

## Chapter 5

# Signal Transduction and Control of the Cell Cycle in Yeast, *Saccharomyces cerevisiae* A Collaborative Laboratory Exercise

*Barbara Hoopes, Nancy L. Pruitt,  
Kathleen Baier, and Sherry Brooks*

Biology Department  
Colgate University  
Hamilton, NY 13346-1398  
(315) 824-7888, kbaier@center.colgate.edu  
FAX: (315) 824-7997

Dr. Hoopes received her B.S. degree in Zoology and Chemistry from the University of Maryland, College Park, and her M.S. and Ph.D. in Biochemistry from Harvard University. She did post-doctoral work in the Department of Biochemistry and Biophysics of the University of California, San Francisco and the Institute of Molecular Biology of the University of Oregon. Barbara is an Assistant Professor in the Department of Biology at Colgate University. She teaches courses in Introductory Biology, Cell Biology, Recombinant DNA Techniques and Molecular Biology. Her research interests are how the DNA sequence of promoter regions determines patterns of gene expression.

Dr. Pruitt received her B.A. in Biology from Gettysburg College, her M.A. in Biology from Wake Forest University, and her Ph.D. in Physiology from Arizona State University. She is an Associate Professor of Biology at Colgate University, where she teaches courses in Introductory Biology, Cell Biology and Animal Physiology. Her research interests are in comparative physiology, particularly the adaptations of some multicellular organisms that enable them to withstand freezing of tissue water in winter.

Ms. Baier received her B.S. degree in Microbiology from Rutgers University. She is a technician for the Molecular Biology program in the Department of Biology. She also teaches laboratory sections of Cell Biology and Recombinant DNA Techniques.

Ms. Brooks received her B.A. in Zoology from Indiana University. She is a Laboratory Instructor at Colgate University where she coordinates the Introductory Biology course laboratory. She also teaches labs in Ecology and Vertebrate Zoology.

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**Reprinted From:** Hoopes, B., N. L. Pruitt, K. Baier, and S. Brooks. 1998. Signal transduction and control of the cell cycle in yeast, *Saccharomyces cerevisiae* a collaborative laboratory exercise. Pages 61-80, in *Tested studies for laboratory teaching*, Volume 19 (S. J. Karcher, Editor). Proceedings of the 19<sup>th</sup> Workshop/Conference of the Association for Biology Laboratory Education (ABLE), 365 pages.

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## Introduction

Science is rarely a solitary activity. Working scientists rely on many different approaches and areas of expertise, and answer difficult questions by developing collaborations within the scientific community. In contrast traditional laboratory exercises in biology courses are carried out in single 2- or 3-hour lab periods among students within one course, presumably with similar backgrounds and levels of expertise. We have challenged this unrealistic approach in our laboratory curriculum by developing an exercise that requires students enrolled in our sophomore/junior level Cell Biology course to work side-by-side with students (many of them non-science majors) enrolled in our Introductory Biology course. The exercise asks students to bring their different expertise to bear in identifying the sites in a signaling pathway affected by mutations in two strains of yeast. Students spend two full lab periods, each 3 hours long, in this pursuit (2 weeks).

For this collaborative project we have chosen to address a scientific topic that is currently the focus of much research: how, at the molecular level, do cells respond to an external signal? Response to an external signal is important at all levels of biology, from bacterial chemotaxis (Shaw, 1991; Stock et al., 1992) to the development of multicellular organisms (Perrimon and Desplan, 1994) to understanding the complex series of events that leads to oncogenesis (Lee, 1995; Nigg, 1995). The simple unicellular eukaryote, *Saccharomyces cerevisiae* (baker's yeast), has played an important role as a model system for understanding how external signals lead to cellular responses. In particular, a signal transduction pathway required for mating of yeast has been a particularly useful model for signal transduction in general (Neiman, 1993). In this pathway, exposure of a yeast cell of one mating type to a peptide pheromone synthesized by cells of the opposite mating type results in a complex series of cellular events leading to mating. These include change in cell appearance from round to pear shaped (“shmooing”), increase in the level of transcription of genes whose products are required for mating, and arrest of the cell in the G<sub>1</sub> phase of the cell cycle (Fields, 1990).

The laboratory exercise employs three strains of yeast, two of which (strains, “X” and “Y”)

contain mutations in genes involved in the mating signal transduction pathway, and one strain with no pathway mutations. The students are asked to design and carry out an experiment to identify which parts of the signal transduction pathway are affected by the mutations in strains “X” and “Y”.

In the first week the students are given an introduction to the molecular biology of signal transduction and the biology of yeast, as well as the various tools and techniques they will have available to carry out the experiment. These include techniques for transformation of yeast with recombinant plasmids, an assay to determine the proportion of cells which are arrested, and an assay for quantifying gene expression. The initial task for each student group is to generate a testable hypothesis and then to design an experiment to determine what part of the signal transduction pathway is affected by the mutations in strains “X” and “Y”. After they have given serious thought to what comprises a testable hypothesis, an appropriate protocol, and a meaningful control (for about 45 minutes), their proposals are discussed as a class and the actual experimental protocol is then distributed. Students might benefit from constructing a flow chart of the protocol.

The first week the combined introductory/cell biology groups (groups of 4, approximately 2 students from each course) perform a genetic transformation of yeast cells. The second week the introductory students use compound light microscopes in assaying the yeast cultures to determine the proportion of cells that are unbudded, an index of the number of cells that have arrested and are no longer actively dividing. The cell biology students assay the cultures for  $\beta$ -galactosidase ( $\beta$ -gal), an index of the activation of gene expression during mating. In the interest of time, the instructors compile and statistically analyze the data (analysis of variance and a multiple comparisons test, where applicable) and the results are distributed to the students. The instructors also lead a discussion on how the results can be interpreted.

Although this exercise would lend itself to writing a collaborative paper (as would be done within the scientific community), we ask the students to write individual reports in the form of a scientific paper. This gives each student experience in scientific writing and also allows us to assess each individual's mastery of the concepts involved more easily.

We have found that this collaborative exercise is valuable in several respects. First, students are exposed to experimental design in a meaningful context that is interesting to them. Secondly, this laboratory exposes students to a problem near the forefront of biological research, to recombinant DNA techniques, and to data collection using simple microscopy and a simple enzyme assay. In addition, conceptual connections not previously considered by students are developed between two courses in our curriculum. The exercise leads to a significant interaction between students of introductory biology and more advanced upperclass students. Although we have developed this laboratory as a collaborative exercise between two classes, it could also be used to reinforce most of these points within a single class.

## Materials

### Equipment for the entire class

microcentrifuges  
 30°C waterbaths (2-3)  
 42°C waterbath  
 30°C incubator with a test tube roller  
 vortexes  
 floating racks for microfuge tubes

### Supplies required for each group

sterile microfuge tubes (9)  
 sterile wooden applicator sticks (6)

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bent glass rod

16x150 mm test tubes, **sterile** (6)

13x100 mm test tubes (12)

Spectronic 20 or equivalent

Bunsen burner and striker

hemacytometer

compound light microscope

adjustable volume automatic pipettors (20  $\mu$ L, 200  $\mu$ L and 1000  $\mu$ L capacity)

sterile pipettor tips

### **Solutions and Media for each group** (see Appendix A for recipes)

streaked YEPD plate of each yeast strain (WT, Mutant "X", Mutant "Y" - 1 each) or 1 plate "patched" with all 3 strains

One Step Buffer (0.3 mL)

pBH315 plasmid (15  $\mu$ L at 0.2  $\mu$ g/ $\mu$ L)

carrier DNA (denatured salmon sperm DNA, Sigma Cat. # D1626) (15  $\mu$ L)

TE (0.3 mL)

SD-ura broth (30 mL)

SD-ura plates (3)

100  $\mu$ M  $\alpha$  factor (60  $\mu$ L), Sigma Cat. # T 6901

Z buffer (15 mL)

chloroform (0.3 mL)

0.1% SDS (0.2 mL)

ONPG (5 mL), Sigma Cat. # N1127

1 M Na<sub>2</sub>CO<sub>3</sub> (3 mL)

0.5 M EDTA, pH 8 (2 mL)

### **Notes to Instructors**

A good portion of the preparation for this exercise can be done well in advance of the scheduled lab time. Yeast strains and the yeast shuttle plasmid, pBH315 (Hoopes, unpublished) are available from the authors (Appendix B). A large scale DNA preparation of the plasmid must be prepared. One inexpensive method is using one of the protocols in Sambrook et al. (1989). A faster, but pricier option is to use a kit such as the Qiagen Plasmid Maxi Kit (Qiagen Corp., Cat. no. 12162, 10 preparations for \$135).

The recipe for the carrier DNA can be found in Ausubel et al. (1996). An ample supply for use in many transformations can be prepared once and stored frozen for future use. We originally used a traditional lithium acetate yeast transformation protocol (Ausubel et al., 1996), but have recently tried the simpler One Step Transformation with good success (Chen et al., 1992). This eliminates the need to monitor cell growth and prepare competent cells immediately prior to class.

The second week of this lab requires the following preparations outside regularly scheduled class time: 1) overnight cultures must be started the day before the scheduled lab; 2) the cultures must be diluted approximately 5 hours before class to restart cell growth; and 3)  $\alpha$  factor must be added to half of each culture 2 hours before class. We usually ask the students to do tasks one and three, and the staff usually does task two. To guard against student forgetfulness, we prepare one set of overnight cultures ourselves. We also have available freshly streaked, transformed cells which students can use to start their overnight cultures, if, for example, their cells did not transform.

It is, of course, possible to have the staff do any or all of the out-of-class tasks. Although we grow cells on a roller in a 30°C incubator, cells could also be grown at room temperature if an incubator is not available.

For the  $\beta$ -gal assay the students use chloroform. Be sure to stress safety because not only is chloroform a harmful irritant if inhaled, exposed to skin or ingested, but it is also a teratogen and possible cancer hazard. Have students wear gloves and work in a fume hood if possible. We collect any liquid waste to dispose with hazardous materials.

We find it helpful to have several 30°C waterbaths to avoid crowding during the  $\beta$ -gal assay incubation. The assay is quite rapid and timing is critical.

Distinguishing between budded and “shmooing” yeast cells can be difficult for the beginning students (See Appendix C for pictures). We provide yeast slides for them to examine during the first week of the experiment. We also have photos of various states of budding and “shmooing” cells.

The mutations are as follows ( See Appendix B for genotypes):

Mutant “X”: *far1*, the arrest factor

Mutant “Y”: *ste2*, the receptor

## Student Outline

### Introduction

All cells have ways of responding to their environment. There are many examples for all kinds of organisms. For instance, bacteria need to detect the presence of nutrients or noxious substances so that they can move toward or away from them. When you cut your finger, skin cells need to start dividing rapidly to replace damaged cells and close up the wound. Both of these responses of cells have the same general components:

- 1) a signal
  - many different ones are possible; in the case of our examples above, the signals would be:
    - nutrients or noxious substances in the media (for the bacteria)
    - factors released by the damaged cells (for the cut finger)
- 2) a receptor for the signal
  - proteins in the cell membrane that bind the nutrients or factors
- 3) transducers
  - proteins within the cell that change as a result of the binding of the signal to the receptor and pass the signal along to other proteins
- 4) a response
  - a change in the behavior or metabolism of the cell.
  - Examples include: changes in transcription of particular genes or the activity of particular enzymes that give the appropriate response to the particular signal.

In the case of the bacterial cell, binding of nutrients to the receptor leads to a change in the beating of the flagella that move the bacteria through the media. This allows the bacteria to move forward, toward nutrients. In the case of your cut finger, binding of factors released by the damaged cells leads to the transcription in the surrounding cells of particular genes whose products affect the cell cycle. This causes your skin cells in the area of the wound to start dividing more rapidly.

The response of cells to cues in their environment is called *signal transduction*. It is a very

important area of current research in biology as signal transduction is critical for many different biological processes. Physiological responses to hormones or drugs involve signal transduction pathways, as do the changes during development of a multi-cellular organism from a single cell. Defects in signal transduction pathways are also often found when a cell becomes a tumor cell. Understanding signal transduction pathways is critical to our understanding of biological processes from development to cancer.

How do scientists learn how a signal transduction pathway works? How do they identify the proteins that act as the protein receptor and the transducers? These pathways are usually complicated and can have a number of proteins or chemical signals involved. One way scientists have dissected the components of signal transduction pathways is to use genetics. They generate mutations in organisms or cells by treating them with DNA-damaging substances, then look for cells that can no longer respond to the particular environmental cue. Characterizing the specific proteins that are affected by these mutations gives insights into the components of the entire pathway.

One pathway that has been extensively studied as a model for understanding signal transduction in general occurs in baker's or brewer's yeast, *Saccharomyces cerevisiae*. This single-celled organism can exist in two different haploid "cell types" called **a** and  $\alpha$  cells. In the absence of the other cell type, each type will divide mitotically, proceeding through one full cell cycle in about an hour. Such vegetatively dividing cells are easily distinguished microscopically. Actively dividing cells appear as large spheres with smaller or equal-sized spheres budding from their periphery. If the two cell types, **a** and  $\alpha$  cells, are in close proximity, they can mate by fusing to form a diploid cell. Mating between two cells requires the following things:

- 1) arrest of the cell cycle in the G<sub>1</sub> phase before DNA is synthesized.
- 2) changing cell shape (called "shmooing") to bring the cells closer together. (Unlike most college students, but similar to 14-year-olds, yeast are not motile, so they can't date easily).
- 3) increasing transcription and translation of genes involved in cell fusion.

How does an **a** cell know there is an  $\alpha$  cell in the vicinity with which it can mate? The  $\alpha$  cells release a 13 amino acid peptide "factor" called  $\alpha$  **factor**. This peptide binds to a receptor,  $\alpha$  **factor** receptor, on the cell surface of the **a** cells. Binding of the  $\alpha$  **factor** to the receptor results in all three of the effects above. At the same time **a** cells also produce  $\alpha$  **factor** which binds to an **a factor** receptor on  $\alpha$  cells. This exercise will examine the effect of  $\alpha$  **factor** on **a** cells.

The  $\alpha$  **factor** receptor belongs to the G-protein-linked family of cell surface receptors (Marsh and Herskowitz, 1988). When  $\alpha$  **factor** binds to the receptor on the surface of an **a** cell (indicating the proximity of a potential mate), a G-protein complex within the cell is activated. The activated G-protein complex subsequently turns on a protein kinase enzyme, which has the ability to phosphorylate other cellular proteins. Among the targets of the newly activated protein kinase are a protein that affects cell cycle arrest, and a transcription factor that is specific for genes involved in mating. Both the cell cycle arrest factor and the transcription factor specific for mating genes lie dormant in the cell until they are activated by the addition of phosphates by protein kinase, an event that occurs in response to the binding of  $\alpha$  **factor** at the cell surface. What we know about the signal transduction pathway for yeast mating is diagrammed in Figure 5.1. Note that the signal transduction pathway branches at the end, with one transducer protein (the arrest factor) stopping the cell cycle and a different transducer protein (the transcription factor) affecting transcription.

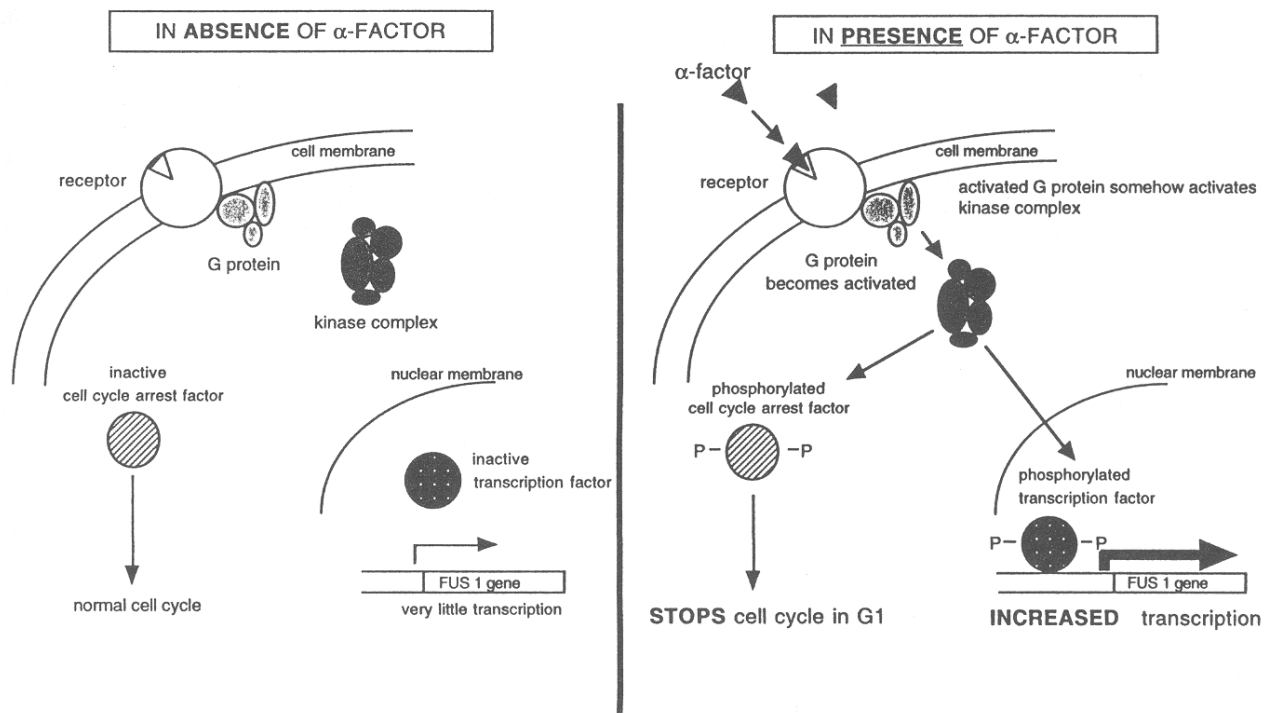
All levels of this complex signal transduction pathway involve proteins; the  $\alpha$  **factor**, the **factor** receptor, the G protein, the protein kinase, the cell cycle arrest factor, the transcription factor, and several intermediates that have been omitted for the sake of clarity. Proteins are coded

in genes, and genes are subject to mutations (damage to DNA or errors that occur during DNA replication) that render the protein products of the genes nonfunctional.

In this lab exercise, three strains of yeast are presented: one “wild type” with no mutation in the cell signaling pathway and two mutant strains, each with one mutation in a gene coding for a protein involved in this signaling pathway. The students' task is to devise and conduct an experiment to identify the pathway site which is affected by the mutation in each of the respective mutant strains.

**Experiment**

You and your partners from the other laboratory will be given three strains of yeast. One of these strains is a normal (“wild-type”) a cell. Each of the other two strains contains a mutation that inactivates *one* of the genes involved in the signal transduction pathway for mating. Your group's job is to figure out which gene(s) could contain the particular mutation based on what you know



**GENE NAME LEGEND** (note: STE stands for “sterile”) :

Receptor is STE2

G protein includes GPA1, STE4 and STE18

Cell cycle arrest factor is FAR 1

Kinase complex probably includes:

Transcription factor is STE12

STE5, STE20, STE7, STE11, KSS/FUS3

about this signal transduction pathway.

**Figure 5.1.** Representation of the signal transduction pathway of mating in a type cells of yeast (*Saccharomyces cerevisiae*).  $\alpha$  factor is a 13 amino acid peptide released by the opposite mating type cell,  $\alpha$  cells. Binding of  $\alpha$  factor to the  $\alpha$  factor receptor on the surface of a cells activates a G-protein complex which in turn activates a kinase complex. The activated kinase complex turns on a protein kinase enzyme which then phosphorylates two proteins, the cell arrest factor and the transcription factor. In response to these two phosphorylated proteins, cells arrest at the G<sub>1</sub> phase of the cell cycle and transcription of genes involved in mating is initiated.

How will you do this? Your first job is to design your experiment. You have the following available:

**Yeast strains:**

a *ura3<sup>-</sup>*

a “pathway mutant X” *ura3<sup>-</sup>*

a “pathway mutant Y” *ura3<sup>-</sup>*

All three strains contain a mutation in the URA3 gene. This gene codes for an enzyme required for the synthesis of uracil. Strains with mutations in URA3 (*ura3<sup>-</sup>* strains) cannot grow on media lacking uracil.

**Recombinant DNA molecule:**

*pBH315 plasmid*

Plasmids are small circular molecules of DNA that are capable of replicating in a cell. The pBH315 plasmid was made by recombinant DNA techniques and contains the promoter region from a gene involved in cell fusion during mating (the FUS1 gene). Promoters are sequences of DNA that direct the beginning of transcription of a gene by serving as a binding site for the RNA polymerase enzyme. Instead of directing the transcription of the mating gene (FUS1), the promoter on the pBH315 plasmid is attached to the gene for the enzyme  $\beta$ -galactosidase. This promoter is *only* active when  $\alpha$  **factor** is detected by the cell. When the promoter is active in this plasmid, the enzyme  $\beta$ -galactosidase is transcribed and translated; therefore, the amount of  $\beta$ -galactosidase produced is a measurement of the amount of transcription directed by the FUS1 promoter.  $\beta$ -gal activity can be measured using a simple enzyme assay.

This plasmid also contains a functional copy of the URA3 gene. Thus any yeast cells that contain this plasmid, even those lacking a functional enzyme of uracil synthesis (*ura3<sup>-</sup>*), can grow in the absence of uracil in their medium. This enables us to identify *ura3<sup>-</sup>* yeast cells that have taken up the plasmid.

**Supplies:**

Petri plates containing yeast media *without* uracil (SD-ura plates)

Synthetic  $\alpha$  **factor** peptide

**Techniques** (directions included with this handout):

*Transformation of yeast with plasmid*

It is simple to put the appropriate plasmid into yeast cells and to select for cells containing the plasmid molecule using the appropriate plates. For instance, if a cell that cannot grow in the absence of uracil (for example, a *ura3<sup>-</sup>* strain) is transformed with a plasmid containing the URA3 gene, then only cells that have taken up the plasmid can grow on plates that do not contain uracil.

*Assay for  $\beta$ -galactosidase enzyme*

Using a simple enzyme assay can determine quantitatively how much  $\beta$ -galactosidase enzyme is being made by yeast cells. Because the promoter for a mating gene has been linked to the gene for  $\beta$ -galactosidase using recombinant DNA techniques, an assay for  $\beta$ -gal activity is a good indicator of transcription of the mating genes.



*Assay for cell cycle progression*

The cell cycle is a combination of the stages through which a cell passes from one cell division to the next. The cycle is divided into the M phase (which includes mitosis and cytokinesis), and interphase. Interphase is subdivided into three stages: a growth period ( $G_1$ ) prior to, and in preparation for, DNA replication, a synthesis stage (S) when DNA is replicated and a second growth stage ( $G_2$ ) that precedes cell division.

*S. cerevisiae* is a “budding yeast” that divides by growing a bud on the cell (see figure 2 in the procedure for assaying cell division). These cells can be easily seen through a microscope. When cells are in  $G_1$ , they do not contain a bud. When they enter S phase, they begin budding. As they progress through the cell cycle, the bud grows until, at the end of the  $G_2$ , the cell looks like a “dumbbell”. During the M phase the bud separates from the cell to give two new cells. The percentage of unbudded cells is a measure of the number of cells *not* progressing through the cell cycle, but arrested in  $G_1$ .

Introductory Biology students have experience in using microscopes. Cell Biology students have experience in doing enzyme assays. You will have 2 weeks in which to do this experiment. As you design your experiment you should consider:

- how could you compare transcription and cell cycle arrest for yeast cells in the *presence and absence of  $\alpha$  factor*?
- how would mutations in the *receptor* affect transcription and cell cycle arrest?
- how would mutations in the *G protein* affect transcription and cell cycle arrest?
- how would mutations in the *protein kinase complex* affect transcription and cell cycle arrest?
- how would mutations in the *transcription factor* affect transcription and cell cycle arrest?
- how would mutations in the *arrest factor* affect transcription and cell cycle arrest?

### Transformation Of Yeast With Plasmid DNA

1. From the front of the room, get one plate of each strain of yeast cells (WT, X, Y).
2. Label three microfuge tubes with your initials and with the designations for the three strains (WT, X, Y). Scrape 3–4 colonies of yeast off each plate with a sterile wooden stick. Transfer these cells to the appropriately labeled microfuge tube by rolling the stick on the inside wall of the tube. *Please try to keep this procedure as sterile as possible.* To avoid confusion complete the procedure for each strain before beginning the next strain.
3. Add 100  $\mu$ L of One Step Buffer to each microfuge tube and resuspend the cells by mixing with the pipette tip.

*One Step Buffer contains lithium acetate and polyethylene glycol. The lithium salts change the cell envelope to make the cells permeable to DNA. The polyethylene glycol precipitates the plasmid and single stranded DNA you add in step 4 onto the yeast cell surface.*

4. For each transformation, add 5  $\mu$ L of pBH315 plasmid and 5  $\mu$ L of denatured salmon sperm DNA to the yeast cells.

*The single stranded DNA (salmon sperm DNA) is referred to as “carrier” DNA; it is*

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*necessary for high frequency transformation of the yeast cells with the plasmid, although the exact mechanism is unknown.*

5. One “no DNA” control for each lab section should also be done for each strain. This will contain only the salmon sperm DNA and not the plasmid DNA. What is the purpose of the “no DNA” control?
6. Incubate the transformation mixture in a 42°C water bath for 30 minutes, mixing occasionally to resuspend the yeast cells in the lithium acetate/PEG buffer.

*It is thought that heating the cells creates a thermal gradient that enhances the uptake of the DNA.*

7. Recover the cells by spinning them for 5–10 seconds in the microcentrifuge. Using a pipette tip discard the supernatant solution. Resuspend the cells in 0.1 mL of TE (Tris-EDTA) by mixing with the pipette tip.

*Lithium salts are detrimental to yeast cell growth. Resuspending the cells in TE dilutes the remaining lithium acetate.*

8. Put each of the transformation mixtures on a separate SD-ura plate and spread the cells evenly around the plate using a bent glass rod. Again keep things as sterile as possible. Remove the glass rod from the beaker of ethanol. Flame the glass rod and allow it to cool before spreading the cells. Replace the rod in the ethanol container. Label the plate with your names, the yeast strain (WT, X, or Y) and whether or not plasmid DNA is present.

*SD-ura plates contain a synthetic growth medium lacking the base uracil. Untransformed cells will not grow on these plates because they contain a mutation in the *ura3* gene. However, transformed cells will grow, because the *pBH315* plasmid contains a normal version of the *URA3* gene which, once the plasmid enters the nucleus, will be transcribed and subsequently translated into the *URA3* gene product.*

9. Incubate the plates at 30°C to allow colonies to grow for approximately 2 to 3 days.

### Growth Of Cells For Assay

1. The day before assaying the yeast cultures, they must be transferred to liquid media. Using separate sterile wooden sticks remove a single colony from each culture plate and add it to separate test tubes each containing 1 mL of SD-ura liquid media. Allow the yeast to grow overnight by placing the tubes on a roller in a 30°C incubator.

*A single colony of yeast will generally grow “to saturation” overnight. This means that the cells will divide until they generate a culture that has as many cells as the media can support. At saturation, there are about  $2 \times 10^8$  cells/mL.*

2. Approximately 5 hours before assaying the cultures, restart growth by adding 9 mL of SD-ura to each of the overnight cultures. Replace the tubes on the roller in the 30°C incubator.
3. Two hours before you wish to assay the cells, add  $\alpha$  **factor** as follows: remove 5 mL of each

culture to a fresh culture tube; label with the culture name. Label one of each of the 5 mL cultures “+ factor” and the other “- factor”. To the “+ factor” tubes, add 20  $\mu\text{L}$  of the 100  $\mu\text{M}$   $\alpha$  **factor** stock provided. You will now have 6 tubes.

- Put all six cultures on the roller in the 30°C incubator until time for the assays.

*The cells require a period of time to detect and respond to the  $\alpha$  factor.*

### **Measuring $\beta$ -Galactosidase Activity In Yeast** (Modification of technique in Ausubel et al., 1996)

- Estimate the cell density of the culture by measuring the absorbance at 600 nm (use SD-ura liquid media as a blank). If the absorbance is over 1, use a portion of the culture to make a 10-fold dilution of the cells with SD-ura liquid media and repeat the absorbance measurement. This absorbance measurement will be used in the calculation of  $\beta$ -gal activity (see equation at the end of the procedure). If you made the above dilution, you must multiply the absorbance measurement by 10 before using it in the equation.

*Cell density can be estimated by measuring the amount of light scattering that occurs for a particular culture of microorganisms. The amount of scattering that occurs depends on the size and shape of the microorganisms as well as on their number. The relationship between light scattering and absorbance is linear to a value of about 1.*

- Place 1.5 mL of the original, undiluted culture in a separate microcentrifuge tube. Pellet the yeast cells by centrifuging them in the microcentrifuge for 30 seconds. Using a pipettor remove and discard the media.
- Resuspend the cell pellet in 150  $\mu\text{L}$  of Z buffer. Add 50  $\mu\text{L}$  of chloroform and 20  $\mu\text{L}$  of 0.1% SDS. Vortex the tubes vigorously for 30 seconds.

*Z buffer is a solution of buffered salts favorable for  $\beta$ -galactosidase enzyme activity which also contains  $\beta$ -mercaptoethanol (a sulfhydryl reducing agent). The chloroform (organic solvent) and SDS (detergent) act to permeabilize the yeast cells so that we can add the substrate for  $\beta$ -galactosidase enzyme to them.*

- Put the tubes in a 30°C waterbath for the assay. (This is a timed assay and the reaction may go quickly and need to be stopped. Read through step 5. and plan accordingly before initiating the reaction by addition of ONPG.) Add 0.7 mL of ONPG solution (1.2 mg/mL ONPG in Z buffer) to each tube and record the exact time at which you added this substrate.
- Continue the incubations at 30°C until a yellow color is observed. When the reaction appears yellow, stop it by the addition of 0.5 mL of 1 M  $\text{Na}_2\text{CO}_3$ . Record the exact time at which you add the  $\text{Na}_2\text{CO}_3$ . If the reactions do not turn yellow, stop them after 5 minutes.

*O-nitrophenylgalactopyranoside (ONPG) is a colorless compound which is cleaved by  $\beta$ -galactosidase to o-nitrophenol and galactose. The resulting o-nitrophenol is yellow. The amount of yellow color produced is proportional to the  $\beta$ -galactosidase activity present in the cells. If there is a lot of  $\beta$ -galactosidase enzyme in the cells, the reaction will turn yellow within a minute. The  $\text{Na}_2\text{CO}_3$  raises the pH of the reaction to 11, which inactivates*

*β-galactosidase*.

- Remove the cell debris from the reactions by spinning the tubes in the microcentrifuge for 2 minutes. Remove the top 1 mL from each reaction tube and add it to 2 mL of Z buffer in a test tube. Measure the absorbance of the reaction at 420 nm using the Spectronic 20 (use Z buffer as a blank).
- The amount of *β-galactosidase* activity is generally expressed in Miller (1972) units:

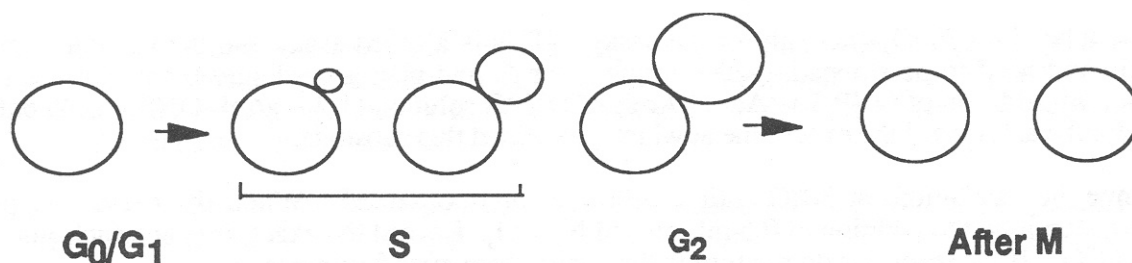
$$\text{Miller units} = \frac{\text{Absorbance (420 nm)} \times 1000 \times 3}{\text{Absorbance (600 nm)} \times (\text{volume culture}) \times (\text{time of reaction in minutes})}$$

The factor of 3 in the numerator of this equation is to correct for the dilution made when measuring the absorbance at 420 nm in step 6. The presence of the Abs(600) and the volume of culture (1.5 mL for our reactions) in the denominator allows reactions done with different numbers of cells to be directly compared. Remember to multiply the ABS(600) by 10 if you used a dilution to make the reading. The number of Miller units for gene fusions in yeast can vary from 0.1 unit to 1000 units or more.

### Assaying cell division in *Saccharomyces cerevisiae*

*S. cerevisiae* divides by budding (Figure 5.2). We can tell something about where yeast cells are in the cell cycle by observing the size of the bud. Bud formation begins in the S phase, and the bud enlarges as the cell cycle progresses. After mitosis, the mother and daughter cells separate. In a rapidly growing yeast culture, cells will be found in all stages of the cell cycle. About 60% of cells will contain a bud, and buds of all sizes will be seen in the culture. If the cells have been depleted of nutrients or are under conditions that arrest their cell cycle in the G<sub>0</sub> or G<sub>1</sub> phase, most cells will be unbudded, round cells. In contrast, if cells are stopped in G<sub>2</sub> right before mitosis, most cells will look like “dumbbells”, with two equal buds.

Budding of *S. cerevisiae*



**Figure 5.2.** Budding in the cell cycle of yeast (*Saccharomyces cerevisiae*). Budding begins in the S phase of the cell cycle and is completed after mitosis with the separation of the mother and daughter cells.

Scientists who work on yeast use these observations to identify yeast strains containing mutations in genes that control the different stages of the cell cycle. These observations will also allow you to determine whether  $\alpha$  factor is capable of arresting the cell cycle in G<sub>1</sub> for a particular strain of yeast.

*Procedure*

1. Remove 0.5 mL of each culture into a labeled tube.
2. Pipette 200  $\mu$ L of each culture into a separate tube which has been labeled appropriately.
3. Add 200  $\mu$ L of 0.5 M EDTA to each culture. (EDTA decreases cell clumping.)
4. For each culture, begin by vigorously vortexing the tube to break up clusters of cells. Place a drop on a hemacytometer slide. Observe the yeast cells using the 40X objective. Find a square containing 16 smaller squares. Designate all the cells in these 16 squares as budded or unbudded. (This may not be as easy as it sounds; sometimes mother cells and daughter cells will not be separated. If you have any doubts about how to assign cells, ask your laboratory instructor.) Have your partner keep track of how many cells you say are budded and how many are unbudded. Have your partner determine the number of budded and unbudded cells for a 16-square grid on another part of the slide. Record a combined total for the number of budded cells observed for the culture and the number of unbudded cells observed for the culture. Each student should count a minimum of 50 cells for each culture.
5. Repeat this procedure for each of the six yeast cultures.
6. Record the number of budded cells and the number of unbudded cells for all the cultures on the class data sheet.

### Student Bibliography

#### *Review articles*

Fields, S. 1990. Pheromone response in yeast. *Trends in Biochemical Science*, 15:270-272.

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**Suggested Experimental Protocol** (This is distributed ONLY after students have designed their own experiment)

**General scheme**

- transform all three yeast strains with pBH315 plasmid and grow on SD-ura agar plates
- grow cells containing this plasmid in liquid culture (three cultures/group)
- split each culture into two tubes (six tubes/group)
- add synthetic  $\alpha$  **factor** to ONE tube of each pair of cultures
- measure cell cycle arrest by  $\alpha$  **factor** by determining percent unbudded cells microscopically
- measure transcriptional activation by a factor by measuring  $\beta$ -galactosidase activity

*First week:*

1. Technician will provide culture plates of the three strains of yeast, “wild-type” (WT), “mutant X” and “mutant Y”.
2. Each group will perform three transformations
 

<i>strain</i>	<i>plasmid</i>	<i>carrier DNA</i>
wt	5 $\mu$ L pBH315	5 $\mu$ L
mutant X	5 $\mu$ L pBH315	5 $\mu$ L
mutant Y	5 $\mu$ L pBH315	5 $\mu$ L
3. Three control transformations will be carried out for each lab section by adding *only* carrier DNA to each strain.
4. Each transformation mix will be put on a plate containing yeast media lacking uracil (SD-ura) to select for the presence of the plasmid by complementation of the *ura3<sup>-</sup>* mutation in the strain. Plates will be incubated at 30°C and colonies of transformed cells should be visible in 2 days.
5. Each group should decide who is going to come in the day before lab next week to start cultures growing.

*Second week:*

*The day before lab:*

1. Take *one* colony from each of the transformation plates with a sterile wooden stick and add it to a separate test tube containing 1 mL of SD-ura liquid media. Put the test tube on the roller in the 30°C incubator to allow the cells to grow overnight.

*Lab day:*

1. Two hours before assaying, recover your test tubes from the incubator (there should be 10 mL of culture; 9 mL of SD-ura was added to each tube in the morning). Remove 5 mL from each culture to a fresh test tube. Add 20  $\mu$ L of  $\alpha$  **factor** to one of the test tubes for each yeast strain and label this tube “+ factor”. Label the other tube “- factor”. Put all six test tubes back on the roller for 2 hours to allow the cells to respond to the presence of  $\alpha$  **factor**.

2. After 2 hours, analyze the cells for cell cycle arrest and transcription of mating genes:
  - the Introductory Biology students should remove 0.5 mL of each of the six cultures to a labeled tube and assess cell division using the protocol provided.
  - the Cell Biology students should use the remainder of each of the six cultures to assay for  $\beta$ -galactosidase activity using the protocol provided.
3. Record your data on the data table for the class.

### Acknowledgments

The development of this exercise was partially supported by NSF Grant DUE 9354728

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## Appendix A

### *Instructions for the preparation of solutions and chemicals.*

All solutions should be sterile unless otherwise indicated

#### **YEPD Plates** (40 of 100x15 mm)

20 g peptone  
10 g yeast extract  
20 g agar  
(or use YPD agar, Difco Cat. # 0427-17-6)  
920 mL H<sub>2</sub>O  
*autoclave and add 80 mL 25 % glucose*

#### **One Step Buffer**

0.2 M lithium acetate  
40 % polyethylene glycol 3500, Sigma Cat. # P3640  
100 mM dithiotheitol

#### **TE**

10 mM Tris-HCl, pH 8.0  
1 mM EDTA

#### **SD-ura Broth**

1.7 g yeast nitrogen base without amino acids or ammonium sulfate and 5g ammonium sulfate.

870 mL H<sub>2</sub>O  
*autoclave and add 80 mL 25% glucose and 50 mL 20x-ura mix (see below)*

#### **20x-ura Mix**

0.168 g adenine  
0.084 g arginine  
0.41 g aspartic acid  
0.41 g glutamic acid  
0.084 g histidine  
0.25 g leucine  
0.124 g lysine  
0.084 g methionine  
0.21 g phenylalanine  
1.55 g serine  
0.83 g threonine  
0.16 g tryptophan  
0.12 g tyrosine  
0.62 g valine  
200 mL H<sub>2</sub>O  
*autoclave - will go into solution during autoclaving*

#### **SD-ura Plates** (40 of 100x15)

add 20 g agar to SD-ura Broth



**Z Buffer**

8.53 g Na<sub>2</sub>HPO<sub>4</sub>  
 5.5 g NaH<sub>2</sub>PO<sub>4</sub>•H<sub>2</sub>O  
 0.75 g KCl  
 0.25 g Mg SO<sub>4</sub>•7H<sub>2</sub>O  
 H<sub>2</sub>O to 1 liter

*Just before use, add 2.7 μL/ml β-mercaptoethanol to the volume to be used.  
 (no autoclaving needed, nonsterile)*

**ONPG**

1.2 mg/mL o-nitrophenyl β-D galactopyranoside in Z Buffer  
*Prepare just before use.*  
 (no autoclaving needed, nonsterile)

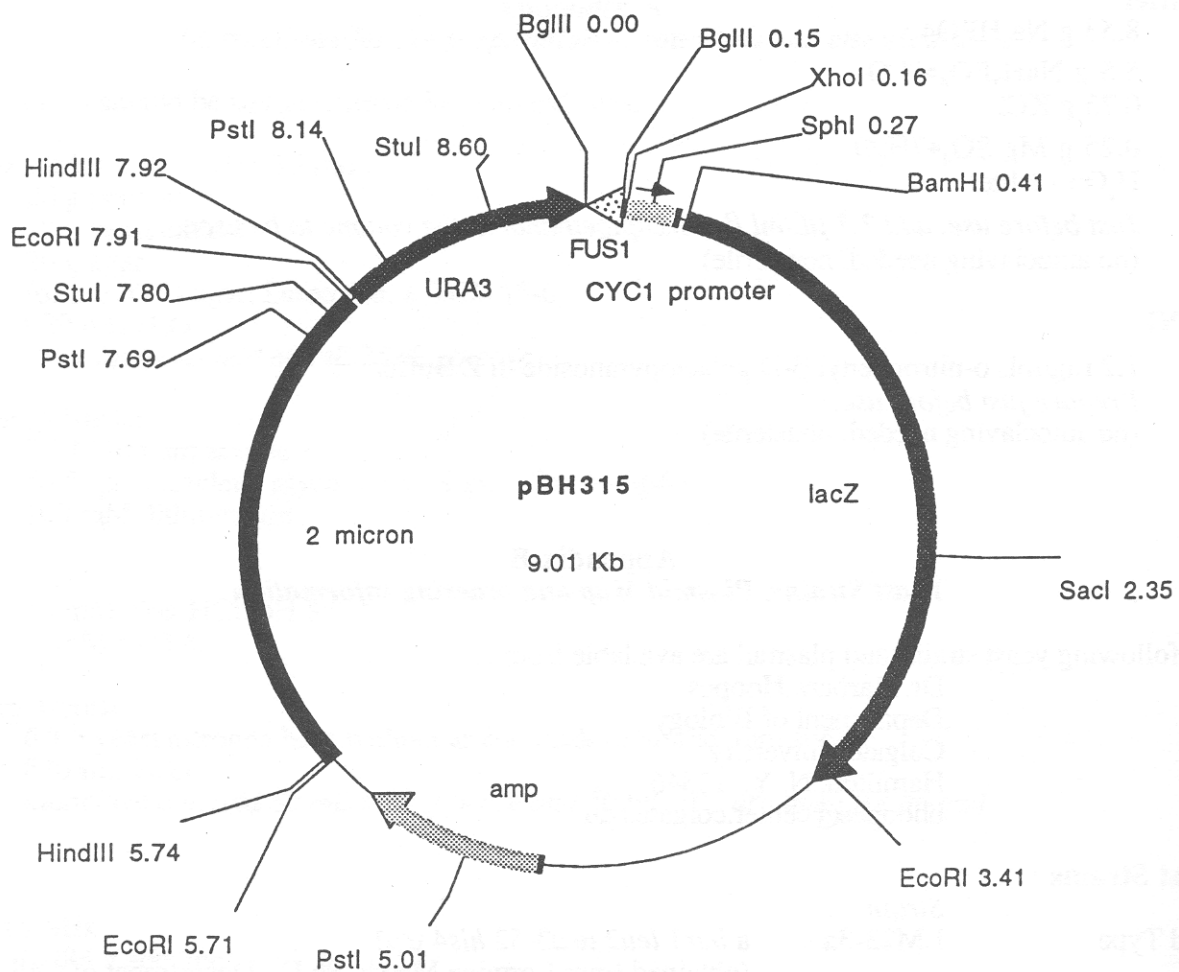
**Appendix B*****Yeast Strains, Plasmid Map and ordering information***

The following yeast strains and plasmid are available from :

Dr. Barbara Hoopes  
 Department of Biology  
 Colgate University  
 Hamilton, N. Y. 13346  
 bhoopes@center.colgate.edu

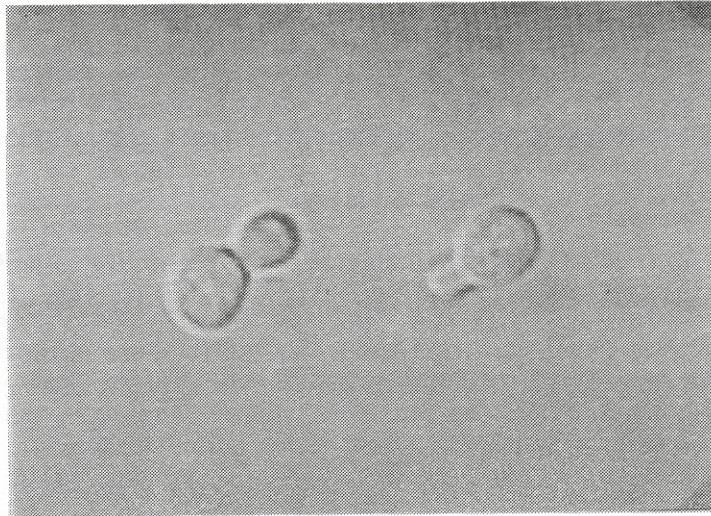
**Yeast Strains**

	<i>Strain</i>	
Wild Type	LM23-3a	a <i>bar1 leu2 ura3-52 his4 trp1</i> (obtained from Lorraine Marsh, Ph.D., Department of Cell Biology, Room C-440, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, NY 10461-1924)
Mutant Y	LM-16a	a <i>ste2ΩTRP1 bar1 leu2 ura3-52 his4 trp1 (ste2)</i> (obtained from L. Marsh)
Mutant X	FC-D1	a <i>far1-1 bar1-1 ura3-52 met1 lys<sup>-</sup> (far1)</i> (Chang and Herskowitz, 1990)

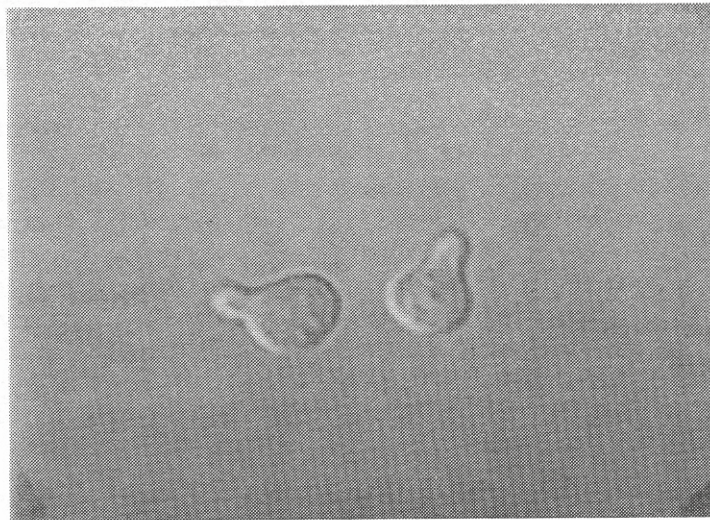


**Figure 5.3.** Plasmid map for pBH315. The plasmid pBH315 contains the upstream activation site of FUS1 from position -73 to -209 relative to the start site for translation (Trueheart et al., 1987) inserted in the yeast reporter vector  $\Delta$ SS-BglII (B. J. Andrews, unpublished). The plasmid  $\Delta$ SS contains the core promoter region from the yeast CYC1 gene fused to  $\beta$ -galactosidase and shows only negligible  $\beta$ -galactosidase activity due to the lack of an upstream activation sequence for the promoter (Johnson and Herskowitz, 1985). The FUS1 DNA is inserted in an orientation opposite to that found in the natural FUS1 promoter, but confers a large amount of transcriptional activity in the presence of mating factor. This plasmid contains both yeast (2 micron) and bacterial replication origins and genes that can be used as selectable markers in both yeast (URA3) and bacteria (ampicillin resistance).

**Appendix C**  
*Photographs of budding and “shmooing” yeast cells*



**Figure 5.4.** Photograph of budding and “shmooing” yeast cells. The cell on the left is budding and the one on the right is “shmooing”. Budding occurs in the haploid cells of yeast and is the non-sexual method of reproduction. “Shmooing” is one of the responses to the signal transduction pathway initiated in a haploid yeast cell by a mating factor from the opposite mating type. It allows the cells to come together and fuse to form a diploid cell.



**Figure 5.5.** Photograph of two “shmooing” yeast cells.

**Appendix D**  
*Expected Results*

Wild Type:

The percent of unbudded cells should *increase* in the presence of  $\alpha$  **factor** due to the increased number of cells arrested at G<sub>1</sub> phase of the cell cycle. The  $\beta$ -galactosidase activity should also *increase* in the presence of  $\alpha$  **factor** indicating increased transcription and translation of mating genes.

Mutant “X”:

The mutation is in *far1*, the cell arrest factor gene, so the percent of unbudded cells should *not change* in the presence of  $\alpha$  **factor** since the arrest factor will not be activated and, therefore, cells will not arrest. The  $\beta$ -galactosidase activity should *increase* in the presence of  $\alpha$  **factor** indicating increased transcription and translation of mating genes as this part of the pathway remains intact.

Mutant “Y”:

The mutation is in *ste2*, the  $\alpha$  **factor** receptor gene, so the percent of unbudded cells should *not change* in the presence of  $\alpha$  **factor**. The  $\beta$ -galactosidase activity should *not change* in the presence of  $\alpha$  **factor**, indicating no increase in transcription and translation of mating genes.