BIOL313: Genetics
Spring 2008
Identification of conserved sequences in casein kinase 1 protein kinases
Determination of mutations to perform

Protein kinases, or enzymes that phosphorylate proteins, regulate almost every aspect of eukaryotic biology. Genes that encode kinases are abundant in diverse eukaryotes, including the yeast species *Saccharomyces cerevisiae* and *Schizomyces pombe*, the fruit fly species *Drosophila melanogaster*, the mouse species *Mus musculus*, and the human species *Homo sapiens*. For example, the human genome contains more than 500 genes that encode protein kinases (1). Most kinases are characterized as part of the eukaryotic protein kinase superfamily due to conserved primary sequence features—or features that are found in all or most family members. Specifically, kinases in this superfamily have a catalytic domain that is characterized by 12 conserved subdomains (see Figure 1, taken from 1). Subdomains I-IV make up an amino terminal lobe that is involved in ATP binding, while subdomains VIA, VIB, VII, VIII, IX, X, and XI make up a carboxy-terminal lobe that is involved in peptide binding and phosphotransfer (2). Within these subdomains, specific amino acids are considered to be highly conserved, and therefore necessary for function of a kinase. Specifically, all or most kinases in this superfamily have the following highly conserved features (2):

1. The sequence GxGxxG (where G is glycine and x is any amino acid) in subdomain I, which may be important in anchoring the nontransferable phosphates of ATP.
2. An invariant lysine (K) in subdomain II that is thought to anchor and orient ATP.
3. And invariant glutamate (E) in subdomain III that helps stabilize ATP binding.
4. The sequence DxxxxN (where D is aspartate, N is asparagine, and x is any amino acid) in subdomain VIB, which is involved in catalysis.
5. The sequence DFG (D = aspartate, F = phenylalanine, G = glycine) in subdomain VII, which appears to bind the Mg^{2+} that helps orient the transferable ATP phosphate group.
6. The sequence APE (A = alanine, P = proline, E = glutamate) in subdomain VIII.
7. The sequence DxxxxG (D = aspartate, G = glycine, x = any amino acid) in subdomain IX, which helps stabilize the catalytic loop of the enzyme.

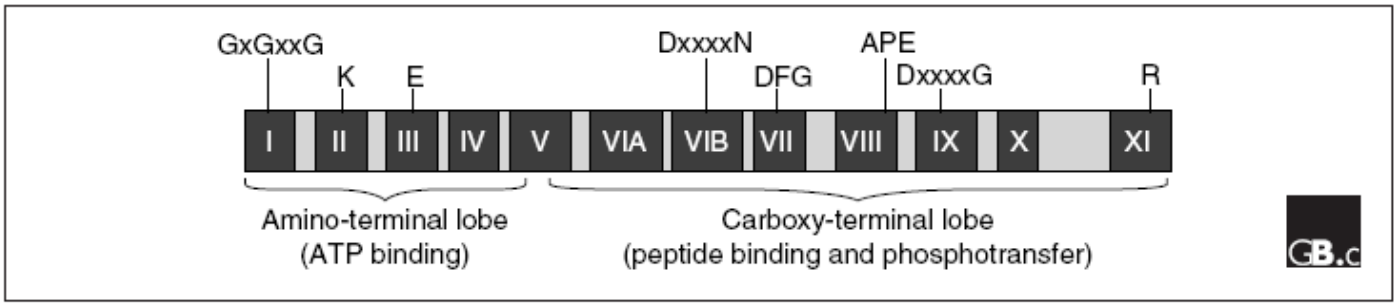


Figure 1
The ePK catalytic domain. The 12 conserved subdomains are indicated by Roman numerals. The positions of amino-acid residues and motifs highly conserved throughout the ePK superfamily are indicated above the subdomains, using the single-letter amino-acid code with x as any amino acid. Crystal structures show that ePK domains adopt a common fold consisting of amino-terminal and carboxy-terminal lobes connected by a hinge region. Binding of Mg-ATP is largely the function of the amino-terminal lobe and hinge region, while peptide-substrate binding is mediated by the carboxy-terminal lobe. Particularly important for catalytic function are the invariant lysine in subdomain II and the invariant aspartate in subdomain VII that function to anchor and orient ATP, and the invariant aspartate in subdomain VIB which is the likely catalytic base in the phosphotransfer reaction. More detailed discussions of ePK subdomains and conserved residues in relation to crystal structures and catalytic function can be found in [3,4,12,13].

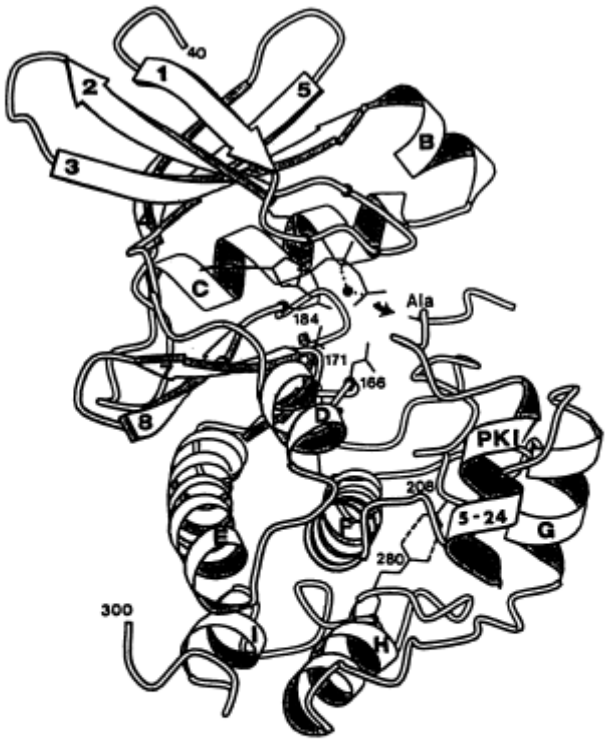
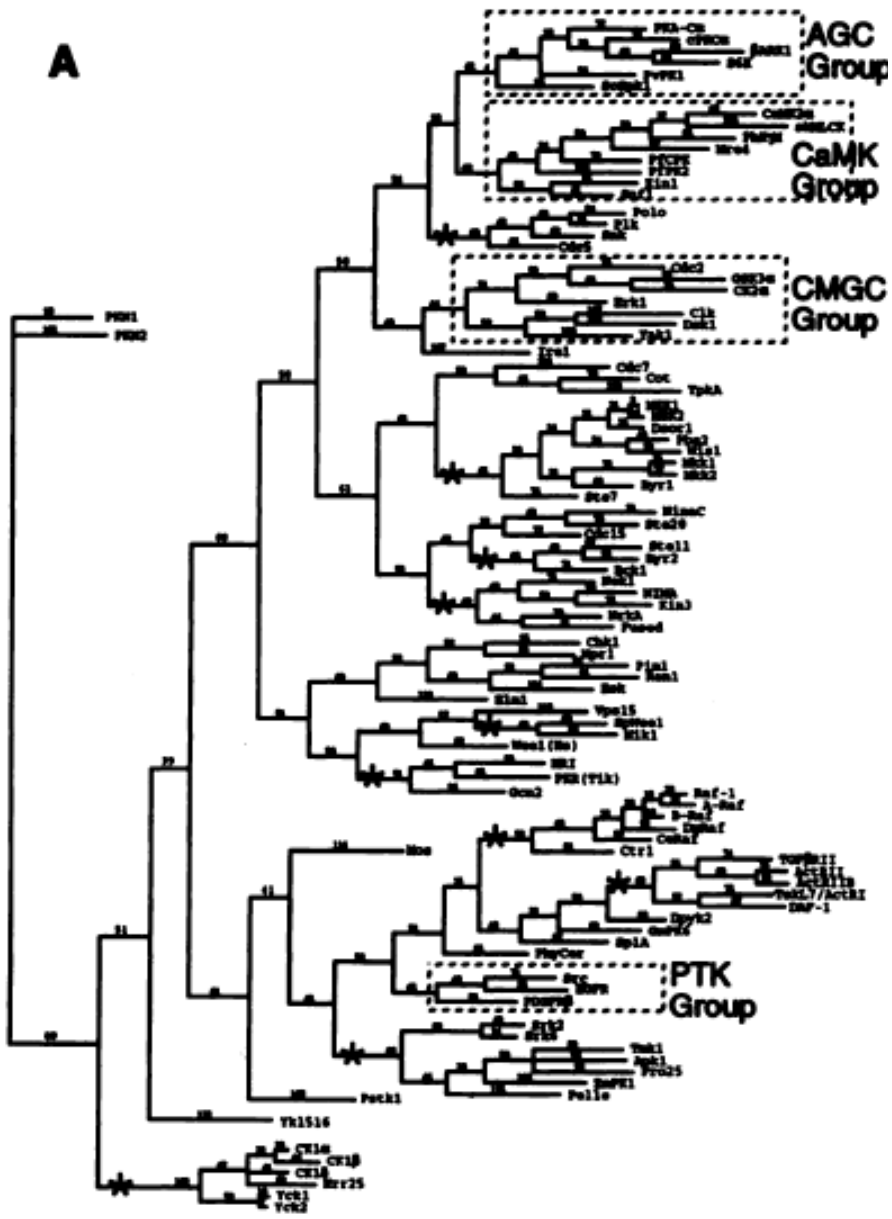


Figure 2. Ribbon diagram of the catalytic core of PKAα (residues 40–300) in a ternary complex with MgATP and pseudosubstrate peptide inhibitor (PKI -5–24). Invariant or nearly–invariant residues (Gly50, Gly52, Gly55, Lys72, Glu91, Asp166, Asn171, Asp184, Glu208, Asp220, and Arg280) are indicated by dots along the ribbon diagram. Side chains are shown for Lys72, Asp166, Asn171, Asp184, Glu208, and Arg280. β–strands and α–helices are indicated by flat arrow and helices, respectively, and are numbered according to Knighton et al. (26). The small arrow indicates the site of phosphotransfer with the Ala in PKI substituting for the phosphoacceptor Ser in the true substrate. (Reproduced, with permission, from Taylor et al. (36)).

The figure at left shows a model of PKAα as a representative protein kinase; the invariant residues are shown by dots along the ribbon diagram. (Reproduced from 3.)

The conserved features described above were identified by aligning the amino acid sequences of many protein kinases and determining which amino acids were found in most or all of these kinases. This work was reported in a review by Steven Hanks and Tony Hunter in reference 3. The aligned sequences that reveal the conserved features are attached at the end of this handout; you will use them in planning your experiment.

Examining these amino acid sequences allows the definition of the evolutionary relationships between kinases as well as the identification of more closely related kinase families within the superfamily. A phylogenetic tree showing the evolutionary relationships within the superfamily is shown on the next page; it is taken from reference 3. This phylogenetic tree reveals that there are several major groups of kinases within the protein kinase superfamily. These include the AGC group, which contains protein kinase A, protein kinase G, and protein kinase C; the CaMK group, which contains calcium/calmodulin-regulated kinses; the CMGC group, which includes the cyclin-dependent kinases; the PTK group, which is the tyrosine kinase group. At the bottom of the tree is the casein kinase 1 (CK1) family of kinases, which includes the yeast enzymes Yck1 and Yck2 (3). The family members in a group are more closely related to each other than to kinases in other groups, and therefore share greater sequence similarity and greater structural similarity.


A
AGC
Group
CaMK
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CMGC
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PTK
Group

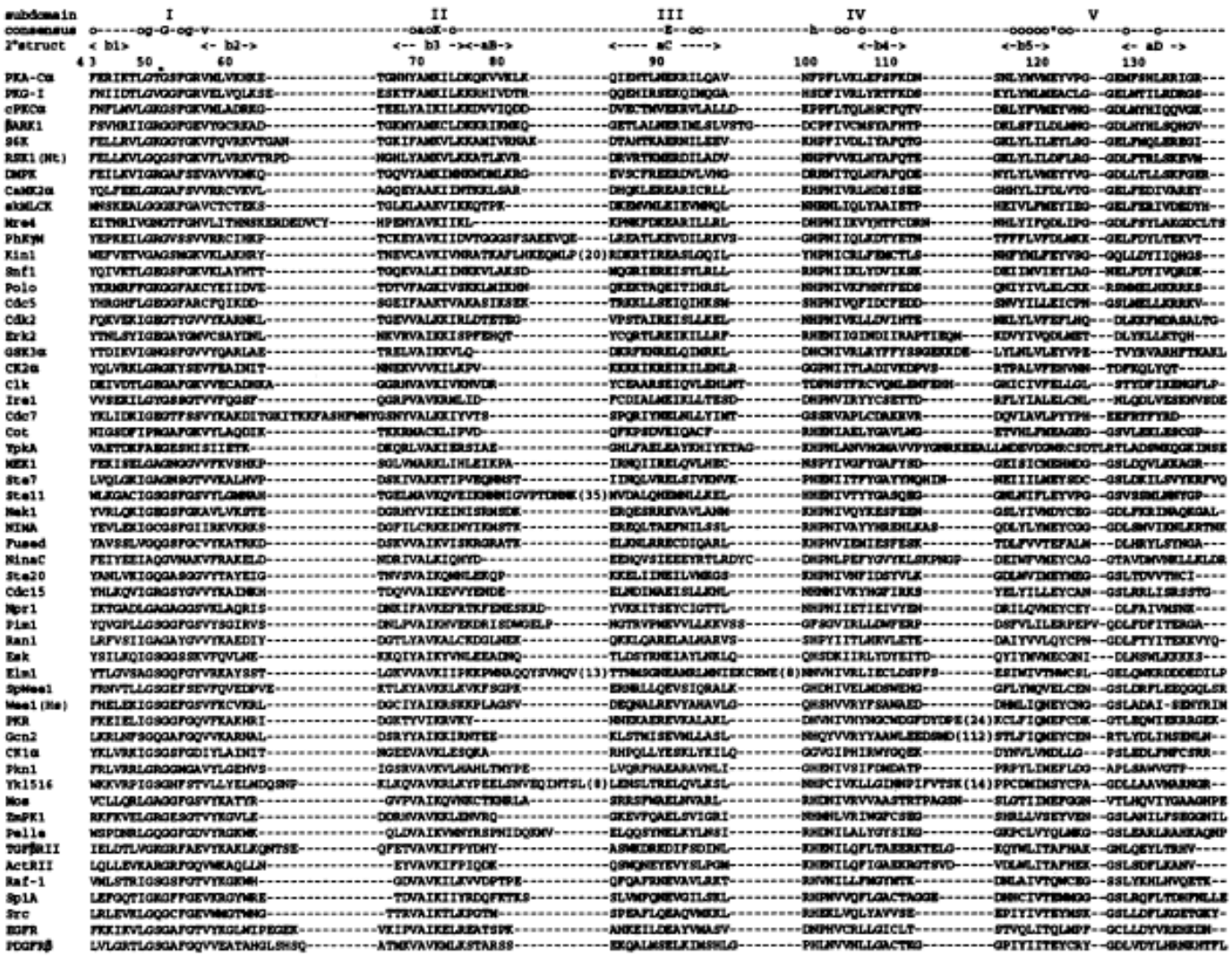

Figure 1. Multiple alignments of 60 kinase domains representative of members of the eukaryotic protein kinase superfamily. The abbreviated names used are as defined in Table 1. The single letter amino acid code is used and gaps are indicated by dashes. The entire sequences for the larger inserts are not shown, but excluded residues are indicated as numbers in brackets. Twelve distinct subdomains are indicated by Roman numerals. The consensus line is given according to the following code: uppercase letters, invariant residues, lowercase residues nearly invariant residues; o, positions conserving nonpolar residues; *, positions conserving polar residues; +, positions conserving small residues with near neutral polarity. Residues corresponding to the numbered β–strands (b) and α–helices (a) in PKA–Cα are indicated in the 2· structure line.

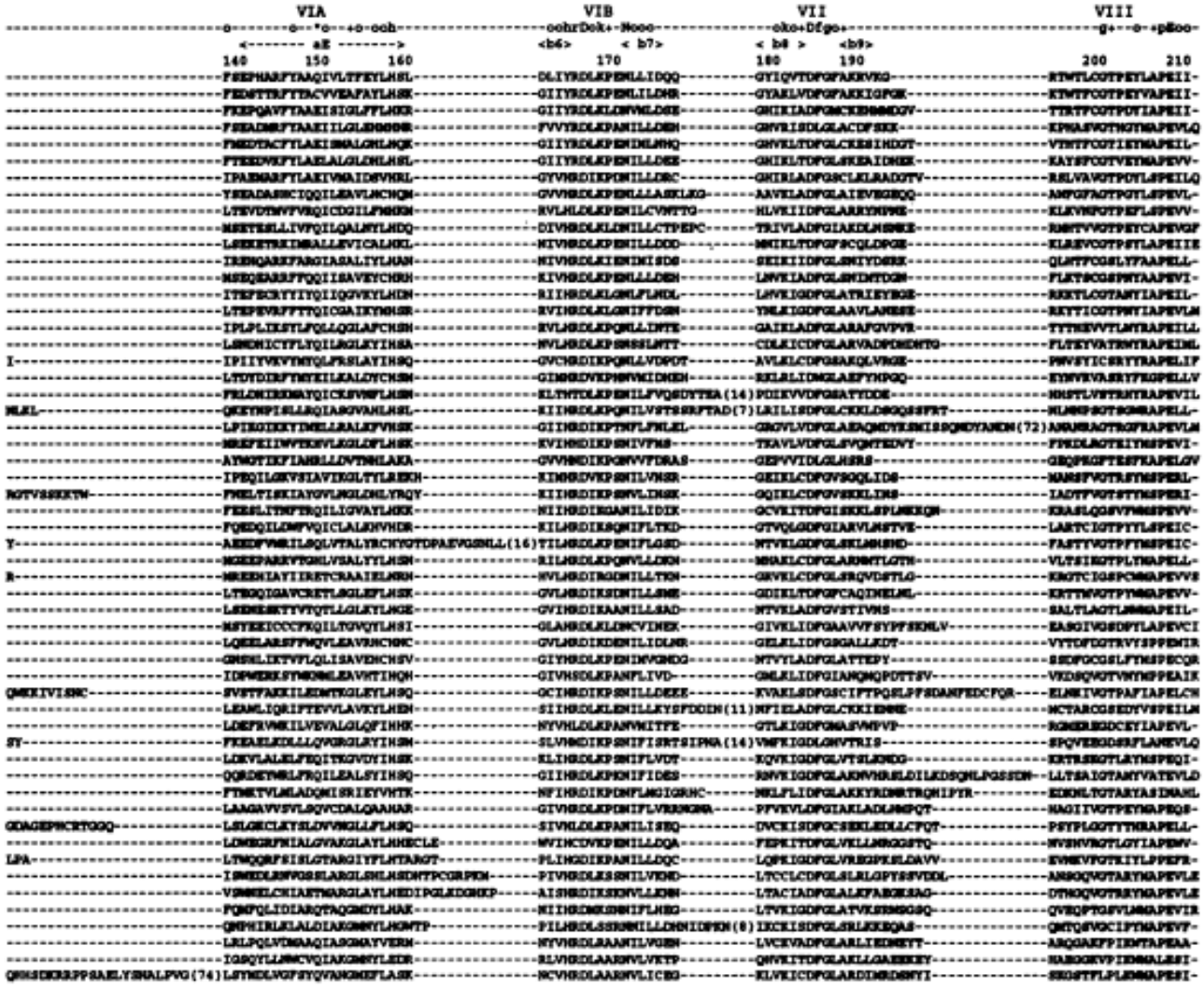

Figure 1 (contd.).

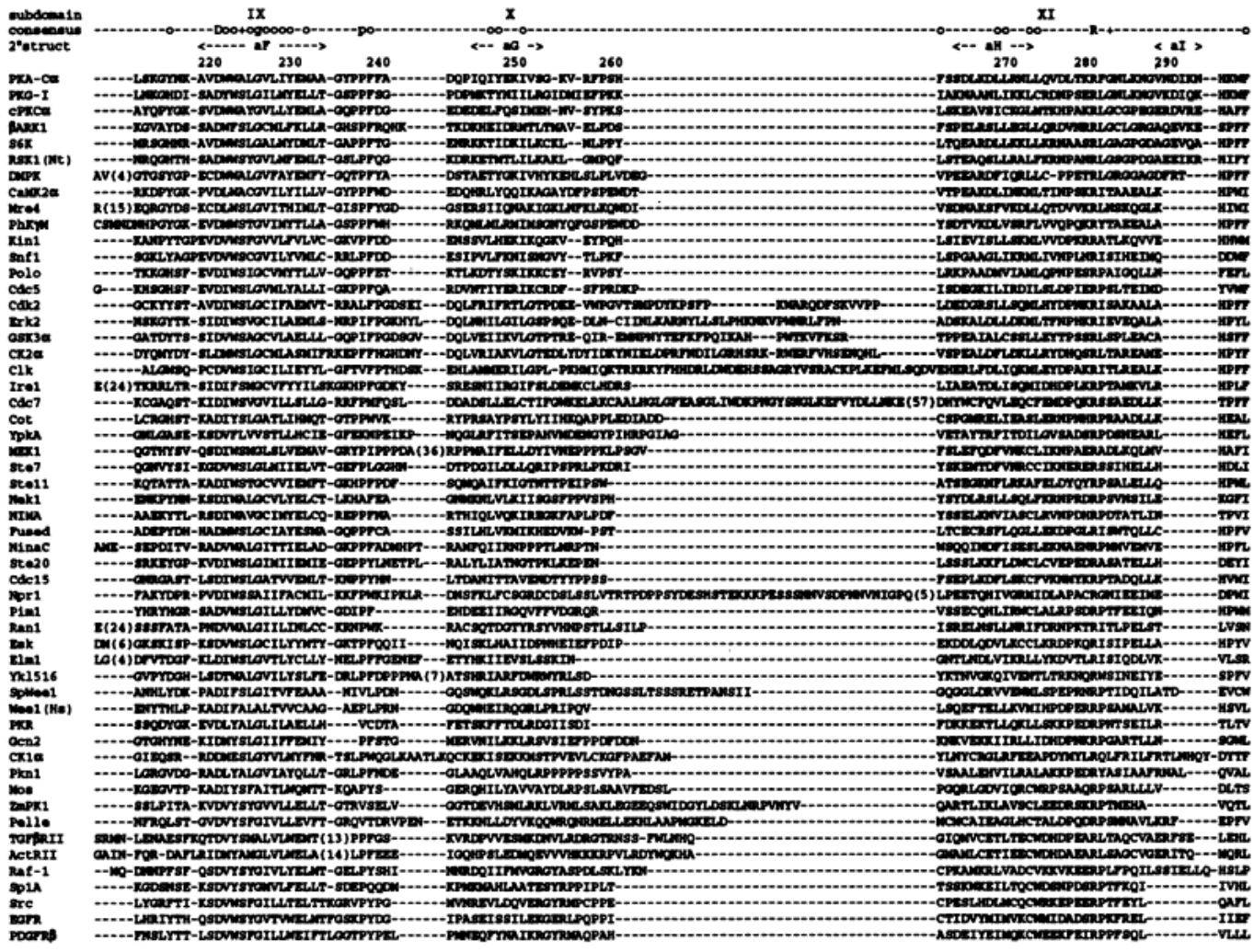

Figure 1 (contd.).

In this class this semester, we are going to examine structural features that appear to be important to the function of CK1-family kinases. Specifically, you are going to identify amino acid sequences that are highly conserved in CK1 enzymes but are not conserved in non-CK1 kinases. You will then determine mutations to make within the conserved sequences that will allow you to investigate the function of those amino acids, design primers to introduce these mutations into Yck2 (a CK1 enzyme), and perform an experiment to determine the effect of your mutation.

Within the CK1 family, there are five major groups of CK1s—the alpha group, the beta group, the gamma group, the delta group, and the epsilon group (4). To determine amino acid sequences that are conserved within the CK1 family, you are going to align the amino acid sequences of a CK1-alpha, a CK1-beta, a CK1-gamma, Yck1, and Yck2. (Yck1 and Yck2 are essential and redundant CK1 enzymes in *Saccharomyces cerevisiae*. We will use Yck2 as a model CK1 enzyme in this experiment. More about Yck2 later.) To align the amino acid sequences, you will use ClustalW, a program that performs multiple sequence alignments. You will also use the protein database provided by the National Center for Biotechnology Information to find CK1 amino acid sequences.

1. Go to www.ncbi.nlm.nih.gov. You will search the protein database for various CK1 sequences.
    a. To do this, select "Protein" in the pull-down menu at the top left of the page.
    b. Enter Yck2 into the blank to the right of the Protein selection and press Go.
    c. You will see multiple Yck2 entries. Choose one of them that is from the species *Saccharomyces cerevisiae*, clicking on the hyperlink. This will give you the Yck2 sequence and accompanying information in GenPept format. Change the GenPept format to FASTA format using the pulldown menu at the top left of the page.
    d. Copy the Yck2 sequence starting at the carrot at the left of the identification line. Paste the sequence into a new Microsoft Word document and save it in a folder marked with your lab day and your group's initials.
    e. Repeat for Yck1, saving the sequence in the same Word document used for your Yck2 sequence.
    f. Repeat for a CK1-alpha. Choose any CK1-alpha sequence from any species—just make sure you get the name along with the amino acid sequence. Save the information in the same Word document as above.
    g. Repeat for a CK1-beta. You will need to omit the hyphen and will need to read the titles of the proteins carefully so that you will get a CK1-beta.
    h. Repeat for a CK1-gamma.
2. Go to http://align.genome.jp/, which allows multiple sequence alignments using the ClustalW program. Copy all five of your sequences from above and paste them into the box for protein sequences. Make sure that your output format is set to "CLUSTAL", that your pairwise alignment is set to "FAST/APPROXIMATE," and that the sequence setting is set to "PROTEIN." Press the "Execute Multiple Alignment" button.
3. Within a couple of minutes, the program will give you the aligned protein sequences. Copy them (and all accompanying information) and paste them into a new Word document. In addition, you should print the alignment. Change the margins on this page to 0.8 inches. This will allow your document to be displayed in a more readable format.
4. Examine your multiple sequence alignments to determine highly conserved amino acid sequences. Identify all stretches of six or more amino acids that are completely conserved in the CK1 enzymes you chose. Highlight the stretches of six or more completely conserved amino acids, both on your electronic and your printed copy. The code indicating full or partial sequence conservation is indicated below:

```
'*' indicates positions which have a single, fully conserved residue
':' indicates that one of the following 'strong' groups is fully conserved:-
STA      NEQK     NHQK     NDEQ     QHRK     MILV     MILF     HY       FYW
'.' indicates that one of the following 'weaker' groups is fully conserved:-
CSA   ATV   HFY   SAG   STNK   STPA   SGND   SNDEQK   NDEQHK   NEQHRK   FVLIM
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5. Determine whether any of these sequences are conserved in other kinases. (Use the tables aligning different kinases shown above.) Are any of these sequences conserved in other kinases? If so, disregard them in further investigations; we are only interested in sequences that are specific to CK1 enzymes.
6. You are now going to examine where these sequences are located within the structure of a CK1 enzyme. Go to www.ncbi.nlm.nih.gov. Choose "Structure" as the database to search, and search the Structure database for casein kinase 1. Choose the CK1 complexed with Mg^{2+}-ATP.
    a. Click on the picture of the structure to get an interactive three-dimensional depiction of the enzyme.
    b. Notice that you can rotate the enzyme to see it from different angles.
    c. Note the presence of the Mg ion and the ATP. You can identify the ribose in the ATP (red labels) as well as the adenine base (blue labels) and the phosphate groups (bound to the Mg ion).
    d. Note the sequence in the box that accompanies the picture of the enzyme. Find one of the conserved sequences within this sequence and box it in using the cursor. This will highlight those amino acids in the three-dimensional depiction of the protein. Rotate the protein until you can see the localization of these amino acids clearly. What does it look like those amino acids are doing? Does it look like they're all doing the same thing, or might one part of the sequence have one function and another part have another function?
        i. Make notes for yourself about what you think these amino acids are doing.
        ii. Save an image that you think clearly shows these amino acids by exporting it as a PNG file.
    e. Repeat for the other conserved sequences you've identified.
7. As a class, choose one sequence to focus on and determine the mutations you will make. As a class, you should choose six mutations to make in one conserved sequence. You may one of two types of mutations:
    a. Deletion: You may choose to delete all or part of your sequence.
    b. Point mutation: You may change one or more amino acids. If you change an amino acid, it is a good idea to make both a conservative mutation (changing to a similar amino acid) and a nonconservative mutation (changing to an amino acid with a very dissimilar structure). For example, if you chose to mutate a glutamate, you could make a conservative mutation to an aspartate and a nonconservative mutation to an alanine.
    c. You should come to next week's lab knowing which mutation your group is going to make. We'll talk about site-directed mutagenesis and you'll design the primers for your experiment.

References

1. Hanks, S.K. (2003) *Genome Biology* **4**, 111.
2. Hanks, S.K., A.M. Quinn, and T. Hunter. (1988) *Science* **241**, 42-52.
3. Hanks, S.K. and T. Hunter (1995) *The FASEB Journal* **9**: 576.
4. Zhai, Z., P.R. Graves, L.C. Robinson, M. Italiano, M.R. Culbertson, J. Rowles, M. H. Cobb, A. A. DePaoli-Roach, and P. J. Roach. (1995) *J. Biol. Chem*. **270**, 12717-12724.

B**IOL313: Genetics**
Spring 2008
Mutagenic primer design
Site-directed mutagenesis

As you learned last week, Yck2 is a member of the casein kinase 1 family of protein kinases. The casein kinase 1 family has been conserved throughout evolution and is therefore found in diverse organisms. This family of protein kinases is thought to be important in determining membrane protein stability, glucose metabolism, synaptic transmission, receptor signaling, circadian rhythm, DNA repair, nuclear import, and cell division (1).

Yck2 and its homolog Yck1 were first reported by Dr. Lucy Robinson and coworkers in 1992. Together, they are essential for cell division in yeast. That is, strains of yeast that express either Yck1 or Yck2 survive and grow, but strains of yeast that express neither Yck1 nor Yck2 do not complete cell division (and therefore do not "grow" into colonies) (Figure 1).

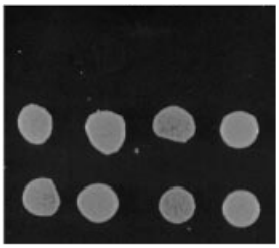


Figure 1. Yck activity is required for cell division and colony formation. Rows 2 and 3 represent colonies formed by yeast with functional Yck1 and Yck2 respectively. The top row demonstrates that yeast lacking both Yck1 and Yck2 do not divide and form colonies. Robinson et al., *Molecular Biology of the Cell*, 1999, **10**: 1077-1092.

The defect in cell division appears to result at least in part from a defect in cytokinesis. In yeast, cell division involves formation of a "bud" off the mother cell. This bud grows, mitosis occurs, and the bud is separated from the mother cell during cytokinesis (see Figure 2).

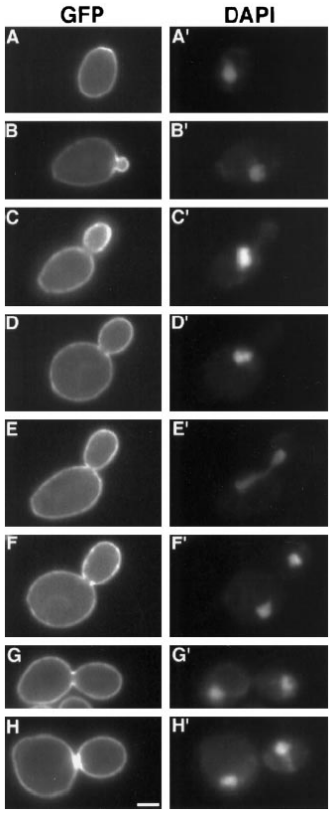
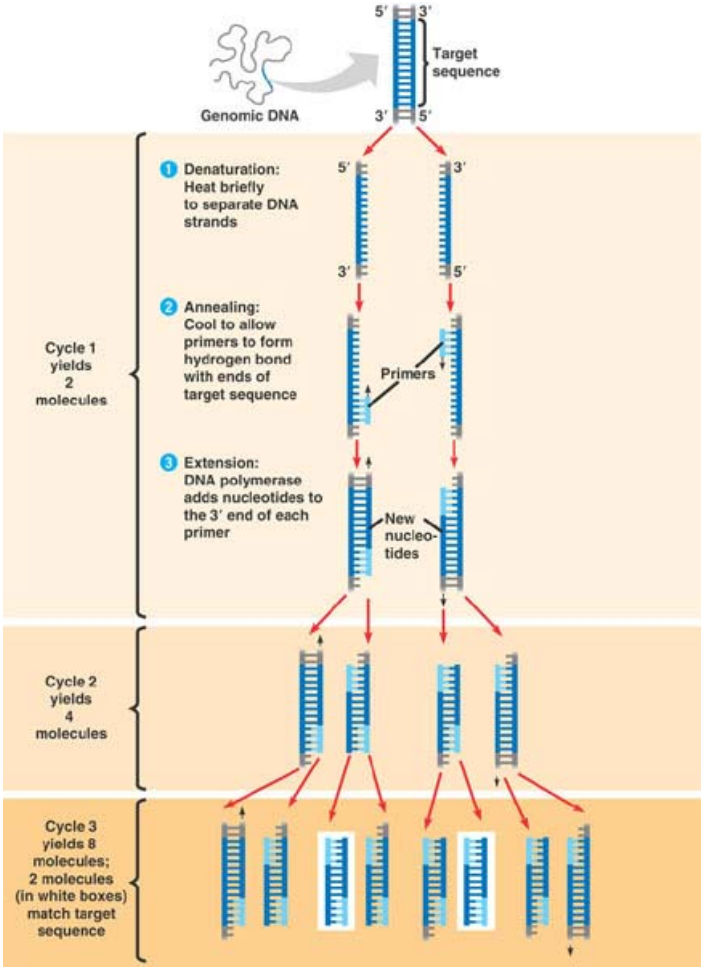


Figure 2. Cell division in yeast involves bud formation and cleavage. The process of cell division is shown in sequential images from top to bottom. Images on the left are stained for Yck2 (shown by light regions); images on the right, which represent the same cells, are stained for DNA. Note that in images A-D there is one nucleus, but in F-H, mitosis is completed and there are two nuclei. Robinson et al., *Molecular Biology of the Cell*, 1999, **10**: 1077-1092.

Yck2 appears to be essential for this process, and may help in bud site selection, polarization of the actin cytoskeleton, and proper formation of a septin ring that is essential for cytokinesis in yeast.

Last week, you identified amino acid sequences that are conserved in CK1 enzymes, including Yck2, but that are not conserved in other kinases. You chose one of these conserved sequences to work with for the rest of the semester and determined mutations to make in that sequence. To make those mutations, you will use a technique called site-directed mutagenesis.

To do site-directed mutagenesis, you will work with the YCK2 gene, which has previously been cloned by Dr. Lucy Robinson. When something is "cloned," many copies are made of it. Typically, gene cloning refers to placing a copy of a gene into a circular piece of DNA called a vector that is defined by a site that allows its replication in cells as well as a selectable marker. The gene/vector construct is then placed in a host organism that replicates it many times. Thus Dr. Robinson has isolated YCK2 from the yeast genome and placed it in a vector, creating a YCK2/vector construct that can be amplified in *E. coli* or in yeast.



To introduce your mutations, we are using a QuikChange site-directed mutagenesis kit from Stratagene. The site-directed mutagenesis reaction is very similar to polymerase chain reaction (PCR). In PCR, a sequence of DNA is exponentially amplified by multiple rounds of replication in a test tube. (The process is diagrammed at left.) Basically, the test tube containing the DNA and other PCR components is heated to break the hydrogen bonds and denature the DNA double helix. The test tube is then cooled to allow primers—which are short pieces of DNA that are specific to the region of DNA to be amplified—to bind (or anneal) to the parental DNA. A special, heat-resistant DNA polymerase (usually, Taq polymerase from *Thermus aquaticus*) then extends the primers using the parental DNA as template. The process is then repeated—the sample is heated to denature the DNA, cooled to allow primer binding, and incubated to allow primer extension. This process allows exponential amplification of the area of DNA bounded by the primers.

Site-directed mutagenesis works in a very similar fashion, but introduces a mutation that you design. How does this work? As you remember, replication requires primers because DNA polymerases do not have *de novo* synthesis activity—that is, they cannot begin forming a nucleic acid without having a previous nucleotide onto which to add. In site-directed mutagenesis, the primers used contain the desired mutation. A DNA polymerase extends the primers, forming a gene/vector construct with the desired mutation.

As in PCR, this process is repeated multiple times, allowing multiple copies of the mutant gene/vector construct to form. Again, the replication is repeated through a process of heating and cooling: The reaction mix is heated to break apart double-stranded DNA; the reaction mix is cooled to allow primer annealing; the reaction is heated slightly to the optimum temperature of the heat-stable DNA polymerase (in this case, *PfuTurbo* DNA polymerase). Site-directed mutagenesis differs from PCR in that only the parent strands are copied, not the newly formed strands as well, so there is no exponential amplification occurs.

After temperature cycling to produce multiple copies of the mutant gene/vector construct, the reaction mix is treated with an enzyme that will digest (= degrade) the parent DNA but will not digest the newly formed daughter gene/vector constructs. (This enzyme recognizes methylated DNA but not unmethylated DNA. You may recall that *E. coli* methylates its DNA after replication, so the gene/vector construct we added as our template, which was grown up in *E. coli*, is methylated.) Since we did not include a methylating enzyme in the reaction, the new DNA is not methylated. Thus the only DNA that remains is the mutant gene/vector construct.

The mutant gene/vector construct is then transformed into *E. coli*, which basically means the mutant gene/vector construct is placed into the media of *E. coli* that can take up the construct. The *E. coli* will then maintain the construct in their cytoplasm, replicating it as they grow. We will therefore be able to harvest a large amount of the mutant construct from the *E.coli* to work with at a later date.

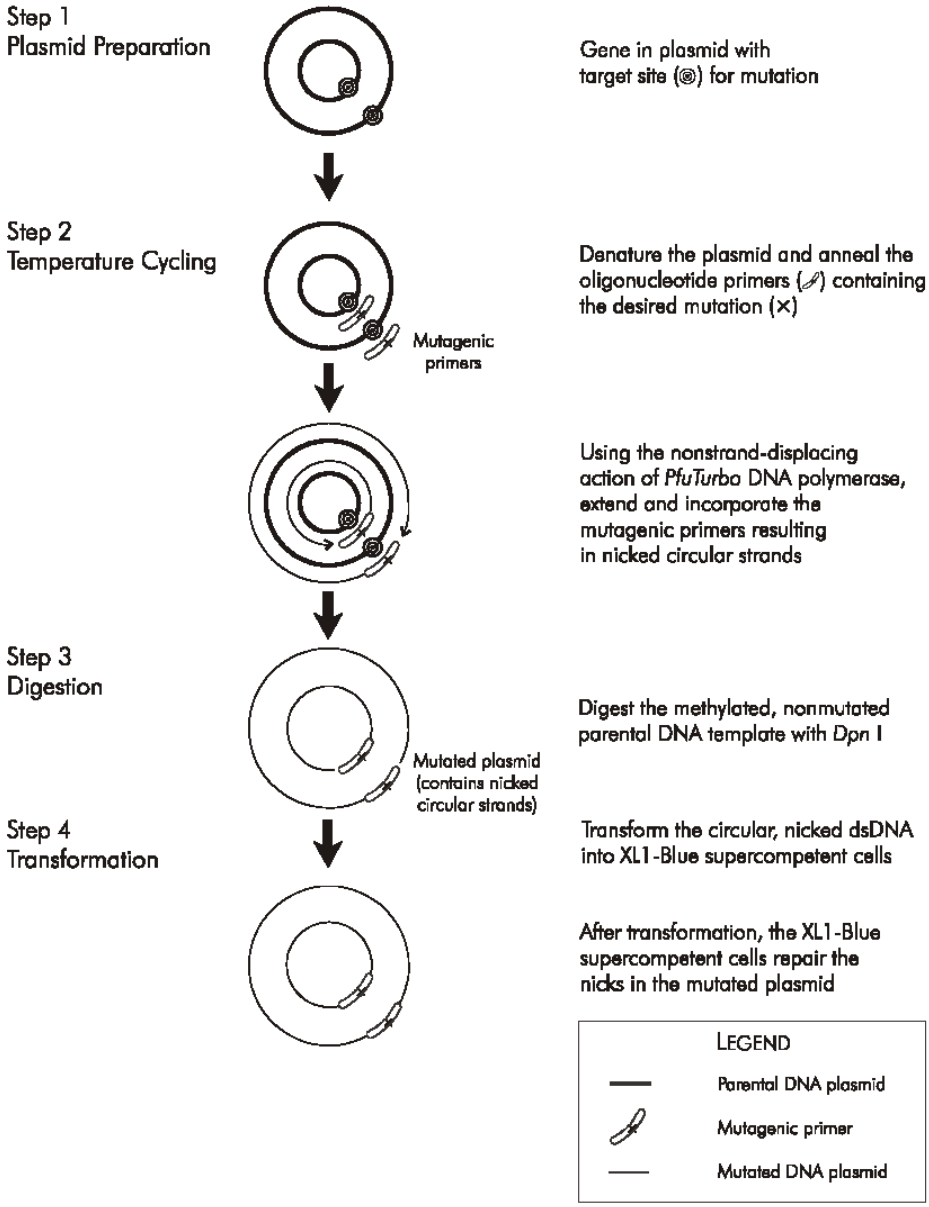


Figure 4. Schematic of site-directed mutagenesis strategy we will use. Taken from Stratagene instructions for QuikChange kit.

Designing your primer:

1. Find the coding sequence for Yck2 by going to www.yeastgenome.org and searching for Yck2. After the Yck2 entry comes up, retrieve the coding sequence by selecting from the pull-down menus on the right side of the page.

2. Copy and paste the YCK2 sequence into a Word document. The best way to get started is probably to align Yck2's nucleotide sequence with its amino acid sequence. To get you started:

Met	Ser	Gln	Val	Gln	Ser	Pro	Leu	Thr	Ala	Thr	Asn	Ser	Gly	Leu	Ala
ATG	TCT	CAA	GTG	CAA	AGT	CCT	TTG	ACA	GCA	ACG	AAC	TCT	GGT	TTA	GCT

3. Identify the mutation you are going to make.
    a. Example 1: Convert Thr9 to a serine. There are six codons for serines: UCU, UCC, UCA, UCG, AGU, AGC. In this mutation, we will therefore convert the codon 9 (ACA) to one of these six codons. The codon that is most similar to ACA is UCA (TCA in the DNA). We will therefore convert codon 9 from ACA to TCA.
    b. Example 2: Delete Thr9. In this instance, we will delete all three nucleotides found in codon 9.

4. Design the primers.
    a. Because DNA is double stranded, you will need to make two primers—one that leads to amplification of the coding strand, and one that leads to amplification of the template strand. Both primers should contain the desired mutation and should anneal to the same sequence on opposite strands of the gene.
    b. Stratagene provides guidelines for primer design.
        i. Primers should be between 25 and 45 nucleotides in length, with a melting temperature (T_m, or the temperature at which this sequence in a double helix will denature) of $\geq 78^oC$.
            $T_m = 81.5 + 0.41$ (%GC) – 675/N - % mismatch
            where N = the primer length in nucleotides and values for %GC and % mismatch are whole numbers.

            To calculate T_m for primers intended to introduce deletions, use this modified formula:
            $T_m = 81.5 + 0.41$ (%GC) - 675/N
            where N does not include the nucleotides being deleted.
        ii. The desired mutation should be in the middle of the primer with ~10-15 nucleotides of correct sequence on both sides. (This is the most important feature.)
        iii. The primers optimally should have a minimum GC content of 40% and should terminate in one or more C or G bases. (This is the second most important feature.)
    c. Example 1: Converting codon 9 from ACA to TCA:

        i. Primer 1: 5'-G CAA AGT CCT TTG **T**CA GCA ACG AAC TC-3'

        ii. %GC = 13 GC/ 27 nt total = 48.1%

        iii. Percent mismatch = 1/27 = 3.7%

        iv. $T_m = 81.5 + 0.41$ (48.1) – 675/27 – 3.7 = 81.5 + 19.7 – 25 – 3.7 = 72.5^oC

        v. Note: The primer is not perfect, as the melting temperature is lower than is strictly desirable. However, as long as the primer begins and ends with a C or a G and there are 10-15 nucleotides on either side of the mutation, it will probably work.

vi. Primer 2: 3'-GTT TCA GGA AAC **A**GT CGT TGC TTG AG-5'

vii. Notice that the primers are complementary to each other. One will be used to initiate formation of the mutant coding strand, while the other will be used to initiate formation of the mutant template strand.

d. Example 2: Deleting codon 9.

i. To delete a sequence, a primer that is complementary to the regions around the desired deletion is made. The primer will anneal and the portion of the cloned gene that is not included in the primer will "bubble out." Since the primer is what initiates formation of the newly replicated sequence, the new sequence will not include the bubbled out region. The picture below attempts to demonstrate.

ii. Wildtype (non-mutant) YCK2 with codon to delete bolded and underlined:
ATG TCT CAA GTG CAA AGT CCT TTG **ACA** GCA ACG AAC TCT GGT TTA GCT

iii. Primer 1: 5'- CAA AGT CCT TTG GCA ACG AAC TCT G- 3'

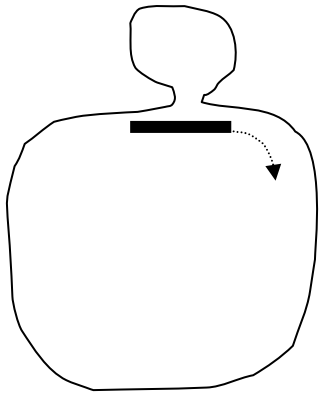
iv. %GC = 12/25 = 48%

v. $T_m = 81.5 + 0.41\ (48) - 675/25 = 81.5 + 19.7 – 27 = 74.2^oC$

vi. Note: Again, the primer is not perfect, as the melting temperature is lower than is strictly desirable.

vii. Primer 2: 3' – GTT TCA GGA AAC CGT TGC TTG AGA C – 5'

viii. Again, notice that the primers are complementary to each other. One will be used to initiate formation of the mutant coding strand, while the other will be used to initiate formation of the mutant template strand.



After designing your primers, show them to your instructor to let her check them. We will order the primers today after class so they will be ready for use next week. Designate at least one member of your group to come in to set up your mutagenesis reaction at 5 p.m. the night before your lab next week. The reaction will run overnight, and during lab next week, you will digest the template and transform *E. coli* with your mutant gene.

To set up the site-directed mutagensis reaction:

1. Talk within your group to remind yourself about how to use pipettemen and which pipettemen are used for different volumes.
2. Set up two site-directed mutagenesis reactions in two thin-walled 0.5 ml microfuge tubes:
   5 ul of 10 x reaction buffer
   X ul of ds DNA template (one reaction with 5 ng, one with 10 ng of the YCK2/vector construct)
   X ul (125 ng) of primer #1
   X ul (125 ng) of primer #2
   1 ul dNTP mix
   ddH2O to a final concentration of 50 ul

   Briefly centrifuge.

   Add 1 ul *PfuTurbo* DNA polymerase (2.5 U/ul)

   Briefly centrifuge.

3. Label your tubes and place them in the Thermocycler, which is set to perform the following cycles:

Segment	Cycles	Temperature	Time
1	1	95°C	30 seconds
2	18	95°C	30 seconds
		55°C	1 minute
		68°C	6.5 minutes

   The reaction will then be cooled and frozen until next week.

References

1. Knippschild U., A. Gocht, S. Wolff, N. Huber, J. Lohler, and M. Stoter (2005) Cell Signaling 17, 675-689.

B**IOL313: Genetics**
Spring 2008
Digestion of template DNA used to make mutant YCK2 (mYCK2)
Transformation of *E. coli* with mYCK2/vector constructs

Restriction enzymes

Restriction enzymes are enzymes isolated from bacteria that cleave ("digest") DNA. It is thought that bacteria have these enzymes to protect them from invading DNA. Typically, these enzymes recognize specific palindromic sequences (a palindrome is a sequence that reads the same forward and backward) and are named for the bacterial species from which they are harvested (e.g., *EcoR*1 is from *E. coli*). Each restriction enzyme cleaves at a specific 4-, 6-, or 8-basepair (bp) sequence. Some make blunt-ended cuts, meaning that they cut both strands of dsDNA at the same site; others leave one of the strands longer than the other, producing "sticky ends" that will basepair with complementary sequences. For example, *Pst*I performs a staggered cut at the 6-bp sequence

5'CTGCAG3'
3'GACGTC5'

producing two DNA fragments with sticky ends: 5'CTGCA G3'
3'G ACGTC5'

Complementary sticky ends will form hydrogen bonds with each other, making it easy for two complementary digestion fragments to cling together.

Typically, restriction enzymes cleave unmethylated DNA but not DNA containing methylated nucleotides. You may remember that many bacteria, including E. coli, methylate their DNA soon after replication. (During mismatch repair, this allows the bacterium to recognize the "good" old strand, remember?) This methylation allows bacteria to distinguish between their own DNA and invading viral DNA. Today, however, we're going to use a restriction enzyme that cleaves methylated DNA but not unmethylated DNA. The restriction enzyme we are using is from *Diplococcus pneumoniae* and is therefore called *Dpn*I. It digests DNA as indicated below (where ^{me} indicates a methyl group):
G^{me}A TC
CT ^{me}AG

The gene/vector construct that we used as our template DNA was grown up in *E. coli* and so is methylated. The mutated gene/vector construct, which was produced in a test tube by site-directed mutagenesis, is not methylated. By treating our reaction mixtures with *Dpn*I, we will therefore digest the template DNA while leaving the mutated gene/vector construct intact.

Transformation and selection

Transformation is a process by which a living cell takes up small pieces of extracellular DNA. It occurs naturally in some species of bacteria, which express receptors that take up extracellular DNA under conditions of nutritional stress. Cells that can take up DNA are termed "competent." Most species of bacteria and all eukaryotic cells are not naturally competent, but can be made competent in the lab. This is most frequently done by chilling the cells in the presence of divalent cations such as Ca^{2+} or Li^{2+}, which makes the cell become permeable to plasmid DNA (remember, we're working with YCK2 that is incorporated into a plasmid, which I

more frequently call a vector). The cells are incubated with the DNA and then briefly heat shocked, which causes the DNA to enter the cell.

Another way to make holes in cells is via electroporation, in which cells are briefly shocked with an electric field of 100-200 volts. The DNA enters through holes created in the plasma membrane created by the shock, and natural membrane-repair mechanisms will close the holes.

It's important to be able to tell which cells were transformed—that is, which cells took up the DNA. This is accomplished by the presence of a selectable marker—in our case, an antibiotic resistance gene on the plasmid vector. If the cells take up the plasmid, they become resistant to a particular antibiotic and so can grow in its presence; if they do not take up the plasmid, they cannot grow in the presence of that antibiotic. This allows the experimenter to select only cells that took up the plasmid, which carries the gene of interest.

In our case, the plasmid carries a gene that confers resistance to ampicillin, making transformed cells Amp^r. We will therefore grow our transformed cells on plates that contain ampicillin, allowing only the cells that picked up the YCK2/vector construct to form colonies.

How does the gene confer ampicillin resistance? It encodes a protein called beta-lactamase, which degrades a four-atom ring containing a lactam bond within ampicillin. When the ring is degraded, ampicillin is no longer antibacterial.

Sterile technique

In today's lab, you are going to need to use sterile technique. What does this mean? In practice, it means that you need to perform your work such that you do not introduce bacteria or other contaminants into your samples. Only sterile instruments should touch your sample or the inside of a container into which your sample will be introduced. Since you are covered with bacteria, you have to be especially careful about touching, breathing into, or placing your face or hair over your sample or the inside of a container.

Why is this so important? Today we're going to be transforming bacteria with our mutant YCK2/vector construct and then growing those bacteria, first on a plate, then in liquid media. If other bacteria get introduced to the plate or the liquid media, they may grow happily and compete with the bacteria containing our gene. Later, then, when we try to recover our mutant YCK2/vector construct, we may be trying to recover it from these contaminant bacteria—which won't contain our gene at all!

In lab I'll show you how to plate your transformed cells and pick a colony for growth in liquid media. In general, however, remember 1) to introduce only sterile instruments to the inside of containers in which your samples are placed, 2) to keep these sterile containers closed or covered at all possible times, and 3) to never lean over, breath on, or touch the inside of one of these containers.

Procedure

Dpn I Digestion

Note: Begin the *Dpn* I digestion as soon as you arrive in lab. We will talk more about the lab while the digestion is proceeding.

1. Add 1 ul of the *Dpn* I restriction enzyme (10 units/ul) directly to each amplification reaction. The *Dpn* I restriction enzyme is kept in the cooler at the front of the lab and should be kept in the cooler except exactly when you are pipetting it.
2. Gently and thoroughly mix each reaction mixture by pipetting the solution up and down several times. Spin down the reaction mixtures in a microcentrifuge for one minute and immediately incubate the reaction at 37^{o}C for one hour to digest the parental (i.e., the nonmutated) DNA.

Transformation

Note: We have bought competent cells, which are very delicate. They must be thawed and aliquotted on ice and kept strictly on ice until heat-shocked. I will do the thawing and aliquotting of the competent cells, but I include the instructions below anyway.

1. Gently thaw the competent cells on ice. For each reaction to be transformed, aliquot 50 ul of the competent cells to a prechilled 14-ml BD Falcon polypropylene round-bottom tube.
2. Transfer 1 ul of the *Dpn* I-treated DNA from each reaction to separate aliquots of the competent cells. Freeze the remainder of your *Dpn* I-treated DNA.
3. Swirl the reaction mixtures gently to mix and incubate the reactions on ice for 30 minutes.
4. Heat pulse the transformation reactions for 45 seconds at 42^{o}C and then place the reactions on ice for 2 minutes.
5. Add 0.5 ml SOC broth (a very rich medium that allows the cells to recover from the heat shock) that has been preheated to 42^{o}C and incubate the transformation reactions at 37^{o}C for 1 hour with shaking at 225-250 rpm.
6. Carefully place 250 ul of each reaction on a prepared LB-Amp plate that you have labeled appropriately (initials, identifying code for your sample). Be very careful to only open the plate for a few seconds and to not touch the inside of it.
   Note: Each group will have two plates, one for the 5 ng mutagenesis reaction and one for the 10 ng mutagenesis reaction.
7. Carefully pour 5 or 6 sterile beads onto the plate containing the transformation mix. Re-cover the plate, and then move it back and forth on the bench (without tilting it) so that the beads carry the transformation mix all over the plate. Continue this process for about 2 minutes, then empty the beads into the plastic beaker on your bench.
8. Give your labeled plates to Dr. Brame for incubation at 37^{o}C overnight. They should develop colonies, each of which formed from a single bacterial cell that picked up your mYCK/vector construct.
9. Label the tubes containing your unplated transformation mix and store in the refrigerator. If plating is successful, we will not need this mix again.

Growing colonies in liquid broth as preparation for recovering our mutant YCK2 gene

Each group should have two plates that contain dozens to thousands of colonies after incubation of the transformation mix overnight at 37^{o}C. The next time we meet, you will recover the YCK2/vector construct from selected colonies. To have enough copies of the YCK2/vector construct to recover, we need to grow selected colonies in liquid medium so they will reproduce asexually, making billions of bacteria from the selected colonies—and thus billions of copies of the YCK2/vector construct.

It's very easy to grow these colonies in liquid medium. The only challenge is using sterile technique.

You'll need to come in to pick colonies and grow them up in sterile medium before the next lab. I'll show you how, but I also include instructions:

1. Pick up a sterile toothpick from an autoclaved tube. Wear gloves, and be sure that the end of the toothpick that you are not holding touches nothing that is not sterile.
2. Open the lid of your plate just a little. Using the sterile end of the toothpick, pick up a single, isolated bacterial colony. Re-cover your plate.
3. Open the lid of a tube of liquid broth in a sterile manner. It is best to hold it in your left hand and remove the lid with the thumb and forefinger of that left hand. If you cannot do this, have your partner open the tube and hold the lid so that it does not become contaminated.
4. Tilt the tube and gently swirl the toothpick in the broth.
5. Quickly cover the tube and plate in the 37^{o}C shaking water bath for overnight incubation.

BIOL313: Genetics
Spring 2008
Examine results from mutagenesis reactions/transformations
Miniprep of plasmid DNA containing mYCK2
Analysis of recovered DNA by restriction digest and gel electrophoresis
Preparation of DNA for DNA sequencing

This week in lab, you will recover your mYCK2/vector DNA from the bacterial cultures I started using colonies from the plates you created by transforming competent *E. coli* with your mutagenesis reactions. After you recover the DNA, you need to examine the DNA to determine if you have actually recovered the mYCK2/vector construct. You will do this by performing a restriction enzyme digest on a tiny aliquot of your recovered DNA. We know the sequence of the mYCK2/vector construct, so we know where specific restriction enzymes should cut. If we have recovered our mYCK2/vector construct, we therefore will see bands of a particular size after the restriction enzyme digest. We will visualize these bands by agarose gel electrophoresis.

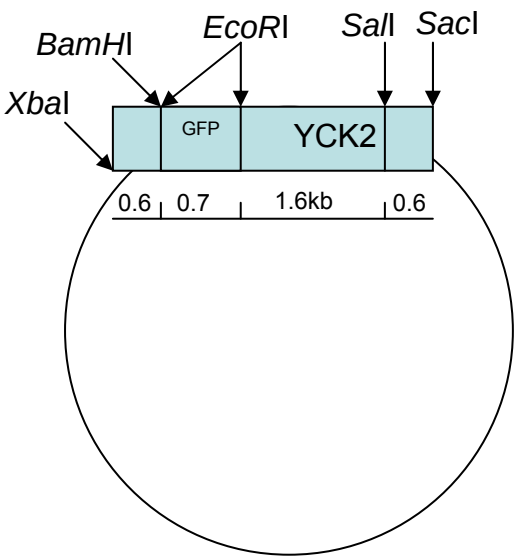
If the digest shows us the bands we expect, you will prepare your DNA to be sent off for sequencing by Retrogen.

Miniprep purification of plasmid DNA

The miniprep procedure is described beautifully at http://csm.jmu.edu/biology/courses/bio480_580/mblab/miniprep.html:
"[The miniprep] procedure is used to extract plasmid DNA from bacterial cell suspensions and is based on the alkaline lysis procedure developed by Birnboim and Doly (Nucleic Acids Research 7:1513, 1979). The procedure takes advantage of the fact that plasmids are relatively small supercoiled DNA molecules and bacterial chromosomal DNA is much larger and less supercoiled. This difference in topology allows for selective precipitation of the chromosomal DNA and cellular proteins from plasmids and RNA molecules. The cells are lysed under alkaline conditions, which denatures both nucleic acids and proteins, and when the solution is neutralized by the addition of Potassium Acetate, chromosomal DNA and proteins precipitate because it is impossible for them to renature correctly (they are so large). Plasmids renature correctly and stay in solution, effectively separating them from chromosomal DNA and proteins."
In our case, the plasmid DNA (=mYCK/vector construct) is then purified using a spin column. For reasons we will talk about later in the semester, the DNA sticks to the column in the presence of salt, but can be washed off (eluted) in the absence of salt. Thus we load our plasmid DNA onto the spin column in the salty buffer in which we isolated it, wash away the salt, and then elute our mYCK/vector construct in pure water.

Restriction enzyme digest of our vectors

Every sequencing reaction we perform costs $10. Therefore we want to verify that the DNA we are sending to be sequenced is our mYCK2/vector construct. To do this, we will perform a restriction enzyme digest of a small aliquot of the plasmid DNA we've recovered. Because we know the sequence of our vector and YCK2, we know where different restriction enzymes will cleave and thus the size of the DNA fragments that will be produced. We can examine the results of our restriction enzyme



digests via gel electrophoresis and see if the fragments correspond to our expectations.

The YCK2/vector construct you used as your template DNA is called pLR10, a partial map of which is shown at right. (The plasmid also contains the BLA gene that encodes beta-lactamase and that confers ampicillin resistance; this gene is not shown.) The plasmid itself is 2.7 kb (= 2.7 kilobases = 2700 basepairs). If we cut this gene/vector construct with *BamH*I and *Sal*I, we should get two fragments: a 2.3 kb fragment (from the 0.7 kb GFP segment and the 1.6 kb YCK2 segment,; 0.7 + 1.6 = 2.3) and a 3.9 kb fragment (the plasmid plus the 0.6 kb *Xba*I to *BamH*I fragment and the 0.6 kb segment between the *Sa*lI and *Sac*I restriction sites; 2.7 + 0.6 + 0.6 = 3.9). If we see this pattern, we can be reasonably confident that we have isolated our YCK2/vector construct from our bacterial culture.

We could also use different restriction enzyme combinations to get different fragments. For example, we could use just *EcoR* I to get a 0.7 kb fragment and a 5.5 kb fragment.

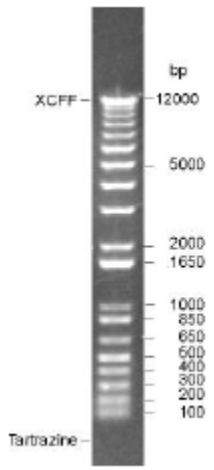
Agarose gel electrophoresis

One of the most globally useful techniques that we will use this semester is gel electrophoresis. In gel electrophoresis, molecules are placed in a gel between a cathode and an anode and subjected to a current. If positively charged, the molecules move toward the negatively charged cathode (black); if negatively charged, toward the positively charged anode (red). How quickly they move is theoretically determined by two characteristics: their net charge and the resistance to movement presented by the gel, which is basically a mesh through which they must migrate.

Two types of gels are commonly used: agarose and polyacrylamide. Agarose is typically used to separate larger molecules because it has larger pores—it is essentially a more porous sieve than polyacrylamide, which is used to separate smaller molecules. Typically, agarose is used to separate DNA fragments while polyacrylamide gels are used for protein separation (three nucleotides—each of which is slightly larger than the average amino acid—are required to encode each amino acid, so proteins are smaller than their corresponding genes). An exception: polyacrylamide gels are used to separate the very small DNA fragments produced in sequencing reactions.

Although the speed with which molecules migrate through a gel is theoretically determined by both net charge and resistance from the gel, in practice fragments of DNA are separated based on size. The sugar-phosphate backbone of all DNA molecules contains negatively charged phosphate groups; all DNA fragments therefore migrate toward the anode. Smaller fragments experience less resistance to movement and thus migrate more rapidly, allowing separation of DNA fragments by size.

The density of the gel determines how quickly DNA fragments move through it. The density can be altered to allow analysis of molecules of various sizes. In agarose gels, the density is determined by the percentage of agarose in the gel: a 3% gel (3% agarose/100 ml) has three times as much agarose as a 1% gel (1 g agarose/100 ml). A 3% gel is therefore much more dense and can be used to separate smaller DNA fragments than can a 1% gel. For example, small fragments would not experience much resistance to movement in a 1% gel and thus would not separate from fragments of similar size, but in a 3% gel, the higher resistance would allow separation of small, similarly sized fragments. A 1% gel is much more useful in the separation



of larger fragments, which would experience great resistance and would not move very far on a 3% gel and thus would not be separated from fragments of similar size.

The separation of DNA fragments based on size is easy to see in a DNA mass ladder, shown at right. The largest fragments are shown at the top of the gel, closest to the wells; the smallest have migrated the farthest from the wells (by convention, the wells are always placed at the top of the figure). Ladders such as these are used to determine the size of unknown DNA fragments. The ladder that you see has fragments of known size, ranging from 12,000 bp to 100 bp. If a fragment in a sample lane migrates the same distance down the gel as one of the bands in the DNA ladder, then it is the same size—has the same number of base pairs.

You can use this ladder to roughly quantify your DNA as well as using it to determine that the DNA fragments are the expected size. We will load 2 ul of the ladder, which corresponds to about 200 ng DNA per band. If your DNA fragment is as bright one of the mass ladder bands, then you have approximately 200 ng in the volume of DNA you loaded; if it appears twice as bright, then you loaded approximately 400 ng of DNA.

For DNA to be visualized, it must be stained in some way. As in the figure above, the DNA is often visualized by staining with ethidium bromide, a dye that intercalates between the bases in the center of the DNA double helix. Ethidium bromide fluoresces when exposed to UV light, allowing easy visualization of the DNA fragments into which it has intercalated. ***Ethidium bromide is a potent mutagen. It can enter your cells through your skin and intercalate in your DNA and should be handled with care—wear gloves and dispose of the dye in designated containers.***

Procedure

Miniprep of mYCK2/plasmid construct

1. Perform the following for each of the three cell pellets.
2. Add 200 ul of buffer P1 and resuspend pellet completely by vortexing.
   The cells are now resuspended in a buffered solution with RNase. When the cells are lysed in the next step, the RNase will catalyze hydrolysis of all RNA molecules into nucleotides, but the DNA will not be affected.
3. Add 200 ul of buffer P2 and mix by inverting and swirling the microfuge tube for 4-6 times. When the cells are lysed, the solution looks clear and viscous.
   This buffer consists of SDS and NaOH. SDS is an acronym for sodium dodecyl sulfate. It is an ionic detergent which disrupts cell membranes and destabilizes all hydrophobic interactions holding various macromolecules in their native conformation. The high pH of the 0.2 M NaOH also denatures macromolecules by changing the condition of ionizable groups (ionizing certain groups and deionizing others). The clearing you see is because the cells are lysing. The viscosity of the solution is increased by the increase in concentration of macromolecules in solution (a result of the cell lysis).
4. Add 400 ul of buffer P3 and mix thoroughly but gently by shaking the inverted tube. Do not vortex hard. A white precipitate will form which consists of SDS, denatured chromosomal DNA, and denatured proteins.
   This is really the key step in the alkaline lysis procedure. The low pH of the potassium acetate solution neutralizes the NaOH and when the pH returns to near-neutrality then the macromolules renature. The proteins and large DNA molecules do not renature correctly however. They form hydrophobic, ionic and hydrogen bonds with each other nonspecifically because the correct conformation of the molecule

was not maintained during denaturation. The plasmid DNA molecules, however, never really fully denatured because they are small circular molecules which are supercoiled. Even though the hydrogen bonds between base pairs were broken by the high pH, they reform correctly when the pH is lowered. The large DNA molecules (chromosomal DNA) and proteins form precipitates because they bind to each other in a large aggregate but the plasmids don't precipitate because they renature correctly and don't become part of the large multi-molecule aggregates. Thus plasmid DNA remains in solution while proteins and other DNA molecules precipitate.

5. Centrifuge the tube for three minutes.
6. Load the supernatant into the Zymo-spin column, either pouring or using a pipet to transfer the supernatant. Be careful not to disturb the white pellet, and avoid carrying over white debris to the column.
7. Centrifuge the Zymo-spin column with the collection tube for 30 seconds.
8. Discard the flow-through in the collection tube. Make sure the flow-through does not touch the bottom part of the column as it would contaminate the DNA inside the column.
9. Add 600 ul of Wash Buffer onto the column with the collection tube and spin for 30 seconds.
10. Add 40 ul water to the column and place the column into a labeled 1.5 ml microfuge tube. Centrifuge for 10-15 seconds to elute the plasmid.

Analytical digest of recovered DNA

1. Choose a set of enzymes to use for your analytical digest:

EcoRI alone	Buffer H
BamHI and Sal I	Buffer D
Xba I and Sal I	Buffer D

2. Set up a digest for each of your minipreps as follows by mixing the following in a microfuge tube (one microfuge tube for each miniprep):
   1 ul 10x buffer (either H or D, depending on which enzyme(s) you're using)
   1 ul DNA from your miniprep
   1 ul each enzyme
   X ul water (to a final volume of 10 ul)

3. Place your tubes in a float and incubate at 37^{o}C for 1 hour.
4. Place the remaining DNA from your minipreps in your freezer box.

Gel electrophoresis

1. Prepare your gel
   a. You are making a 1% gel. Weigh out 0.2 g agarose and place it in an Erhlenmeyer flask with 20 ml TBE.
   b. Microwave on high until the mixture begins to boil.
   c. Stop the microwave, swirl the agarose mixture, and microwave again until the mixture begins to boil.
   d. Stop the microwave, remove the agarose mixture, and swirl.
   e. Allow the agarose to cool until it is easily handled. Add 2 ul ethidium bromide (10 mg/ml), being sure to wear gloves. Swirl to mix.
   f. Pour the agarose into a prepared gel tray.

    g. When the agarose has solidified, remove the dams and the comb. Cover the gel completely with TBE.
2. After your digest is completed, add 2 ul loading buffer to each sample. Load each sample into a separate well. Into another well, load 2 ul of mass ladder.
3. Run the gel at 120 volts for about 1 hour.
4. Visualize using ChemiDoc XRS in the research lab. Save a picture in your folder, and print pictures for your lab notebook.

Preparing DNA for sequencing

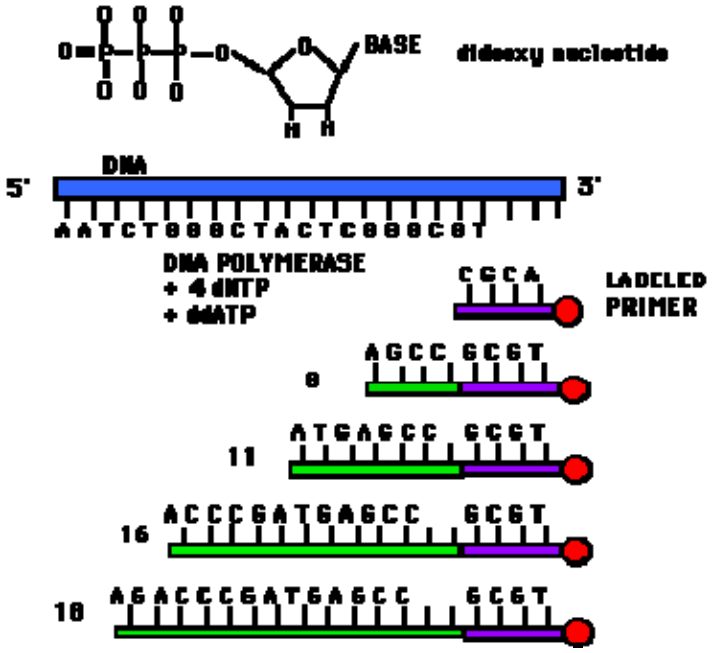
If you see the expected bands on the gel, we will prepare your recovered DNA for sequencing. I will probably do this work so that you are not in the lab late.

1. Determine the approximate concentration of your recovered DNA.
    a. Match the intensity of your plasmid/gene bands to a band in the mass ladder. Use one of the bands larger than 1.65 kb fragment.
    b. If the fragment from your digest is approximately as bright as the band from the mass ladder, then you know you have about 200 ng DNA in that fragment. Since you digested 1 ul of your miniprep DNA and loaded all of that digest, you know that your miniprep DNA is approximately 200 ng/ul.
    c. Place 3 ug of your recovered gene/vector construct in a clean, sterile, small microfuge tube. If necessary, add water so that it is at a concentration of 200 ng/ul.
    d. Label your tube using an abbreviation for your mutation and a number to indicate which miniprep the tube corresponds to (e.g., DelSer-1, DelSer-2, etc.).
    e. Place the tubes at the front of the room in the rack provided by Dr. Brame.
    f. Store your remaining DNA in your freezer box.

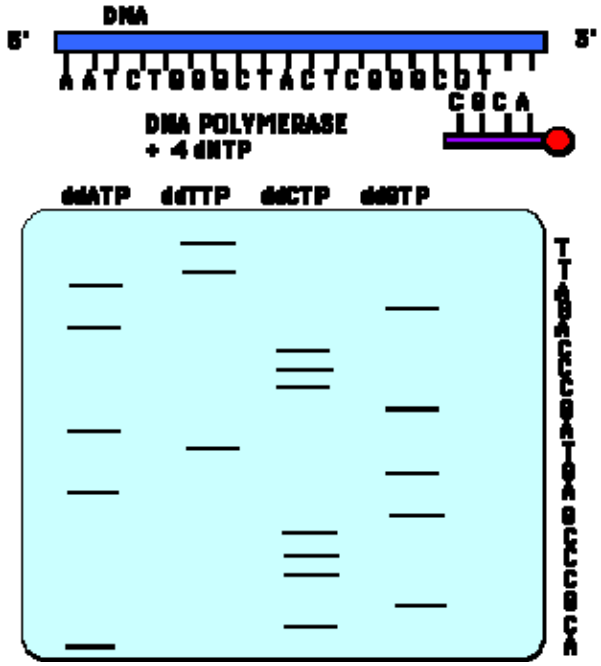
BIOL313: Genetics
Spring 2008
Analysis of sequencing results
Digest of pLR10 to separate GFP-tagged mYCK2 from plasmid
Digest of pRS316, which can use either *E. coli* or yeast as a host

Two weeks ago, we sent your mutant YCK2/vector constructs to a company called Retrogen that specializes in DNA sequencing. Today, we'll examine the sequences they send us to determine if we formed the particular mutations we wanted.

Background on sequencing



The most common method for sequencing DNA is the Sanger dideoxy method. Traditionally, the template DNA is denatured and mixed with a labeled (radioactive or fluorescent) primer that is complementary to DNA adjacent to the 3' end of the DNA of interest. The template and primer are mixed with other components necessary for primer extension, such as dNTPs, DNA polymerase, Mg^{2+}, etc. These components are mixed in four tubes; to each is added a different dideoxynucleotide (ddATP, ddCTP, ddGTP, ddTTP), which lacks the 3'-OH required for primer extension. In each tube, primer extension occurs on each of the many template molecules until a dideoxynucleotide is incorporated, generating a series of labeled oligonucleotides of varying lengths that are complementary to your gene of interest. See the figure at right, taken from www.bio.davidson. edu/ courses/Bio111/seq.html (10). The products of these reaction mixtures are run in four separate lanes of a gel, where they separate according to size. Oligonucleotides that differ by a single base can be distinguished, and the terminal nucleotide in each oligonucleotide can be determined by the lane it is in. For example, the lane loaded with the ddTTP-containing reaction mixture will contain only oligonucleotides terminated by a thymidine. Thus the gel shows a series of oligonucleotides differing by one base; by "reading" what this terminal base is, one can determine the sequence of your gene of interest. The figure below demonstrates these features and is taken from www.bio.davidson.edu/courses/ Bio111/seq.html. Because the smallest fragments are found at the bottom of the gel, the 5'->3' sequence is read from the bottom up (at right, 5'ACGCCCG...3'). This gives the 5'->3' sequence of the DNA complementary to the template; the template sequence would be 5'TTAGA...3'.



Currently, a slight modification of this method is most frequently used: the chain-terminating nucleotides are labeled with different colored fluorescent dyes, rather than the primer being labeled, so all four reactions can be done in the same tube. The products can then be separated in a single lane of a gel, with a detector placed near the bottom of the gel recording the color of the fluorescent label on each band as it passes through a laser beam.

We do not have the equipment to perform DNA sequencing (the sequencers cost about $600,000!), so we sent our DNA offfsite for analysis and will analyze the data that is returned to us.

Analyzing our sequences

Retrogen posts our nucleotide sequences to a website wehre we can download them. We will align these sequences with the wildtype YCK2 sequence to see if we introduced the desired nucleotide. To do this, we will use a tool that is known as BLAST, which stands for Basic Local Alignment Search Tool. BLAST, as its name implies, finds areas of homology between a sequence of your choice and the sequences in a database. It can look for protein homology (alignment of amino acid sequences) or nucleic acid homology (alignment of nucleotide sequences). It can also search for homology in different databases.

We're going to use a database consisting of yeast genes (called "ORF DNA, Coding Sequences of defined ORFs"). The most convenient way to access this database is through a website called the *Saccharomyces* Genome Database (www.yeastgenome.org; *Saccharomyces* is the genus name of the yeast we are using, which is the most commonly used yeast in molecular biology). This website provides access to a variety of features; we'll only use a subset of these features.

One useful feature allows you to search for specific genes, such as YCK2. If you search for YCK2 on the SGD homepage, you will get a page with a variety of links that can take you to literature on YCK2, the genomic sequence of YCK2, the protein sequence of Yck2, *etc*. The only feature we will use today is the BLAST feature, however.

To use BLAST, click the "BLAST" link at the top of the SGD homepage. This will take you to a page where you can input the YCK2 sequence that you were sent by Retrogen. Copy your sequence and paste it into the box labeled "Type or Paste a Query Sequence." Then choose BLASTN at the pulldown box that directly follows the Query Sequence box. (BLASTN searches nucleotides; BLASTP searches protein; BLASTX will search nucleotides against protein databases by generating a predicted translation.) In the next pulldown box, choose the "ORF DNA, Coding Sequences of defined ORFs" database. (The other databases are also yeast databases, but may include intron sequences, noncoding intergenic sequences, or protein sequences.) After making these choices, hit the "Run BLAST" button.

The results that emerge will list all yeast genes that have significant homology to the sequence you entered. YCK2 should be at the top of this list, indicating it has the highest homology. As you scroll down, you will see how the sequence you entered (the query sequence) aligns with YCK2 (the subject sequence). Lines between the Query sequence and the Subject sequence indicate homology; no lines indicate a difference. You should be able to find your mutation using this alignment. We'll do some of this work together, so don't worry if you have questions.

Digestion of pLR10 to separate GFP-tagged mYCK2 from plasmid and pRS316, which can use either *E. coli* or yeast as a host

This week, we also begin the second part of the YCK2 project. Currently, each of you should have one or more clones of your desired mutant YCK2 (mYCK2) in pLR10. As we've seen, pLR10 can be replicated by *E. coli*, producing multiple copies of the mYCK2 gene. It cannot, however, be replicated by yeast. We therefore need to transfer the mYCK2 into a plasmid that has a yeast origin of replication (called ARS, for autonomous replication sequence). This plasmid is called pRS315.

The plasmid into which we will transfer our mYCK2 has several other notable features:

1) It is a yeast/*E. coli* "shuttle vector," meaning it can be replicated in both yeast and *E. coli*. We can therefore grow up the mYCK2-containing shuttle vector in *E. coli* (which grow quickly and therefore will produce many copies of the vector), do a miniprep to recover the mYCK2/shuttle vector construct, and then place the mYCK2/shuttle vector construct into yeast.
2) It has a beta-lactamase gene (Amp^r) that inactivates ampicillin. We can therefore select for bacteria containing this plasmid by growing them on ampicillin.
3) It has a LEU2 gene that allows for leucine synthesis. We can therefore select for yeast containing this plasmid by growing them in the absence of leucine.

The steps for transferring mYCK2 from pLR10 to pRS315 are as follows:

1. Perform a restriction digest to cleave mYCK2, the GFP tag, and the flanking regions out of the plasmid. One flanking region contains a promoter sequence that allows GFP-tagged YCK2 to be expressed in yeast, while the second flanking region contains a terminator.
2. Perform the same restriction digest on pRS315 to create an opening with the appropriate sticky ends to receive our mYCK2 fragment.
3. Run the digests on a gel to separate the mYCK2 fragment from the plasmid fragment and to isolate the appropriate pRS315 fragment.
4. Cut out the gel band that corresponds to mYCK2 and the gel band that corresponds to the desired pRS315 fragment.
5. Purify the DNA out of the agarose gel.
6. Ligate the mYCK2 gene into the cut pRS315.

To express the mYCK2/shuttle vector constructs in yeast, we will then perform the following steps.

7. Transform competent *E. coli* with the mYCK2/shuttle vector construct. Plate the transformed *E. coli*.
8. Pick colonies; grow liquid cultures.
9. Perform miniprep procedure to recover mYCK2/shuttle vector constructs.
10. Perform an analytical digest to check recovery from miniprep.
11. Transform yeast with mYCK2/shuttle vector constructs.
12. Analyze mYck2 function and localization.

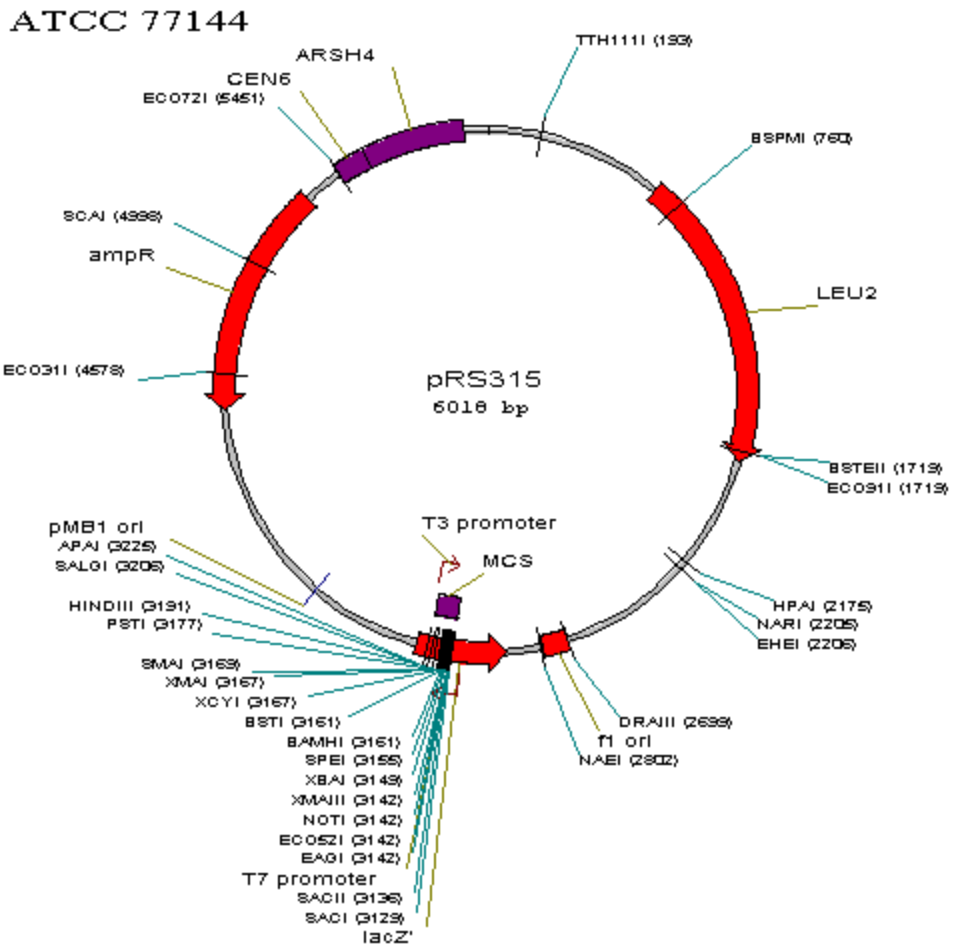
Today you will digest the mYCK2/plasmid constructs to cleave the GFP-tagged mYCK2 out of the plasmid. Next week, you will run the fragments on a gel and cut out the desired bands and purify the DNA away from the agarose gel.

To determine the restriction enzymes that you will use, examine the map of pLR10 that is in your week 3 handout. Which restriction enzymes will allow you to cut out the GFP-YCK2 fragment including both flanking regions?

Will those two restriction enzymes each cut in a single place in pRS315? Examine the map below to answer this question.

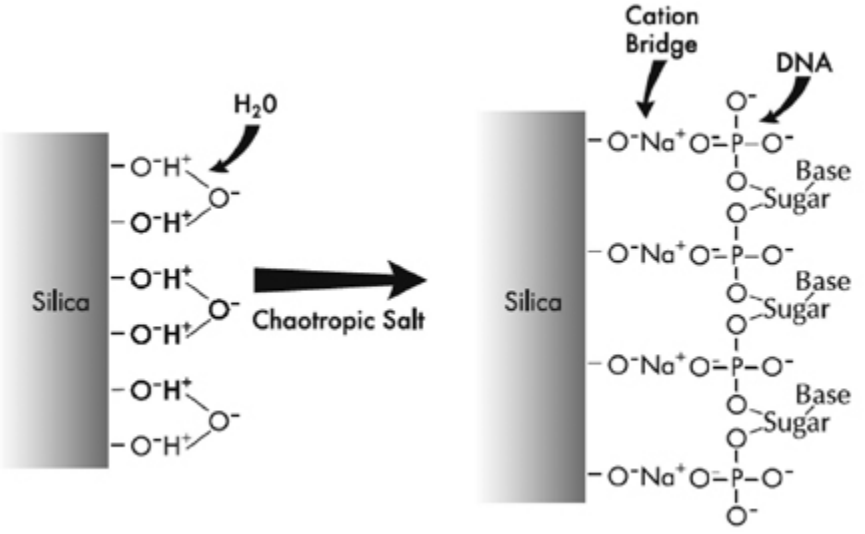
Procedure

1. Choose the mYCK2 clone with which you will work and retrieve it from your freezer box. I suggest you pick the clone that both has the desired mutation and has the highest concentration.
2. Briefly centrifuge the tube to bring all the liquid to the bottom.
3. Set up a 25 ul digest using 2.5 ug of your mYCK2 DNA.
    a. You will need to include
        i. enough 10x buffer to give a final concentration of 1x
        ii. 1 ul of each enzyme (be sure to record how many units this is)
        iii. enough water to bring the digest to 25 ul
4. Set up a 25 ul digest using 2.5 ug of pRS315. You will have a tube containing 2.5 ug pRS315 (in 0.5 ul water) in your freezer box. Simply add your enzymes, buffer, and water to this tube.
5. Incubate the digest at 37°C for 2 hours.
6. While digesting your DNA, pour a 1% gel. Use the comb that gives larger wells.
7. After letting your digest proceed for 2 hours, add 5 ul loading buffer to each sample and briefly centrifuge. Store the samples in the refrigerator until the next lab period.



B**IOL313: Genetics**
Separation of fragments by gel electrophoresis
Purification of GFP-mYCK2 fragment and purification of digested pRS315
Ligation
***E. coli* transformation**

This week, you will separate the DNA fragments you generated in your preparatory digest last week using gel electrophoresis. You will then purify the GFP-mYCK2 fragment that you generated by restriction digest and separated from other DNA fragments by gel electrophoresis. You will also purify your digested pRS315. After purifying the fragments, you will ligate them together.



The purification procedure you are using is based on the observation that DNA binds silica in the presence (but not the absence) of chaotropic salts. In brief, the agarose gel will be melted in the presence of guanidine HCl, a chaotropic salt. This mixture will be passed over a silica minicolumn, which will bind the DNA, but not the agarose. The column will be washed to remove the chaotropic salts, at which point the DNA is eluted with nuclease-free water. The mechanism of DNA-silica binding has not been fully investigated but is hypothesized to be as shown at left (taken from www.qbiogene.com/ products/geneclean/ geneclean-overview.shtml).

Procedure

1. Cast a 0.7% agarose gel. Remove your restriction digests from the freezer and briefly centrifuge. Load the samples into the gel. In addition, load 5 ul of mass ladder in a separate lane. Run the gel at approximately 100 volts for about 2 hours.
2. Visualize the gel with UV light. Take a picture and use a scalpel to excise the band containing your mYCK2 gene (how big is this fragment?) and the band containing the desired pRS315 fragment (how big is it?).
3. Place the gel bands in labeled, pre-weighed tubes.

DNA purification

1. Determine how much agarose gel you have (by weight) for each of your DNA fragments. *How will you do this?*

2. Melt the agarose gel in Membrane Binding Solution (4.5 M guanidine isothiocyanate and 0.5 M potassium acetate, pH 5.0), using 10 ul MBS for every 10 mg of agarose and incubating at 60^{o}C. Vortex every couple of minutes to speed melting. The gel should be melted within 10 minutes. Make sure the gel is completely melted before proceeding further.

3. For each of your samples, follow the procedure described in steps 4-7.

4. Load the DNA onto the silica column by doing the following:
    a. Transfer the dissolved gel mixture to the SV minicolumn assembly and incubate for 1 minute at room temperature.
    b. Centrifuge the SV minicolumn assembly in a microcentrifuge at top speed for 1 minute. Remove the SV minicolumn from the spin column assembly and discard the liquid in the collection tube. Return the SV minicolumn to the collection tube. Why are you discarding the "flow-through"?

5. Wash the column by adding 700 ul of Membrane Wash Solution (10 mM potassium acetate, pH 5.0, 16.7 uM EDTA, pH 8.0 and 80% ethanol) to the SV minicolumn. Centrifuge the SV minicolumn assembly for 1 minute at high speed. Empty the collection tube and repeat the wash with 500 ul of Membrane Wash Solution, centrifuging for five minutes.

6. Remove the SV minicolumn assembly from the centrifuge, being careful not to wet the bottom of the column with the wash solution. Empty the collection tube and recentrifuge the column assembly for one minute. The final spin removes all the liquid and chaotropic salt from the column and prepares it for elution.

7. Transfer the SV minicolumn to a clean 1.5 ml microcentrifuge tube. Apply 50 ul of water directly to the center of the column without touching the membrane with the pipette tip. Incubate at room temperature for one minute. Centrifuge for two minutes at high speed. Why will this elute the DNA?

B**IOL313: Genetics**
Ligation
***E. coli* transformation**

This week, you will ligate your purified GFP-mYCK into pRS315. You will then use your ligation mixture to transform *E. coli*.

Ligation procedure

1. Estimate the concentration of each of your DNA fragments using the gel you ran last week. Remember that since you used 5 ul of mass ladder, each band on the mass ladder is equal to 500 ng (rather than the 200 ng we saw when we ran 2 ul). Assume that you recovered 80% of each band using the procedure described above.

2. Convert your concentration from "ng/ul" to "pmol/ul" using the following information:
    a. For a 1 kb fragment of double-stranded DNA, 1 ug DNA is approximately equal to 1.5 pmol of DNA.
    b. Thus 1 ug of your GFP-tagged mYCK2 is approximately equal to 5.25 pmol (3.5 kb * 1.5 pmol/ug).
    c. Thus 1 ug of your pRS315 is approximately equal to 9 pmol (6 kb * 1.5 pmol/ug).

3. To perform your ligation, you need to use a 1:2 molar ratio of vector: insert DNA. Plan to use 0.15 pmol of your insert DNA (GFP-tagged mYCK2) and 0.075 pmol of your vector DNA (pRS315). How many microliters of each will you need to use?

4. Mix the ingredients for your ligation reaction in a sterile microfuge tube.

    X ul GFP-tagged mYCK2 fragment
    Y ul pRS315
    Z ul 2X rapid ligation buffer
    1 ul T4 DNA ligase (3 units/ul)
    W ul water (if necessary)
    16 ul total volume

5. Incubate for 10 minutes at room temperature.

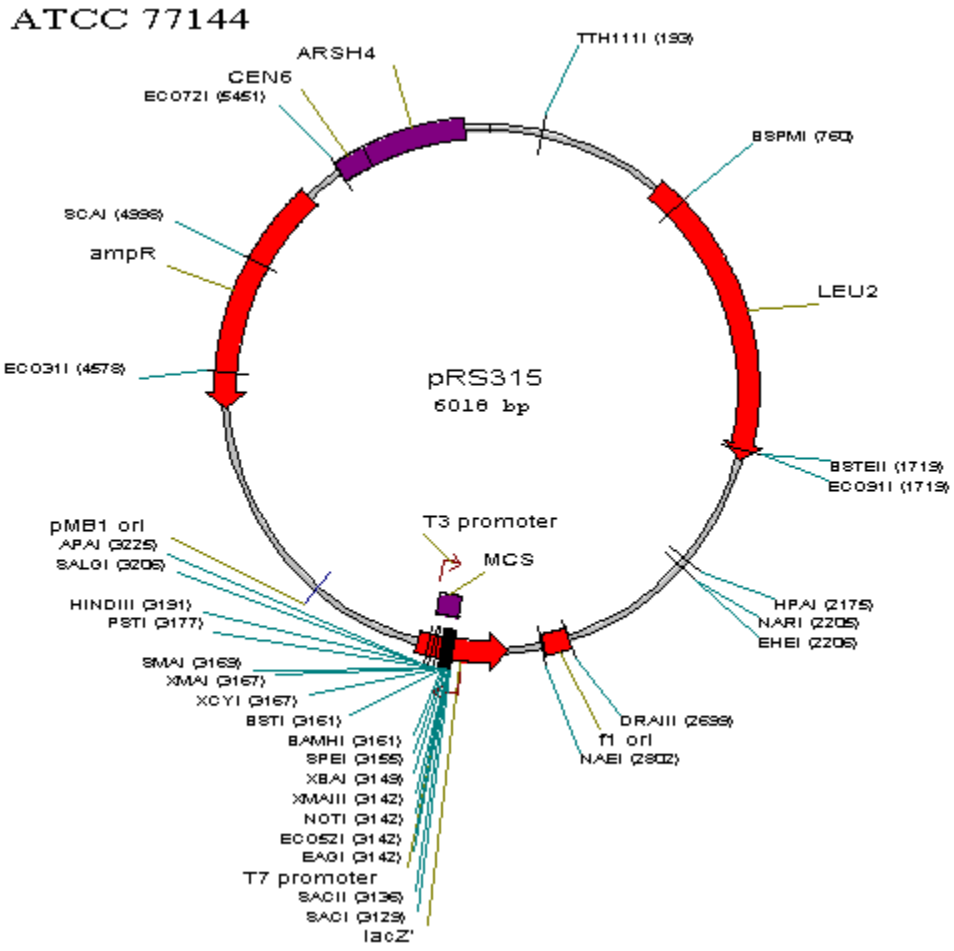
6. Transform a 100 ul aliquot of competent *E. coli* with your ligation mix, using all of your ligation mix. I will provide the competent *E. coli* and the other materials you will need; you will provide the procedure.

B**IOL313: Genetics**
Growth of liquid *E. coli* cultures
Miniprep of plasmid DNA
Analytical digest
Gel electrophoresis

Last week, you ligated GFP-tagged mYCK2 into pRS315. You then transformed competent *E. coli* with this ligation mix and grew the transformed cells on LB-Amp plates. Your goal this week is to pick three colonies from this plate, growing each as a liquid culture overnight. You'll then perform a miniprep to isolate the plasmid DNA from each culture. Finally, you will perform an analytical digest on an aliquot of your miniprep DNA, checking the results by gel electrophoresis. This will allow you to determine if you have isolated pRS315 containing the GFP-mYCK2 fragment and, if so, to determine the concentration of the miniprepped DNA.

A representative of your group will need to come into the lab very briefly the afternoon before your lab meets to pick colonies and set up your liquid cultures. When you come to lab that week, you'll harvest your cells by centrifugation (super easy: just pour them into a microfuge tube, spin for two minutes, and repeat until you have the whole culture), and then do your miniprep. Because you've done minipreps, analytical digests, and gels before, you will provide the procedure for the day.

BIOL313: Genetics
Transformation of *Saccharomyces cerevisiae* with mYCK2/pRS315 construct



This week, you'll transform yeast with pRS315 containing GFP-tagged mYCK2 to test the functionality of the mutant allele you made. A map of pRS315 is shown at left; remember that the GFP-mYCK2 fragment was ligated into the gap made when *Sac*I and *XbaI* were used to digest pRS315.

You will be doing two different types of functionality tests.

Functionality assay #1

The first involves a yck^{ts} strain of yeast. This yeast strain is $leu2^{-}$, meaning it has a mutation in a gene required for the synthesis of leucine and cannot make leucine. This yeast strain therefore cannot grow unless leucine is supplemented in the medium *or* it picks up a functional LEU2 gene. Note that pRS315 contains a functional LEU2 gene. The yeast strain we are using is also a yck^{ts} strain. In this strain, the YCK1 gene is deleted, and the YCK2 gene has a point mutation that renders Yck2 temperature-sensitive. Specifically, Yck2 functions (somewhat) normally at 24°C (about room temperature), but does not function at 37°C. The yeast can therefore grow at 24°C but cannot grow at 37°C. This feature will be essential for testing the functionality of your mutant Yck2.

Initially, you will plate the transformed cells at 24°C on a –leucine (-Leu) medium, which will allow any yeast that took up pRS315 to grow—whether the mYCK2 is functional or nonfunctional. You are actually going to do four transformation reactions, providing us with several controls to allow us to interpret our final results. The four transformation reactions you will perform:

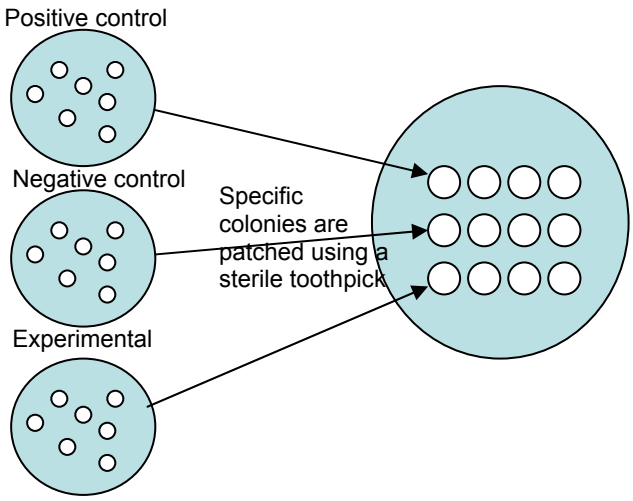
Yeast are transformed with:	Expected result in –Leu medium at 24°C:	Function of the sample
Carrier DNA only	Should produce no colonies	To determine rate of spontaneous Leu+ reversion

Carrier DNA + wildtype GFP-YCK2 in pRS315	Should produce colonies	Positive control (will see how later)
Carrier DNA + empty shuttle vector (pRS315)	Should produce colonies	Negative control (will see how later)
Carrier DNA + GFP-mYCK2 in pRS315	Should produce colonies	Our experiment! (will see result later)

After about four-five days' growth at room temperature, you should see colonies on all three plates that were transformed with the shuttle vector (+/- YCK2). We can now assess our mYCK2 function.

To assess mYCK2 function, you will patch colonies to separate plates, and will then replica plate the colonies to determine whether your mYCK2 can function at 37^{o}C.

Specifically, after colonies form on the plates on which you grew your transformed yeast, four colonies from each plate will be patched onto another plate. This plate will be allowed to grow overnight so that the patched cells will divide and produce colonies. See below:



The plate onto which the colonies are patched will be –leucine.

After the transferred cells are allowed to grow overnight and form colonies, two replica plates will be made onto identical –leucine plates. One of these plates will be placed at 24^{o}C and the other will be placed at 37^{o}C. What result do you expect for each of your samples? How will this allow us to determine if the mutation you introduced to Yck2 inhibits Yck2's function?

<u>Functionality assay #2</u>

The second assay we will use also examines whether the mutant YCK2 allows growth. In this assay, we will use a strain of yeast that has both the YCK1 and YCK2 genes deleted and is $leu2^-$ and $ura3^-$. Specifically, the YCK1 gene has been replaced with a gene that allows the yeast to be resistant to the antimycotic G418 (Kan^r), and the YCK2 gene has been replaced with a gene that allows the yeast to be resistant to nourseothricin (Nat^r). As you know, yeast cannot grow in the absence of both Yck1 and Yck2. To allow them to grow, this yeast strain contains a high-copy plasmid that carries both a wild type YCK2 gene and a URA3 gene, which allows synthesis of uracil by this strain.

You will transform these yeast with pRS315 containing your mutant YCK2 gene (plus carrier DNA as above). As shown above, the pRS315 plasmid contains a LEU2 gene, allowing leucine synthesis. We can therefore select for yeast carrying the pRS315 plasmid by growing them in the absence of leucine; since this strain of yeast is $leu2^-$, only those that contain pRS315 will be able to make leucine and grow on this medium. In addition, we will use a medium with 5-fluoroorotic acid (5-FOA). Cells that have a functional URA3 gene convert 5-FOA to 5-fluorouracil, which is toxic and kills cells. Thus, the 5-FOA in the plate selects for cells that have lost the plasmid containing the wild type YCK2 and the URA3 genes. We will therefore only see growth if the mutation you generated in the YCK2 gene allows formation of a functional Yck2 protein.

In this experiment, you will also do control transformations with carrier DNA only, carrier DNA plus "empty" pRS315, and carrier DNA plus pRS315 containing wildtype YCK2.

A summary of this experiment:

	Medium without leucine	Do we see growth in presence of 5-FOA?
Starting yeast strain (yck1Δkan; yck2Δnat; LEU2-; URA3-) transformed with plasmid containing URA3 and wild type YCK2 ("plasmid 1")	No growth	No
Starting yeast strain transformed with pRS315 with no YCK2 gene	Growth (LEU2 provided by pRS315)	No—because the only YCK2 gene available is on plasmid 1, which must be lost to allow growth on 5-FOA
Starting yeast strain transformed with pRS315 with wildtype YCK2	Growth	Yes—the pRS315 vector in this case contains a functional YCK2 gene, so the URA3-containing plasmid 1 can be lost
Starting yeast strain transformed with pRS315 with mutant YCK2	Growth	??? We will see growth if the mutation in YCK2 does not interfere with protein function

Procedure

Making yeast cells competent:

For each group, two cultures of yeast cells (one, the yck^{ts} strain; the other, the yck1Δ yck2Δ strain) were started last night by inoculating 5 ml of a rich medium (YPD) with the cells. The cultures were grown overnight at 24°C with shaking. At 11:00 a.m., the cultures were diluted to 50 ml with the same rich medium and grown at 24°C with shaking until 2 p.m. The remainder of the steps will be carried out in lab.

1. Cells are centrifuged at 2000 rpm for 5 minutes in 50 ml conical tubes to pellet cells. The supernatant is discarded. (You will use the Sorvall centrifuge for this step.)
2. The cells are resuspended in 5 ml sterile water and centrifuged as above. Again, the supernatant is discarded. (Again, you will use the Sorvall centrifuge.)

3. The cells are resuspended in 0.5 ml lithium acetate/TE and placed on ice until ready for use.

Preparing carrier DNA for transformation:

Because we are transforming the yeast cells with very small amounts of DNA, we will also add DNA that is unrelated to our experiment. It is called "carrier" DNA because it helps to carry our DNA into the cells, and it has been found to make transformation into yeast significantly more efficient. We are using DNA from calf thymus (because it's cheap and easy to get) as our carrier DNA. This DNA needs to be single-stranded to function effectively as carrier DNA, so we will denature it.

1. Wrap the lid of the microfuge tube securely with Parafilm.
2. Boil the carrier DNA 10 minutes.
3. Place on ice immediately for 15 minutes. The DNA will remain single-stranded as long as it is kept on ice; if it is warmed to room temperature, the procedure must be repeated.

Transformation and plating:

1. For all steps, work quickly and use sterile technique. Always use sterile tips, and make sure not to introduce undesired components (like bacteria from your skin) to the tubes.
2. Transfer DNA to clean microfuge tubes and place the tubes on ice to chill. You will have eight tubes:
   Tube 1: 7.5 ul of carrier DNA (7.5 mg/ml)
   Tube 2: 7.5 ul of carrier DNA (7.5 mg/ml)
   Tube 3: 7.5 ul of carrier DNA + 2 ul "empty" pRS315
   Tube 4: 7.5 ul of carrier DNA + 2 ul "empty" pRS315
   Tube 5: 7.5 ul of carrier DNA + 6 ul pRS315 containing wildtype YCK2
   Tube 6: 7.5 ul of carrier DNA + 6 ul pRS315 containing wildtype YCK2
   Tube 7: 7.5 ul of carrier DNA + 7 ul pRS315 containing your mutant YCK2
   Tube 8: 7.5 ul of carrier DNA + 7 ul pRS315 containing your mutant YCK2
3. Add 100 ul competent cells to each tube. Add the yck^{ts} strain to tubes 1, 3, 5, and 7; add the yck1Δ yck2Δ strain to tubes 2, 4, 6, and 8. Flick the tubes to mix.
4. Add 600 µl polyethylene glycol/lithium acetate/TE (PEG/LiAc/TE) to each tube. Invert the tubes until the two layers mix, watching to make sure that the layers do mix together.
5. Incubate at room temperature with agitation for 30 minutes. (A moving platform will be available at the front of the lab.)
6. Add 70 ul dimethylsulfoxide (DMSO) to each tube and gently mix.
7. Heat shock the transformation mixtures at 42°C for 6 minutes.
8. Incubate the transformation mixtures on ice for 2 minutes. Do not keep them on ice for more than three minutes.
9. Centrifuge the cells at 3000 rpm for 3 minutes in your microfuge.
10. Pour off the supernatant. Some sup may remain on the cells; this does not harm the procedure.
11. Add 1 ml –Leu medium to each tube. Resuspend the cells by drawing them up and down in the pipet.
12. Transfer 100 ul of each cell suspension to a clearly labeled (date, group, yeast strain, plasmid DNA added) –Leu plate. Add 5-6 sterile beads and plate the cells, discarding the beads after plating.
13. Incubate the cells at room temperature for up to five days (until you see 2-3 mm colonies).

Patching for yck^{ts} strain (tubes 1, 3, 5, and 7 above):

1. Label the bottom of a –Leu plate. Be sure that your label can be used to establish orientation. You will have three rows of colonies on the plate, and you should be able to tell which row corresponds to the

negative control, which corresponds to the positive control, and which corresponds to your experimental sample.

2. Using a sterile toothpick, pick a colony from your negative control plate. Rub cells from the toothpick in a small circular spot on the new –leucine plate. Repeat, forming a total of four patches in a row from the negative control plate.
3. Repeat step 2, creating a row of four patches from the positive control plate.
4. Repeat step 2, creating a row of four patches from your experimental plate.
5. Incubate at room temperature overnight.

Replica plating yck^{ts} strain (tubes 1, 3, 5, and 7 above):

1. Obtain two –Leu plates and label so that the orientation is clear. Also label one "24°C" and one "37°C."
2. Place a piece of sterile velvet over the replica-plating block. Carefully press the velvet onto the patch plate that you created above.
3. Gently press the velvet onto each of the two –Leu plates you just obtained and labeled. Replace the lids. Incubate the 24°C plate at room temperature overnight. Incubate the 37°C plate at 37°C overnight.

Patching for yck1Δ yck2Δ strain (tubes 2, 4, 6, and 8 above):

1. Label the bottom of a -Leu plate. Be sure that your label can be used to establish orientation. You will have three rows of colonies on the plate, and you should be able to tell which row corresponds to the negative control, which corresponds to the positive control, and which corresponds to your experimental sample.
2. Using a sterile toothpick, pick a colony from your negative control plate. Rub cells from the toothpick in a small circular spot on the new –leucine plate. Repeat, forming a total of four patches in a row from the negative control plate.
3. Repeat step 2, creating a row of four patches from the positive control plate.
4. Repeat step 2, creating a row of four patches from your experimental plate.
5. Incubate at 30°C overnight.

Replica plating yck1Δ yck2Δ strain (tubes 2, 4, 6, and 8 above):

1. Obtain one -Leu plate and one 5-FOA plate and label each so that orientation is clear.
2. Place a piece of sterile velvet over the replica-plating block. Carefully press the velvet onto the patch plate that you created above.
3. Gently press the velvet onto each of the two plates you just obtained and labeled. Replace the lids. Incubate the plates at 30°C overnight.

B**IOL313: Genetics**
Scoring results of functionality assays
Examining transformed yeast for morphological abnormalities
Examining transformed yeast for Yck2 localization

Results of functionality assays

1. Examine the patches you made from the yck^{ts} strain on the –Leu plates you incubated at 24^{o}C and 37^{o}C overnight last night.
    a. Do you see growth of your positive control at 24^{o}C? 37^{o}C? Is this what you expected? Why?
    b. Do you see growth of your negative control at 24^{o}C? 37^{o}C? Is this what you expected? Why?
    c. Do you see growth of yeast expressing your mutant Yck2 at 24^{o}C? 37^{o}C? If you see growth, is it as much growth as you see with the positive control? Does this mean that your mutant protein is functional or nonfunctional?
2. Examine the patches you made from the yckΔ strain on the –Leu and 5-FOA plates you incubated at 30^{o}C last night.
    a. Do you see growth of your positive control? Is this what you expected? Why?
    b. Do you see growth of your negative control? Is this what you expected? Why?
    c. Do you see growth of yeast expressing your mutant Yck2? If you see growth, is it as much growth as you see with the positive control? Does this mean that your mutant protein is functional or nonfunctional?

3. Do the results you obtain in the two assays correspond? Overall, what does it mean about the function of your mutant YCK2 allele?

Examining transformed yeast for morphological abnormalities and Yck2 localization

1. Heat a tube of 2% agarose in a flask of water until it is melted. (There may be a tube in the lab that is already melted; if so, use it.) Drop 100 µl of the agarose on a slide and immediately place a second slide on top to flatten the agarose pad. After 10-15 seconds, gently slide the top slide off the lower slide. The agarose pad may stay on the top or the bottom slide. If necessary, turn the slide with the agarose pad so that the agarose pad faces up.
2. Repeat, making six slides with agarose pads.

Examining morphology

1. To examine the morphology of your transformed yeast, obtain a sterile toothpick and very gently take a VERY SMALL amount of your yckΔ yeast transformed with pRS315:GFP-YCK2. Using a very, very light touch, slide the tip of the toothpick across the agarose pad to spread the yeast on it. Place a coverslip on the agarose pad and gently press down.
2. Repeat with another slide and your yckΔ yeast transformed with pRS315:GFP-mYCK2.
3. Using one of the microscopes in the lab, take pictures of the yeast.
    a. Turn on your microscope on the right side. You should see the light come on at the bottom. You can adjust the intensity of the light by turning the knob labeled 1-10 on the right side of the arm of the microscope.

b. Turn on the software that you will use to observe your specimens on the computer by going to Start→Programs→Infinity software→Infinity Capture. You should then have a box that lets you control the software and a box that shows whatever your microscope is seeing.
c. Slide the 10X objective into place and then place a specimen on the microscope stage. Use the knobs to the right and below the stage to position the specimen right over the light source. Look into the oculars of the microscope and slowly move the coarse focus knob until you see your sample. *Note: the microscopes should be very close to being in focus. You should not have to move the coarse focus knob much, if at all.*
d. Slide the 40x objective into place. If necessary, sharpen the image using the fine focus knob and adjust the light source to let in more light. To change from brightfield to phase contrast microscopy, turn the flywheel at the front and bottom of the microscope stage to Ph2. You will probably need to adjust the light source to let in more light.
e. Find a yeast cell that is budding, place it in the middle of your field of view, and capture an image by pressing the camera button on the software control box. Another box showing your image will appear. Save it with an appropriate name—telling what the specimen is, what mode you're using, and what the magnification is.
f. When you're ready to observe your specimen at higher power, place a budding yeast cell right in the middle of your field of view. VERY CAREFULLY, slide the 40X objective over such that neither the 40X or the 100X objectives is in place. Place a very small drop of oil directly on the light shining through the specimen, and then carefully slide the 100X objective into place. Slide the flywheel to PH3.
g. If necessary, sharpen the focus with the fine focus knob. Do not move it more that a quarter of a turn in either direction. Capture images as desired.
h. Repeat for your yckΔ yeast transformed with your mutant Yck2 allele.

Examining Yck2 localization

To examine Yck2 localization, you will use a fluorescence microscope located in the small room off the genetics research lab. The YCK2 gene that you subjected to mutagenesis has a green fluorescence protein (GFP) tag on its 5' end, so the Yck2 protein produced has a GFP tag on its amino-terminus. This allows us to determine where within the cell Yck2 is localized by examining the cells for the green fluorescent color.

1. As described above, make streaks of your yck^{ts} yeast transformed with pRS315: GFP-YCK2 and pRS315: GFP-mYCK2 on two of the agarose pads you made.
2. Place the yeast with the wildtype Yck2 on stage of the fluorescence microscope. Turn on the microscope using the switch on the back of the microscope arm. If you're the first group, turn on the laser located to the front left of the microscope.
3. Open the Microsuite software on the adjacent computer.
4. Go to Acquire, and select Acquire. This will allow you to observe the specimen in real time.
5. Go to Acquire and select camera control. While you are doing brightfield microscopy (not looking at fluorescence, just focusing on the cells), you will adjust your exposure time to 10-20 msec.
6. Using the 10X objective, focus on the yeast cells. If necessary, adjust the light source using the up-and-down light switch on the right bottom of the microscope arm.
7. Move to the 40X objective and, if necessary, sharpen your focus.
8. Slide the 40X objective to the side and place a very small drop of immersion oil directly on the light shining through the specimen. Slide the 100X objective into place and, if necessary, sharpen your focus. Do not adjust the fine focus knob more than a quarter turn in either direction.

9. When you are focused on a field of view with several yeast cells, take a fluorescent image.
    a. Turn the light source all the way down.
    b. Make sure all the light is directed to the camera. To do this, pull the top front lever on the camera all the way out.
    c. Set the exposure time on the Cameral Control to 1 second.
    d. Push the shutter lever labeled with red tape all the way in, and immediately take a snapshot by going to Acquire and selecting Snapshot. As soon as the image appears on the computer screen, pull the shutter lever back out. GFP quenches quickly as it is exposed to blue light, so we want to reduce its exposure.
    e. If your image is in focus and shows you green fluorescence, save the image. Compare the image to one Dr. Brame or Dr. Pruitt will show you. Can you tell that Yck2 is localized to the membrane?
    f. If you're not happy with your image, repeat. If you want to recheck your focus, you will need to go to Acquire and select Acquire again. In addition, to use brightfield, you will need to turn the light source back up and reduce your exposure time.
    g. When you are satisfied with your wildtype Yck2 images, repeat for your mutant Yck2. Compare your image to one showing a Yck2 mutant that is localized to the cytosol as well as the wildtype, membrane-associated Yck2. Can you tell where your mutant Yck2 protein is localized?