Question 1. You are interested in understanding the residues of a specific group I intron that function specifically in catalysis of the splicing reaction. To probe this question, you mutagenize the intron and, using an *in vivo* screen, isolate mutant variants that are splicing-defective.

Because group I introns contain many regions of secondary structure that are important for folding of the RNA but probably are not directly involved in catalysis, you decide to initially examine your mutant RNAs for those defective in folding. These mutants will be set aside, as they are unlikely to have specific defects in the catalytic steps.

1a (6 points). You find that it is relatively easy to isolate the unprocessed RNA from your mutant cell lines. With this in mind, suggest a type of analysis you could use to probe for mutants that have defects in RNA folding. Explain what controls you will use, and how you will decide if the mutant is a folding defect.

1b (7 points). After setting aside the folding mutants, you wish to further screen your mutants for those that are good candidates for having specific catalytic defects. You decide it will be easier to look for mutants that have defects in the second splicing step. Your idea is to look for mutant RNAs that accumulate the intermediates expected of an RNA that can catalyze the first step successfully, but show a complete defect in the second step.

Given the schematic of the full length pre-RNA shown below, draw the structure of the RNAs you would expect to see after an *in vitro* splicing reaction for a mutant RNA that is defective in the second step.

```
   900  ntds              3800  ntds          1200  ntds
  |―――――――|―|――――――――――――――――――――――――――|―|――――――――|

5'―| Exon 1 |―――――――――――――――――――――――――| Exon 2 |―
```
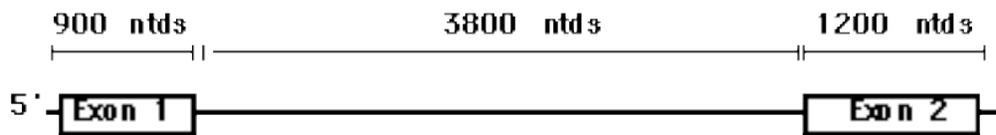
Also, in addition to your purified pre-mRNA, what components would you add to the *in vitro* splicing reaction?

1c (7 points). Using the strategy outlined above, you successfully identify several mutant RNAs that are specifically defective in carrying out the second splicing reaction. You are most interested in mutants that identify residues that are truly part of the active site that catalyzes this reaction. Therefore, you next want to determine if the mutant RNAs are or are not defective in the tertiary structure rearrangement that is necessary prior to the second splicing step, as mutants defective in this rearrangement reaction are less interesting to you.

Suggest a genetic or biochemical method that will allow you to screen out mutants that are defective in the rearrangement . A general description of the method, without experimental details is sufficient.

Using this method, what result would you expect to see for the mutants that are truly defective in catalysis?

What result would you expect for the rearrangement-defective mutants?

Question 2. You are studying the pathogenic fungus *C. albicans*, and when you sequence one of its mitochondrial genes you find that it has a novel intron. Analysis of its sequence suggests that it may be related to the group II self-splicing introns. Your first step in studying the intron's structure is to mutagenize it.

2A. You identify a series of mutants which can no longer splice. Shown below is the sequence of the region of the intron (NOT the whole intron) containing these mutations.

```
5'-...AUAGGGAUGCAAAAGCAUCCCAAAGCUACCGCUUACGCUUUUGCGUAAGCGGAAUUUAA...-3'
         ↑    ↑        ↑             ↑         ↑       ↑
         U    G        G⬚            A         A       U⬚
        Mut  Mut      Mut           Mut       Mut     Mut
         A    B        C             D         E       F
```
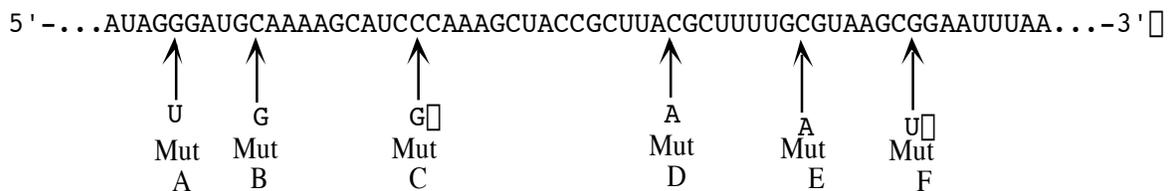
What is a possible explanation for the failure to splice in these mutant strains?

2B. You decide to further analyse these mutant introns by treating fragments containing this region with a set of RNA nucleases.   You use an RNA fragment corresponding to the intron sequence shown above, labelled at its 5'end, as a substrate.   You visualize the digested products by autoradiography. The smallest piece you can distinguish on the gel is 5 bases in length, based on control size standards.

Wild type   Mut. A   Mut. D

| S1 | Snake Venom | S1 | Snake Venom | S1 | Snake Venom |

60 bp

Region I →
Region II →
Region III →
Region IV →
Region V →
Region VI →
Region VII →
Region VIII →

5 bp
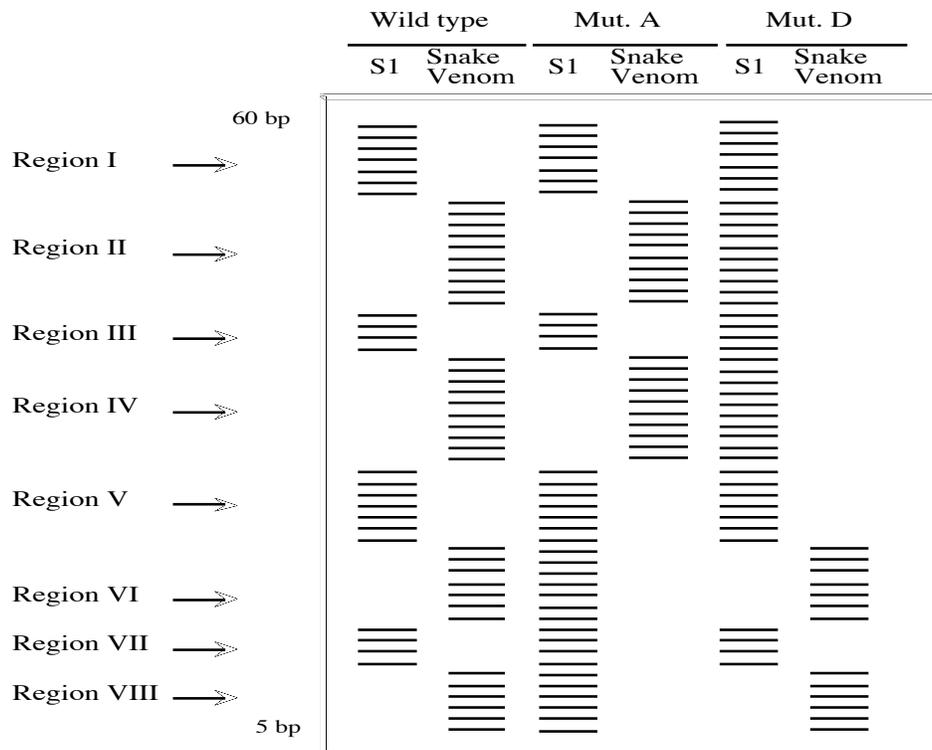
You find that Mutants B and C give similar patterns to Mutant A, while Mutants E and F give similar patterns to Mutant D. From these data, draw the following:

    i. The secondary structure of the wild type intron

    ii. The secondary strucuture of the Mut. A-like mutant introns

    iii. The secondary structure of the Mut. D-like mutant introns

Indicate on each drawing regions I-VIII. Also indicate on the wild type structure where the individual muations A-F map.

i.

ii.

iii.

2C. You decide to generate new mutations which suppress the mutant phenotypes and restore normal splicing. You isolate two different suppressors, identified as Sup1 and Sup2. Sup1 specifically suppresses only Mut A, and Sup2 specifically suppresses only Mut E. Each suppressor turns out to map to a location within the intron, but neither is a revertant. Why are these suppressing mutations so specific? Can you guess what the specific mutations might be, and where they might be located?

2D. If you repeated the nuclease protection experiments with both S1 nuclease and snake venom shown above using similarly labeled intron fragments from the two suppressed strains (one of which carries the Sup1 and Mut A mutations, while the other carries the Sup2 and Mut E mutations), what would you predict the digestion patterns to be? Explain your reasoning.

Question 3. You are interested in studying eye development in mice and your lab has recently discovered a gene, BLG1, that causes mice to develop abnormally large eyes. You have a suspicion that BLG1 might be involved in tissue proliferation in other developmental pathways so you decide to purify BLG1 from a variety of different developing mouse tissues. You separate these proteins on a SDS polyacrylamide gel and then do a western blot with an primary antibody to BLG1. You use a radioactive secondary antibody (this will recognize the primary antibody) and expose your gel to film. You see the following results on film:
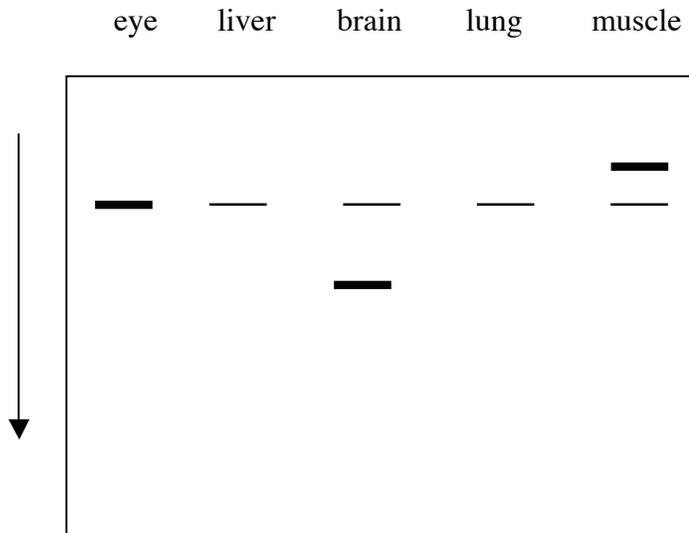
3A. In what tissues is BLG1 most likely to be playing an important developmental role? Why?

3B. Give two possible explanations that could account for the difference in the mobility of the BLG1 protein purified from **brain** tissues. (Hint: Don't worry about degradation products.)

3C. You decide to test your hypotheses by comparing the cDNA prepared from brain tissue and eye tissue. You have been given cell lysates from both tissues that contain all of the DNA, RNA, and protein these two tissues. Describe in detail how you would isolate the BLG1 cDNA from these two lysates.

Question 4. You are studying the molecular basis of a neuromuscular disease of genetic origin. The major symptom of the disease is droopy eyelids, causing students to have difficulty studying for exams. You find that a specific muscle protein is poorly expressed in the eyelid cells of students suffering from the disease. You name this protein pSNOZ and the corresponding gene Snoz1.

4A. You wish to determine why pSNOZ is poorly expressed in the eyelid cells of affected individuals. Assuming you have a cDNA clone of the wild-type Snoz1 gene and RNA samples isolated from the nuclear and cytoplasmic extracts of both wild-type and mutant cells, how would you determine if problem with expression is due to a defect in: (1) transcription; (2) RNA processing or (3) translation?

Specifically describe the results you would expect and draw a schematic of the predicted data from your experiment for the following three possible explanations:

(1) the gene is poorly transcribed;

(2) there is a defect in mRNA processing; and

(3) there is a defect in translation of the Snoz1 mRNA.

4B. You determine that the problem in pSNOZ expression is in some stage of RNA processing; only a low level of the mature mRNA is generated in the mutant cells. To analyze the nature of this splicing defect you decide to characterize splicesome assembly on the mutant Snoz1 pre-mRNA (you have determined that the gene has only two exons). To your surprise, you find that all of the snRNPs (U1, U2, U4/U6 and U5) and U2AF interact with the mutant pre-mRNA at least as well as they do with the pre-mRNA from the wild-type gene. The assembled splicesomes, however, never generate active complexes or catalyze even the first step of splicing.

Based on these results, in which of the splicing signals on the pre-mRNA (5' splice site, branch site, polypyrimidine track, or 3' splice site) would you expect the mutation

preventing splicing to lie? Justify your answer by suggesting a molecular explanation for the splicing defect.

4C. Your lab has identified a drug that treats the SNOZ disease. You find that splicing of pre-mRNA is partially restored in cells from these treated individuals. What type of splicing factor might, if highly expressed, generate this recovered phenotype? Briefly justify your answer.

4D. Design a simple *in vitro* assay that could be used to purify such a factor from the recovered cells.

Question 5. You are working in a lab that studies differences in maternal and zygotic control of the fly embryo. In many multicellular organisms, mothers provide all of the proteins and RNAs required for the new organism to go through early embryonic development. Later in development, the zygote/ new organism begins producing its own RNAs and proteins while the maternal ones are degraded. You are studying a gene called *sum1*. Its predicted structure is shown below. The lines represent intronic or untranslated sequences, and the boxes represent exonic sequences. The nucleotide length is written below each segment.

5'   300   200   500   125   550   150   400   220   800   400   300   3'
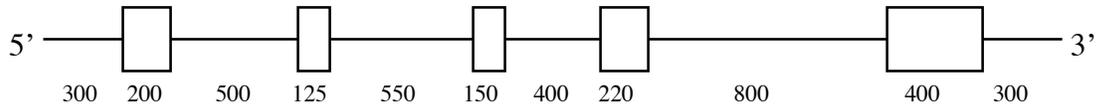
To look at expression of this gene, you decide to do a Northern blot with cytoplasmic RNA from different tissues. You use a probe that will detect exon 1.
The key indicates the origin of RNA in each lane.

1 = oocyte
2 = early embryo
3 = brain
4 = eye
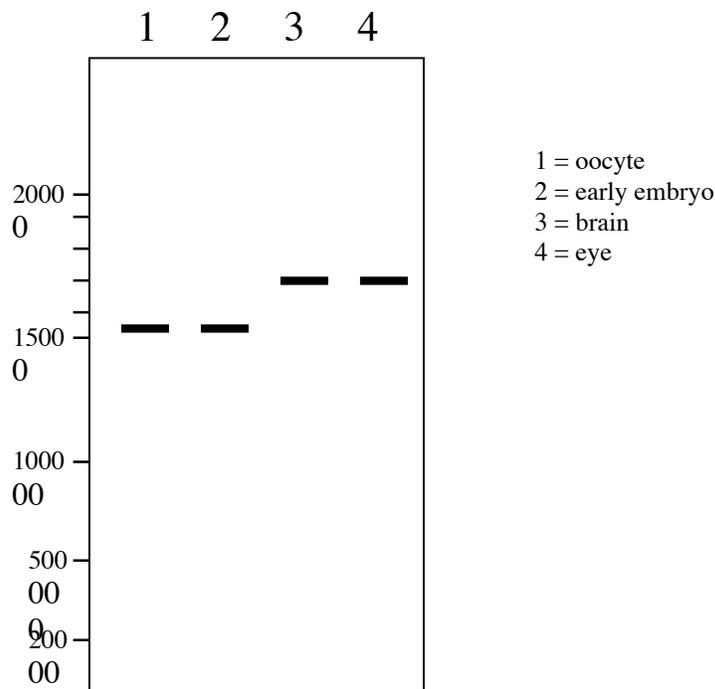
5A. Draw your predictions of the RNA structure for each lane.

5B. You decide to use RT-PCR to confirm your predictions about the exon organization of the 2 mRNAs made in part A. Describe the experiment you would do and what results you would expect.

Luckily, your labmate has made an antibody to the protein encoded by *sum1*. You do a Western blot on the same tissues you used in the Northern blot and find that the protein in the brain and the eye tissue is about half the size of the protein made in the oocyte and early embryo.

5C. Explain this result considering the relative mRNA sizes in these tissues.

Next, you look through the useful database of fly mutant collections and find 2 mutants that exist in sum1, m1 and m2. You order these mutant stocks and isolate RNA from oocytes and the brain. You run a Northern blot similar to the one shown earlier in the problem.
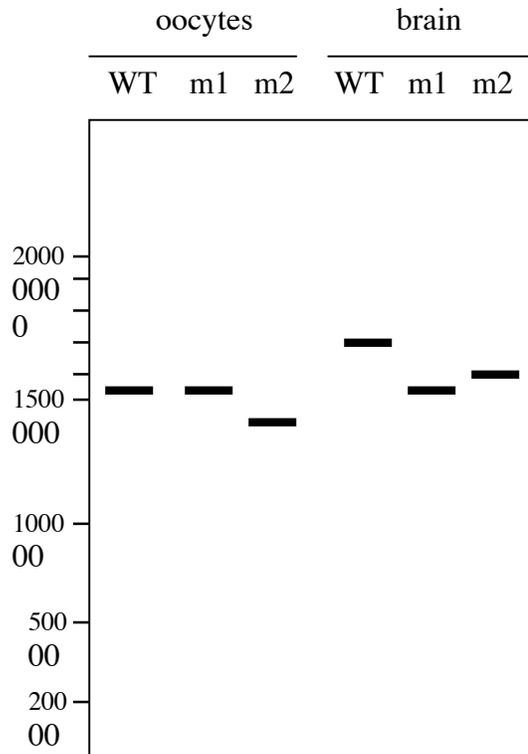
5D. Draw the altered mRNAs and hypothesize the location of the mutations in m1 and m2.

You do a screen to look for suppressors of the m1 mutation that restore WT function of *sum1* in the brain. You find a suppressor, m3, which restores the mRNA in m1 back to the WT size. You identify the gene disrupted by m3, and do a Northern blot to determine its expression pattern. You find that this gene is only expressed in zygotic tissues.

5E. What is this gene likely to encode? Propose a model for how the m3 mutation suppresses the splicing defect seen in m1.

Question 6. After graduating, you decide to work in a lab at a large Agrotech firm for a summer. You are surprised to learn that production of the amino acid Lysine is a major effort at the company. Indeed, your advisor assures you that even a 1% increase in the amount of Lysine produced by a production bacterium is worth ~$10,000,000 a year for the company.

Your advisor has identified a new bacterium, *K. expressus*, that holds promise as a high Lysine expressor. You are given the task of determining the regulation of lysine biosynthesis operon in these bacteria.

You first set out to determine how addition of amino acids to the growth media effects expression of the operon. You perform both a northern blot of the Lys operon mRNA and a western blot using an antibodies to two different proteins (LysA and LysC) expressed from the mRNA. You obtain the following results:

Amino Acids + −   + −   + −

Lys Operon
Northern Blot   LysA Western Blot   LysC Western Blot
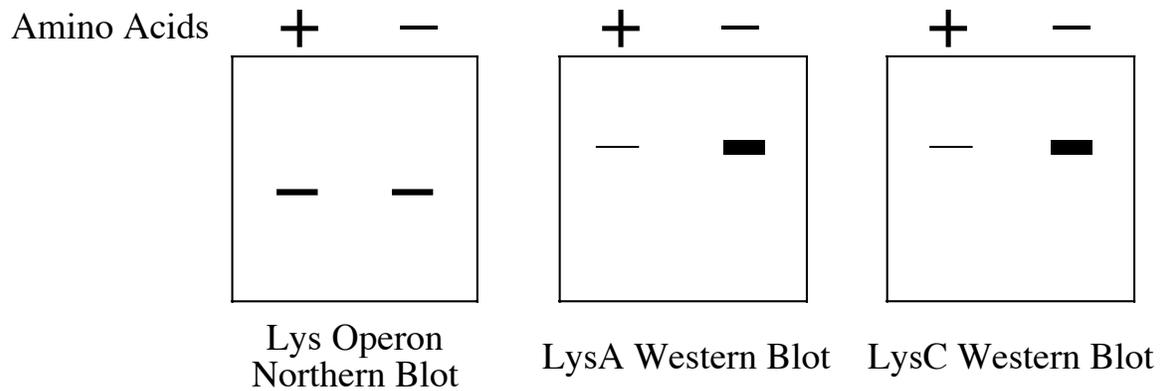
6A Based on these observations what can you conclude about how the expression of the *K. expressus* lysine biosynthetic genes are regulated?

You next determine the sequence of the entire expressed mRNA and find that there is a short open reading frame upstream of the three Lys biosynthetic genes. The structure of the mRNA and the sequence of the short open reading frame are shown below.


```
AUG GCU AAA AAG AAA AUU CUG GCC GAU UUU AGU CGU UGA GCAACAGGAGG
Met Ala Lys Lys Lys Ile Leu Ala Asp Phe Ser Arg Stop
```
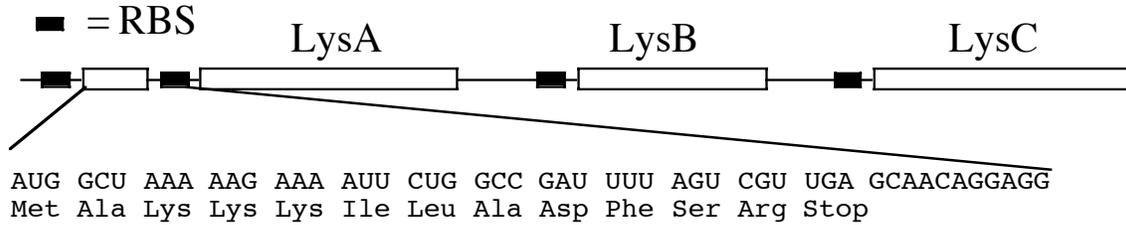
6B. You are initially excited that the short open reading frame could act as a transcriptional attenuator, however, you rapidly discard this notion based on the data above. What data allows you to exclude this hypothesis? Why?

The presence of three Lys codons in the leader peptide still seems suspicious. Although there are no potential hairpins adjacent to the short open reading frames, you notice that the RBS of LysA is unusually close to the stop codon of the leader peptide.

To address whether the leader peptide functions in the regulation of the Lys genes, you construct a hybrid operon in which the LacZ open reading frame (ORF) is precisely substituted for the LysA ORF (see image below). You then make three mutations in the leader peptide coding region. Finally, you re-introduce your constructs into *K. expressus* and assay the LacZ hybrids for expression (using a β-galactocidase assay) with and without amino acids in the growth media.

β-Galactosidase
Activity Units
Amino Acids

	+	−

■ = RBS   LacZ

Sequence	+	−
AUG GCU AAA AAG AAA AUU CUG GCC GAU UUU AGU CGU UGA Met Ala Lys Lys Lys Ile Leu Ala Asp Phe Ser Arg Stop	20	600
UUG GCU AAA AAG AAA AUU CUG GCC GAU UUU AGU CGU UGA Leu Ala Lys Lys Lys Ile Leu Ala Asp Phe Ser Arg Stop	600	600
AUG GCU GAA GUC AAC AUU CUG GCC GAU UUU AGU CGU UGA Met Ala Glu Val Asn Ile Leu Ala Asp Phe Ser Arg Stop	20	25
AUG GCU GAA GUC AAC AUU CUG GCC GAU AAA AAG AAA UGA Met Ala Glu Val Asn Ile Leu Ala Asp Lys Lys Lys Stop	25	25

6C. Based on the results of your β-Gal assays and your knowledge of translational regulation, propose a model that explains your results.

Although your advisor likes your model, she feels that additional mRNA mutations outside of the leader peptide coding sequence will be necessary to test your hypothesis.

6D. What type of mRNA mutation do you propose to construct to address the coupling between the leader peptide and LacZ/LysA expression? What results would you expect if your model is correct? (hint: you can make insertions, deletions, or substitution mutants)

As a further test of your hypothesis you mutate the stop codon of the leader peptide from UGA to UAA. You find that this increases the level of β-Gal expression in the presence of amino acids by two-fold (from 20-25 to 50-60 units).

6E. How does your hypothesis explain this observation? What effect on LacZ expression would you expect if you over-expressed RF-1 in cells with the *unmutated* LacZ-fusion construct? RF-2?

Your advisor is happy with the results of your experiments and it is clear that you understand the regulation of LysA by the leader peptide.

6F. Can your observations and model concerning the function of the leader peptide directly explain the co-regulation of LysC? Why or why not?

Question 7: You are a graduate student in a lab studying the human protein YCG1. From a previous graduate student's work, you know that there are different levels of YCG1 in different tissues and you are interested in understanding the mechanisms determining the levels of YCG1 in these different tissues. You decide to start by comparing levels of YCG1 mRNA and protein in liver cells and kidney cells.

7A. How would you go about purifying RNA PolII transcripts from each cell type (both pre-mRNA and mRNA) and what assay would you use to look at YCG1 mRNA levels?

7B. How would you determine levels of YCG1 protein in the different cells?

Curiously, you find that the levels of YCG1 pre-mRNA and mRNA are the same in the two tissues, but that there is eight times more YCG1 protein in kidney cells than in liver cells. You also notice that the mRNA from the kidney cells is longer than in the liver cells but that the pre-mRNA is the same length.

7C. You suspect that the difference in mRNA length is due to alternative splicing. What experiment would you do to test this hypothesis?

You determine that the difference in mRNA length is not due to alternative splicing. Your next hypothesis is that the YCG1 mRNA poly-A tails are different lengths in the two cell types.

    7D. How could the difference in poly-A tail length lead to different levels of protein in the two tissues?

    7E. You would like to test your hypothesis. Based on your knowledge of the role of the poly-A tail in translation, what protein would you propose to delete to test your hypothesis?

Question 8. You are studying the regulation of the gene encoding release factor 3 (RF-3) from a recently isolated single cell eukaryote. You first assess the abundance of the RNA encoding the RF-3 as well as the RF-3 protein. You obtain the following results.

Amino Acids   Amino Acids

   +   **−**   **+**   **−**

(the + and − indicate the amount of amino acids in the growth media)?

Northern Blot   Western Blot
                                 (anti-RF-3
                                 antibody)
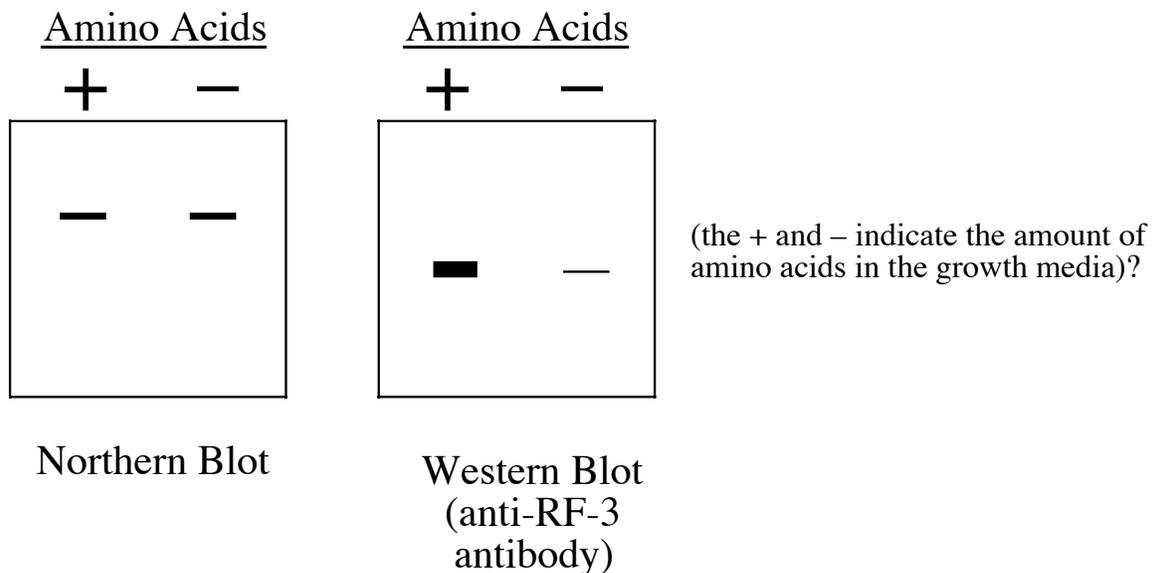
    8A. At what stage is RF-3 synthesis regulated by amino acids?

To gain insights into the mechanism of control you want to isolate mutants that alter the control of RF-3 expression. **You only have the sequence of the first 75 bases of the mRNA available to you but the first AUG is present in this region.**

You make two fusions to LacZ. One precisely places the AUG of LacZ in the same place as the first AUG of the RF-3 gene. The second uses a convenient restriction site located 250 bases into the mRNA (see below) but you cannot tell whether it is in the same reading frame as the first AUG or not.

You test each fusion for beta-galactosidase activity with high and low amounts of amino acids present in the growth media. You obtain the following results.

	β-Galactosidase Activity Units	
	Amino Acids	
	+	−
AUG ... UAA LacZ (50 bp)	600	600
AUG AUG ... UAA RF-3 LacZ (250 bp)	300	10

8B. What can you conclude about the mechanism of control of RF-3 expression based on these results?

You use the 2nd construct shown above to screen for mutants that are mis-regulated. You have some difficulty but eventually identify 3 mutations that alter RF-3 expression and call them RRT1, RRT2, and RRT3. They have the following characteristics:

RRT1 cis, constituitive
RRT2 trans, uninducible
RRT3 cis, uninducible

Having identified two cis-acting mutants you decide to sequence the rest of the WT and mutant mRNAs. All instances of either start (AUG) or in frame stop codons are shown.

UAG

AUG | AUG   UAA
Wild Type ◆ — [ ] | RF-3 | LacZ ████████████ —

UAG

A**C**G | AUG   UAA
RRT1 ◆ — [ ] | RF-3 | LacZ ████████████ —

GGA

AUG | AUG   UAA
RRT3 ◆ — [ ] | RF-3 | LacZ ████████████ —
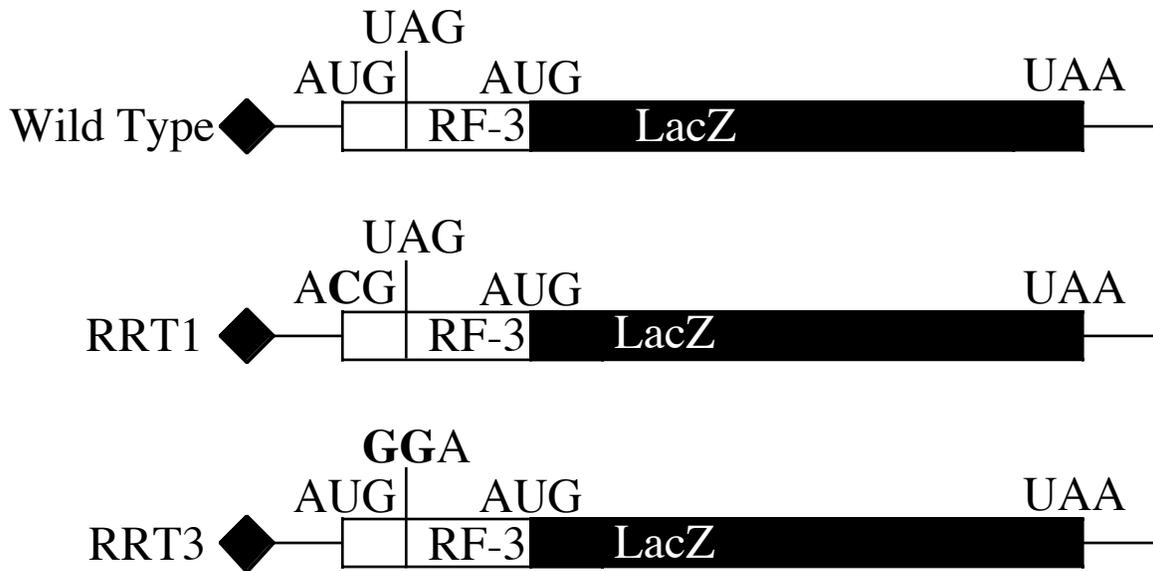
8C. Using the above information, explain the phenotypes of the RRT1 mutation. Propose a model to explain the RRT3 mutation.

To be sure that the phenotypes that you observe are relevant to the normal regulation of RF-3 expression, you make the same mutations in the normal RF-3 gene (without the LacZ fusion) and find the following results.

Western Blot (anti-RF-3 antibody)
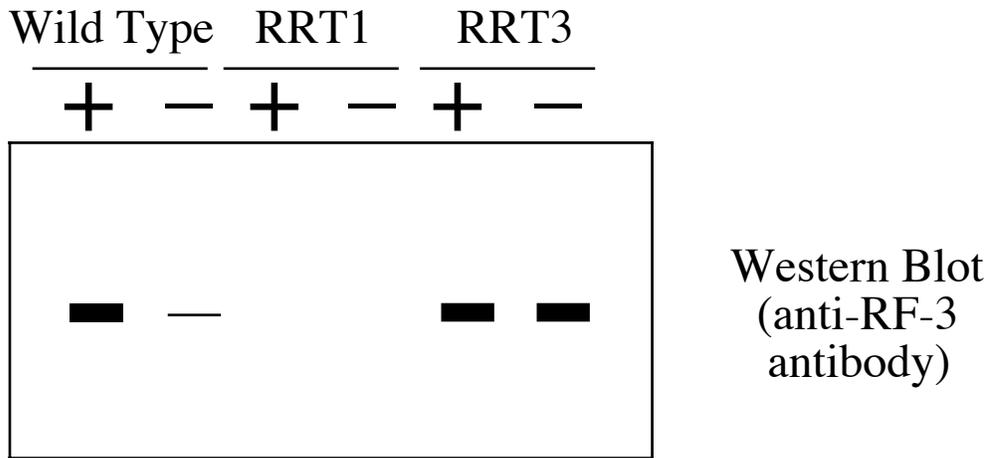
You also sequence the entire wild type mRNA and find that there is only one AUG in the entire coding region (all AUGs and Stop codons are shown below).
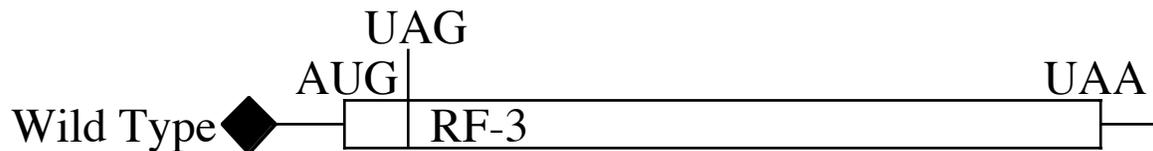
Finally, you also clone the gene that is mutated in RRT2. You find that it encodes a Tyr-tRNA that is normally expressed at low levels. Interestingly, the anti-codon of the tRNA recognizes the UAG stop codon.

8D. Given that reduced amino acid levels result in substantial reductions in the level of translation (and therefore an increase in the amount of available RF-3), propose a model to explain the regulation of the expression of the RF-3 protein.

8E. How does your model explain the different effects of the RRT1 and RRT3 mutants on protein expression in the normal RF-3 gene compared to the LacZ fusion situation?

8F. Based on your model, what would you expect would be the consequence of deleting the RRT2?

Question 9

You are working for a Pharmaceutical company in an effort to identify new antibiotics against tuberculosis bacterium. You have recently identified a new antibiotic derived from soil bacteria you isolated in the Amazon rain forest that is very effective against tuberculosis. Thinking like the pharmaceutical executive you hope to one day be, you name the antibiotic CoughNot. Before starting clinical trials you need to determine the target of the antibiotic.

9A. Describe the experiments you would use to determine if the antibiotic inhibited DNA replication, transcription, or translation.

DNA Replication:

Translation:

        9B. After outlining your experiments, you send your technicians to perform each assay. They assay translation first and find that it is inhibited by CoughNot treatment. They want to proceed to more detailed assays for the exact target in translation, but you explain that they must continue with the replication and transcription assays. Briefly explain why this is necessary?

After completing the other assays, your technicians have convinced you that translation is the target of CoughNot. You next want to determine what step in the translation process is inhibited by this antibiotic. To this end you add CoughNot to *in vitro* translation assays that include radioactive ^{35}S-Methionine to detect new translation. You obtain the following results.
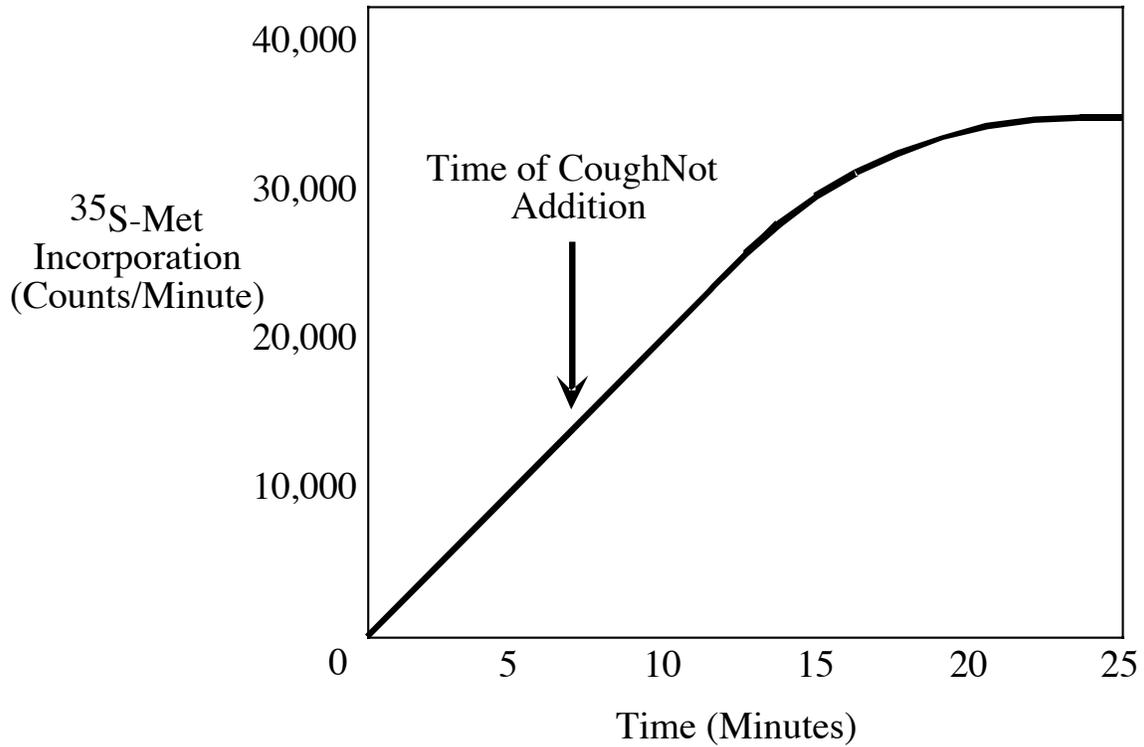
9C. Based on this graph, what conclusions can you make about the step(s) in translation that could be inhibited by CoughNot. You should assume that the antibiotic inhibits it target within seconds. Explain how the graph supports your conclusions.

To further distinguish between the different possible steps of translation that could be inhibited by CoughNot, you perform a different translation reaction. In this case you use radiolabeled ^{35}S-Methionine and you add the CoughNot **before** you start the *in vitro* translation reaction by the addition of an mRNA encoding a 50 kd protein.

After several different time intervals, you **isolate the ribosomes and any associated proteins**. You separate the resulting proteins on an SDS-polyacrylamide gel and expose the resulting gel to X-ray film. You obtain the following results.

Time (seconds)	10		20		30		40		60	
CoughNot	–	+☐	–	+☐	–	+☐	–	+☐	–	+
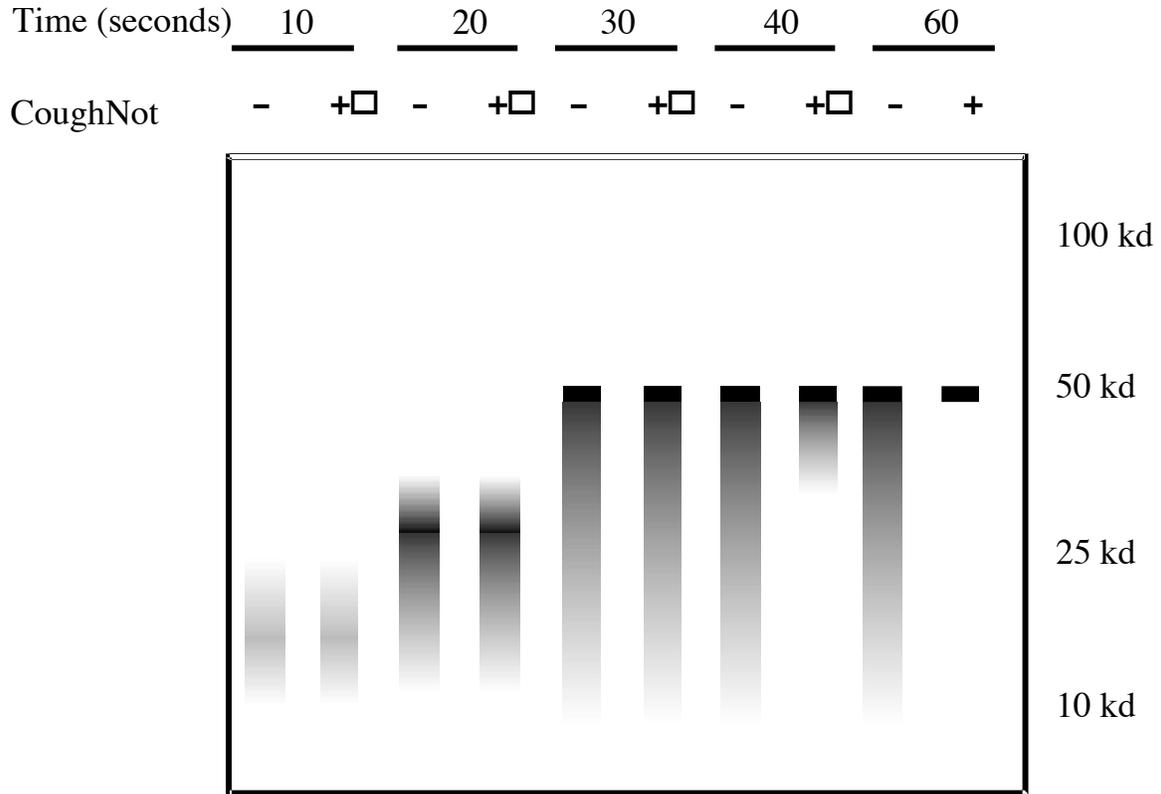
9D. How does this data change your hypothesis for the step in the translation reaction that is inhibited? Propose a model for CoughNot action based on these data.

9E. You want to determine if there are any accessory factors associated with the Ribosome after addition of CoughNot. Based on your model above, describe what accessory factor(s) you would test for and how you would monitor for their association with the ribosome.

Question 10 You are interested in developing technology to incorporate a chemically-modified Serine into proteins at a defined position. Your approach will be to use an *in vitro* translation extract and program the extract with a single mRNA for the protein you want to modify.

10A. The first important decision that you need to make to accomplish your goal is to decide what codon you want the tRNA coupled to the modified Serine to recognize. You want to ensure that the modified Serine is incorporated into all of the protein synthesized in the *in vitro* reaction. What codon would you choose so that you could accomplish this goal? Provide two reasons supporting your choice of codon.

10B. Your next step is to modify the anticodon of one of the Serine tRNAs to recognize the codon that you have chosen. Given your choice of codon, what anticodon would you choose? Keep in mind that you want to recognize a single codon.

10C. Once you have decided on the codon choice, and modified the anticodon of the tRNA, you now have to determine how to attach the modified Serine to the tRNA. Given that you have changed the tRNA anti-codon and want to couple a modified Serine to the tRNA, what domains of the appropriate Serine amino-acyl tRNA synthetase might you have to modify to allow charging of your modified tRNA with the chemically-modified Serine?

You are worried about whether you can change the amino-acyl tRNA synthetase appropriately to allow for charging with the modified Serine.   For this reason, you want to investigate a second method for generating a tRNA charged with the modified Serine.

10D. Describe an alternative method you could use to generate a tRNA coupled to the modified Serine.

Using the crystal structure of the tRNA bound to its cognate amino-acyl tRNA synthetase, you are able to make several key mutations that allow the amino-acyl tRNA synthetase to bind to the modified tRNA with high specificity and charge it with the modified Serine. Excited, you want to test the functionality of your modified Serine charged tRNA in an *in vitro* translation extract.

10E. To test your modified Serine charged tRNA *in vitro* you need to program the *in vitro* translation extract with the appropriate mRNA. Describe the mRNA you would use, including any changes you would need to make to ensure that the modified Serine is only incorporated at a single position.

 10F. You are disappointed to find no evidence of incorporation of the modified Serine into your target protein. Give two explanations for the lack of incorporation based on your knowledge of translation. Briefly describe how you could experimentally determine if these explanations are responsible for the lack of incorporation.