Biology 7.06 Exam 3 1994

Question 1 (25 points)

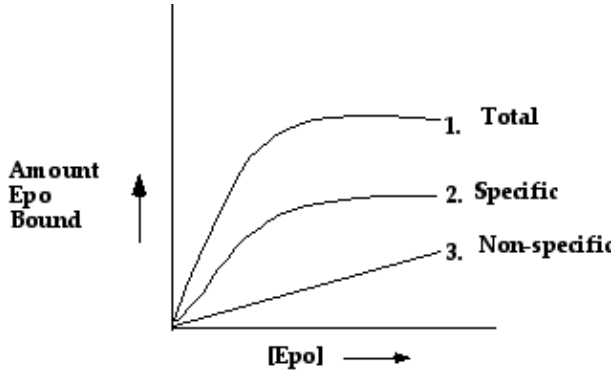
The population of red blood cells is dependent on the growth/differentiation factor erythropoietin (epo), which is produced in the kidneys and stimulates the differentiation of erythroid precursors into mature red blood cells. The epo receptor is present only on the cells of the erythroid (red blood cell) lineage.

A. (5 points) Harry the hematologist wishes to determine the number of epo receptors on the surface of erythroid cells. He carries out an experiment that shows that there are approximately 1000 molecules of the receptor per cell. Knowing that Harry used the K562 erythroid cell line and both radiolabeled and unlabeled epo in his experiment, describe how Harry came to his determination. Hint: Harry knows the specific activity (or amount of radioactivity per mole) of the radiolabeled epo. Feel free to include a graph in your answer.

1. Use radioactive epo in increasing concentrations to find total binding activity.

2. Use radioactive epo in increasing concentrations with a large excess of unlabelled epo to find non-specific binding activity.

3. Subtract 2 from 1 to get specific binding.



See figure 19-6 in text for more detail.

B. (5 points) Define in general terms the dissociation constant (K_D) of receptor/ligand binding. Based on your answer in Part A, explain how you could determine the K_D of the epo receptor present on K562 cells.

KD is a measure of the relative affinity between two molecules - in this case between the receptor and ligand.

$$K_D = \frac{[R][L]}{[RL]} \qquad [RL] \rightleftharpoons^{K_D} [R]+[L]$$

You can either use this or another equation derived from it and solve for KD. You can also determine KD by knowing that the KD = [L] (ligand concentration) when 50% of the receptors are bound and looking on the graph for this place.

C. (5 points) Harry next decides he would like to clone the gene encoding the epo receptor. Since he has shown that the receptor is present in low abundance on the cell surface, he decides against a biochemical purification and, instead, opts for an expression cloning strategy. Outline what Harry would do. Be sure to include enough experimental detail so that we know you know what you're talking about.

There are a couple of ways to do this.

You could use the method outlined in figure 19-10 in the text. The basic idea is that you attach ligand to the bottom of a dish or flask and select for cells that attach. You then isolate the plasmid from these cells.

The other method is the FACS analysis discussed in class:

-transfect EpoR- (Epo Receptor -) cells with cDNA library from EpoR+ cells

-expose EpoR- transfected cells to Epo conjugated to FITC (Not radioactivity for FACS)

-use FACS to isolate cells which are bound to fluorescent Epo

-isolate plasmid and transfect into EpoR- cells

-confirm that retransfected cells bind Epo with FACS as above

or

-expose the EpoR- transfected cells to a flask with Epo attached to the bottom of the flask

-isolate adherent cells

-extract plasmids

-retransfect plasmid into EpoR- cells and confirm that they can adhere to a flask coated with Epo

D.) (5 points) Having cloned and sequenced the epo receptor gene, Harry makes a series of point mutated versions of the gene and expresses them in K562 cells. One such mutant converts an arginine residue present on the extracellular side of the receptor molecule to a cysteine. K562 cells transfected with this epo receptor gene spontaneously differentiate in the absence of added epo. Drawing on his knowledge of signalling of other receptor signalling systems, Harry correctly suggests that this effect can be explained by intramolecular disulfide bonding. Explain why Harry makes this suggestion and describe one experiment that would allow him to test it. (You can assume that Harry has made an antibody against the epo receptor by now.)

He suggests this because he knows that in many signaling systems, two receptors dimerize when exposed to ligand to initiate the pathway by causing the phosphorylation of both of them. The intermolecular disulfide bond mimics this ligand induced dimerization.

One way to show this would be to isolate Epo-receptor protein from K562 cells and look on a western blot under conditions where disulfide bonds stay intact (don't add DTT or β -Mercaptoethanol (β ME)). This is nondenaturing SDS - polyacrylamide electrophoresis. Under these conditions, the apparent molecular weight of Epo-R should be twice that seen in wild-type cells.

Many people got most of the credit by suggesting growing cells in the presence of B-ME which would reduce disulfide bonds. This, however, may also affect things besides the epo receptor.

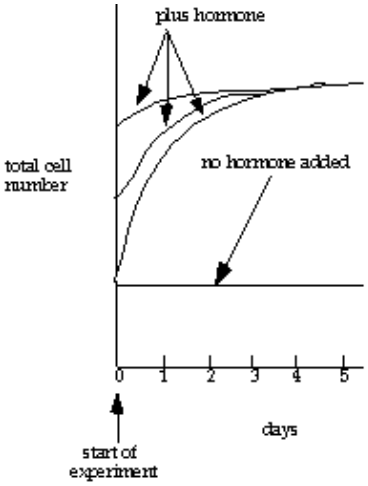
E.) Stimulation of the epo receptor by epo binding is thought to initially activate a non-receptor tyrosine kinase. Subsequently, a tyrosine phosphotase becomes associated with the receptor and becomes activated. A former Olympic champion athelete has a mutation in one of his epo receptor genes in the region required for the binding of this phosphatase. Provide a reasonable explanation as to why this athelete has greater than the normal number of red blood cells in his circulation.

The phosphatase normally inactivates the non-receptor tyrosine (NRTK) kinase by removing the phosphate on it. If the phosphatase can no longer bind it can't remove the phosphate. Thus, the NRTK stays activated and causes the production of more red blood cells than normal.

Question 2 (25 points)

Mitt the MIT undergraduate is studying growth control in a cell line derived from the tongue of a newt. Mitt knows that a newt hormone called notaxin stimulates the proliferation of this tongue cell line.

A.) (3 points) As shown in the graph below, Mitt examines the growth of the newt cells in tissue culture in response to added notaxin. He starts the experiment by plating different numbers of newt cells on tissue culture dishes and then determines the total cell number over the next several days. All of the dishes are fed with tissue culture medium containing the same concentration of notaxin and the medium is replaced every day. Explain why each of the curves reaches the same maximum. What general principle does this observation support?



When (non-transformed) cells growing in culture reach confluence, they stop growing due to cell-cell signaling. This general principle is called contact inhibition.

B.) (3 points) As his sophmore UROP project, Mitt clones the gene encoding the notaxin receptor. Based on the predicted amino acid sequence of the recptor, Mitt is nearly certain that it is coupled to a trimeric G protein. What makes him so sure?

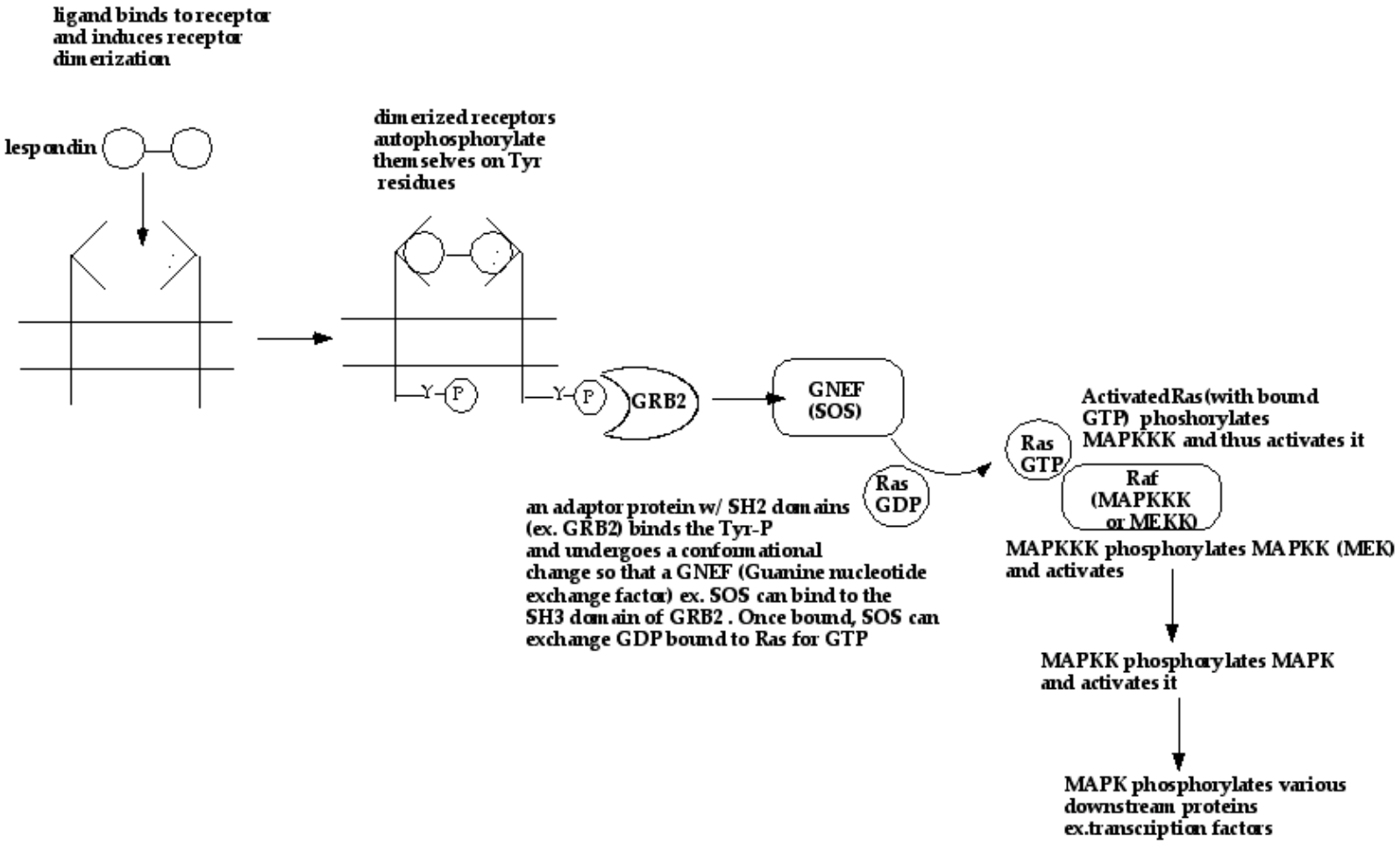
From the a.a. sequence he sees it has seven transmembrane (i.e. highly hydrophobic stretches of ~ 20 a.a's.) sequences suggesting it is a seven-membrane-spanning receptor homologous to the ß-adrenergic receptor.

C.) (5 points) Mitt wishes to understand the signal transduction pathway between the notaxin receptor and the stimulation of cell growth. He immunoprecipitates a number of different protein kinases from untreated and notaxin treated cells and determines their activity. Mitt discovers that protein kinase A activity is greatly increased following notaxin treatment. What second messenger is likely to be produced following notaxin binding to its receptor. Also , Mitt observes that the addition of cholera toxin to the newt cells along with notaxin results in even higher levels of protein kinase A (PKA) activity. Explain. (BE SPECIFIC).

(2 points)Protein kinase A (PKA) is the cAMP - dependent kinase (i.e. directly activated upon binding cAMP) therefore cAMP is the likely second messenger.

(3 points)Cholera toxin binds specifically to the Gsα subunit, locking it in the GTP-bound state. Given our knowledge of the ß-adrenergic pathway, notaxin is binding to its receptor which causes GTP to bind to Gs and the α-GTP bound subunit dissociates and activates adenylate cyclase to produce cAMP from ATP. Thus in the presence of cholera toxin adenylate cyclase is permanently on, leading to even more cAMP production and thus more PKA activity.

D.)(8 points) Mitt further observes that a second newt hormone, lespendin, also stimulates the growth of the tongue cells. Having cloned the lespendin receptor, Mitt notes that it is highly related to the human epidermal growth factor (EGF) receptor. He then shows that binding of lespendin to its receptor results in activation of a MAP kinase. Diagram the likely molecular pathway that links the lespendin receptor to the activation of MAP kinase.



E.) (6 points) Mitt wishes to isolate mutant cell lines which can grow in the absence of added hormone. He first exposes one thousand of the newt cells to the mutagen ethylmitrosourea and then places each mutagenized cell into its own small tissue culture dish. The dishes are supplemented with tissue culture medium without added hormone. 998 of these cells fail to grow, but two cells, A and Z, do form colonies under these conditions. Interestingly, when Mitt removes the growth medium from A cells and places it on unmutagenized newt cells, the unmutagenized cells are now also able to grow in the absence of added hormone. In contrast, placing unmutagenized newt cells in growth medium isolated from Z cells results in no growth.

1. What is the explanation for the serum independent growth of A cells?

2. Describe an experiment to test your suggestion.

3. Name two possible explanations for the serum independent growth of Z cells.

1. The A cells have sustained a mutation which causes them to secrete notaxin, lesperdin, or an agonist of these hormones. This leads to growth. (The main clue is that the media the cells were grown in is then capable of stimulating the growth of unmutagenized newt cells). This is called autocrine signaling.

2. There are several possible answers that were acceptable here:

-to classically determine if an agonist was present one could add swamping amounts of specific notaxin or lesperdin antagonists to the media the A cells are growing in and observe whether growth is now inhibited due to competition for receptor binding sites.

-one can also assay for the presence of either hormone in the media from A cells by immunoprecipitation with antibodies against either. Alternatively one can use affinity chromatography coupling the notaxin or lesperdin receptor to the column and passing over the media from A cells, and then eluting whatever bound - note that it is important in this case to assay whatever binds to the column for its ability to stimulate growth of unmutage cells to show that it is not just nonspecific junk sticking to the column.

3. There are tons of possibilities here. Essentially any mutation that activates growth will give the phenotype. Since we know two pathways that operate to signal growth in the cells the mutations are likely to occur in proteins in these pathways. It is important here to give specific examples.

Ex: Ras permanently bound in GTP form, permanently activated Raf, MAPKK, receptor active in absence of hormone.

Question 3 (20 points)

In the early 1980's analysis of the chromosomes in cancer cells from patients with follicular lymphoma, a type of cancer affecting B cell precursors, revealed a characteristic translocation involving chromosome 14 and 18. As in other translocations present in different types of B cell lymphoma and leukemia, the affected region on chromosome 14 was known to contain the immunoglobulin heavy (IgH) chain gene. The affected region on chromosome 18 was later shown to contain a presumed proto-oncogene called bcl-2.

A.) (5 points) Draw a rough diagram that shows the likely structure of the junction point between chromosome 14 and 18 in this 14;18 translocation. Your diagram should show the relevant portions of the IgH and bcl-2 genes present at the translocation breakpoint. You should also describe in word how this translocation would affect the expression of the bcl-2 gene in the tumor cells. Note: you should not concern yourself with the reciprocal 18;14 chromosomal translocation.

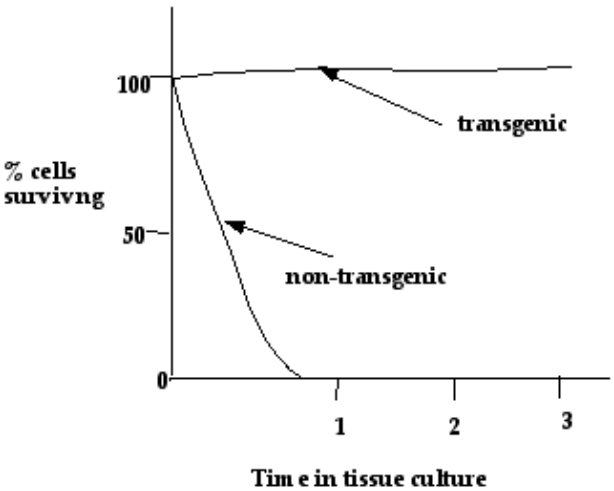
This results in overexpression of bcl-2.

B.) (5 points) Carol the cancer geneticist decides to make an animal model for human follicular lymphoma. She generates a transgenic mouse strain in which the human bcl-2 gene is expressed at high levels in all B cell precursors. After several months of age, these transgenic mice developed a B cell disease very similar to follicular lymphoma. How would you explain why it took several months for this tumor to develop? Also, when these same transgenic mice are given water from the Charles river in place of regular drinking water, they develop lymphoma at a much earlier age. Provide an explanation of this observation as well.

It took more time because you need other mutations ("hits") besides bcl-2 overexpression to cause tumor growth. Note: bcl-2 is not a tumor suppressor gene (it is an oncogene) therefore you do not need to lose the other copy to initiate tumorigenesis.

Charles River water contains many mutagens which accelerates the development of other mutations ("hits").

C.) (5 points) In order to begin to address the effects of bcl-2 overexpression, Carol isolates B cell precursors from transgenic and normal (non-transgenic) mice. When she places these cells in tissue culture in the absence of any added growth factors and follows the fate of the cells over time, Carol makes the obervations that are summarized in the graph shown below. Describe the effects of bcl-2-overexpression in this setting and suggest what biological process the bcl-2 protein might be affecting.

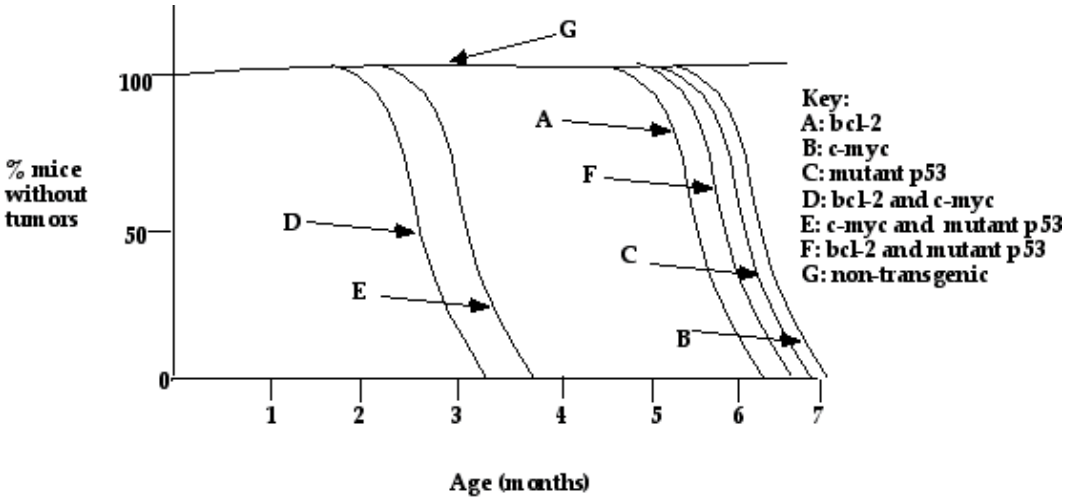


Bcl-2 overexpression is allowing cells which would normally die in culture without growth factors to remain alive. Note: The cells are not growing - only surviving since there are never more cells than when you started the experiment on day 0.

Bcl-2 is probably inhibiting apoptosis or programmed cell death.

D.) (10 points) Carol next creates two additional transgenic strains of mice. Using the same promoter/enhancer region she used previously, Carol recreates animals that over-express the c-myc proto-oncogene and animals that over-express a mutant allele of the p53 tumor suppressor gene that encodes a mutant

p53 protein which can inhibit the function of the normal p53 protein. Through interbreeding of these different strains, she is able to construct animals that carry two of the transgenes. The graph shown below summarizes the rates of tumor formation in animals that carry one or two of the transgenes.



1. Provide an explanation for the difference in the rate of formation of the animals shown in curve D versus curves A and B.

2. Provide an explanation for the difference in the rate of tumor formation of the animals shown in curve E versus curve B and C.

3. Provide an explanation for the similarity in the rate of formation of the animals shown in curves F, A and C.

1. The two mutations act synergistically to affect tumor growth. Myc alters the cell cycle control of cells while bcl-2 overexpression interferes with cell death.

2. Basically the same explanation as in #1. (mutant p53 blocks apoptosis).

3. Since p53 (mutant p53) and bcl-2 affect the same process (apoptosis), altering both of them in a cell does the same thing as affecting one of them. Therefore there is no synergistic or cooperative effect.

Question 4 (25 points)

A.) (6 points) Describe antigen presentation to CD4+ T celss. Your answer should describe what cell types do this, where the presented antigen comes from, and the organelles and molecules that are involved.

CD4+ T cells are T helper cells (for the most part).

(1.5 points)Macrophage and B cells endocytose (by phagocytosis and Ab mediated receptor endocytosis respectively) foreign extracellular particles(1.5 points) which get degraded in the lysosome(1.5 points) where they bind to the MHC Class II protein(1.5 points) and are then presented on the surface as MHC Class II bound peptides to CD4+ T cells.

B.) (6 points) Describe antigen presentation to CD8+ T cells. Your answer should describe what cell types do this, where the presented antigens come from, and the organelles and molecules that are involved.

CD8+ T cells are CTLs (cytotoxic T lynphocytes). Every (most) cells of the body express MHC Class I molecules which bind to peptides in the ER and then present them to CD8+ T cells on the cell surface. The peptides are degraded in the ER and the cytosol from all intracellular proteins (cytosolic peptides are then pumped into the ER).

C.) (6 points) After graduating from MIT, you become a hot-shot physician interested in diseases that appear to be due to immunological disorders. You examine a patient who is frequently ill with infections. A blood exam reveals that the levels of B and T cells in the serum are normal. You decide to begin studying the patient's CD8+ Cytotoxic Lymphocytes (CTLs) in detail. After one visit, when the patient has evidence of infection with the MIT virus, you decide to clone the CTLs that recognoze MIT virus-infected cells, but discover that these CTLs are not very good at killing the infected cells. To examine this in more detail, you decide to clone the cDNA for the α and β chains of the T cell receptor from one of these T cell clones. To your surprise, there is only one

type of cDNA for the α chain, but two different cDNAs for the β chain. Further investigation shows that the cells are producing two different β chain proteins.

Explain why this observation is surprising and provide a possible explanation for this observation. How might this account for the weak killing activity of the CTL clone?

(1 point)What is surprising is that there are two different β chains being expressed. Normally, there is one α and one β TCR being expressed.

(3 points)Allelic Exclusion is the mechanism by which T (+B) cells prevent further rearrangement of - in this case - the other TCR allele, once the first rearrangement yields a functional (productive) rearranged TCR β chain. This mechanism ensures that each cell will express only one type of TCR that is capable of recognizing one unique MHC and antigen peptide combination, thereby ensuring the specificity of each T-cell. Obviously something is defective in the patient's allelic exclusion mechanism.

(2 points)The weak killing could be a result of the expansion of the CTL clone that recognizes two different antigens. One of the two TCRs will be specific for the MIT virus, but will only be present in half the normal amount on the T-cell surface. As we heard in lecture, there is a critical number of TCR-MHC specific antigen interactions necessary to trigger an immune response by the T-cell (we referred to this as epitope density because normally it is the amount of antigen that is limiting) here there may not be enough of the correct TCRβ with the TCR that recognizes the MIT virus to ever trigger a response.

D.) (7 points) You realize that a better understanding of the mechanism that is defective in this pateint (described in C) might send you to Stockholm for the big prize. You decide to find the gene that could complement the defect. You know that mice with Severe Combined Immunodeficiency Disorder (SCID) lack an immune system and that you can establish a functional human immune system in SCID mice by transplanting human bone marrow cells into them (these transplanted bone marrow cells contain the precursors for all the important cells of the immune system). You transplant bone marrow cells from your patient into one set of SCID mice and bone marrow cells from a patient without the defect (control patient) into another set of SCID mice. You find that SCID mice that had received bone marrow transplants from the patient described in part C died after exposure to the MIT virus whereas the SCID mice that had recieved the bone marrow from the control patient survived infection with the MIT virus.

Describe how you could identify the gene that would improve the function of your patient's (described in part C) CTLs in the mouse model. How would you confirm that you had isolated the correct gene?

You can use a complementation approach using the patient's bone marrow cells (there are several different ways this can be done - I will only outline one approach).

You need to transfect the affected patients bone marrow cells with either a genomic or pre-T cell cDNA library from the normal individual. There is an additional issue here: you need either retrovirally transfect or somehow otherwise flank your insert sequences in your library because you need the library plasmids to integrate into the bone marrow genome so that you can ensure the introduced cDNA will be stably inherited as the bone marrow cells grow and differentiate to T-cells and then you need to be able to isolate the inserted sequence from a CTL clone, so the DNA must be flanked with some sequences you can PCR from.

Next place the patients bone marrow cells transfected with the normal individuals cDNA library into SCID mice and expose the mice to the MIT virus. Look for mice that can now survive the virus.

Since these mice can now survive they must have acquired a gene which corrects the allelic exclusion - since the CTL against the MIT virus will be clonally expanded, isolate CD8 + cells (CTLs) and clone the portion of DNA flanked by the sequences chosen to mark the integrated cDNA.

To confirm you have isolated the correct gene, after cloning it from the bone marrow cells, go back and isolate and clone this gene from the patient's DNA and check to see if there is a mutation which would result in a loss of that gene's function.