1. (5 pts) You are a fifth year graduate student whose Ph.D. thesis concerns the cloning and analysis of a novel transcription factor that regulates drug metabolism. After struggling for a few years, experiments are going just great and you are nearly ready to publish a major paper and start postdoctoral study. Your advisor has just returned from a scientific meeting and has some very disturbing news. She heard a rumor that Dr. Evil has a) also cloned your gene b) has similar results to yours and c) has a manuscript under review at a leading journal. You have a brief time window to avoid being scooped (i.e., time is of the essence). The final experiments for your paper require the use of a liver cell line that stably expresses the promoter of your gene driving an appropriate reporter. Outline the approach you would use to generate this cell line. Be sure to mention how you would get the DNA into the cells, what type of selection you would use (positive or negative), which antibiotic or other selection would be preferred and what reporter would work best in these experiments. The reporter should be sensitive and have a large dynamic range. There is more than one way to accomplish your goal - full credit depends on how well justified your chosen approach is.

Prepare a reporter gene construct fusing the promoter of your gene with luciferase or b-lactamase. Others do not have the combination of sensitivity, dynamic range and speed. Transfect cultured cells with this reporter using any method except DEAE dextran (doesn’t work well for stables). Use positive selection only. Puromycin or blastocidin are preferred since speed is of the essence.
2. (5 points) Highlight some of the important issues to consider when selecting a reporter gene to use for gene expression assays

- required sensitivity
- dynamic range
- speed and ease of assay
- expense of required reagents
- availability of equipment
- requirement for in vitro or in vivo
- throughput requirements
- local expertise
- what reagents are already available vis a vis the number of assays, etc

3. (5 points) What is the fundamental mechanism shared among most transfection methods that allows cells to take up DNA from the surroundings?

phagocytosis

4. (5 points) Retroviral gene transfer is a useful and efficient technique for getting cloned genes into cells or embryos. Would this be the method of choice for introducing cloned genes into early mammalian embryos? If this is not the method of choice, what would be? Justify your answer.

Retroviral mediated gene transfer will not work in the early embryo since the viral LTRs are not active. The method of choice would be to use a standard transgenic approach (inject DNA into egg, etc). The gene could also be knocked into ES cells and these injected into a blastocyst but this approach is slower, trickier and more costly.
5. (6 points) Transgenic mice are important experimental models to evaluate gene function. What are three important things we would like to know about any founder animal before undergoing a large scale breeding project? Describe how would you test to ensure that these criteria are met?

- **How many copies of transgene are present?** Southern blotting of tail DNA
- **Is the transgene expressed?** Western or Northern blots of various tissues
- **Is the transgene expressed as predicted?** Tissue Northerns, in situ hybridization, quantitative RNA blotting or RT-PCR
- **Is the transgene transmitted faithfully, i.e., does copy number change with breeding?** – Southern blotting of tail DNA
- **Are expression levels similar in progeny of founders?** – Quantitative RNA analysis or western blotting

6. (4 points) What are some problems and pitfalls in targeted gene disruption experiments in mice?

- Incomplete knockout of gene function (weak alleles created)
- Alteration of expression of adjacent genes (removal of regulatory elements)
- Interference from selection cassette
- May not be possible to study dominant lethal phenotypes
- Non-specific embryonic lethality due to placental defects is common
7. (5 points) Discuss the features of the sterol regulatory element binding proteins that distinguish them from other closely related transcriptional regulatory proteins and describe how these features specifically influence cholesterol regulation in the cell.

The answer should include:

1). SREBPs are bHLH proteins that are membrane bound in the ER/nuclear envelope in a precursor state

2). low cholesterol levels result in their proteolytic release and nuclear accumulation

3). The basic region of the DNA binding domain has a key tyrosine residue that allows the proteins to have a dual DNA binding specificity:
   a). They bind to both a classic bHLH E-box inverted repeat (like virtually all bHLH proteins which have an arginine in place of the tyrosine)
   b). they also bind to a direct repeat sterol regulatory element site. The key is the arginine to tyrosine substitution that allows binding to both E-boxes and SRE's

4). Cholesterol regulated genes have SRE's and not E-boxes which provides specificity in gene activation. This is because no other SRE binding proteins are in the nucleus before cholesterol depletion (unlike the case for E-box binding proteins where there are lots of them in the nucleus independent of the cholesterol level in the cell).

8. (4 points) Compare and contrast conventional, confocal and two photon stimulated fluorescence microscopy. Be specific.

Conventional fluorescence has the disadvantages that photons can be out of focus and phototoxicity. Confocal fluorescence uses a pinhole to select for photons in the focal plane but still has the problem of phototoxicity since the entire sample is being excited. In contrast, two photon microscopy deals with both problems. Since only the focal plane is excited, all emitted photons are collected and in focus. There is no phototoxicity to the sample above and below the focal plane because the excitation photon density is not great enough.
9. (3 points) What is a chromosomal translocation? Describe two types of chromosomal translocations that can lead to the formation of cancers.

A chromosomal translocation occurs when two chromosomes are broken and hybrid chromosomes formed by fusing different chromosomes at these breakpoints. Cancers could occur when the rearrangement causes the activation of an oncogene near the breakpoint or if the rearrangement leads to the production of a hybrid protein that causes, or allows tumor formation.

10. (3 points) With a diagram, explain the physics and principles of laser tweezers.
11. (10 points) You are a molecular biologist who really loves eating shrimp after work as much as you enjoy working in the lab. To combine your two loves, you have decided to create a biotech company (www.jumboshrimp.com) based on producing the largest shrimp anywhere. You took an invertebrate biology course at UCI and learned that shrimp and related animals express an, as yet, unidentified growth inhibitory gene when cultured at high density. You really need to a) clone this gene, b) figure out where this gene is expressed to begin to understand how it works and c) knock it out so that your shrimp will grow to large size in aquaculture. How would you go about accomplishing these goals? How would you prove to the patent office that your gene is the decisive factor in regulating shrimp growth in culture? Assume that all of the standard sorts of methods we discussed in class will work in shrimp (except targeted gene disruption). Assume that shrimp heterozygous for disruption of the growth inhibitory gene will be large enough to be detected but not big enough for your company.

Several approaches are possible. Here is the one I was looking for:

Since you want to knock out a gene before you have cloned it and also determine its expression pattern, a gene trap approach would be a good choice. One would construct a retroviral vector that contains a splice acceptor sequence 5’ to a reporter gene such as GFP or b-gal. Infect a large pool of fertilized eggs and allow the larvae to develop. Identify shrimp that grow significantly better than the rest. Expand these lines by breeding. Test whether the homozygous animals grow to large size in culture. If they do, you have a candidate. Clone the sequence flanking the insertion (e.g. by inverse PCR, screening a genomic library or simply cutting with a rare enzyme also present in the plasmid and recircularizing). Identify where the gene is expressed during development and in the adult by assaying for your reporter (by staining, UV, etc). Use the genomic clones to identify cDNAs. Create transgenic shrimp with the cDNA under the control of an inducible promoter to demonstrate that the amount of expression is inversely related to growth rate. Taken together with the evidence from knocking out the gene, this is sufficient to demonstrate the role of your gene in growth control.
12. (10 points) In the example above, you have identified a putative gene that can regulate the growth of shrimp when they are cultured at high density. You also know where and when the gene is normally expressed. The next step is to understand the factors that regulate expression of this gene. Describe the approach you would take to characterize the promoter of this gene and identify elements required for correct temporal and spatial expression in both cultured cells and transgenic animals. What reporter gene constructs would you make and why? Assume that transgenesis in shrimp is possible.

The first thing you need to do is obtain the 5' flanking sequence of the gene. You would do this by screening a genomic library with the cDNA. Map the 5' end of the cDNA to determine the transcriptional start site. Fuse the promoter to the reporter gene. For use in cell culture, luciferase or β-lactamase are preferred reporters due to their sensitivity and wide dynamic range. For use in transgenic animals, GFP or β-gal are preferred for ease of visualization. You could be clever and use a chimeric reporter with GFP fused to luciferase so that you can detect either one. Make deletion constructs to identify regions of the promoter required for expression in cultured cells. Make transgenic animals to evaluate whether these elements are required in vivo, particularly in response to high density culture.
13. (10 points) The two scenarios above have provided you with an important gene and information on what regions of its promoter are required for temporal and spatial expression. Unfortunately, although the shrimp grow to large size when the gene is knocked out using the approach you described in question 11, their fecundity is impaired. One thousand fold fewer embryos are produced per mating, despite the increased size of the animals. Oops. Fortunately, the consultant you have hired (your former Ph.D. advisor) has a bright idea. Why not just identify the promoter element responsible for regulating expression of the gene in response to high density culture and then modulate its activity rather than knocking the gene out and getting 1000 fold less shrimp? You described how to do this in your answer to question 12, right? As it turns out, you identify a single promoter element that is required for the activation of the gene when the shrimp are cultured at high density. The sequence of the element is the following: AGGTCATCAGATGACCT. What can you deduce about the protein(s) from the sequence of the binding site? Describe how you could identify a protein(s) that binds to this site. How would you identify cDNAs encoding the protein that binds to this element?

The sequence has dyad symmetry. This means that the factor which binds to it is a dimer. You can identify putative binding proteins from cell, tissue or embryo extracts by gel shift experiments. The proteins could be purified biochemically, microsequenced and then cloned. Alternatively, you could use an expression cloning method to identify the binding protein directly. The dimeric nature of the protein implies that you may need both partners for high-affinity binding. If the protein is a homodimer, this will not be a problem. Any expression cloning strategy is likely to work. If the protein is a heterodimer, direct oligonucleotide screening of an expression library may be problematic. In this case, one could create a reporter construct with the binding element upstream of a minimal promoter driving luciferase. Transfect pools of cDNA expression libraries into cultured cells either together with the reporter or make a stable reporter cell line that you transfect. Alternatively, IVEC could be used to identify a cDNA that encodes a binding protein in the presence of cellular extracts.
14. (5 points) OK, you are really making progress now. Genes closely related to your shrimp growth inhibitory gene have just appeared in the EST databases from *Drosophila* and human. The *Drosophila* gene maps to a region on chromosome 2 where a previously uncloned gene required for germ cell formation is located. Loss-of-function mutations at this locus produce embryos lacking most of their germ cells. Cool. This suggests a possible model for why the shrimp deficient in this activity are less fecund. Which of the known embryonic signaling pathways is most likely to be involved? How would you go about determining where your gene fits into the pathway?

The production of germ cells in Drosophila is regulated by the posterior patterning pathway. Since most of these genes are involved in localizing each other, one could test which mutants in the pathway are rescued by microinjection of mRNA encoding the putative Drosophila homolog of the shrimp gene. The mRNA should rescue genes that are upstream of it in the pathway.
15. (5 points) It gets better. The human EST is from a testis cDNA library. Considering that the shrimp produce fewer embryos and that flies produce fewer germ cells, you hypothesize that the protein is required for the formation of sperm. Wow, perhaps one could use this gene as a target to develop new male contraceptives. Describe an experimental approach that would demonstrate that sperm are produced when the gene is on but not produced when the gene is off?

This could be accomplished by making a transgenic mouse model that expresses either the human cDNA (or the mouse homolog) under the control of a promoter that can be modulated (e.g. tet, rev-tet, ecdysone). Any inducible system should work but the best choice might be the standard tet system since the target gene is normally on and then turned off by the addition of tet.