NAME_____TA_____

7.013 Spring 2005 Problem Set 3

## FRIDAY March 11, 2005

Problem sets will NOT be accepted late.

Question 1

You'd like to clone the 3 kb *YFG* gene from the '*pOld-YFG*' plasmid into the '*pNew*' plasmid. The *p*New plasmid carries the **lac Z** gene encoding β-Galactosidase that can cleave a substrate, X-gal, into a blue colored compound. When grown on medium with X-Gal, cells without β-Galactosidase form white colonies, while cells with β-Galactosidase form blue colonies.

$$X\text{-Gal} \xrightarrow{\beta\text{-galactosidase}} Blue$$

Below are drawings of the plasmids and the inset shows the linear *YFG* gene.
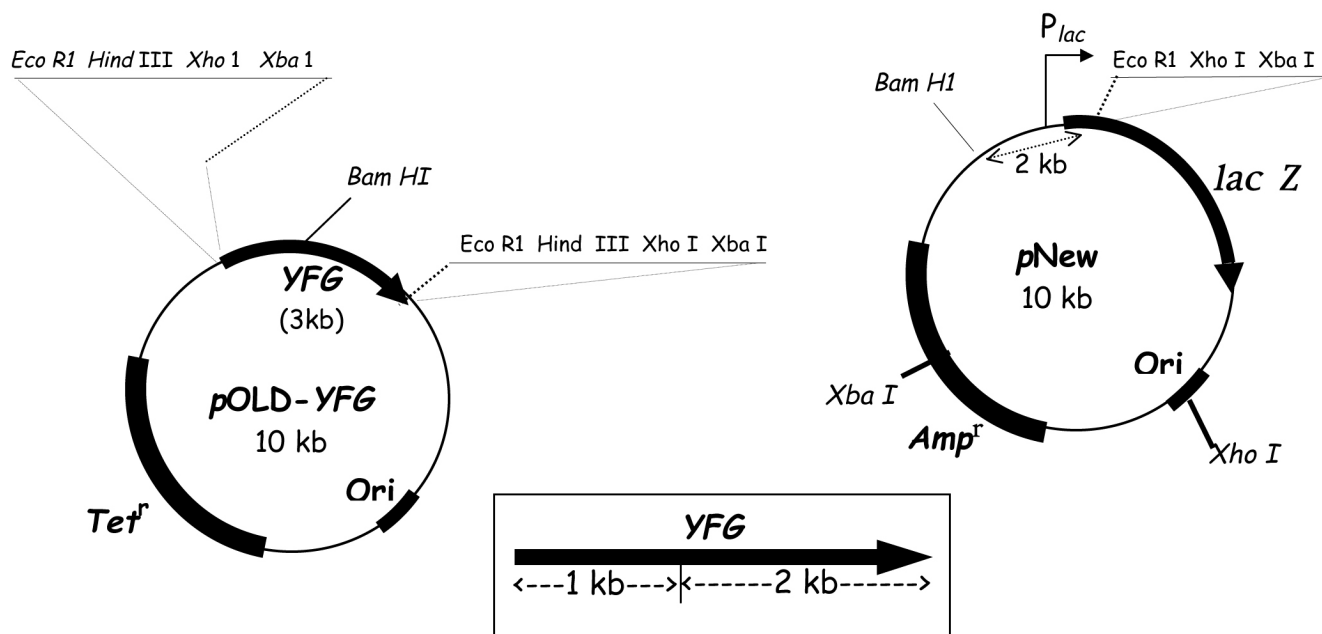
a) Of the following enzymes, which would or would not be useful for cloning the *YFG* gene from *p*Old-YFG to the *p*New plasmid. Assume that each of the cut restriction sites can only ligate with the same kind of cut restriction site (ex: *Bam* HI with *Bam* HI, *Xho* with *Xho* I, etc.)

i) Would *Eco* RI be useful? Why or why not? Sure. *Cut both plasmids with Eco RI. Ligate and transform.*
ii) Would *Hind* D III be useful for cloning? Why or why not? *No. There's no Hind III site in pNew*

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iii) Would *Bam HI* be useful cloning? Why or why not? *No. There's only one Bam site in smack inthe middle of YFG.*

iv) Would *Xba I* be useful for cloning? Why or why not? *No. There's an Xba I site in the middle of the Ampicillin resistance gene.*

v) Would *Xho I* be useful cloning? Why or why not? *No. There's a Xho I site in the Ori of pNEW.*

You digest both plasmids with the appropriate restriction enzyme(s) and combine digests in a single tube and add DNA Ligase. You transform appropriate bacteria with this ligation mix.

b) How would you identify transformants that have taken up *pNew* but not *pOld-YFG*? (Hint: 2 steps) *Select transformants on Ampicillin plates. Replica plate colonies onto tetracycline medium to screen Amp^R colonies for tetracycline sensitivity.*

Now that you have colonies that have taken up *pNew*, you realize that the *YFG* gene could have inserted in either orientation. You take 10 transformants, isolate plasmid DNA from them, and perform restriction digestion using *Bam H1*. You get the following results after running the digests out on an agarose gel.

c) Fill in the electrode charges in the circles depicting how the gel was run.

BAM H1 – DIGESTED PLASMIDS
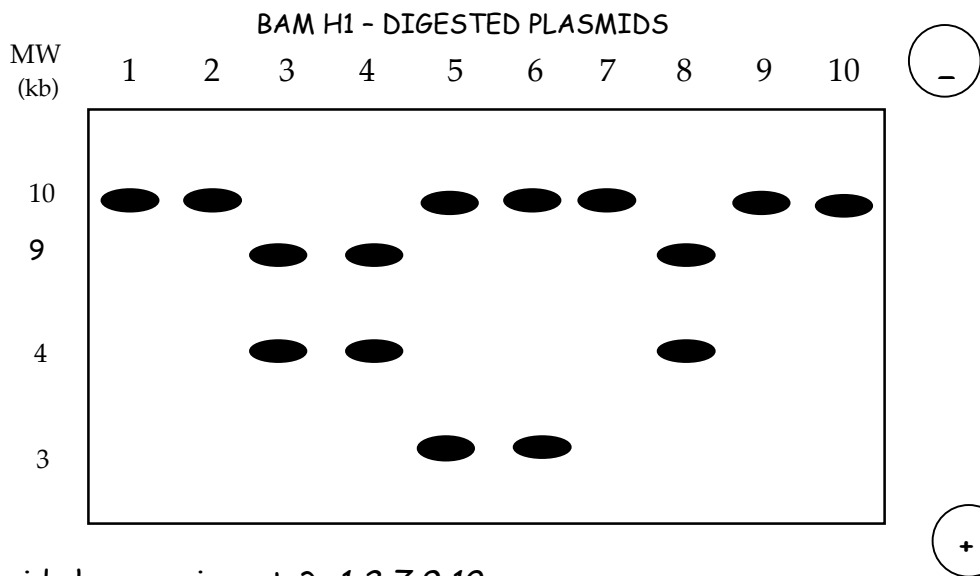
d) Which plasmids have no inserts? *1 2 7 9 10*

e) Which plasmids have YFG inserted? *3 4 5 6 8*

f) Of the plasmids in e) which colonies have *YFG* inserted in a way that it will be expressed off P$_{lac}$? *5 6*

g) How could you have avoided choosing colonies harboring plasmids without inserts? How could you have screened the transformants for ones that had plasmids with the *YFG* gene? *Plate the transformation mix onto Ampicillin X-gal plates. Pick white colonies.*

2

Question 2

You want to study protein targeting in yeast, but first you need to construct a strain that will help you with your research. Your goal is to clone the gene encoding the Fructose Receptor (FruR), a plasma membrane protein, and fuse it with a gene encoding Green Fluorescent Protein (GFP).

a) You first make a cDNA library from yeast that is able to grow fructose. This is done by using the enzyme Reverse Transcriptase (RT) to make a DNA copy of each of the messenger RNA molecules in this yeast strain. RT, like other DNA polymerases, needs a primer to initiate elongation. You recall from earlier lectures that eukaryotic mRNA has a cap at the 5' end and is polyadenylated (A_n) at the 3' end.

> $^{5'}$CAP--$^{3'}$polyA tail

i) Which end of an RNA molecule (**5'** or **3'**) would you use as a template to design a primer? Circle one.

3'

ii) Given what you know, as stated above, what would be a good 'universal' primer for an RT reaction? (Assume 10 nucleotides long is sufficient.)

TTTTTTTTTT□

b) Using the primer you just designed you've synthesized millions of cDNA molecules but only a few encode the fructose receptor gene.

i) First you need to clone these cDNAs into vectors. What needs to be upstream of your cDNA in the vector to insure strong expression of the fructose receptor?
Promoter

ii) You consider expressing the protein in bacteria. Why would this plan make it critical to have made a cDNA library rather than making genomic DNA library?

Bacteria are unable to splice out the introns that would occur in a genomic sequence from eukaryotic DNA

iii) You decide to transfrom your library into yeast. How would you isolate the plasmid clone expressing the fructose receptor? What yeast strain would you transform into?

Wild-type Yeast   Fructose Receptor Mutant Yeast

iv) What medium could you use to plate the transformants that would select for the correct plasmid clone. *Minimal Fructose medium (Fructose as the only carbon source.)*

c) Now that you have the *FruR* gene cloned, you want to amplify the *gfp* gene encoding Green Fluorescent Protein from some jellyfish cells. Your first step is to design primers so that you can use polymerase chain reaction (PCR) to amplify the region. Below is highlighted the sequence at each outside end of *gfp*.

```
5'...ACGTAAACGGCCACAAGTTCAGCGT.......ACCCCGACCACATGAAGCAGCACGACT...3'
     |||||||||||||||||||||||||  (800)  ||||||||||||||||||||||||||||
3'...TGCATTTGCCGGTGTTCAAGTCGCA.......TGGGGCTGGTGTACTTCGTCGTGCTGA...5'
```

i) Of the potential primers listed below, which 2 can you use to amplify the DNA of interest? (Circle the correct 2.)

A: 5'-GTAAACGGCCACAAG-3'
B: 5'-CATTTGCCGGTGTTC-3'
C: 5'-CTTGTGGCCGTTTAC-3'
D: 5'-AATGGCATATGCCGT-3'
E: 5'-ATGAAGCAGCACGAC-3'
F: 5'-GTCGTGCTGCTTCAT-3'
G: 5'-CAGCACGACGAAGTA-3'

ii) Schematically, using lines as DNA and filled in boxes and open boxes as primers, draw the products of the reaction starting from the double stranded piece of DNA below after it has gone through each of 2 rounds of replication. The binding sites of the primers are shown on the template below.

5'☒ 3' Primer1 (in excess)

5'▭ 3' Primer2 (in excess)
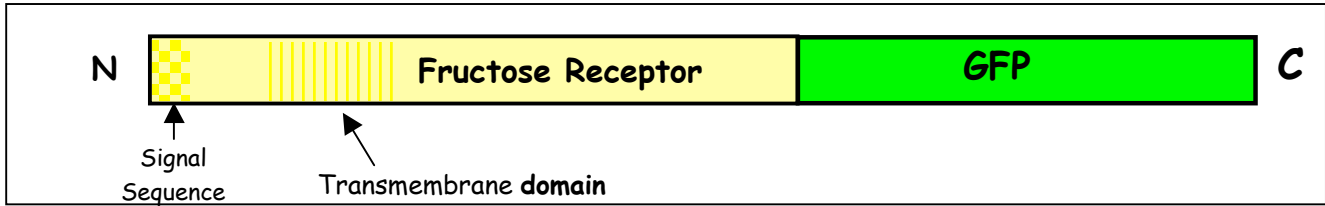
After 1 round of PCR   After 2 rounds of PCR
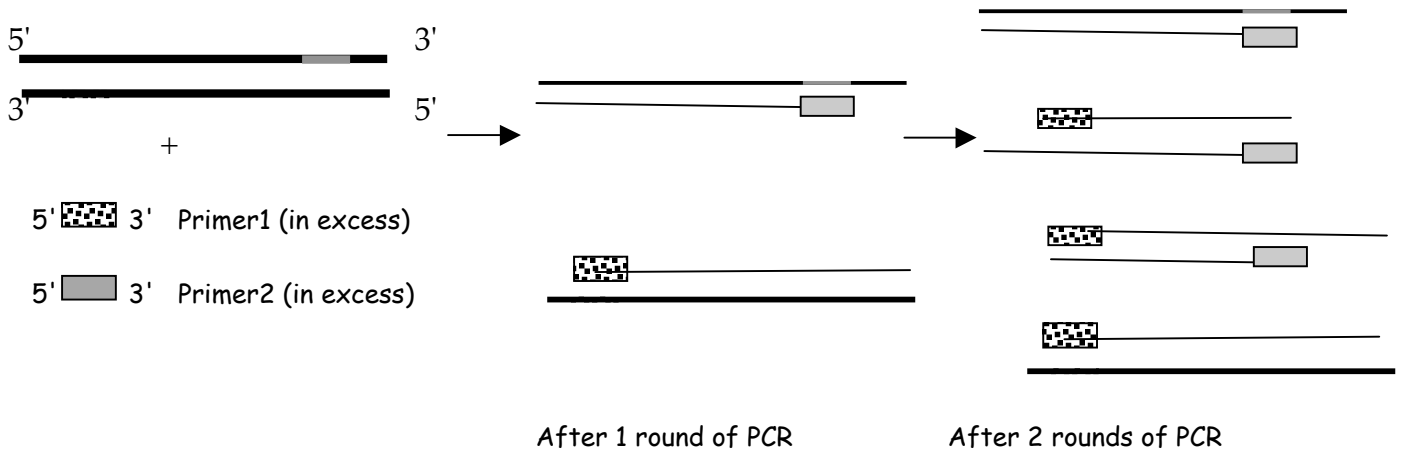
iii) Using PCR, you successfully amplified a piece of DNA. To confirm that it is indeed *gfp* you sequence it. What is the sequence of the DNA on the gel below? (Be sure to label the 5' and 3' ends.)

dATP dCTP dGTP dTTP

5'-AGCTGTATAGGTTGTC-3'

4
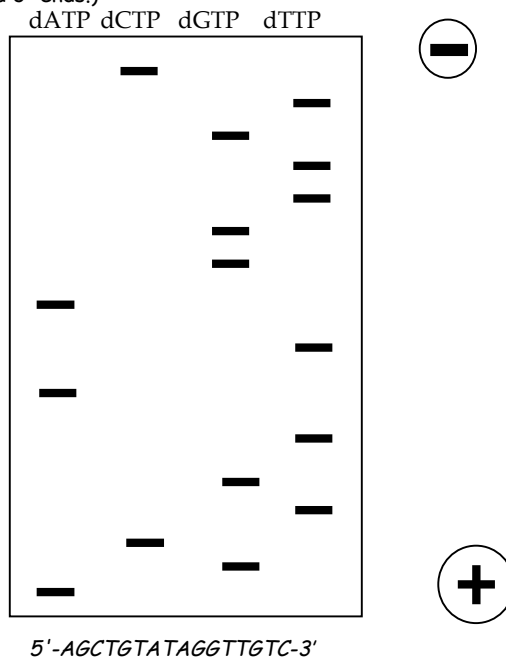
Question 3

You are pleased to discover that this is indeed a sequence of *gfp*. Your goal is to make a fusion protein where the N-terminus of Green Fluorescent Protein is replaced by Fructose Receptor. Green Fluorescent Protein (GFP) is a protein isolated from jellyfish that fluoresces green when exposed to blue light (It has been demonstrated that some proteins do not require their extreme C terminus or their N terminus to function.)

In this case, the first half of the fusion protein (the NH_3 end) will be a fully functional Fru R protein, and the second half (the COOH end) will be a fully functional GFP protein.

```
  N    [Signal]  ||||||||||  Fructose Receptor          GFP                    C

       Signal
      Sequence            Transmembrane domain
```

The sequence for the extreme 3' end of *fruR* and the 5' start of *gfp* is shown below. For both, the underlined indicates an in frame codon. For *gfp*, it corresponds to the start codon.
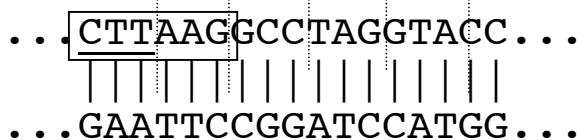
3' end of *fruR*

```
5'...CTTAAGGCCTAGGTACC...3'
     |||||||||||||||||
3'...GAATTCCGGATCCATGG...5'
```

5' end of *gfp*

```
5'...AGGCCTTAAGCCTAGGCTAGCAATGGTACC...3'
     ||||||||||||||||||||||||||||||
3'...TCCGGAATTCGGATCCGATCGTTACCATGG...5'
```

*Afl*II:	*Avr*II	*Stu*I	*Nhe*I	*Kpn*I
C^TTAAG	C^CTAGG	AGG^CCT	G^CTAGC	GGTAC^C
GAATT^C	GGATC^C	TCC^GGA	CGATC^G	C^CATGG

Determine what enzyme(s) you would use to fuse the 5' end of *gfp* with the 3' end of *fruR* such that the resulting gene fusion would result in the expression of an in-frame hybrid protein. Remember it's ok to fuse the genes in a way that they are missing a bit off the ends or even adding some nucleotides between them. The goal is to make an in-frame fusion protein.

```
...CTTAAGGCCTAGGTACC...
   |||||||||||||||||
...GAATTCCGGATCCATGG...
```

5' end of *gfp*

```
...AGGCCTTAAGCCTAGGCTAGCAATGGTACC...
   ||||||||||||||||||||||||||||||
...TCCGGAATTCGGATCCGATCGTTACCATGG...
```

The way I do this problem is that I delineate the codons in both nontemplate coding strands. I then look at where the restriction sites are and how they line up in the reading frames. To keep the frame intact in the fusion, the matching restriction sites have to be exactly like each other. That is if the 6 base site perfectly includes 2 codons then the same site must perfectly include 2 codons in the other piece. If the site starts within a codon by 1 nucleotide, then it has to join the same site that also starts with a codon by 1 nucleotide. Using this strategy there are only two possible answers that line up exactly. –And that is:

Afl II and Kpn I. However since the Kpn I site in the end of the fruR gene is downstream of the stop codon, using this site could never result in a hybrid protein. SO THE ANSWER is Afl II.

Question 4

After successfully making your in-frame GFP fusion, you are ready to use this construct to elucidate new genes involved in protein targeting in the yeast cell. To identify new targeting genes, you create a collection of temperature sensitive yeast mutants. Temperature-sensitive mutations produce functional protein products at low (permissive) temperatures but do not produce functional protein at high (restrictive or nonpermissive) temperatures. This allows a rapid switch from a wild type to a mutant phenotype by a temperature shift from permissive to restrictive. Thus temperature sensitive mutations allow one to study genes whose products are essential for the strain because one can grow and maintain the strain at the permissive temperature.

To determine if any of your temperature sensitive mutations have an effect on protein targeting you introduce your newly constructed gene fusion to each mutant strain to examine the localization of FruR-GFP hybrid protein. The GFP tag allows you to visualize that localization of FruR in the cell using fluorescence microscopy.

Below are the observations from the fluorescence microscopy for the **wild type** yeast strain. Note that all fusion proteins are bound to the plasma membrane as seen in picture below.

Photomicrograph removed due to copyright reasons.

You identify 5 temperature sensitive mutations that are <u>d</u>efective in <u>p</u>rotein <u>t</u>argeted at the restrictive temperature and name them dpt1-5.

dpt1: All ER localized
dpt2: Found in small vesicles
dpt3: Found in the cis Golgi
dpt4: Cytoplasmic
dpt5: No GFP detected

a) For each of the mutants identify which step of the secretory pathway is blocked. (Assume all mutations are in proteins other than FruR.)

Mutant	Step of Secretory Pathway Affected
dpt1	**ER to Golgi transport vesicle budding**
dpt2	**Block of fusion of transport vesicle to Golgi or Secretory vesicles to PM**
dpt3	**Block of formation of Secretory vesicles**
dpt4	**Block of ribosome binding to ER**
dpt5	**Block of protein synthesis**

b) Which of the above mutations could be a result of an internal mutation in FruR? Explain what the mutation would be.
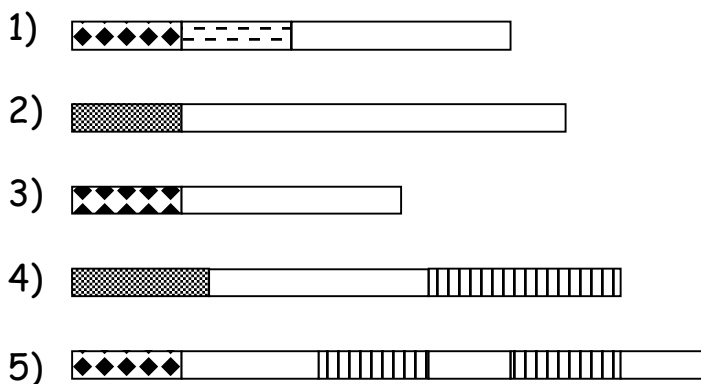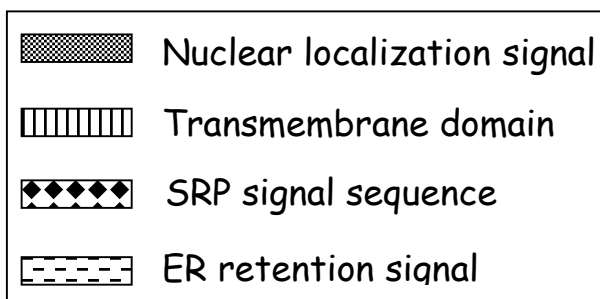
dpt1: **Gain of an ER retention signal**

dpt3: **loss of signal to enter vesicles**

dpt4: **loss of signal sequence**

dpt5: **nonsense mutation**

You are interested in determining the effect of some of the above mutations on the localization of other cellular proteins. Below are the domain structures of the cellular proteins you are interested in (labeled 1-5):

1)

2)

3)

4)

5)

c) In a wild type cell where would each of these proteins be found:

Protein	Cellular Location
1	**ER**
2	**Inside the nucleus**
3	**Outside cell (secreted protein)**
4	**Nuclear membrane**
5	**Plasma membrane**

d) Where would each protein be localized a dpt1 mutation:

Protein	Cellular Location
1	ER
2	**Inside the nucleus**
3	ER
4	**Nuclear membrane**
5	ER

e) Where would each protein be localized a dpt3 mutation:

Protein	Cellular Location
1	ER
2	**Inside the nucleus**
3	Golgi
4	**Nuclear membrane**
5	Golgi

Question 5

Many signaling pathways make use of heterotrimeric G protein coupled receptors. One such pathway is the adrenalin or epinephrine pathway. Production of epinephrine leads to many downstream effects that are part of the "fight or flight" response. Epinephrine binds to β-adrenergic receptors which leads to the activation of adenylate cyclase, cAMP production and the activation of Protein kinase A. In the cell depicted below the net result of epinephrine binding is Calcium influx to the cell.
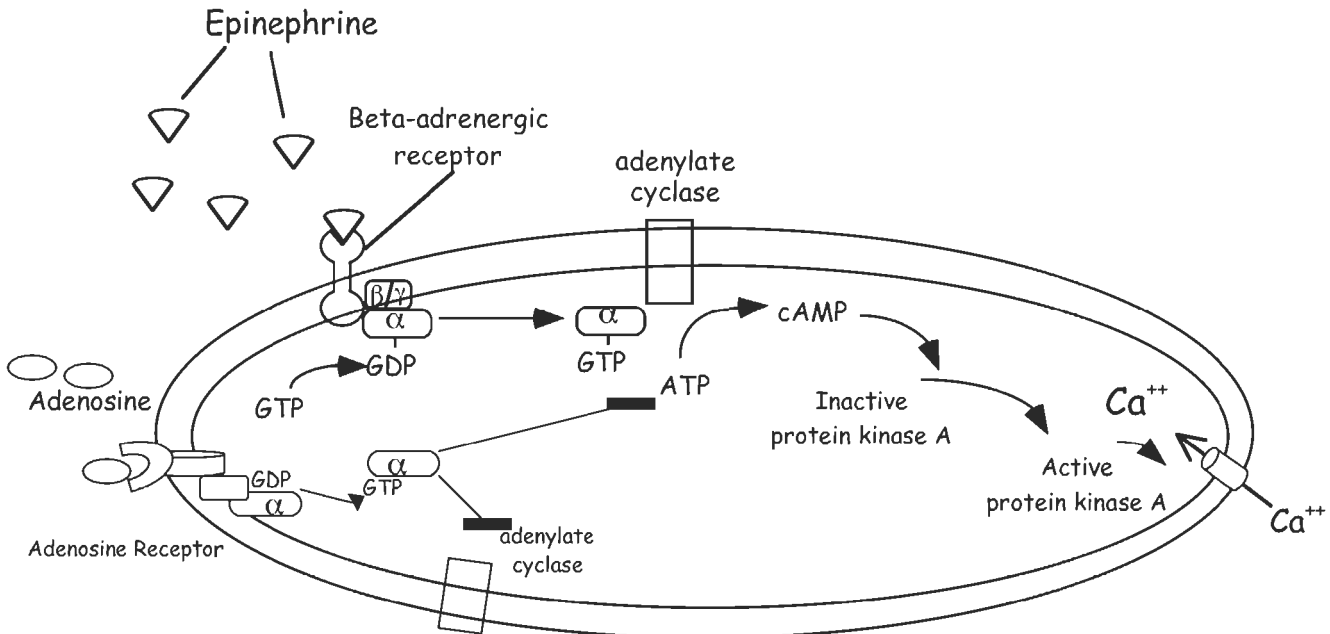
a) List two advantages of having multiple steps in a signal transduction pathway.

Allows for amplification of signal, allows multiple points of regulation of the pathway, and allows for integration of different inputs. Amplification, multiple points of regulation, integration of signals

b) Once the G-protein is active why doesn't it continually activate adenylate cyclase?

The Gα subunit has intrinsic GTPase activity that converts GTP, bound to the Gα subunit, to GDP. This conversion deactivates the Gα subunit of the G-protein. The inactive Gα subunit can rejoin the β and γ subunits to await another signal from the receptor. Also, cAMP is degraded by phosphodiesterase and phosphorylated kinases are dephosphorylated.

c) Some cells have surface adenosine receptors that are also G-protein coupled receptors like the adrenergic receptor depicted above. However, unlike the adrenergic receptor above, the affect of adenosine receptors is to **inhibit** the enzyme adenylate cyclase, decreasing cAMP levels, through an inhibitory G –protein subunit. Draw the adenosine receptor, the ligand, and its downstream pathway in the figure above.

Caffeine is similar in chemical structure to the adenine portion of adenosine. Caffeine is similar enough to adenine to fit into adenosine receptors, but is not similar enough to stimulate those receptors. So the main action of caffeine is to block adenosine from binding to its receptor. Thus caffeine is an adenosine receptor "antagonist."
Interestingly, caffeine ingestion also leads to the production of epinephrine.

d) Given the information above, what would the end result be in a cell that has both the β-adrenergic receptor and the adenosine receptor described, in an animal that has been fed caffeine? *Sustained levels of HIGH Calcium in the cytoplam.*