shuttled to the cytoplasm, and there it is translated into protein. The protein TBP is then transported back into the nucleus, finds its cognate DNA binding site; i.e., the TATA box where it performs its role. This is the basic *modus operandi* of many transcription factors. Just as TBP binds to the TATA box, most *cis*-acting DNA elements have a cognate DNA binding protein capable of binding to that particular combination of sequences. In addition to DNA binding, most *trans*-acting factors perform a second important function, which often but not always is to activate expression of the gene to which it binds. This is why this class of proteins are referred to as transcriptional activators. Thus both *cis*-acting elements and *trans*-acting factors confer regulatory specificity for gene expression; *cis*-acting elements confer regulation to the gene to which the *cis*-elements are molecularly linked, whereas *trans*-acting factors have the capacity to control expression of many unlinked genes.

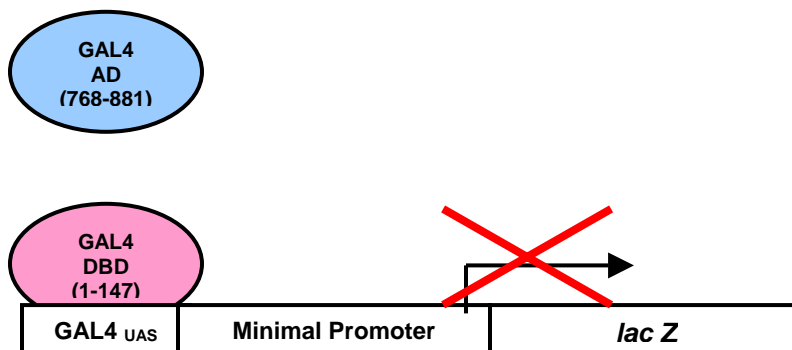
The TATA box and TBP can be considered ubiquitous and generally don't provide any expression pattern specificity. It is the other sequence-specific DNA binding proteins that act on their cognate DNA binding sites that determines gene-specific expression patterns. One current goal of many genome projects is to identify all the *cis*-acting DNA sequence elements in promoters and in turn identify all of their cognate *trans*-acting factors. This is because the ability to manipulate a single *trans*-acting protein has the potential to allow one to control the entire pathway, or battery of downstream genes. Together the regulatory protein and the downstream pathway can be referred to as a regulon. Consider the regulon concept analogous to a room full of lights in which the gene encoding the structural protein comprises each individual light and the regulatory protein acts as the light switch controlling the entire circuit.

Before moving on, the other relevant feature about *trans*-acting transcription factors we must consider is that the DNA binding and *trans*-activation functions are usually compartmentalized into separate modules of the protein. For example let's examine the yeast protein GAL4. GAL4 is the archetypal transcription factor from which most artificial yeast one-hybrid and two-hybrid systems evolved. GAL4 is an 881 amino acid protein. It binds to a 17 base pair sequence deemed the GAL4 Upstream Activation Sequence or GAL4_{UAS}, which resides in the promoter of many yeast genes including the *Gal1* and *Gal10* genes. (The 17bp GAL4_{UAS} sequence to which GAL4 binds is actually a palindrome of two 8bp sequences mirrored around a central nucleotide.) Although the full-length GAL4 protein is 881 amino acids, only amino acids 1-147 of GAL4 are required for binding to the *cis*-acting GAL4_{UAS}. Similarly at another business end of the GAL4 protein, amino acids 768-881, is the portion of the protein that confers the activation properties to the protein (actually GAL4 has two activation regions, ADI, consisting of residues 148-196 and ADII, consisting of residues 768-881). Thus to reiterate, the **NH₃-terminal** portion of GAL4 harbors DNA binding activity whilst the **COOH-terminal** portion of GAL4 harbors *trans*-activation properties.



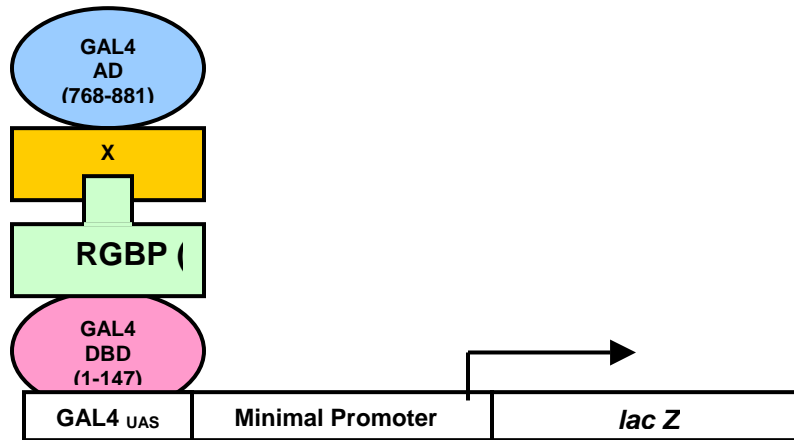
How does all of this relate to yeast one-hybrid and two hybrid screening strategies? Lets define what the one hybrid and two hybrid systems allow us to do. First the two-hybrid because historically this was the first of the two methods described. The two-hybrid method was developed as an *in vivo* method to fish out novel or unknown proteins that might interact with a protein that we already have in hand; i.e., it is a cloned gene encoding some protein of biological interest to us. This will be used as bait in a molecular fishing expedition. The underlying goal of this fishing expedition is two fold: (1) By identifying interacting partner proteins it is generally thought that this will provide additional clues into the biological function of the bait protein used in the screen and (2) Having the interacting partner protein in hand in turn aids in the overall biological understanding of the entire system.

How does the two-hybrid work? Simple. The two hybrid works on the principle that these *trans*-acting transcription factors can be compartmentalized into separate modules. Thus we can separate the DNA binding domain of GAL4 (amino acids 1-147 = GAL4_{DBD}) from the activation domain of GAL4 (amino acids 768-881 = GAL4_{AD}) and express each as separate proteins in the same yeast cell. Although the cognate DNA binding site for GAL4 is present on the yeast chromosome, the GAL4_{DBD} protein is incapable of activating expression of this gene because it lacks the GAL4_{AD}, because it is no longer molecularly joined to the GAL4_{DBD} within the same protein molecule.

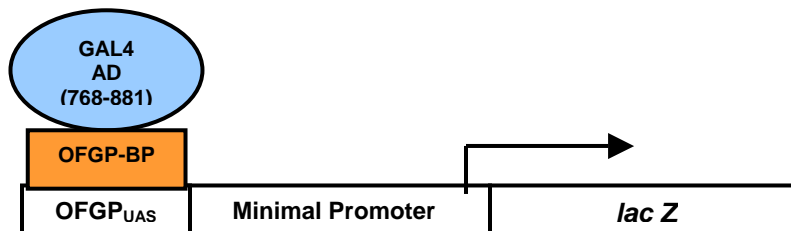


The strategic objective of the two-hybrid is to reconstitute the DNA binding and trans-activating properties of the native GAL4₁₋₈₈₁ protein by bringing these two domains back together in the yeast nucleus. This is accomplished not through direct interactions of the GAL4_{DBD} with the GAL4_{AD}, but rather through the interactions of Our Favorite Protein, which will now be abbreviated as OFP, and a second protein that (we hope) is a natural partner of OFP in the organism from which it came. This is accomplished by the use of chimeric proteins. One chimeric protein is the GAL4_{DBD} fused to OFP. The GAL4_{DBD}-OFP chimeric fusion protein is then used as bait in the molecular fishing expedition. The fish,

or prey consists of an entire library of unknown protein encoding sequences fused to the GAL4_{AD}. If OFP finds an interacting partner in the same yeast cell, then we have brought the GAL4_{DBD}-OFP into juxtaposition with some unknown protein X, fused to GAL4_{AD}; i.e., GAL4_{AD}-X. A result of this GAL4_{DBD}-OFP:: GAL4_{AD}-X interaction the gene harboring the GAL4 UAS in its promoter is now activated and expressed.



The yeast one-hybrid system evolved out of the two-hybrid system and was designed to use the *cis*-acting sequence as bait to directly capture a DNA binding protein capable of binding to and recognizing this *cis*-acting sequence (Li and Herskowitz, 1993; Wang and Reed, 1993). Instead of using the GAL4_{UAS} as the *cis*-acting element upstream of the minimal promoter-*lacZ* fusion, we insert the *cis*-acting element from our favorite gene's promoter (OFGP_{UAS}). Now we simplify things. Instead of requiring protein-protein interactions to drive expression of the *lacZ* reporter gene, we require only that a piece of DNA encoding a protein that is capable of binding to OFGP_{UAS}. We create a population of proteins; i.e., a library in which each unique protein is fused to the GAL4_{AD}. If a protein capable of binding to OFGP_{UAS} (for the sake of simplicity, we will deem that as of yet uncloned protein OFGP-BP for Our Favorite Gene Promoter Binding Protein) is fused in the same reading frame as that of the GAL4_{AD}, then we should be able to fish it out of the library using our molecular bait, the OFGP_{UAS}. This is because the GAL4_{AD} will activate; i.e., turn on the *lacZ* reporter gene when it is in proximity of that gene.

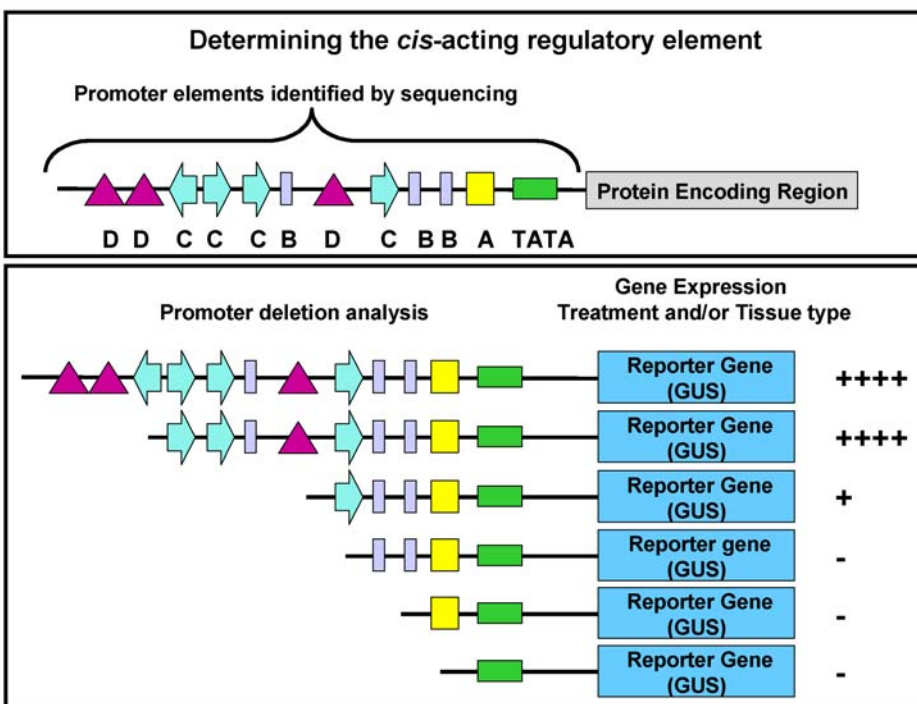


If you think about it, the one hybrid system could also be used to identify the unknown target site of a suspected DNA binding protein. In this instance the OFGP-BP is in hand (cloned) and is fused to the GAL4_{AD}. Our library would then consist of a population of oligonucleotides that we created sitting upstream of the minimal promoter *lacZ* gene. While this is all that will be covered regarding this concept, this is exactly what has already been done (Wilson et al., 1991). The rest of the following is presented in

the context that we are after the DNA binding protein binding to a defined *cis*-acting DNA regulatory element.

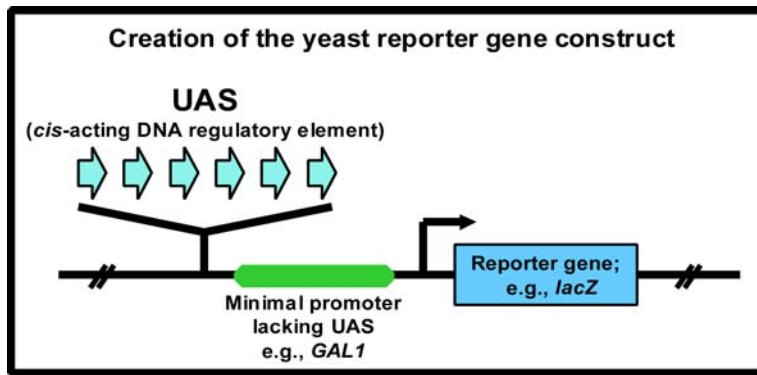
Defining the *cis*-acting regulatory element

Ideally before embarking on a one-hybrid screen for a DNA binding protein, the *cis*-acting DNA regulatory element will have been clearly defined. This is generally accomplished by using combinations of the following: (a) fusion of the promoter to a reporter gene and transformation of the chimeric construct back into the organism from which it was isolated, (2) subsequent nucleotide substitution within the minimal promoter deletion construct to identify essential nucleotides, and (3) gel electrophoretic mobility shift assays (gel retardations) using nuclear (or whole cell) protein extracts and radiolabeled DNA fragments harboring the *cis*-acting DNA regulatory element. The more clearly the *cis*-acting element is defined up front, the greater the ease at which interpretations can likely be made later on.



Creation of the yeast reporter gene construct

Once the DNA binding site is determined (and hopefully clearly defined), the next step is to substitute this as a UAS in a yeast minimal promoter. One could use either a portion of the original native gene promoter or a synthetic oligonucleotide representing the defined minimal *cis*-acting element and then subclone this upstream of the yeast minimal promoter (i.e., consisting of not much more promoter sequence other than a TATA box and the +1 sites). This “hybrid” promoter then drives expression of a *lacZ* reporter gene. The product of the *lacZ* gene, β -galactosidase cleaves the substrate, X-gal, which will result in blue color formation in that yeast expressing the gene (Guarente, 1983; Rose and Botstein, 1983).



Creation of the yeast reporter host strain

In order to create the reporter gene construct, all manipulations with plasmid DNAs are performed in *E. coli*. Once the *cis*-acting element of interest is cloned upstream of the yeast minimal promoter, this plasmid construct is then introduced into yeast cells. We then select for the presence of the plasmid in yeast cells by leaving an essential nutrient, either an amino acid or an enzyme in the biosynthetic pathway of a base, out of the yeast growth medium. The plasmid we introduced carries a gene encoding the missing component. Thus the only yeast cells capable of growth are the ones that also have the plasmid. In our particular example it happens to be gene encoding *URA3*, the third protein in the Uracil biosynthetic pathway. Here's how it works: The yeast host strain that we are using, GGY1 has a defective *URA3* gene and cannot grow unless uracil is added as a supplement to the growth medium. The correct descriptive terminology for this lesion is that GGY1 is a uracil **auxotroph** and the standard yeast genetic notation writes this as lower script; i.e., *ura3*. When we introduce a plasmid encoding *URA3* we no longer need to add uracil to the growth medium because *URA3* is encoded on the plasmid and takes care of this for us. The *ura3* genotype of yeast strain GGY1 harboring the plasmid with the wild type *URA3* gene is now considered a uracil **prototroph**. One other additional feature of our plasmid construct is that the *URA3* phenotype is only possible if our introduced plasmid integrates into the yeast chromosome. This is because our plasmid construct is devoid of a yeast replication origin and so the only way the *URA3* phenotype is possible is if the plasmid DNA integrates into the yeast chromosome. This is through homologous recombination, which is performed very efficiently by yeast cells if there are about 250 nucleotides of identity between the chromosomal DNA and the exogenously added DNA (Rothstein, 1991). When the *URA3* gene on the plasmid integrates into the defective *ura3* gene, it also takes with it the entire plasmid and thus our reporter gene construct. Restoring the uracil lesion results in a uracil prototroph. For a great primer on yeast check out the chapter "Getting Started with Yeast" in the Methods in Enzymology series (Sherman, 1991). (Note that there is one critical typo in the Sherman reference in Table V; the stock per 1000 ml should be stock per 100 ml.) Transformation of the yeast may be made by either electroporation (Becker and Guarente, 1991) or LiCl mediated DNA transfer (Schiestl and Gietz, 1989). (For our transformations we will use LiCl transformation technique.) We have now created a yeast reporter construct harboring the *lacZ* reporter gene. This newly created yeast strain will now become the host strain into which a library will be transformed. This is the "bait." The *lacZ* gene should be expressed at low basal levels such that the yeast cell should not turn blue when provided the X-Gal substrate in a β -galactosidase assay.

Creation of the activation domain fusion library

The other resource needed for the screen is the construct expressing the DNA binding domain. We'll refer to this construct as the "prey." For the one-hybrid screen this is a cDNA library prepared from the appropriate cells, tissues and/or under the appropriate conditions in which you would expect to find this protein in its normal environment and then cloned into a vector that will constitutively express these

cDNAs as fusion proteins with an activation domain (in our case the yeast GAL4 protein activation domain II, GAL4_{AD} = GAL4₇₆₈₋₈₈₁). Any fragment which encodes a DNA binding motif capable of binding to our defined *cis*-acting element and cloned in frame with the GAL4_{AD} can now bind to the UAS upstream of the *lacZ* reporter gene and should turn the yeast cell blue in a β -galactosidase assay. In theory we don't need the complete cDNA of the unknown target protein, just the region encoding the DNA binding motif. Several Arabidopsis cDNA libraries (CD4-10 and CD4-22) are available and may be obtained from the Arabidopsis stock center (<http://aims.cps.msu.edu/aims/>). Since rather large quantities of plasmid DNA of the library constructs are required for transformation into yeast a large-scale preparation of DNA (i.e., CsCl, Qiagen kits etc.) is recommended.

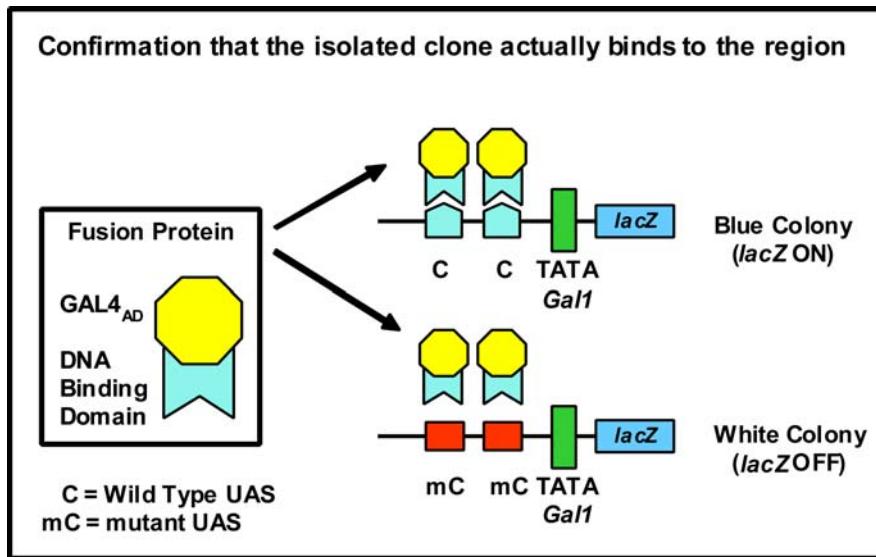
Transformation of the yeast host reporter strain with the activation domain fusion library

With all the above tools in hand the next step is to transform the yeast host reporter strain with the library of cDNA constructs cloned into the activation domain vector. These transformed yeast cells are then plated onto medium in which only those cells transformed with our DNA can grow. To do this the host strain is propagated in rich medium (YPAD) to about mid-log phase, which is $\sim 5-10 \times 10^6$ cells per ml or an OD₆₀₀ of about 2 based on an OD₆₀₀ approximating $\sim 2 \times 10^6$ cfu/ml for yeast strain GGY1 and its derivatives. The yeast cells are then made competent to take up DNA, which they should do once we add it and then plated onto medium lacking the appropriate constituent. In this case the plasmid has an origin of replication (i.e., there is no homologous recombination) and the plasmid replicates inside the yeast host cell as an autonomous element. Cells are allowed to grow on the plates for approximately 48 hours. A nitrocellulose membrane (or Whatman 50 filter paper) is then overlaid on top of the yeast. The yeast are returned to the incubator for another 12 – 24 hours during which time they grow into the membrane. The membrane is lifted off the plate, dropped into liquid nitrogen, freezing the yeast and then returned to RT to thaw. This freezing and thawing event effectively lyses the yeast cells. These filters are then incubated in a solution containing X-gal. If the product of the *lacZ* gene, β -galactosidase was expressed in those yeast cells, then they will turn blue.

Confirmation that the isolated clone actually binds to the region

During one-hybrid (and two-hybrid) screens many false positives arise. For example there might be 10 different yeast colonies that turn blue during a β -galactosidase assay. Yet when the plasmid DNA is isolated from each of these different yeasts and then transformed back into the original yeast host reporter strain, they fail to activate expression of the *lacZ* gene. Who knows why this occurs. Thus, transformation back into a fresh virgin host strain is a very important control before proceeding too far. Basically this entails growing a small 5 ml overnight culture of the positive yeast clone in an appropriate selectable medium, performing a yeast plasmid mini prep (Strathern and Higgins, 1991), transformation of this plasmid DNA into an *E. coli* host strain, isolating the plasmid DNA from the *E. coli* and transforming this back into yeast. **This is where we will begin our exercise.**

If this clone is indeed a true positive, then it becomes critical to establish that the clone specifically binds to the target site and it is not just some spurious non-specific DNA binding protein. This is accomplished by transforming the clone into one or more different yeast host reporter strains. Ideally one of these reporter strains should be one in which the *cis*-acting promoter sequence of interest has been mutated. This mutated version should also have been previously shown to be non-functional in some other assay (e.g., transgenics and/or a gel shifts). Thus when a true positive clone isolated from the one-hybrid screen is transformed into a yeast strain with a wild type UAS that yeast cell should turn blue. The same clone when transformed into a yeast strain with a mutant UAS should not turn blue but should remain white.



In the previous set of experiments conducted last week, we were testing whether our protein was capable of specifically recognizing a DNA sequence. In the following set of experiments we will test whether our protein can function as an activator. Together these two exercises will hopefully demonstrate the modularity of transcriptional activator proteins. In other words, one portion of the protein provides the DNA binding component and the other region provides the activation component. To demonstrate this we have created a chimeric protein consisting of the DNA binding domain of the GAL4 protein (GAL4₁₋₁₄₇ = GAL4_{DBD}) and the activation domain of another protein CBF1 (CBF1₁₁₅₋₂₁₃ = CBF1_{AD}). Remember from the above introduction that despite the fact that the GAL_{DBD} might be expressed in the yeast cell nucleus, it cannot activate a gene unless it is juxtaposed to the GAL_{AD} region. This will be demonstrated quantitatively by measuring the quantity of a reporter gene induced by the GAL_{DBD}, and that same reporter gene induced by the GAL_{DBD}CBF1_{AD} fusion. This involves preparing protein extracts from yeast and spectrophotometric measurement of the amount of substrate cleaved by the product of the *lacZ* gene, β -galactosidase protein. We then normalize that value against the total yeast protein (using Bradford assays) and this provides us with the specific activity of the β -galactosidase protein.

Timetable and Experimental Objectives:

Week of Monday May 10, 2004

| | Activity | Time | Experimental Objective(s) and Technical Purpose(s) |
|--------------------------------|---|-------------|--|
| Day 1 Tuesday 05/11/04 | Transform Yeast with plasmid constructs | 3-4h | To introduce plasmid DNA molecules into yeast cells. One plasmid consists of only the vector whereas the other plasmid consists of the vector expressing CBF transcription factor. |
| Day 2 Wednesday 05/12/04 | Lay filter on plate | ~5 min | Allows yeast to grow into the nitrocellulose filter. |
| Day 3 Thursday 05/13/04 | Perform Fields Screen | ~2h | To visually demonstrate that the CBF protein will activate expression from the $_{WT}CRT/DRE$ but not the $_{MUT}CRT/DRE$ yeast reporter. |

Week of Monday May 17, 2004

| | Activity | Time | Purpose(s) |
|-------------------------------|---|-------------|---|
| Day 4 Monday 05/17/04 | Pick yeast overnight cultures | ~10 min | During exponential growth yeast require ~140 min to divide in minimal medium (~90 min in YPD rich medium). Overnight growth in a 5ml culture will produce sufficient quantities of yeast cells for the Bradford and β -gal assays. |
| Day 5 Tuesday 05/18/04 | Isolation of Yeast Proteins using glass beads | 2-3h | Glass beads disrupt and permeabilized the yeast cell wall (and also shear the DNA) but leave proteins intact. |
| Day 6 Thursday 05/20/04 | Bradford assays and β -gal assays | ~3h | This experiment should reveal the requirement of the activation domain for gene activation. The vector alone and the construct expressing just the $GAL4_{DBD}$ (pEJS933) should not result in <i>lacZ</i> reporter gene activation whereas the $GAL4_{DBD}$ fusion construct $GAL4_{DBD}CBF_{AD}$ (pEJS591) should activate the <i>lacZ</i> reporter gene. The other biological phenomenon revealed is the modularity of transcription factors. Bradford assays are used to quantitate total yeast protein and β -gal assays are used to quantitate only β -galactosidase, the protein product of the <i>lacZ</i> reporter gene. |

II. METHODS

A. Yeast Transformation

Steps 1 – 6 below have been completed in advance.

1. Streak out (from the -80°C) the yeast host reporter strains $_{\text{WT}}\text{CRT/DRE}$ (EJS151), $_{\text{MUT}}\text{CRT/DRE}$ (EJS216) and Y190 onto YPAD plates. It generally takes 3-4 days to get good-sized yeast colonies.

Note that yeast strain Y190 will need to be transformed with the GAL4_{DBD} (pEJS933) and GAL4_{DBD} fusion construct $\text{GAL4}_{\text{DBD}}\text{CBF}_{\text{AD}}$ (pEJS591) and also with the Vector (pDB20.1) alone by Eric or one of the TAs prior to the second week.

Sunday, 05-08-04:

2. Pick a single colony of each into 5 ml YPAD. Grow overnight.

Monday, 05-10-04:

3. Inoculate 250 ml YPAD with the 5 ml overnight culture. Grow overnight.

(**Note:** The 250 ml overnight culture is sufficient for numerous transformations or in our case, for the entire class.)

Tuesday, 05-11-04:

4. Spin cells down at 4000 X G (5,000 rpm in Sorvall SLA1500 rotor).

5. Resuspend cells in 500ml fresh media.

6. Grow cells for approximately two additional generations (3 – 4 hours).

Note: steps 5 and 6 are optional but when performed result in a significant increase in yeast transformation efficiency.

The above steps will be taken care of by Eric prior to Tuesday's class.

The following will be performed by each group:

7. Transfer ~ 30 ml of each yeast culture ($_{\text{WT}}\text{CRT/DRE}$ and $_{\text{MUT}}\text{CRT/DRE}$; AKA yeast strains EJS151 and EJS216, respectively) to an Oak ridge tube. Spin cells down at 6000 X G (7,000 rpm in Sorvall SS34 rotor) for 5 min at 4°C (RT is okay too).

8. Resuspend cells in 10 ml sterile dH_2O (this is a washing step).

9. ~~Transfer cells to a 50 ml Oak ridge tube.~~

10. Spin cells down at 6000 X G (7,000 rpm in Sorvall SS34 rotor) for 5 min at 4°C (RT is okay too).

11. Resuspend cells in 0.5ml ml freshly made 1X TE / 1X LiAc (~1-2ml of a very dense yeast suspension should be obtained). Mix well.

| 1X TE / 1X LiAc | | |
|--------------------|-------------|-------------|
| Stock | Vol for 1ml | Final Conc. |
| ddH ₂ O | 800μl | |
| 10X TE | 100μl | 1X |
| 10X LiAc | 100μl | 1X |

12. Incubate ~ 60 min at 30°C with gentle shaking/agitation.

Note: The following volumes are for transforming a single DNA construct into a yeast host (which, in our case is what we are doing); if a library screen was the objective then volumes are simply scaled up. With this method ~1.5% of the yeast cells are transformed and ~ 1 X 10⁵ transformants per μg are obtainable. Also realize that rather large quantities of library plasmid DNA is required for transformation into yeast; therefore a large-scale preparation (i.e., CsCl, Qiagen kits etc.) is recommended.

13. Meanwhile prepare a mixture of 10uL transforming DNA and 10uL carrier DNA (one for each _{WT}CRT/DRE and _{MUT}CRT/DRE yeast strains).

| DNA Mixture | | |
|----------------|---------------------------|---------|
| DNA | Vol for 2 transformations | |
| | pDB Vector | pDB-CBF |
| Carrier DNA | 20μl | 20μl |
| pDB Vector DNA | 20μl | |
| pDB-CBF DNA | | 20μl |

Aliquot 20μl of the Vector-Carrier DNA mixture to one microfuge tube labeled _{WT}CRT/DRE/Vector and another 20μl to a tube labeled _{MUT}CRT/DRE/Vector. Aliquot 20μl of CBF-Carrier DNA to one microfuge tube labeled _{WT}CRT/DRE/CBF and another 20μl to a tube labeled _{MUT}CRT/DRE/CBF.

14. At the conclusion of the 60-minute incubation, add 200uL of the yeast cell suspension to the 20μl volume of carrier and transforming DNA.

15. Incubate ~ 30 min at 30°C with gentle shaking/agitation.

16. To the yeast/DNA mixture add 1.2 ml 1X TE / 1X LiAc /40% PEG solution. Mix well.

| 1X TE / 1X LiAc / 40% PEG | | |
|---------------------------|---------------|-------------|
| Stock | Vol for 1.2ml | Final Conc. |
| 10X TE | 120 μ l | 1X |
| 10X LiAc | 120 μ l | 1X |
| 50% PEG | 980 μ l | 40% |

17. Incubate ~ 30 min at 30°C with gentle shaking/agitation.

18. Heat shock yeast DNA mixture for 15 min at 42°C in a water bath.

19. Quick spin cells in microfuge (~10-12 sec; if you spin too long, the next step of resuspending the cells is much more difficult).

20. Resuspend cells in 500uL T₁₀E_{0.1} pH 7.5 (this is a washing step to remove LiAc and PEG).

21. Quick spin ~8s in microfuge.

22. Decant off the supernatant.

23. Repeat yeast cell T₁₀E_{0.1} pH 7.5 washing (steps 19- 21).

24. Resuspend with 200uL T₁₀E_{0.1} pH 7.5.

25. Create a 10-1 dilution of each transformation mixture by transferring 10 μ l of the resuspended cells to 90 μ l of T₁₀E_{0.1} pH 7.5.

26. Label 8 SD Δ LEU plates according to the yeast host strain genotype, the transforming DNA construct, the dilution, the group and also the date:

Host/DNA Construct Dilutions

| | |
|--------------------|--------------------------------------|
| WT CRT/DRE/Vector | 10 ⁰ and 10 ⁻¹ |
| MUT CRT/DRE/Vector | 10 ⁰ and 10 ⁻¹ |
| WT CRT/DRE/CBF | 10 ⁰ and 10 ⁻¹ |
| MUT CRT/DRE/CBF | 10 ⁰ and 10 ⁻¹ |

27. Plate the two serial dilutions 10⁰ and 10⁻¹; i.e., 100uL & 10uL)

(For library screening approximately 50,000 (5 X 10⁴) colonies per plate is a good target. (~ 1 X 10⁵ transformants per μ g are possible). However when transforming a single DNA construct all you need are isolated colonies on the plates; e.g. plating serial dilutions makes more sense if you were transforming mini prep DNA that you did not know the exact concentration or your cells were not at the ideal growth stage etc.)

Normally the yeast should be allowed to grow ~ 2 days. However for the purpose of the class we will cut this short by one day. So the next step, which would normally be performed after 48 h will be performed after 24 (Wednesday).

B. Fields Screen

Wednesday, 05-12-04:

1. Label each piece of nitrocellulose (N/C) membrane according to the plates:

| | |
|--------------------|----------------------|
| WT CRT/DRE/Vector | 10^0 and 10^{-1} |
| MUT CRT/DRE/Vector | 10^0 and 10^{-1} |
| WT CRT/DRE/CBF | 10^0 and 10^{-1} |
| MUT CRT/DRE/CBF | 10^0 and 10^{-1} |

2. Lay the corresponding N/C membrane over the yeast colonies. To do this, fold the membrane (without creasing it) such that the folded part is centered over the plate. Touch the membrane to the plate and let adhesion pull the membrane down onto the plate (this is performed the same way as if you were laying a membrane onto a gel for Southern Blotting). Don't lift the membrane and then lay it back down, if you make a mistake in which things are not absolutely perfect, proceed anyway because they don't need to be. Alternatively discard the membrane and use a new one. Realize however that the N/C membranes cost several dollars each. Ideally there should be no air bubbles under the N/C paper disc.

3. Return the plate to the 30°C incubator and allow the yeast colonies to grow into the N/C. Incubate the plates overnight at 30°C.

Thursday, 05-13-04:

4. Asymmetrically mark the membrane and yeast plate poking a hole through both with a syringe needle (filled with India ink).

5. Peel the N/C filter off and drop it into a container of liquid N₂ for ~ 5 sec to freeze the filter and the cells (a small Styrofoam ice bucket works great).

6. Remove the membrane from the N₂, lay it on a piece of filter paper yeast side up and allow it to thaw at RT for a few minutes.

7. Meanwhile prepare the required volume of Z buffer/X-gal/2ME:

| <u>Z buffer/X-gal/2ME</u> | | | |
|---------------------------|--|--|--|
| Stock Solution | Vol for one 100 x 15mM (small) petri dish plate | Vol for four plates (One group) | Vol for 16 plates (Four groups) |
| Z buffer | 3.0ml | 12.0ml | 48ml |
| 3% X-gal in DMF | 30µl | 120µl | 480µl |
| 2-ME | 6.75µl | 27µl | 108µl |

8. Lay the circular Whatman 3MM inside the bottom of the petri dish. Add the Z buffer/X-gal/2ME.

9. Once the membrane has thawed, transfer it (yeast side up) into the petri dish containing Whatman 3MM filter paper soaked in the Z buffer/X-gal/2ME. (Try and make sure there are no air bubbles under either paper disc.) Seal the petri dish by wrapping it in plastic wrap, black electrical tape or Parafilm.

Incubate the filters on a level surface at 37°C for several hours to overnight (note however that parafilm will fall apart at 37°C).

Notes:

Whatman 50 filter can be used instead of the nitrocellulose to overlay onto the yeast colonies. However even though it may be way cheaper the results are ambiguous. NC is the only way to go. You need the reinforced nitrocellulose. The other stuff will crack from the liquid N₂ freeze.

3% X-gal is used instead of the standard 2% because this improves weak signals. Use fresh X-gal.

C. Yeast Protein Isolation

Monday, May 17, 2004:

1. Pick three single and individual colonies of each Vector (pDB20.1), GAL₄_{DBD}CBF_{AD}T116→STOP (pEJS949), GAL₄_{DBD}CBF_{AD} (pEJS591) and GAL₄_{DBD}VP16_{AD} (pJR92) per group into 5ml of SD ΔLEU. Grow overnight at 30°C with shaking at ~300rpm.

(Each colony will be analyzed separately for β-gal activity from which the mean and standard deviation/error will be determined.)

Tuesday, May 18, 2004:

2. Spin the 5ml culture volume in the clinical centrifuge on high (2000 rpm) for ~5 min.
3. Decant off the spent medium into an appropriate waste container.
4. Add 1.0 ml sterile dH₂O to the cells and resuspend. Transfer cells to a 1.5ml microfuge tube.
5. Spin cells in a microfuge for 2 min at 4°C (RT is also fine) to pellet the cells.
6. Discard the supernatant into an appropriate waste container.
7. Resuspend the cells in 250 μl of breaking buffer.

Note: Keep the cells on ice from this point on.

8. Add sterile glass beads ~0.45mm diameter (Sigma cat# G8772) to just below the level of the meniscus of the liquid. The easiest way to do this is to gradually pour the beads from a test tube into each microfuge tube. Then add 12.5 μl of AEBSF stock solution to each sample.
9. Vortex six times at top speed in 15-second bursts. Place the tubes on ice in between vortexing bursts.
10. Add an additional 250μl of breaking buffer to the disrupted yeast cells and mix well. Insert the tip of a p1000 pipettor to the bottom of the tube and withdraw the liquid extract to a new microfuge tube.
11. Clarify the extract by centrifuging in a microfuge at 4°C, g-max, for 15 minutes. Transfer the clarified supernatant to a new microfuge tube. Store the extract at -20°C for Thursday's lab.

Yeast Protein Concentration Determination using the Bradford Assay:

Background

There are four different constructs (Vector, GAL₄_{DBD}CBF_{AD}T116→STOP, GAL₄_{DBD}CBF_{AD} GAL₄_{DBD}VP16_{AD}) that will be assayed for levels of β-galactosidase (the *lacZ* reporter gene product). To determine β-galactosidase levels we will conduct β-gal assays (next procedure). However to accurately compare the different levels of β-galactosidase between the different constructs and even between the five replicates of each construct, each β-galactosidase measurement level must be standardized, or normalized. To do this we will express β-galactosidase levels relative to the total protein content of each yeast cell extract. This will be accomplished first by determining the total protein content of each yeast cell extract using the colorimetric assay of Bradford (1976). The Bradford assay is the simplest method for protein detection and is based on the principle that Coomassie Brilliant Blue, the dye component of the Bradford reagent binds via strong interactions (electrostatic and van der Waals forces) to basic proteins (proteins with NH₃⁺ side groups, primarily Arginine, (R), Lysine (K), and with lesser affinity to amino acids with aromatic side chains). The number of strong binding sites can vary widely from protein to protein and depends upon the number of amino acids residues in any given protein that possess NH₃⁺ chains groups with reactive NH₃⁺.

1. Dilute the Bradford reagent fivefold in dH₂O (1 part Bradford: 4 parts dH₂O). Filter the diluted reagent through Whatman 540 paper (or equivalent, I use the Millipore filtration unit).
2. Prepare a dilution series of the BSA reference protein into breaking buffer as follows:
 - a) Prepare a 1.0mg/ml stock solution of BSA in breaking buffer (5-10ml should be more than sufficient for the entire class).
 - b) Label microfuge tubes according to the BSA concentration indicated in the table. Prepare a dilution series of BSA using the 1.0mg/ml stock solution as indicated in the following table:

| Volume of 1mg/ml BSA | Breaking Buffer | Final BSA Conc. (mg/ml) |
|----------------------|-----------------|----------------------------|
| 0μl | 100.0μl | 0 (Blank) |
| 2.5μl | 97.5μl | 0.025 |
| 5.0μl | 95.0μl | 0.050 |
| 7.5μl | 92.5μl | 0.075 |
| 10.0μl | 90.0μl | 0.10 |
| 15.0μl | 85.0μl | 0.15 |
| 100.0μl | 0μl | 1.0 |

c) Label 12 microfuge tubes according to the yeast cell extract (Vector, GAL4_{DBD}CBF_{AD}T116→STOP, GAL4_{DBD}CBF_{AD}) and each of the three different replicates; e.g., V-A, V-B, V-C, G4/CBF-T116→STOP-A, G4/CBF-T116→STOP-B... G4/VP16-B, G4/VP16D.

d) Dilute the yeast cell extracts into breaking buffer in a similar fashion:

| Yeast Construct | Vol yeast cell extract | Breaking Buffer | Estimated Conc. (mg/ml) |
|-----------------|------------------------|-----------------|-------------------------|
| V-A | 20.0µl | 80.0µl | |
| V-B | 20.0µl | 80.0µl | |
| V-C | 20.0µl | 80.0µl | |
| G4/CBF-STOP-A | 20.0µl | 80.0µl | |
| G4/CBF-STOP-B | 20.0µl | 80.0µl | |
| G4/CBF-STOP-C | 20.0µl | 80.0µl | |
| G4/CBF-A | 20.0µl | 80.0µl | |
| G4/CBF-B | 20.0µl | 80.0µl | |
| G4/CBF-C | 20.0µl | 80.0µl | |
| G4/VP16-A | 20.0µl | 80.0µl | |
| G4/VP16-B | 20.0µl | 80.0µl | |
| G4/VP16-C | 20.0µl | 80.0µl | |

- e) Add 1.0ml of the diluted Bradford reagent to each microfuge tube (the BSA dilution series and the yeast cell extract test samples).
- f) Mix the Bradford reagent and the samples well by gentle inversion. Avoid vigorous agitation; i.e., do not vortex as this will result in foaming and poor reproducibility.
- g) Allow the samples to sit at 25°C (RT is okay too) for 5 min.
- h) Add the samples to disposable plastic cuvettes and measure the A₆₂₀ (A₅₉₅) of the samples and standards against the breaking buffer blank within one hour after mixing.
- (Color development is complete after 5 min. Prolonged incubation can actually result in microprecipitation of protein that continues with time resulting in visible aggregates and ultimately resulting in the introduction of non-reproducibility. Higher protein concentrations are even more prone to this phenomenon.)
- i) Prepare a standard curve using the BSA standard and use this to estimate the yeast cell extract protein concentration. (Typical extracts prepared in this fashion contain 0.5-1 mg/ml of protein.)
- j) Be sure to print this and to annotate the readings with the sample. This then will get entered into an excel spreadsheet.

β-gal assays:

Label 12 microfuge tubes according to the yeast cell extract (Vector, GAL4_{DBD}CBF_{AD}T116→STOP, GAL4_{DBD}CBF_{AD}) and each of the three different replicates; e.g., V-A, V-B, V-C, G4/CBF-T116→STOP-A, G4/CBF-T116→STOP-B...G4/VP16-B, G4/VP16D. Also label microfuge tube as a blank for each series (V, G4/CBF-STOP, G4/CBF and G4/VP16). (The most convenient tubes for this assay are the 13 X 100 disposable culture tubes.)

Add 100 μl of extract to 0.9 ml of Z buffer.

Incubate the mixture in a water bath at 28°C for 5 minutes.

Initiate the reaction by adding 0.2 ml of ONPG stock solution. Note precisely the time that the addition is made. Incubate at 28°C until the mixture has acquired a pale yellow color (~30 min).

Terminate the reaction by adding 0.5 ml of Na₂CO₃ stock solution. Note precisely the time that the reaction is terminated.

When working with many samples the easiest thing to do is to initiate and terminate reactions at a convenient interval. For example, add 0.2 ml of ONPG to the first sample tube at T = 0 sec, 0.2 ml of ONPG to the second sample tube at T = 10 sec and so on until ONPG has been added to all samples. Then terminate the reactions in the same way; e.g. sample #1 at 30 min 0-sec, sample #2 at 10 min 10-sec, etc.

Using a spectrophotometer measure the optical density at 420 nm (this can be done using the *vis* light source; i.e., the *uv* is not required). Do this similar to the way you do the Bradford assays:

Read several of your blank samples as well. Usually I do this after reading all of my samples. Most of the protein samples come out in the range 0.09 to 0.6. The numbers that we get from the blank will be averaged and then that average will be subtracted from each samples' OD₄₂₀ reading.

Be sure to print this and to annotate the readings with the sample. This then will get entered into an excel spreadsheet.

Express the specific activity of the extract according to the following formula:

$$\frac{\text{OD}_{420} \times 1.7}{0.0045 \times \text{protein} \times \text{extract volume} \times \text{time}}$$

OD₄₂₀ is the optical density of the product, o-nitrophenol, at 420 nm. The factor 1.7 corrects for the reaction volume. The factor 0.0045 is the optical density of a 1nmole/ml solution of o-nitrophenol. Protein concentration is expressed as mg/ml. Extract volume is the volume assayed in ml. Time is in minutes. Specific activity is expressed as nmoles/minute/mg protein.

Safety notes

Phenylmethylsulfonyl fluoride (PMSF) is extremely destructive to the mucus membranes of the respiratory tract, the eyes, and the skin. It may be fatal if inhaled, swallowed, or absorbed through the skin. In case of contact, immediately flush eyes or skin with copious amounts of water and discard contaminated clothing.

General Notes:

From experience and from discussions with others, single copies of the insert do not seem to work well or at all; i.e., a minimum number of two copies of the *cis*-acting element are required for subsequent DNA binding and trans-activation in yeast. Also from experience, the greater the number of insert copies, the faster the blue color development when the lysed yeast cells are placed on the X-Gal substrate. Thus a minimum of two copies is essential and greater sensitivity occurs with additional copies.

To be sure that the *lacZ* gene is not expressed to detectable levels prior to embarking on a massive one-hybrid screen of a cDNA library, it is recommended that the newly created yeast host strain be tested to insure that the *cis*-acting element-Minimal Promoter-Reporter gene fusion (i.e., UAS-GAL₁TATA-*lacZ*) does not express the *lacZ* gene at random. To do this, simply streak out the construct either (or both) on YPAD rich medium (remember that your construct is integrated into the yeast genome, so there is no need to grow it on selective media once you have a pure culture) or synthetic dropout media. Then go through the Fields screening steps described below.

B. DNA preparations

1. Carrier DNA (Herring sperm (HS): 20mg/ml; 2-8 kb; single stranded)

(Prepared in advance.)

a) Dissolve 25 mg of HS DNA (Sigma catalog # D6898) in 2.25 ml sterile dH₂O.
(Gentle heat, vortexing and patience; i.e., several hours helps.

b) Vigorously draw the dissolved DNA up and down in through a 25g hypodermic needle about 25 – 35 times. This will shear the DNA into approximately 2-8kb size fragments.

c) Phenol/Chloroform extract and EtOH precipitate the HS DNA:

- 1) Add an equal volume TE equilibrated phenol.
- 2) Vortex and spin.
- 3) Transfer the aqueous layer to a new tube.
- 4) Add an equal volume of TE equilibrated phenol – Sevag (50:50).
- 5) Vortex and spin.
- 6) Transfer the aqueous layer to a new tube.
- 7) Add an equal volume of Sevag.
- 8) Vortex and spin.
- 9) Transfer the aqueous layer to a new tube.
- 10) Add 1/10 volume 3M NaOAc (pH 5.2-6.0).
- 11) Add 2X volume EtOH.
- 12) Invert to mix well and spin for 1-2 min.
- 13) Decant off supernatant.
- 14) Wash pellet with 70% EtOH.
- 15) Spin, Wash and Dry the DNA pellet.
- 16) Resuspend DNA in T₁₀E_{0.1} pH 7.5 to ~20mg/ml.
- 17) Boil 10 minutes to denature.
- 18) Place on ice; it is now ready for use. Store at –20°C.

d) To check the size of the sheared DNA, run an aliquot on a gel. Optimal size is 2-8kb.

Note: Never use old phenol; otherwise your DNA will be lost to the phenol.

Sevag is Chloroform/Isoamyl alcohol (24:1)

I suspect that any of the different kits that companies sell (e.g., Qiagen) for cleaning up DNA will work just as well and are way less hazardous, although I have never tried them.

2. Transforming DNA

Plasmid DNA to be transformed can be prepared by CsCl₂, Qiagen (or some other Company's kit) or even phenol-extracted mini-prep; i.e., they all work well. Ideally it should be ~1ug per 10uL TE₁₀E_{0.1} pH~7.5.

II. Materials

A. Consumables

YPAD plates

4 X 500 ml YPAD (2 X 500 ml per yeast host)

Sterile dH₂O

10X TE_{pH~7.5}

10X LiAc

50% PEG 3,350

1X T₁₀E_{0.1} pH~7.5

Yeast Nitrogen base

Amino acids

Bacto Agar

Synthetic complete (SC ΔLeu) plates

Synthetic complete (SC ΔLeu) liquid medium

Sterile 250ml centrifuge bottles

Sterile Oak Ridge tubes

Sterile 100 X 15mm petri dish plates

Sterile disposable culture tubes

Sterile microfuge tubes

Sterile pipette tips

Herring testes DNA (Sigma catalog # D6898)

TE saturated phenol (or DNA clean up kit)

Sevag (Chloroform/Isoamyl alcohol; 24:1)

3M NaOAc pH 5.2-6.0

Ethanol (EtOH)

X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactoside; Gold BioTechnology, Inc. catalog # X4281C).

Nitrocellulose (Schleicher and Schuell, Keene NH; S&S catalog # 10439116)

2ME

Whatman 3MM filter paper (or equivalent) cut into circles the diameter of the petri dish.

Liquid N₂

Sterile 425-600 nm Glass beads (Sigma cat # G8772). Autoclave and dry.

Disposable plastic cuvettes

Whatman 540 paper or equivalent (Millipore _____)

Sterile 1ml syringae and 25G needle (one per group)

Sterile 10ml pipettes

Plastic wrap, black electrical tape or Parafilm

B. Equipment

1. 30°C Incubator

2. 37°C incubator

3. 30°C shaker

4. Superspeed centrifuge

(a) Rotor for 250 ml bottles

(b) Rotor for Oak Ridge tubes

5. Microfuge

6. Pipettes

P1000

P200

P20

7. 42°C water bath (or incubator)

B. Recipes

| | |
|---------------------------------|---------------|
| 1. <u>YPAD</u> | <u>1L</u> |
| a) Yeast Extract | 10g |
| b) Bacto Peptone | 20g |
| c) Dextrose* | 20g |
| d) Adenine sulfate ⁺ | 40mg |
| e) dH ₂ O | to 1L |
| f) Bacto agar (Difco 0140-01) | 20g |
| g) Autoclave | 30-40 minutes |

(For liquid media, omit the bacto agar)

⁺The Difco bacto agar is recommended for yeast because it is reported to result in higher transformation efficiencies for some reason.

| | |
|--|------------------------------------|
| 2. <u>10X TE</u> (7.5) | <u>100ml</u> |
| a) 1M Tris (pH 7.5) | 10ml |
| b) 0.5M EDTA | 2.0ml |
| c) dH ₂ O to | 100ml |
| d) Autoclave | |
| 3. <u>10X LiAc</u> | <u>100ml</u> |
| a) LiAc | 10g |
| b) pH w/ acetic acid to | ~ 7.5 |
| c) dH ₂ O to | 100ml |
| d) Autoclave | |
| 4. <u>50% PEG 3,350</u> | <u>100ml</u> |
| a) PEG 3,350 (Sigma p4388) | 50g |
| b) dH ₂ O to | 100ml (heating helps dissolve PEG) |
| c) Autoclave | |
| 5. <u>T₁₀E_{0.1} pH 7.5</u> | <u>200ml</u> |
| a) 1M Tris pH 7.5 | 2.0ml |
| b) 0.5M EDTA | 40μL |
| c) dH ₂ O | 198 ml |
| 6. <u>Synthetic complete (SC) ΔLeu Medium</u> | |
| (a) Liquid | <u>1 liter (1000ml)</u> |
| 1) Yeast Nitrogen Base (Difco 0919-15) | 6.7g |
| 2) Dextrose* | 20g |
| 3) SC ΔLeu Stock Powder | 5g |

- 4) ddH₂O to 1000ml
 5) Autoclave

(b) Plates

1 liter (~40 plates)

- 1) Yeast Nitrogen Base (Difco 0919-15) 6.7g
 2) Dextrose* 20g
 3) SC ΔLeu Stock Powder 5g
 4) ddH₂O to 1000ml
 5) Bacto agar (Difco 0140-01) 20g
 6) Autoclave
 7) Pour the plates when the medium is cool enough to handle.

7. Synthetic Complete (SC ΔLeu) stock powder amino acid stock for yeast strains EJS151 and EJS216 (alternatively a commercial synthetic complete medium lacking leucine may be used):

Stock

| | | |
|---------------------------------|----------------------|-------|
| a) <u>A</u>DE nine | (Sigma cat # A-9126) | 1.0g |
| b) <u>H</u>IS tidine | (Sigma cat # H-8125) | 1.0g |
| c) <u>L</u>YS ine | (Sigma cat # L-5626) | 1.5g |
| d) <u>M</u>ET hionine | (Sigma cat # M-9625) | 1.0g |
| e) <u>T</u>ry Ptophan | (Sigma cat # T-0254) | 1.0g |
| f) <u>A</u>rg inine | (Sigma cat # A-5131) | 1.0g |
| g) <u>T</u>yr osine | (Sigma cat # T-3754) | 1.5g |
| h) <u>P</u>hen ylalanine | (Sigma cat # P-2126) | 2.5g |
| i) <u>T</u>hr eonine | (Sigma cat # T-8625) | 10.0g |
| j) <u>U</u>RA cil | | 1.0g |

- k) Grind all ingredients to a very fine powder in a mortar and pestle.
 l) Store at RT in an opaque or dark glass container
 m) Use ~5g per liter

8. Breaking buffer:

100mM Tris-HCl (pH 8)
 1mM Dithiothreitol (DTT)
 20% Glycerol

9. AEBSF (4-(2-amino-ethyl)- benzenesulfonyl fluoride hydrochloride), which is a non-toxic alternative to PMSF)

AEBSF (100mM) 23.9mg
 dH₂O 1.0 ml
 Store at -20°C

Alternatively PMSF (Phenylmethylsulfonyl fluoride) may be used but it is much more toxic.

Stock For 1 ml

PMSF (100mM) 17.4mg
 2-propanol (iso) 1.0 ml
 Store at -20°C

10. Z buffer (Miller 1972):

| | <u>Stock</u> | <u>Final []</u> |
|---|--------------|-----------------|
| Na ₂ HPO ₄ ·7H ₂ O | 16.1g | 60 mM |
| NaH ₂ PO ₄ ·H ₂ O | 5.5g | 40 mM |
| KCl | 0.75g | 10 mM |
| MgSO ₄ ·7H ₂ O | 0.246g | 1 mM |
| dH ₂ O to a final volume of: | 1 liter | |

pH to 7. Store at 4°C.

Right before using add 2-Mercaptoethanol to 50 mM:

2ME 2.7 ml (per liter Z buffer)

11. ONPG (o-nitrophenyl-β-D-galactoside) stock solution:
 4 mg/ml in Z buffer. Store at -20°C.

12. Na₂CO₃ stock solution:
 1 M in dH₂O (53g/500ml)

13. Bradford reagent (BioRad)

14. 1.0mg/ml stock solution of BSA (bovine serum albumin) in breaking buffer (5-10ml should be more than sufficient for the entire class).

⁺Adenine is not essential but keeps *ade* mutation from reverting

*Dextrose is often prepared and autoclaved separately (as a 50% dextrose solution) from other media components because it tends to caramelize during autoclaving. After autoclaving the dextrose is then sterilely added to the other medium components. For simplicity we are preparing the dextrose with all other media components.

[#]These are examples of selectable SD media; of course the genotype of the yeast and the marker on the plasmid will ultimately determine the required composition of the selectable media.

Genotypes:

GGY1: (*MATa Δgal4 Δgal80 ura3 leu2 his3 ade2 tyr*) (Li and Herskowitz, 1993)

EJS151: (*MATa Δgal4 Δgal80 URA3::CRT-lacZ leu2 his3 ade2 tyr*) (Stockinger et al., 1997)

EJS216: (*MATa Δgal4 Δgal80 URA3::mCRT-lacZ leu2 his3 ade2 tyr*) (Stockinger et al., 1997)

Glossary:

Activation domains (AD) are component regions of proteins that impart the ability to activate transcription when tethered to a DNA binding domain.

Amino-terminal (NH₃-terminal) is that region of the protein encompassing the first amino acid residues incorporated into the polypeptide chain, which includes the initiator methionine and corresponds to the 5' end of the mRNA.

Auxotrophs are yeast mutants unable to grow in the absence of the particular compound; generally an amino acid (e.g., leucine) or a base (uracil) because they lack the ability to synthesize it.

Carboxy-terminal (COOH-terminal) refers to the last residues of the protein ending at the termination codon and corresponds to the 3' end of the mRNA.

Cis-acting elements are specific arrangements of nucleotide (DNA) sequences within a promoter that are physically linked to the transcribed gene and usually confer regulatory specificity to the transcribed gene.

DNA binding domains (DBD) are component regions of proteins that confer the ability to bind to specific arrangements of nucleotide (DNA) sequences (within a promoter).

Exon is any segment of an interrupted gene that is part of the mature translated mRNA.

Five-prime (5') is used to denote upstream directionality to a nucleotide sequence.

Gene is the segment of DNA (chromosome) transcribed into RNA and translated into protein.

Genome refers to the full complement of nucleic acid that constitutes that organism. Plants have three genomes, the nuclear, the chloroplast and the mitochondrion. The genome of a single stranded RNA virus would be a single stranded RNA.

Introns are segments of DNA transcribed into mRNA but are splice out during maturation of the mRNA into the template actually translated.

+1 refers to the first ribonucleic acid incorporated in the transcribed gene.

Promoter is the region of the gene that dictates where the RNA initiates. A promoter also encompasses the *cis*-acting DNA sequence motifs that confer temporal, spatial and environmental responsiveness to the gene.

Prototrophs are yeast auxotroph mutants in which the ability to synthesize the missing compound has been restored via complementation.

Three-prime (3') is used to denote downstream directionality to a nucleotide sequence.

Trans-acting factors are proteins, RNAs and other cellular components encoded on a non-molecularly linked region of the genome that make their way to the site of action, which is often the *cis*-acting element.

Transcription is the synthesis of RNA from DNA.

Transformation of yeast entails the introduction of a DNA molecule into a yeast cell.

Translation is the synthesis of protein from RNA.

UAS-GAL1_{TATA}-lacZ is a synthetic reporter gene construct consisting of an Upstream Activation Sequence; the TATA box from the yeast GAL1 gene promoter and the *lacZ* gene encoding β -galactosidase.

Upstream activation sequences (UAS; also called enhancers) are *cis*-acting elements that confer increased expression of the gene in response to some sort of temporal, spatial and/or environmental cue. A promoter may have multiple UAS elements that act independently and/or coordinately in response to different stimuli.

PROBLEM SET:

The following problem is meant to recapitulate what is written above about genes and their promoters. **Answers are due at the end of class on Thursday May 20, 2004.**

While working in your parents' Pinot Noir (a red grape cultivar) vineyard one day you noticed that all clusters on an entire branch of one particular grapevine are all white. You figure this must be some kind of mutant. Being an astute horticulturist, you decide to propagate this mutant, call it Pinot Blanc and plant a hectare of it. Several years later your efforts have paid off because you have discovered that Pinot Blanc makes a great white wine. By now you are well on your way to obtaining a Ph.D. in grape genetics because of a fascination with this color phenomenon.

Along the way you have learned that over the years different researchers have molecularly isolated most if not all of the genes encoding proteins involved in pigment biosynthesis. They have called these genes Pigment Gene-R (PGR), Pigment Gene-E (PGE) and Pigment Gene-D (PGD). You decide to look at the mRNA levels of PGR, PGE and PGD in the grapes from your original Pinot Noir cultivar and your new Pinot Blanc cultivar. It turns out that PGR, PGE and PGD are not expressed at all in your Pinot Blanc cultivar; i.e., the genes are off. This is in complete contrast to Pinot Noir, in which PGR, PGE and PGD are expressed at very high levels.

Northern Blot Results

| | Pinot Noir | Pinot Blanc |
|------------|---------------|----------------|
| PGR | ████████ | |
| PGE | ████████ | |
| PGD | ████████ | |

Since the PGR, PGE and PGD genes seem to all be on in Pinot Noir and off in Pinot Blanc you hypothesize that there may be a single regulatory factor that controls their expression. Further, that this bud sport that arose in the vineyard is due to a single gene mutation in the regulatory factor gene. To identify that factor you figure that the most expeditious starting point to do this would be to compare the promoter sequences of PGR, PGE and PGD in order that a shared and repeated sequence motif might be identified.

>PGR

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ACTCTCTTTACCCTCGTAAAGTCGCGAGAACAGCAAACGCGTGGACAGTTATATCAGCGTGGTGTACCCGCGCGGATGTGTGCGGGG
TCCCTCTCCGAGTCAGAACGACTTTGCTTTGCGTAGAGTACACTTTTGACAACAGGTTAACAAATGTACGCTCGGCTAGACAATTGGCGG
AGTCAGCCCGACAATCATCGGAGATACGGGGTATCTCATCATCGCCAAACAAGTAGAGACTAGGTACCATGAGTCCCCCTGGTATG
GCAGTCAGCGTCCCGGGATCGGGCGGAATGTGGGATGCGTACTATTGCCAGCCACGTTACGGCTCCTTAGGTTGCCGGTTACGTATA
CTACAACGTCCTCACTCTAGATGTAATAACAATGGAGAGTGGGTGGCTGAGGTGGCCCGCAGCTATATATAAAGGAACGTGATCTG
GATGACCACGCAGGGCACTACGCCAATTACTGTATAATTACACCAGCGAATACTACAGTACCCCGTCTTGGCGAGCCAGATATACGG
GAGAAGCGGCTTACAGGTCGGCTAGTCCCTACCAATTTGTGTCCAGCAGATGATCACCTTGACCCCTCCCGTATCAGGACGCTACTGTT
GCTTGATGTTAATCCAGTAGGTCGACATTTGGAGGAGGCAAGGCAATGTTTCCCGCGGAACGTTCAAGCCTACGAGAGTCAAGTTA
TCTTGAAATCAAAGTTATGTGATTACCACCTCCAAAATGACACGCATGCACACACAATGGACTGCCGCAAGCGGCTCGTCTCTGCGCTG
GTTAGAAATAGCGTATTGTTGCGAGGCCCTGGAGGTCGCTTCCGCTGCACGCCATTTCCGCTCAGGCGTGTCCCTACTGCCTCTTCGG
GGTCAATCTGATCGGTGCAACCATCACAGTTTGATTCTTAGTGGCTCATCCCTCAATGTTGAATCCTGTGGGTAATGACGCTGCGT
GCTTCAACGGAATCCACGATAAAATTTCTTTTCCCGGGATGCCATAAataaacatataagcttgcacttctctcgtcgagttcg
caacagatcagcacacttccacagccacacgtataatctgtagatcgatcactgttcgtagctcgtagcaccacaata
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>PGE

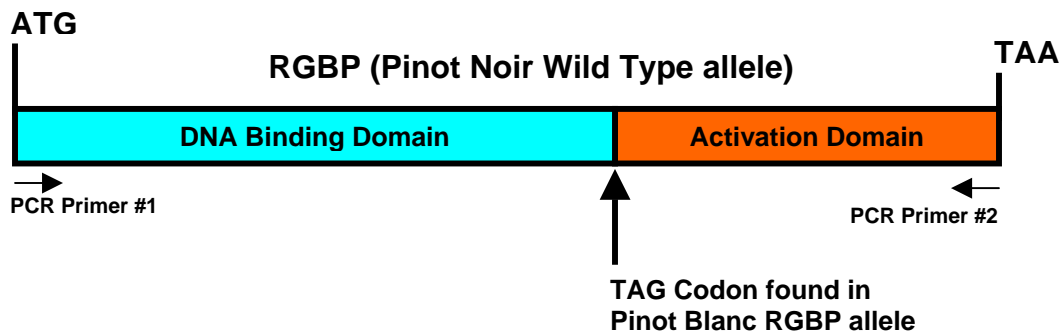
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GGCTCTCCAGAGTTGTGTCAGCTTCTTCAGCTTCTCTTGTGCGGGCGCAGGGCAACCGCAAGGATCATGGCACACTGTCTCACGGCGCA
ACCCAATTGGCTGCCATGAGTCAGCTTACCAGTCCAATAAATGGTTGTTGGCGGCTTTTCTTTCCGAGTCTGATAGTGGAGCACATT
CGTGAAAGAACAATGTGGCAAACGGGGGAGTGGCGGCGTCTGGAATACTGGTCCATCCAAGAGGCAATCATATGCTCCTCATGTAA
TATAGTTACGCGAGTTTGTCTATAAAAAATAGCTGAGTGTGACTTTCAGTAACAGAACAGCGCGCCGATGCAATACCGGGTTC
ACACTACAGCGATGATGTTTTGTGAGGAGGTCGTCGGCAGGGGGGACGGCTAAGTTATTGATCTCGGTATGAACCTGGGGAATTGGAG
ATACAATGTTAGCTACCAGCGGCGTTCACAGCAAAGCGGCTACAACGACTCTGATGAGTTCAACTTTTAAGCGATGCTAGGGTAGCAG
TTTGCAATGATGACTGACCTCGTCCCTGATCGCTCCGTGATAAGCGGCTCACATGTGCTGACCGCAAGCCAGCAACCTGACCGCCAA
ATGTCGCGGGGCTGTAATGTGGGTCGACCCGGCTGCACCGGTGCACGTTAACTCTTCTGATGCGACAGTTAAACCGTATTAGTTA
CCCTGCGGCGTGTGCTGAACCAGCGGCGTAAATGAGCATGTTCTCGACAAGCAACAATAATATAAGTATTGATAGCATACAGTATCTTA
GACTCTACCGAGCAATATTTCTTTCAGTTCATATTTTCCACCTAAACGGCGGCGGGTCTACCACCTAATGCGCGCATCATCGTCT
CCTGAGCTTAAACTAGGCATCAACGATGCGAATAATGCCAATAAAGCCGACGGTTAAATGCAAGGTGTGGTGAACCGCGAGTT
AGACGTAATTTGCAAGCGGCTAGTGGGATCGCACGGGCAAGACAATAacttgcagtcgacttgtctgcatcgcaactcgcagca
gttcacacttacacagccatacacatcatctgtagatcgaccggtgtacgtaaggcgcgacaata
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>PGD

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GATTGACGTTACAAGTTGTGGGACACTGCCAAATTTGCGTGCATATTATTGCCACAAAACGGCGTTTACAGCGACGGCGGCTGTG
TGCGCAGTGGTTACGAATGGCACTGATGGTATTCCTCTAAGCGTTTATTTTACACTCGCAGTCCGCTTTCTAAGAGGGAAATTCATGA
AGAATTCATCAATATGCGGGTCTGTTTCGCCCTGCACTAAGTACGAGCAAGTAATTTGAGCCAAATCCTTCAGGACTGAGCTGTTAG
CGCGAGAAAAGCGGCTTAGCAATGTCACTATGCGCGGCACTAAGTTCGGTATAGGTATCCAGGTGCATAAACAGCTAAGAAACCCAC
AGCCTTGGATTTCCGGTTTACTGCAATGGGTATAGAGGTGGTTTACTCGCCGTGCACATCGGGTAAACCTGTGAACAAGGTAACCTGA
TTTCTTTAGAAGCGATGACAGTCACTCGGTACGGACCGATCATAAATGACGGGGCACCCGGATCCCTACTAGTGTCTTACGGCCGA
TCACAGCAGCTCTGCGTGTAGGAGCGAAGTATAAGTATCGGTCGATGTCCTCGTCCGCTAGCTAGGGCGCAGCTGCGCATTTAG
GTGCCGGTCTTCAAGCGGCTATGGTAAGATCTGTTTCAGATGAGGGGAAACTATTTATCCCGGTAGTTCCTACCGGTGAAGTCACT
TATCCATTGTTCTATTTTTCGGCTAAACAGTAGGGGACATGCAGCGGGGATATGACCACAGCTTTGACCCGGTGGGTACGTCAT
GTATCGCCGATCGCTGAAATGATTGCCTCAACCTCGATGCGTGGTTGAATAATTCGAGAACCTCGACAAGCAGCGCTCGCCTGGTGA
CTAATGTCTGCTCTACGATGGCGCATGTTTACTTAGATTGTCGGCGGGGATCAACACTAGACACAAGGTAATAAATACGGGTTTCC
AGAGGAATCTAAATTAAGCTGCGGGTGGGCTTTGTTATCGCGTATAAactgagcctcgtgctgcaactgcttccacaaccagatc
tgcagagttaactgaggaagacaacacaccagatttctgaggaaccaagatcaacaccactgtgctgtgcaagata
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- (1) What are the common shared sequence motifs between these three gene promoters that might serve as the *cis*-acting regulatory elements for PGR, PGE and PGD expression and why are there two of them (excluding the TATA Box and the Methionine Start codon of each gene which are highlighted in red and green, respectively)?
- (2) Pick one of these three promoters to use as bait in the one hybrid screen; i.e., which promoter do you think will work the best when fused to the *lacZ* reporter gene? Why?
- (3) Using this particular promoter in a one-hybrid screen you identify a transcription factor from the Pinot Noir plant (wild type) that you think binds to your DNA sequence motif. You decide to call this RED-Gene-Binding-Protein (RGBP). Design a synthetic promoter that will prove whether RGBP really binds to the *cis*-acting elements in the PGR, PGE and PGD promoters.

(4) Now that you have molecularly isolated RGPB, the *trans*-acting factor from Pinot Noir that binds to the *cis*-acting DNA sequence motif in the PGR, PGE and PGD genes, you want to determine what mutation is responsible for the Pinot Blanc phenotype. To do this you design PCR primers to amplify the whole RGPB gene from Pinot Blanc and sequence the result. This indicates an in-frame TAG codon midway into the RGPB sequence. Provide a hypothesis consistent with the sequence data to account for the Pinot Noir to Pinot Blanc phenotype that occurred as a bud sport in your family's vineyard.



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