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.

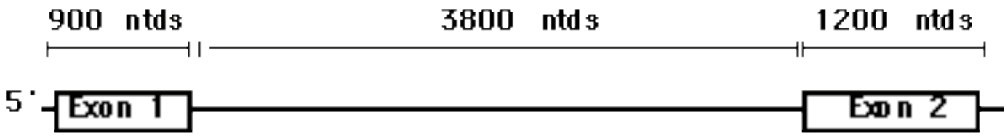
Because group I introns contain many regions of secondary structure that are important for folding of the RNA but probably are not directly involved in catalysis, you decide to initially examine your mutant RNAs for those defective in folding. These mutants will be set aside, as they are unlikely to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

1a 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.

You can identify mutants in RNA folding by isolating the unprocessed RNA from wt and each of your mutants (1 pt), end-labeling it, digesting each with either S1 nuclease or snake venom RNAse individually (1 pt), and running the products out on a denaturing gel (1 pt). S1 nuclease will digest ssRNA and SV will digest dsRNA (1 pt). You will compare the banding pattern in each of the mutants to the wild type pattern. Any 'footprints' that differ from the wt will indicate a mutant in RNA folding (2 pts).

1b 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.



(3 pts)
5'----Exon1----3'OH AND G-O-P-O----------Exon2-----3'

Also, in addition to your purified pre-mRNA, what components would you add to the *in vitro* splicing reaction?

1. (2 pts) Guanine nucleoside or nucleotide
2. (1 pts) Divalent cations (e.g. Mg^{++} or Mn^{++})
3. (1 pts) Monovalent cations (e.g. Na^{+} or K^{+})

1c 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.

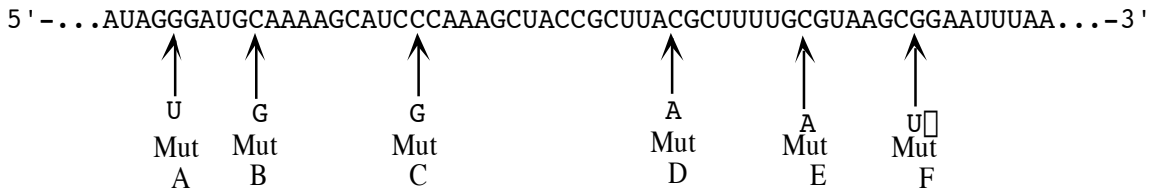
Using this method, what result would you expect to see for the mutants that are truly defective in catalysis?

What result would expect for the rearrangement-defective mutants?

You would like to eliminate mutants that are defective in tertiary structure formation. Normally, regions P1 and P7 come together in step 1 and regions P1 and P9 come together in step 2 of group I intron splicing (2pts). These interactions can be tested by using a crosslinking reagent (3 pts). Since you would like to identify mutants in the active site of the RNA, you will study mutants that crosslink similarly to the wild type RNA, but fail to catalyze the second step (2pts).

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.

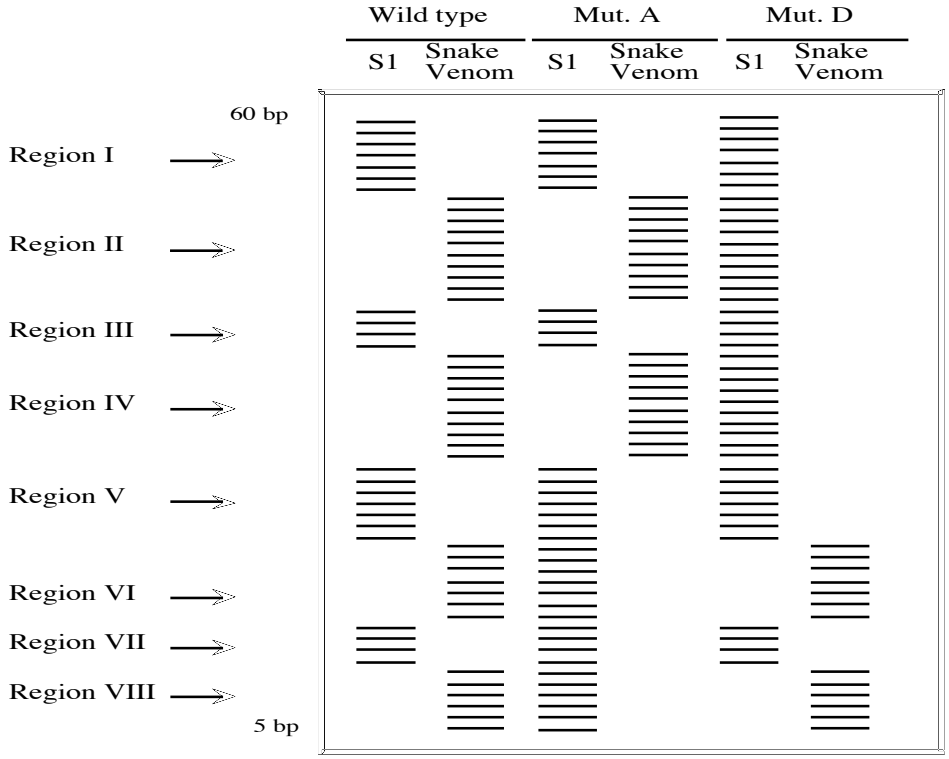
2A. You identify a series of mutants which can no longer splice. Shown below is the sequence of the region of the intron (NOT the whole intron) containing these mutations.



What is a possible explanation for the failure to splice in these mutant strains?

The mutations in the mutant strains may disrupt the secondary structure elements necessary for proper folding of the intron into an active catalytic structure. Inspection of the sequence above reveals several potential stem-loop structures in which the mutations are located.

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.



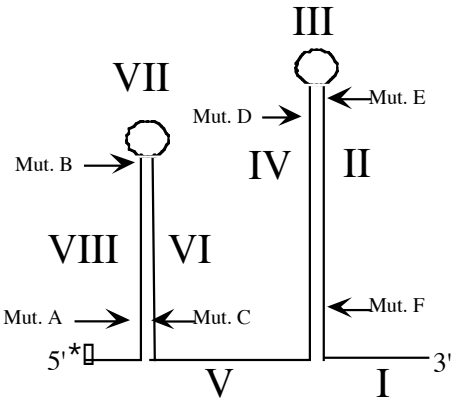
You find that Mutants B and C give similar patterns to Mutant A, while Mutants E and F give similar patterns to Mutant D. From these data, draw the following:

i. The secondary structure of the wild type intron

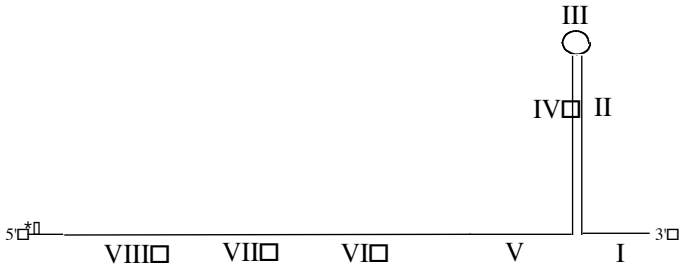
ii. The secondary strucuture of the Mut. A-like mutant introns

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.

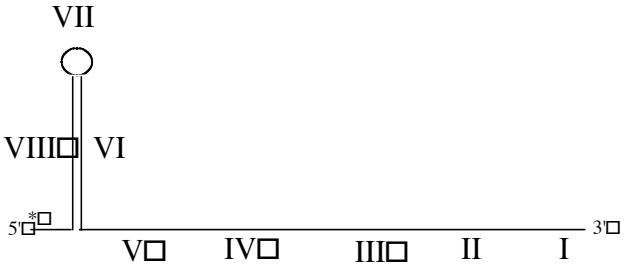
i.



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?

The suppressing mutations are specific because they are individual base changes complementary to the original mutations and restore Watson-Crick base pairing between the two bases in question. This allows reformation of the original secondary structure, although with a different sequence. This is known as "covariation".

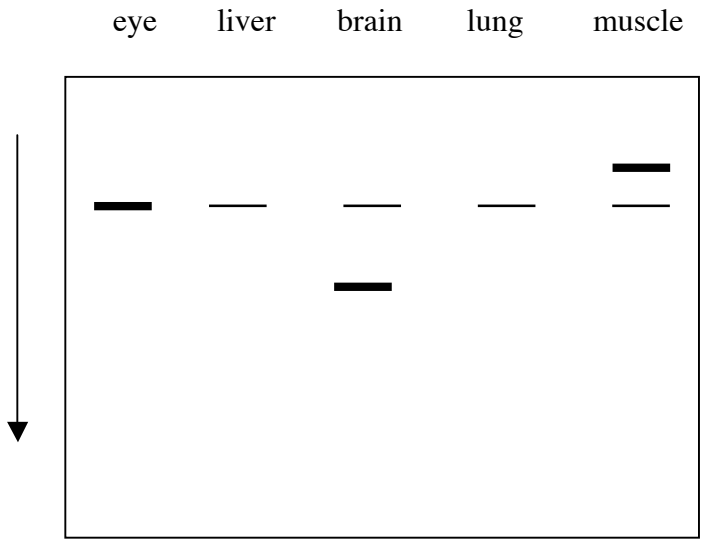
MutA has a U instead of a G at the second position in region VIII (in the first stem structure). Sup1 must therefore have an A rather than a C at the second-to-last position in region VI.

Mut E changes a C to an A at the second position of region II (in the second stem). Sup2 is therefore likely a change from a G to a U at the second-to-last position in region IV.

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.

The two suppressed strains are likely to contain introns which generate digestion patterns with S1 and snake venom nucleases that are identical to the wild-type digestion patterns, since wt intron splicing activity has also been restored.

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?

BLG1 seems to be upregulated in eye, brain and muscle tissues suggesting that the BLG1 protein may be important in these tissues.

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.)

MODEL 1:

You would expect to see a faster migrating BLG1 band if the protein was proteolytically processed to give a smaller protein species.

MODEL 2:

BLG1 mRNA could be spliced in alternative ways in different tissues. In brain tissues BLG1 may be spliced in such a way that not all of the exons are included in the processed mRNA. This would lead to a smaller protein product.

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.

1) **Isolate the mRNA from the cell lysates. (One possibility would be to use a oligo dT affinity column i.e. little beads with $(TTTT)_n$ attached to them. You would pass your cell lysate over the column and mRNAs would stick to the beads due to the base pairing of the poly-A tail to the oligo dT. You would then wash the column and elute the mRNA.)**

2) **Anneal an oligo dT primer to the mRNA to provide a 3'-OH to begin reverse transcription. Add dNTPs and reverse transcriptase.**

3) **Treat the RNA/DNA hybrid with base. The base will destabilize the RNA/DNA duplex and degrade the RNA.**

4) **Reverse transcription of the original mRNA will create a weird little hairpin that can serve as a primer for synthesizing the second cDNA strand. Use DNA Polymerase I to make a double stranded cDNA.**

5) **Treat the double stranded cDNA with SI nuclease to digest the hairpin and create a double stranded cDNA molecule.**

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.

1) To determine if the gene is poorly transcribed, you could perform a northern blot analysis and look at overall mRNA levels in the cells.

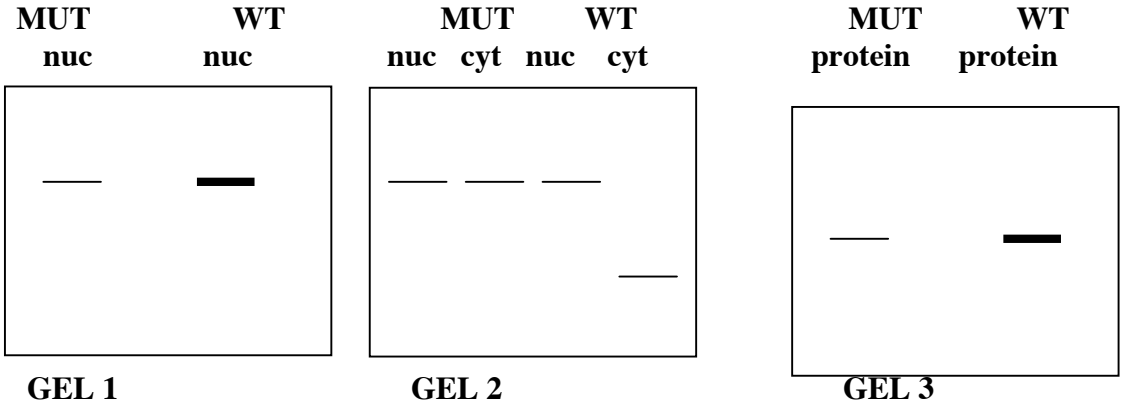
-You would run RNA purified from both mutant and wild-type cells (mRNA purified from nuclear fractions is probably best.) on a denaturing polyacrylamide gel. Transfer the RNA to nitrocellulose and probe with a radiolabled wild-type cDNA. If the mutant gene is poorly transcribed you would see the result shown in gel 1.

-Another possibility would be to do an *in vitro* transcription reaction using the mutant and wild-type DNA (however you were not given the ingredients needed for this experiment.) (1 point for naming the assay, 1 point for a complete explanation.)

2) In order to determine whether the mutant is defective in mRNA processing, you could use a Northern blot (see method in part 1) to compare mRNA isolated from the cytoplasm and nucleus of both mutant and wild-type cells. mRNA isolated from the nucleus will be unprocessed and mRNA from the cytoplasm will be processed. By comparing the two side by side you will be able to determine if the defective step occurs as the mRNA moves from the nucleus to the cytoplasm. GEL 2. (4 points- 1 point for naming the assay, 3 for explanation including proper controls.)

Another possibility would be to do an in vitro splicing reaction purified mRNA and ask whether the mutant mRNA is spliced properly.

3) If there is a defect in translation of the Snoz mRNA you would expect to see no obvious defects in the first two experiments. In order to definitively test whether there is a translation defect, you would want to purify antibodies to the protein and do a western blot to determine the Snoz protein levels in wild-type and mutant cells. GEL 3. (1 point for naming assay; 1 point for proper explanation.)



GEL 1 **GEL 2** **GEL 3**

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.

The Snoz mRNA is not processed properly in the mutant even though the snRNPs and U2AF can bind. There are two possible answers. The 5' splice site could be mutated causing U1 to bind more tightly so that U6 is not able to compete effectively for the 5' splice site. If U6 cannot bind then U2 cannot interact with U6 and the first step of the splicing reaction cannot occur. The second possiblity is that the critical A residue in the branch site is mutated. This mutation does not preclude U2 binding but prevents the 2'-OH from attacking the 5' splice site. There was a hint in the problem to help you avoid this model (a low level of mature mRNA is produced) but it was confusing since later it says that spliceosomes never generate active complexes. Credit was given for this answer even though it was not what we were looking for. To get full credit, you needed to have an answers as well as a clear explanation.)

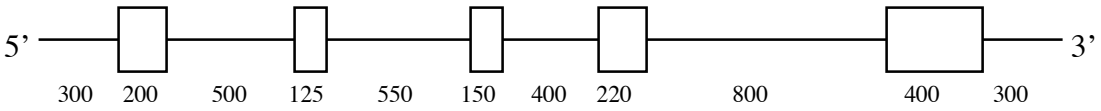
4C. Your lab has identified a drug that treats the SNOZ disease. You find that splicing of pre-mRNA is partially restored in cells from these treated individuals. What type of splicing factor might, if highly expressed, generate this recovered phenotype? Briefly justify your answer.

You have now found that some mutant cells are able to recover and are asked to suggest a type of splicing factor that could rescue the splicing defect found in Snoz mutants. Using the first model from above, the dead box proteins that act as helicases and unwind RNA duplexes could suppress the tight binding of U1. By unwinding the RNA duplex formed by U1 and the 5' splice site, the dead box proteins would allow U6 to bind the 5' splice site and restore splicing. (2 points for a logical answer and 2 points for an explanation.)

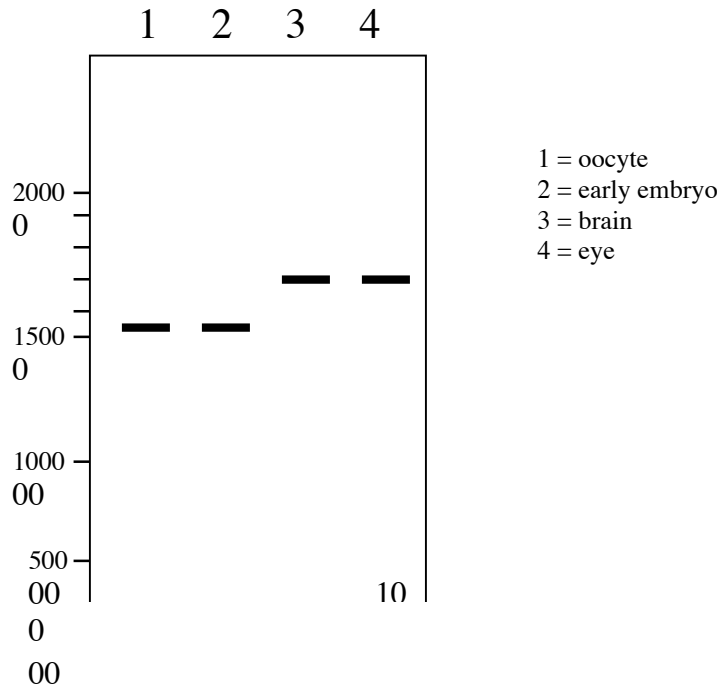
4D. Design a simple *in vitro* assay that could be used to purify such a factor from the recovered cells.

You would want to use biochemical complementation to purify the factor that can suppress the SNOZ mutant. You would fractionate the extracts from restored cells and then add these fractions back to an in vitro assay. The most simple assay would be to set up a helicase assay in which you have a unlabled mRNA and a small labeled fragment (representing the U1 RNA) hybridized to it. By adding back fractions and running the samples on a non-denaturing agarose gel you could identify fractions that contain proteins that can unwind RNA/RNA duplexes and consequently cause the radiolabled RNA to be released.
(Partial credit was given to logical experiments that did not show that the protein purified complemented the mutant phenotype.)

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.

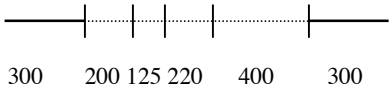


To look at expression of this gene, you decide to do a Northern blot with cytoplasmic RNA from different tissues. You use a probe that will detect exon 1.
The key indicates the origin of RNA in each lane.

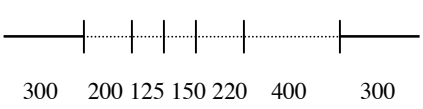


5A. Draw your predictions of the RNA structure for each lane.

Lanes 1 and 2:



Lanes 3 and 4:



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.

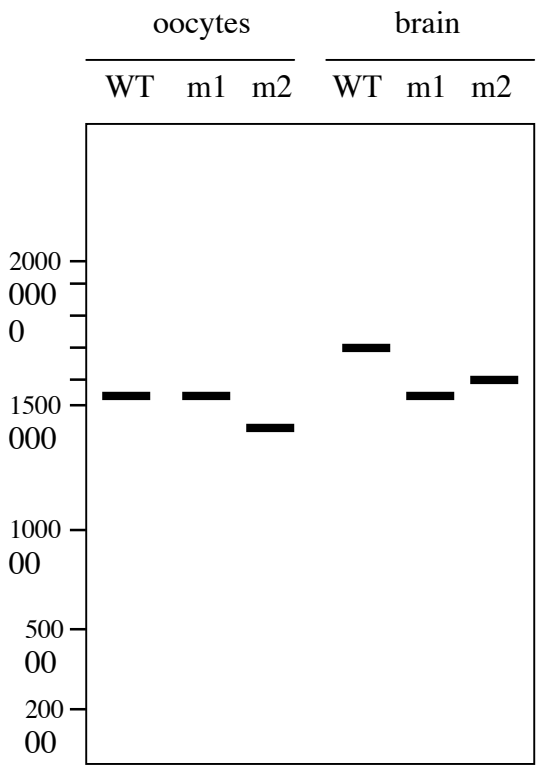
Isolate mRNA from each tissue type.
Add DNA primers complementary to 5' and 3' ends of the third exon.
Add reverse transcriptase and dNTPs to transcribe one strand of DNA.
Remove the RNA strand with RnaseH or alkali.
Add Taq polymerase and dNTPs for normal PCR amplification.
Run PCR reactions on a gel. For lanes 1 and 2 you should not see a band. For lanes 3 and 4 you should see a specific band that was amplified corresponding to the distance between your primers.

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.

Exon 3 must contain a stop codon in frame so that when this exon is included in the zygotic isoform, translation produces a shorter protein than the maternal form(the stop codon for this form is probably in exon 5).

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.

m1 probably is a mutation in the ESE of exon 3 which is required for inclusion of the exon in the zygotic mRNA. Without its function, exon 3 is not spliced into the zygotic mRNA.

m2 is a mutation in the BPS or 3' SS of intron 1 which causes skipping over exon 2 and splicing to the 3'SS of intron 3 in the maternal RNA and to the 3'SS of intro 2 in the zygotic RNA.

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.

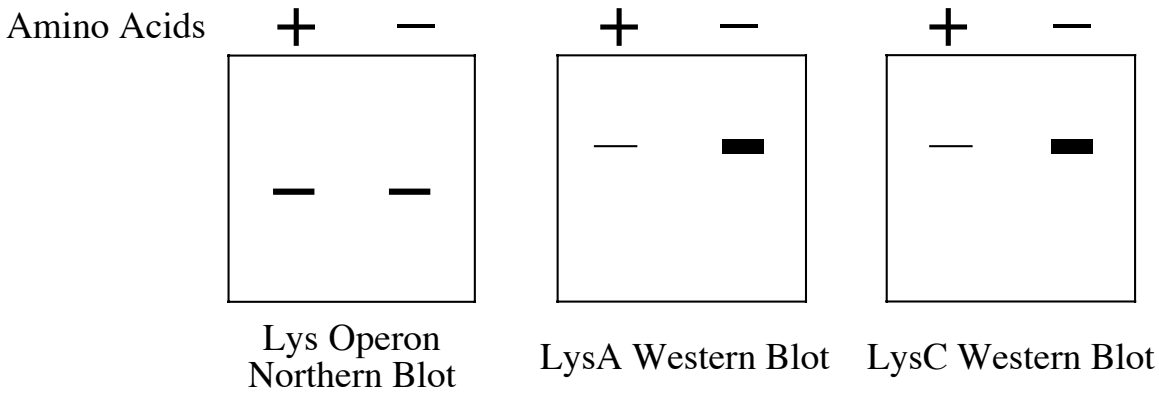
5E. What is this gene likely to encode? Propose a model for how the m3 mutation suppresses the splicing defect seen in m1.

The m3 mutation could be in a gene for a splicing activator which binds to the ESE in exon 3. Since this activator is only expressed in zygotic tissues, it is only in those tissues that exon 3 is included in the mRNA. The mutation must cause a change in the binding site of the activator such that the changed amino acid sequence is able to bind to the mutated ESE sequence.

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.

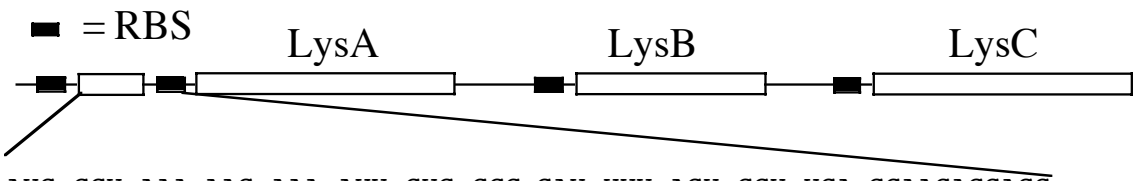
You first set out to determine how addition of amino acids to the growth media effects expression of operon. You perform both a northern blot of the Lys operon mRNA and a western blot using an antibodies to two different proteins (LysA and LysC) expressed from the mRNA. You obtain the following results:



6A Based on these observations what can you conclude about how the expression of the *K. expressus* lysine biosynthetic genes are regulated?

The northern blot shows that lys operon transcription is not regulated by the presence of amino acids. The western blots of Lys A and LysC show that LysA and LysC are regulated post-transcriptionally, with protein expression low in the presence of amino acids and high in the absence of amino acids.

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
```

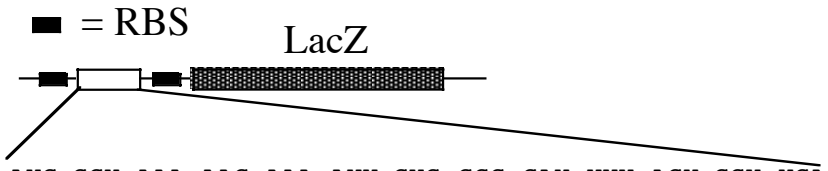
6B You are initially excited that the short open reading frame could act as a transcriptional attenuator, however, you rapidly discard this notion based on the data above. What data allows you to exclude this hypothesis? Why?

If this operon was regulated by transcriptional attenuation, then you would expect the size and amount of the RNA to change in the presence of amino acids. You did not observe this in the northern blots for part A.

Partial credit was given if you listed all the parts of a transcriptional attenuator (stem loop followed by a run of U's in the mRNA) that would be absent based on the coding sequence you were given.

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 +	β-Galactosidase Activity Units Amino Acids −
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.

The model that best fits this data is the following:

The ribosome binding site of the downstream gene (LysA or LacZ) is very closely positioned to the stop codon of the leader peptide. This positioning allows the ribosome translating the end of the leader peptide to block binding of the downstream rbs by another ribosome. In the wild type construct, this situation will occur in the presence of amino acids. In the absence of amino acids, the ribosome will stall at the 3 Lys codons. In this case, the ribosome does not block the downstream rbs, so a new ribosome can bind and translate LysA/LacZ. The phenotypes of the mutants can be interpreted in the context of this model.

Mutant 1(AUG-UUG): Leader peptide is not translated, so no ribosome is present at the end of the leader peptide to block translation of downstream gene. Therefore, you observe high levels of LacZ expression in the presence and absence of amino acids.

Mutant 2 (KKK-EVN): Removing the three Lys codons (KKK) at the beginning of the leader peptide removes the site of ribosome pausing. Therefore, ribosome translates leader peptide in the presence and absence of amino acids and this blocks downstream translation.

Mutant 3 (KKK-EVN, FSR-KKK): Moving the Lys codons to the end of the leader peptide restores pausing of the ribosome, but now it is at the end of the gene, where it is optimally positioned to block translation of the downstream gene.

Other models, if they were plausible and could explain <u>all</u> the mutant phenotypes were accepted.

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)

Based on the model in 1C, the best test of your model would be to insert sequences between the end of the leader peptide and the rbs of the downstream gene. If your model is correct, then increasing the spacing should result in constitutive expression of LacZ.
Other experiments were accepted if they were explained, related to your model, and provided information that was significantly different from the information provided by the mutants in part 1C.

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?

Based on your hypothesis, you would expect that anything that increased translation termination and release of the ribosome would increase expression of LacZ. Switching the stop codon from UGA to UAA could meet this requirement, as UAA is recognized by both RF1 and RF2, while UGA is only recognized by RF2. Based on this data and your model, you would expect overexpression of RF1 to have little affect on LacZ expression in the wild type construct, as RF1 does not recognize UGA. You would expect overexpression of RF2 to result in an increase in LacZ expression in the presence of amino acids, similar to the effect observed when the stop codon is mutated from UGA to UAA.

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?

The best response here, based on your model, was that the regulation of LysA did not necessarily explain the regulation of LysC, as the ribosome that was terminating

on the leader peptide would not also occlude ribosome binding on the LysC rbs, as it was so far away.

The answers for this question varied widely, and credit was given if you came up with a plausible explanation that explained how the regulation of LysA and LysC was or was not coupled.

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?

First, you must separate the nuclear pre-mRNA from the cytoplasmin mRNA, then to purify pre-mRNA or mRNA, use an oligo dT column (oligo dT will bind to the polyA tail of the transcript). You could use a northern blot or RT-PCR to assay mRNA levels

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

Precipitate the protein from each of the different kinds of cells, run on an SDS-PAGE gel and do a western blot (if you don't have antibodies to the YCG1 protein, then you can tag it eg HA, MYC, FLAG, etc and use antibodies that recognize the tag)

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?

Make cDNA: isolate the mRNA from the cytoplasm (using oligo dT), add oligo dT primer (which will bind to polyA tail), RT, and dNTPs. Hairpin acts as primer for second strand, get rid of RNA (eg alkali), add DNA polI and dNTPs to get second strand synthesis and add S1 nuclease to cut hairpin. You can get both length and sequence information from cDNA.

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.

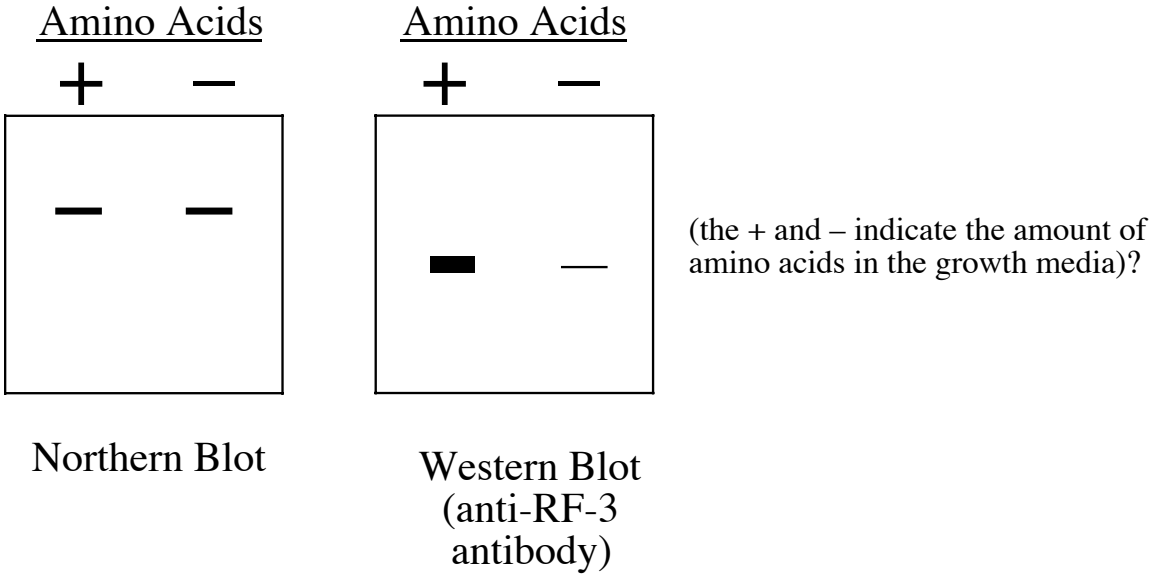
7D. How could the difference in poly-A tail length lead to different levels of protein in the two tissues?

The poly-A tail is important in getting efficient translation of the mRNA. It could be that in liver cells, the YCG1 mRNA poly-A tail is very short and so translation does not occur very efficiently and thus there isn't a lot of YCG1 protein. In contrast, the YCG1 poly-A tail in kidney cells could be longer and thus translation occurs frequently and there is more protein. The mechanism behind the importance of the poly-A tail in translation: The poly-A binding protein coats the poly-A tail and the poly-A binding protein binds the initiation factor eIF4F. This provides a connection between the poly-A tail of the mRNA and initiation of translation.

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?

You would delete the gene that codes for the poly-A binding protein. In this environment, the YCG1 protein levels in kidney and liver cells should be equal.

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:



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

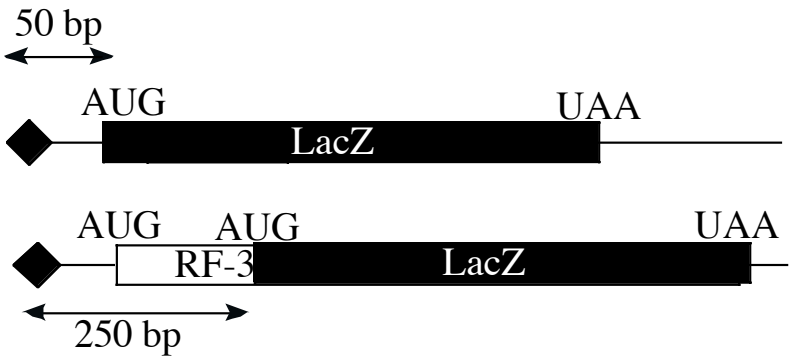
8A At what stage is RF-3 synthesis regulated by amino acids?

RF-3 synthesis is regulated during translation because transcription levels of RF-3 mRNA are identical, but Western protein amount differs.

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	600	600
AUG AUG … UAA	300	10

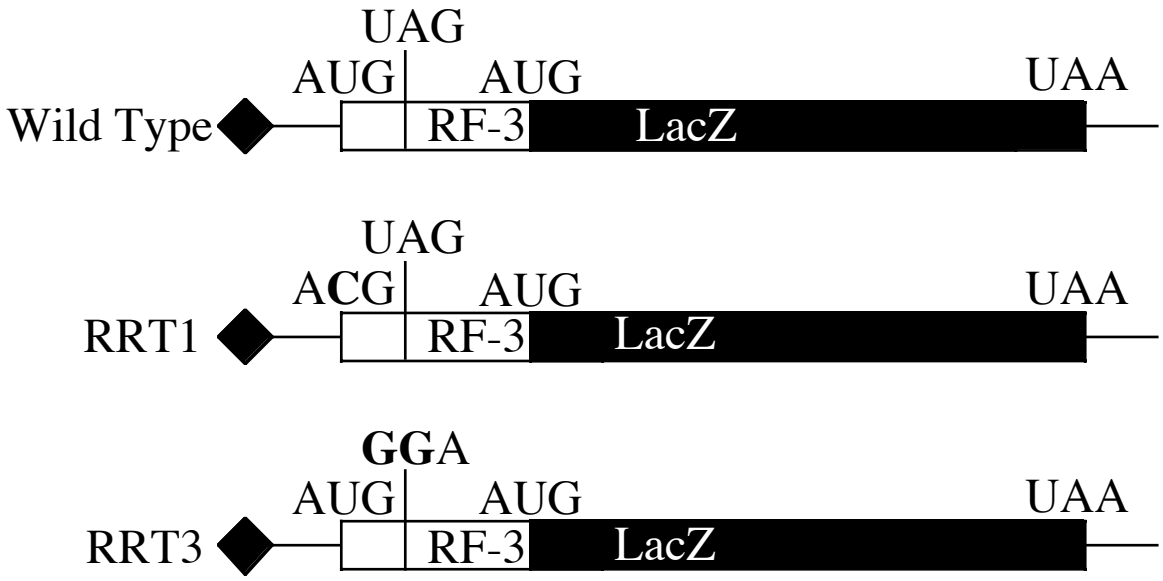
8B What can you conclude about the mechanism of control of RF-3 expression based on these results?

One can conclude that the first 200 bases of RF-3 are required for regulation of the RF-3 mRNA.

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.

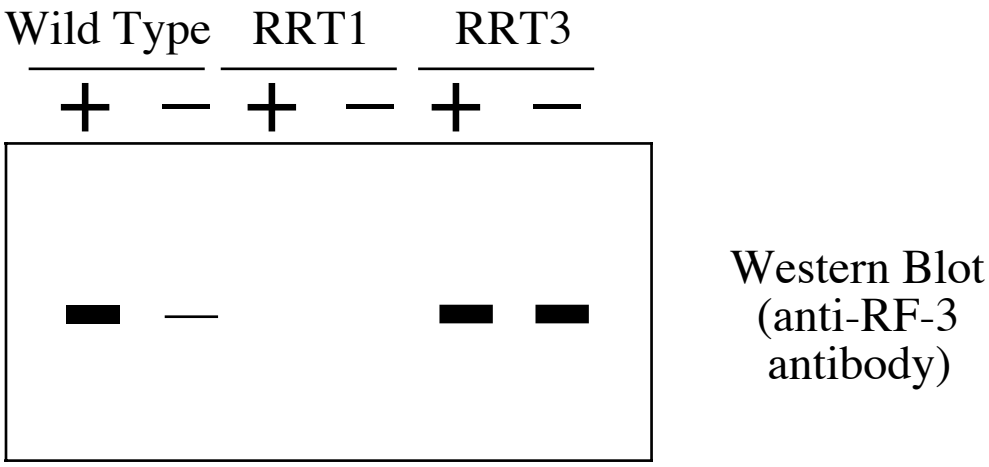


8C Using the above information, explain the phenotypes of the RRT1 mutation. Propose a model to explain the RRT3 mutation.

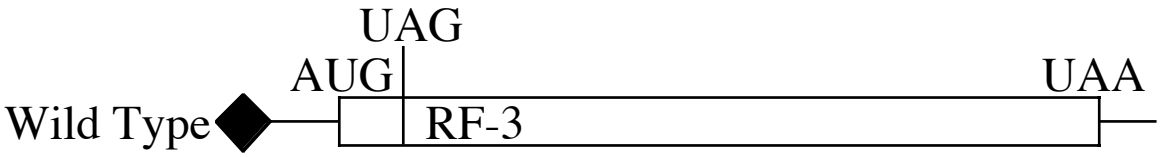
RRT1 mutant lacks the ORF of the WT which is required to negatively regulate the translation. The mutant is constitutie because the ribosome does not react to the aa levels; it binds AUG of LacZ regardless; no negative regulation.

RRT3 mutant lacks the stop codon in the ORF. The ribosome binds the AUG of the ORF and starts going all the way through LacZ, which is most likely out of frame since no product is produced (uninducible AUG of LacZ is not recognized because its inside an ORF.

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.



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.

RF-3 is required to stop translation, for a stop codon to work. When aa levels are low, RF-3 is around to stop translation at UAG, and little RF-3 is produced. When aa levels are high, there is little RF-3 around and Tyr is substituted instead of STOP codon so a full length RF-3 is produced.

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?

Normal RF-3 gene is in-frame and LacZ fusion is out of frame, so the effects are reversed. RRT1 mutant in normal RF-3 has no start codon, so no RF-3 is made. In LacZ it has no ORF, which gives the LacZ start codon a chance. RRT3 in normal RF-3 lacks the regulatory STOP codon, so RF-3 is expressed all the time. RRT3 in LacZ fusion has only 1 ORF which is recognized and since LacZ is out of fram, no LacZ is expressed.

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

Deleting RRT2 would mean the stop codon is recognized as stop, not Tyr. In normal RF-3 it is uninducible, no RF-3 is ever made. In a LacZ fusion, it is regulated: high LacZ when aa high, low LacZ when aa low.

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:

Incorporation assay: Incubate cells or cell extract with radiolabeled dNTPs, isolate DNA, measure radioactivity.

Transcription:

Same, but radiolabeled UTP.

Translation:

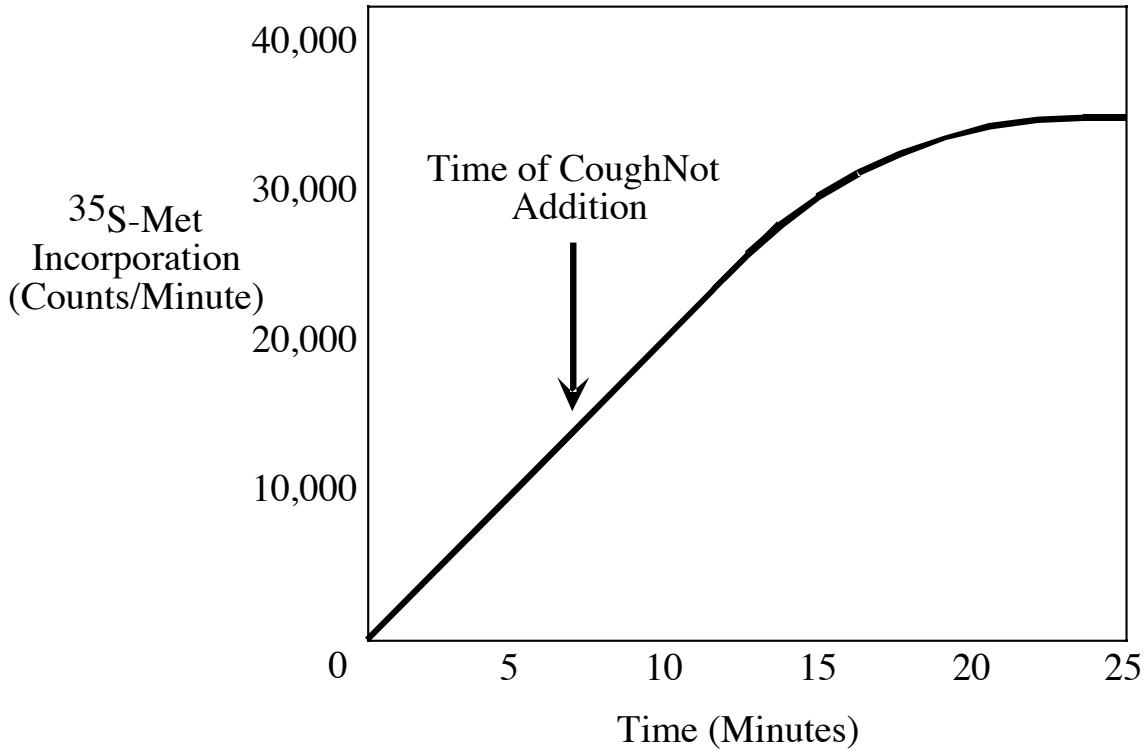
Same, but radiolabeled amino acids. (If an extract, must be from tuberulosis, not from rabbit reticulocytes, as want to inhibit tuberculosis translation, not rabbit translation.)

For all 3 assays, do +/- antibiotic. Half credit was given if you used purified components instead of an extract, because there are many more proteins in vivo that could be inhibited by the drug (for example, for DNA replication, initiator proteins, primase, helicase, etc.)

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?

Besides the fact that multiple processes could be inhibited by the drug, transcription is necessary for translation. Lack of replication will arrest the cell, and although metabolic processes will continue, levels of transcription and translation may be altered (as we didn't talk about this, credit was given for simply realizing that replication comes before translation, or that lack of it may kill the cell.)

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.

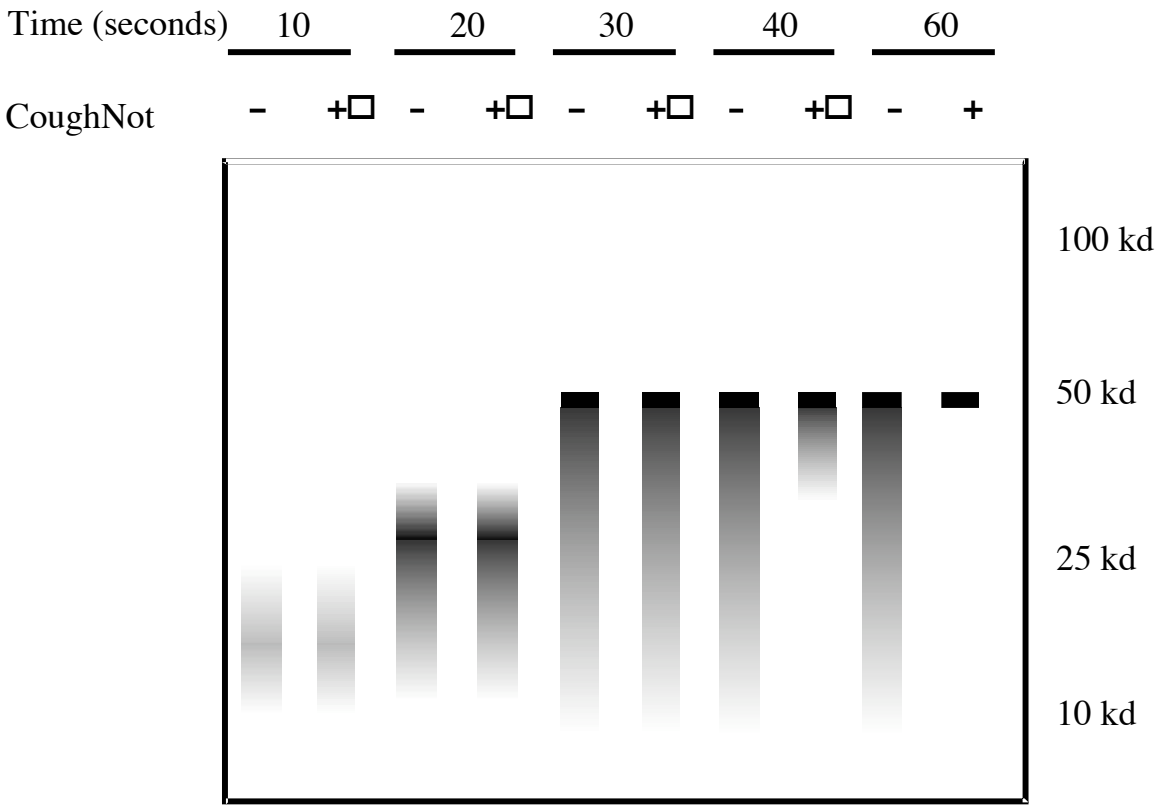


9C Based on this graph, what conclusions can you make about the step(s) in translation that could be inhibited by CoughNot. You should assume that the antibiotic inhibits it target within seconds. Explain how the graph supports your conclusions.

Initiation or Termination. Because translation continues for a while after the addition of CoughNot, elongation is not affected (like a slow stop DNA replication mutant). However, after a while translation is inhibited, as the ribosomes try and either terminate the proteins they were making when the drug was added, or initiate new translation.

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.



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.

The defect is not in iniatiation, as the drug is added before the mRNA is added to start translation. But then, after a certain amount of full length protein is synthesized, there is not new initiaton (40 and 60 seconds). Therefore, there is a problem in termination. After the full length protein is made, the ribosome is not released, and therefore cannot initiate new protein synthesis.

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.

If your model in D was a problem in termination, then you'd want to test for association of the release factors with the ribosome.

Isolate the ribosomes and run on a sucrose gradient. Also, run ribosomes alone, and release factors alone. See what fraction the ribosomes alone run in by running out the fractions on a gel and doing Northerns for rRNA. See what fractions the release factors run in by SDS PAGE and a Western with antibodies against the factors (or have them radiolabeld). Then see if the release factors comigrate with the ribosomes isolated from the extract.

The modification interference assay would also work here, but gel shifts would not.

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.

UAG – Then you (1.) do not have to modify the transcript of your protein to avoid a codon for a specific amino acid and (2) are using a codon that will not be recognized by a tRNA, as UAG is usually recognized by the protein, RF1.

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.

Anticodon: 5' – CUA – 3'

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?

Modify the domain of the tRNA synthetase responsible for binding to the acceptor arm (active site for charging.)

Modify the domain of the tRNA synthetase responsible for amino acid adenylation, if the modification of the serine affects its ability to occupy the active site of this domain.

Because there are so many different Serine codons, and some do not share any of the same three nucleotides in the codon, it is unlikely that you would need to make changes to the anticodon binding domain.

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.

Try using an enzyme that converts the Serine into its modified form after charging. This strategy is used by many organisms for charging a tRNA with glutamine. (tRNA is charged with glutamate and a second enzyme converts glutamate to gluatmine.) This strategy is also used for charging prokaryotic initiator $tRNA^{fMet}$ with a formylated methionine.

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.

You would use an mRNA containing a ribosome binding sequence, a start codon (AUG), a single UAG codon near the AUG, and a non-UAG stop codon (UAA or UGA.)

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.

The modification could affect binding of EF-Tu to the modified tRNA-Ser. Either EF-Tu is (1) not capable of binding, or (2) binds too tightly to EF-Tu.

- **Use a filter binding assay (with filter that binds protein but not RNA) with radiolabelled tRNA-Ser, EF-Tu, GTP, Mg++ and buffer to distinguish these possibilities.**
  - **Determine if EF-Tu binds to modified tRNA-Ser.**
  - **Determine the K_d of binding for modified and wt tRNA-Ser by performing multiple filter binding reactions with different concentrations of EF-Tu.**