

156-201 Principles of Molecular and Cellular Biology
October 20, 2005
Second Exam- Geyer

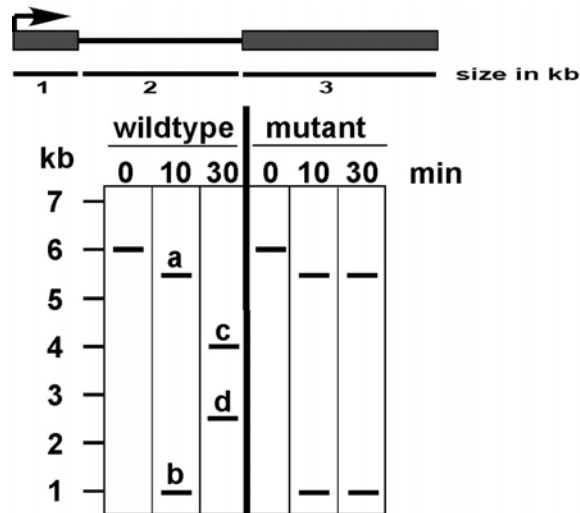
Each question is worth the number of points indicated. It is written as a 2 hour, closed book exam. Answer each question with a short paragraph, table or labeled diagram. Answer in the space provided.

Partial credit will be awarded. Be sure to state any assumptions made in your answer. An unanticipated answer that is the logical consequence of reasonable assumptions will be given partial credit.

PG1 (10 points). Transcription of eukaryotic genes depends upon several RNA Polymerases. Describe each nuclear RNA polymerase found in mammalian cells. Include in your answer the class of genes that each transcribes and any noteworthy characteristic of the Polymerase.

There are four RNA polymerases. RNA Pol I is responsible for transcription of ribosomal RNA and localizes primarily to the nucleolus. RNA Pol II is responsible for mRNA synthesis. The large subunit of RNA Pol II contains a heptad repeat (YSPTSPS) that is repeated 27 to 52 times, depending upon the eukaryotic species. This carboxy-terminal domain (CTD) is important for transcription. Hypophosphorylated CTD is incorporated into pre-initiation complexes, while hyperphosphorylated CTD is associated with transcriptionally active RNAP II. Further the RNAP II CTD associates with the capping, splicing and termination complexes, helping to coordinate mRNA processing. RNA Pol II is responsible for transcription of tRNA and 5S RNA. Finally, the spRNP-IV is a single polypeptide polymerase responsible for transcription of some nuclear mRNAs. This single polypeptide polymerase was initially thought to be responsible for transcription of the mitochondrial genome, but truncated protein produced from translation of a splice variant was found in the nucleus.

PG2 (10 points). RNA splicing can be reconstituted *in vitro* using purified snRNPs. A yeast strain was identified that was defective in RNA splicing at 30°C, but had robust splicing at 22°C. To explore the cause of the RNA splicing defect, snRNPs were isolated from the wild type and mutant strains and *in vitro* splicing reactions were carried out at 30°C using a pre-mRNA corresponding to the gene shown below (note the size of the exons and introns are shown below the structure of the gene). The splicing reactions were carried out for 0, 10 or 30 minutes and the RNA products analyzed on a denaturing RNA gel.



PG2A. (5 points) State a possible structure for each RNA band present in the 10 and 30 minute lanes of the wild type *in vitro* splicing reaction (bands a, b, c, d).

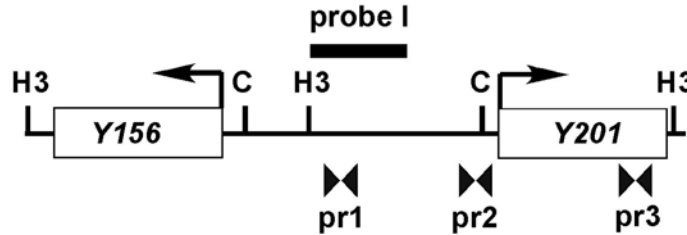
Splicing involves multiple steps catalyzed by snRNPs. The first step is the transesterification reaction resulting from attach of the branchpoint A to the 5' exon-intron junction. Isolation of the RNAs at this point would produce the free 5' exon and the lariat containing intron-exon RNA. The free 5' exon corresponds to the 1 kb band. The intron-exon RNA migrates aberrantly slow for its size (> 5 kb) due to the presence of the lariat.

The next step in the splicing reaction is the second transesterification step between the 3' OH of the 5' exon and the intron-exon junction. This reaction produces the mature mRNA, corresponding to the 4 kb band and the lariat intron, again migrating slower than its molecular weight, at ~2.5 kb.

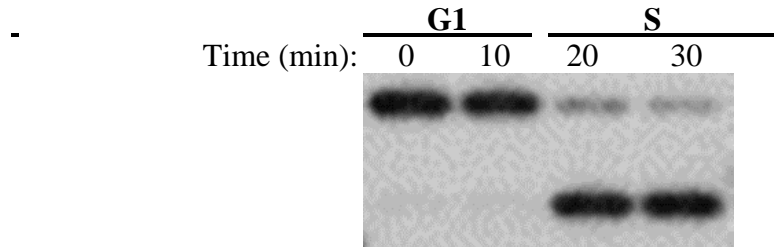
PG2B. (5 points) Propose a model that accounts for the defect in the temperature sensitive mutant. Briefly explain how this defect caused the observed RNA banding pattern in the 10 and 30 minute lanes of the mutant.

The splicing reaction appears to be blocked after the initial attack of the branchpoint A with the 5' exon. As the U6 snRNP is responsible for secondary steps of splicing, it is likely that the mutation is present in this snRNP. The U6 snRNP contains proteins and the U6 small nuclear RNA, either which may be responsible for the temperature sensitivity. A second snRNP that might be involved in the U5 snRNP, which also participates in the second transesterification reaction.

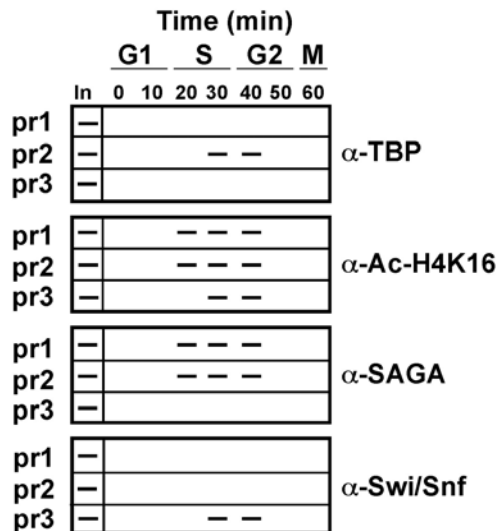
PG3 (30 points). Chromosome 2 in the *S. cerevisiae* genome contains two genes, *Y156* and *Y201*. Transcription of *Y201* is regulated during the cell cycle. To investigate how transcription is regulated, cells were blocked at the end of mitosis. At time 0, cells were released from the block and allowed to synchronously re-enter the cell cycle. Nuclei were isolated and treated as described in Experiments 1 and 2. Use the following data to answer the questions on the next pages.



Experiment 1: Nuclei isolated at the indicated time were incubated with the restriction enzyme Cla I (C) for 10 minutes. The reaction was stopped, DNA was purified, cleaved with Hind III (H3) and Southern analysis was carried out, using probe I. Results are shown below, with the stages of the cell cycle shown:



Experiment 2: Isolated nuclei were treated with formaldehyde, chromatin was sheared and split into five equal samples. Four samples were incubated with one of the following antibodies α -TBP, α -SAGA, α -Swi/Snf, and α -Acetyl H4K16 and the immuno-complexes were isolated. DNA was purified from the four antibody treated samples and the untreated sample (Input, In). The purified DNA samples were amplified with PCR primers (pr1, 2, 3) to the indicated regions. Results from this analysis are shown below. The stages of the cell cycle are shown.



PG3A (10 points). Experiment 1 is called a restriction enzyme accessibility assay. Consider the results and explain the origin of the bands in each lane. Briefly explain what this information tells you about the chromatin structure of *Y201*.

[5 points for explaining G1 phase data; 5 points for explaining S phase data]

A restriction enzyme accessibility assay tests the chromatin structure of a genomic region. This assay determines whether a restriction enzyme physically binds a region in the context of chromatin (protein + DNA) to cleave the DNA. If the chromatin context is open, then the enzyme has access to the DNA and will cleave it. However, if the chromatin context is closed, then the enzyme cannot access and digest the DNA.

In this question, the genomic region contains three Hind III (H3) sites and two ClaI (C) sites. Detection of the bands on the gel relies on a radioactive probe that hybridizes to sequences just 3' to the second H3 site. The Cla I digest is done on chromatin, so its effectiveness depends upon the chromatin structure. In contrast, H3 cleavage is performed after the DNA was purified, so H3 cleavage will be complete. Smaller bands were produced in the S phase digest, versus the G1 phase digest. This suggests that accessibility to the C restriction site changed. In the G1 stage, there is only one band that corresponds to the H3-H3 fragment, suggesting that cleavage by C did not occur and the chromatin structure was compact. In the S phase, two bands were produced, suggesting that C had some access to its site. The bottom band represents the smaller H3-Cla fragment. As the C site is near the *Y201* promoter site, these data suggest that the promoter lies in open chromatin in S phase and that might be the time when the gene is transcribed.

PG3B (12 points). Experiment 2 used antibodies against 4 different proteins. Briefly define each protein, indicating how this protein affects transcription.

(B) [3 points for each antibody/protein]

- **TBP (TATA binding protein)**
 - Recognizes TATA sequence of promoters.
 - Required for Pol I, II & III transcription.
 - General transcription factor involved in TFIID recognition of promoter.
 - Interacts with TAFs to promote transcription.
- **Ac-H4-K16 (acetylated Lys16 on Histone H4)**
 - Histone H4 is member of core histone complex (along with H2A, H2B, H3).
 - Marks active chromatin (Acetylation promotes transcription).
 - Recognized by chromatin binding proteins containing a bromo-domain.
- **SAGA**
 - Gene transcription activator = increases level of RNA production.
 - Histone modifying enzyme because it is a histone acetyl transferase (HAT).
 - In the HO gene paradigm, recruited by Swi/Swf to URS2.
- **Swi/Snf**
 - Uses ATP.
 - Part of histone remodeling complex that opens up chromatin.
 - Involved in altering the contact or "sliding" nucleosomes upstream of genes.
 - In the HO gene paradigm, Swi/Snf recruited by Swi5 to URS1 & URS2.

PG3C (8 points). Consider the data in Experiments 1 and 2. Propose a model for the regulation of *Y201*. Provide a brief explanation for your rationale behind your model. In your answer, you must include an explanation of the PCR data obtained in Experiment 2.

[4 points for explaining PCR/ChIP data of experiment 2 correctly; 4 points for model & rationale]

Experiment 2 represents a ChIP assay of yeast during various stages of the cell cycle. Pr1, 2 & 3 represent the various primer pairs to amplify the DNA that is pulled down by the various antibodies after cross-linking and sonication. In each set of gels, “In” represents DNA isolated from a “mock” immunoprecipitation. Since all of the genomic DNA is present in the “In” samples, this column reports that the primers worked and that the DNA preparation was good. It also can be used to ascertain relative enrichment of a particular DNA region in the immunoprecipitated sample.

TBP: TBP is present only at the Pr2 site, and only at the end of S phase and the beginning of M phase. Taking into account the data from experiment 1 and the role of TBP in the TFIID complex, these data are consistent with the Pr2/C region representing the *Y201* promoter and with the hypothesis that *Y201* is being transcribed during S phase and into early G2 phase.

H4: Acetylated H4 is found at both pr1 and pr2 during all of S phase and into early G2 phase and at pr3 at the end of S phase and into early G2 phase. Pr3 is only acetylated when TBP was bound. These data suggest that the chromatin surrounding and immediately upstream of *Y201* is in an open, accessible state during S phase and into early G2 phase.

SAGA: The SAGA complex is present at pr1 and pr2 at the same time as acetylated H4, consistent with its role as a HAT. SAGA is not present at the pr3 site. These data further re-enforce the idea that *Y201* is actively transcribed during S phase and into early G2 phase.

Swi/Snf: Swi/Snf is recruited to the pr3 site only at the end of S phase and the beginning of G2 phase. Since Swi/Snf is recruited coincident with Ac-H4K16 at Pr3, this implies that it is recruited by the transcribing polymerase and may help remodel nucleosomes to allow polymerase movement through the gene.

Summary: Based on data in both experiments, the chromatin surrounding *Y201* is in a closed, inaccessible state during G1 phase. In contrast, during S phase, first SAGA is recruited, which probably lead to the acetylation of H4K16 causing an opening of the chromatin. Next, TBP/TFIID and Swi/Snf are recruited to help remodel the nucleosomes structures and transcribe the gene. The open chromatin state persists into early G2 phase but is lost by the middle of G2 phase. There is likely an upstream transcription factor (not studied here) that binds and recruits SAGA to initiate opening of the chromatin and to allow for TBP binding and transcription of *Y201*.

GL1a. Acceptable answers used the following ideas. Full credit was determined based on demonstrated knowledge of the GPCR – trimeric G protein – adenylyl cyclase – cAMP – PKA pathway. Mechanisms by which signaling was down-regulated had to be described in terms of cause and effect.

- 1) an inhibitory hormone activates $G\alpha_i$ which down-regulates adenylyl cyclase
- 2) phosphodiesterase breaks down second messenger cAMP resulting in a decrease in PKA activity
- 3) a phosphatase, like PP1, dephosphorylates and down-regulates proteins kinased by PKA
- 4) a kinase, like PKA or GRK, phosphorylates the cytosolic domain of GPCR and decreases its interaction with $G\alpha_s$ protein
- 5) binding of arrestin to GPCR decreases its interaction with $G\alpha_s$ protein
- 6) activation of a GAP down-regulates the active G-protein by increasing its GTPase activity thus leading to less activation of adenylyl cyclase
- 7) inactivation of a GEF down-regulates the active G-protein by slowing the exchange of GDP for GTP thus leading to less activation of adenylyl cyclase

As clarified, we were not asking for experimental manipulations that would interfere with signaling. We were asking for mechanisms that were used by the cell to down-regulate signaling. Therefore, answers that included site directed mutagenesis or antibodies did not receive full credit. Vague answers like “inhibit the enzyme” or “reduce cAMP” did not receive full credit.

GL2a. In the competitive binding assay, runt-y acts similarly to runt in the inhibition of L49 binding. Because the curve for runt-y is shifted to the left, this means that lower concentrations of runt-y are sufficient to achieve the same level of inhibition relative to runt. Therefore, runt-y has a higher affinity than runt for the site.

Runt-x does not inhibit the binding of L49 at all. The conclusion would be that runt-x does not recognize the same site as L49 and runt. Therefore, it must employ a different mechanism to elicit hormone secretion.

GL2b. Radiolabeled protein species 1-4 represent cell surface proteins that bind to the sepharose beads, independent of the ligand, and therefore represent background binding that has no relevance towards runt signaling or growth hormone (GH) secretion. These radiolabeled species would not be the sepharose beads themselves since the gel sample consisted of proteins that were eluted from the sepharose. Also, the beads were not radiolabeled and would not enter the gel anyway.

Radiolabeled protein species 5 is the runt receptor that activates GH secretion.

Radiolabeled protein species 6 is not the runt receptor but is the receptor to which runt-x binds. Because runt-x also elicits GH secretion, species 6 is likely to be another receptor that activates GH secretion. The fact that runt-x does not bind to species 5 is consistent with the competitive binding assay in part a.

Radiolabeled protein species 7 is probably the same as 5 as they co-migrate on the protein gel. This suggests that runt-y is able to bind to the runt receptor. This conclusion would agree with the fact that runt-y is able to inhibit the binding of L49, a known antagonist of the runt receptor and that runt-y is able to elicit GH secretion.

Radiolabeled protein species 8 is a cell surface protein to which runt-y binds. Relative to the information we know regarding the other protein species, there is, at this point, insufficient data to suggest any relationship of species 8 to GH secretion. For instance, it may have nothing to do with GH secretion, it may be a separate receptor for GH secretion, or it may aid runt-y in its interaction with the runt receptor. There is not enough data at this point, although any of these possibilities can be entertained.

GL2c. In the absence of L49, runt-y will activate GH secretion. In the presence of an excess of L49, signaling from the runt receptor (species 7) will be blocked and binding of runt-y to this receptor will be reduced (this is based on the knowledge that L49 is an antagonist that can compete with runt-y for runt receptor binding). Therefore, since GH secretion remains the same under the conditions of L49 excess, this suggests that runt-y may have an alternate route to activate GH secretion that does not involve the runt receptor. Since runt-y also interacts with cell surface protein species 8, one would hypothesize that this protein is a separate receptor that is activated by runt-y binding and leads to GH secretion. The species 8 receptor would not have a binding site for L49.

The idea that 50 nM runt-y would “out-compete” L49 for the activation of the runt receptor was not tenable given that L49 was present in excess. The possibility that protein species 8 would influence the association of runt-y with the runt receptor, in such a way as to prevent L49 binding, is not consistent with the data from the competition binding assay. This assay was performed on cells and since protein species 8 would have been present, runt-y would have inhibited L49 binding differently (not resembling runt as illustrated).

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Points will be awarded for your answer, including the explanation of how you arrived at your response. Be sure to explain your reasoning. State any assumptions that you made in your answer. An unanticipated answer that is the logical consequence of reasonable assumptions may receive partial credit.

Your answer should fit in the space provided for each section. Please write legibly. If the instructor cannot read your answer, then the answer cannot be graded.

MG1. Initiation of translation requires coordinated assembly of ribosomal subunits and protein components. These elements play critical roles in regulation of protein translation. Select FIVE items from the list below, and identify the function that it plays in this process. (10 points)

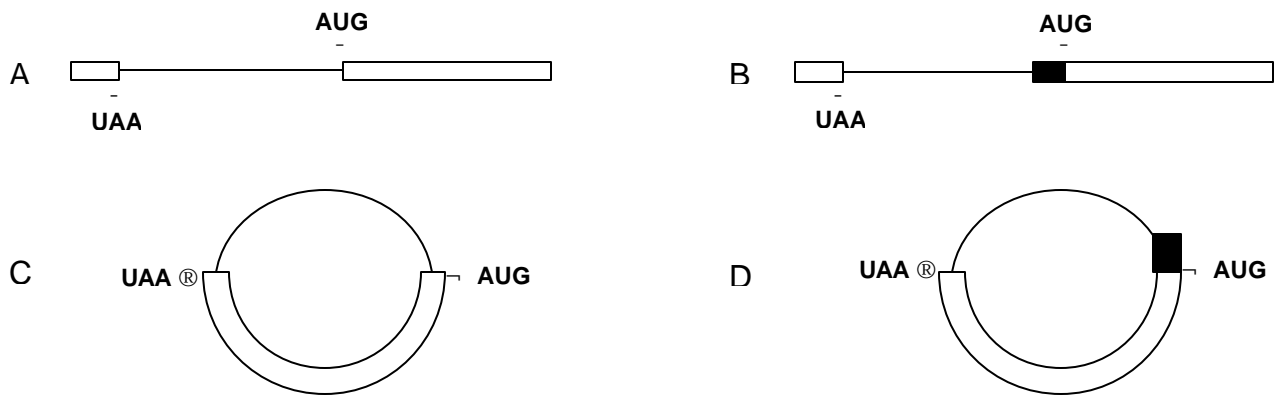
- a) Met-tRNA_i^{Met} – **the charged tRNA carrying in the initiator Met to begin translation of the new protein. The tRNA contains the anticodon that base pairs with the codon on the mRNA transcript. Only this charged tRNA enters directly into the P site of the ribosome, while additional tRNAs deliver subsequent amino acids via the A site.**
- b) Kozak sequence – **this sequence encompasses the AUG start codon and possesses a conserved motif that binds with ribosomal RNA to position the initiation complex as needed to facilitate initiation of translation. It is used by eukaryotic transcripts. The Shine Dalgarno sequence is the prokaryotic counterpart.**
- c) eIF4A – **a subunit within the eIF4F complex; it is a helicase that serves to unwind and remove secondary structure in mRNA transcripts. It allows the ribosomal complex to translocate along the mRNA.**
- d) eIF4E – **a subunit within the eIF4F complex; it is a cap binding protein that associates with the specialized 5' methyl⁷Gppp cap structure on mRNA transcripts.**
- e) eIF4G – **a subunit within the eIF4F complex; it is a scaffolding protein that facilitates association of the eIF4F complex – eIF4A, eIF4E, and polyA binding protein. These protein:protein interactions facilitate circularization of the mRNA transcript to enhance efficiency of translation, allowing ribosomal subunits to off-load at the 3' end and quickly reload on to the nearby 5' end.**
- f) phosphorylated eIF2 α – **phosphorylation of this initiation factor inhibits a GDP/GTP exchange reaction that is necessary to deliver the charged tRNAs into the pre-initiation complex. Thus initiation of translation is down-regulated, except in conditions when cells are growing in a stress-inducing environment (e.g., viral infection, heat shock, hypoxia).**
- g) hyperphosphorylated eIF4E-BP – **hyperphosphorylation of the eIF4E binding protein results in its inability to associate with eIF4E. When eIF4E is left accessible without eIF4E-BP, then eIF4G can associate with eIF4E, and protein translation is facilitated. eIF4E-BP and eIF4G compete for the same binding site on eIF4E.**

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FOR QUESTIONS MG2 AND MG3, ANSWER ONLY ONE QUESTION – IT'S YOUR CHOICE.

NOTE: Questions MG2 and MG3 describe using reticulocyte lysates for *in vitro* translation reactions. These assays provide all components necessary for cell-free protein translation (e.g., tRNA, ribosomes, amino acids, initiation, elongation, and termination factors). Microsomal membranes are not included.

MG2. You are studying a protein that you believe is using a cap-independent translation mechanism. You have identified what you think is the sequence element that serves as the internal ribosome entry site (IRES). You have made four reporter RNA constructs (A, B, C, D) and inserted your IRES sequence (black box) into two of them (B, D). Constructs A and C lack your IRES sequence. Constructs A and B are linear RNAs and would result in a truncated 25 kD protein. Constructs C and D are circular RNAs and would result in a 30kD protein. Each reporter construct contains a 5' untranslated region (black line), an AUG start codon, an open reading frame (white box), and a UAA stop codon. You will add each construct to a cell-free reticulocyte lysate for *in vitro* translation to measure the ability of each construct to express the encoded reporter protein. The reticulocyte lysate provides all components necessary for translation to occur. The results from your immunoblot identifying the expressed reporter proteins are shown in panel E.



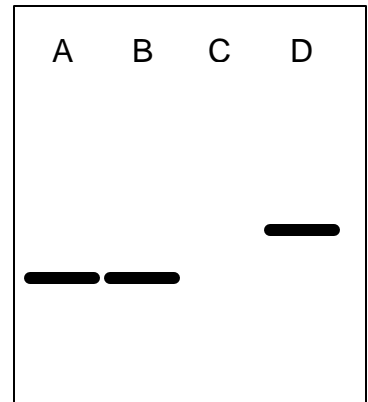
a) What mechanism of translation (cap-dependent or -independent) is likely used by each construct? Add one sentence for each construct to explain your choice. (8 points) **Panel E:**

Construct A – **cap dependent.** Lane A shows a translation product, and the construct does not appear to have an IRES.

Construct B – **cap dependent.** Lane B shows a translation product, and it is likely that it will also use the same mechanism as observed in lane A. **Cap-independent was also accepted, because one cannot rule out that the IRES element might also be active.**

Construct C – **cap independent.** Construct C is circular and thus lacks a 5' methyl cap on the mRNA. Since Lane C shows no translation product, it is presumably due to the lack of an IRES sequence; however, one cannot rule out that it requires a 5' methyl cap. Thus both responses were credited.

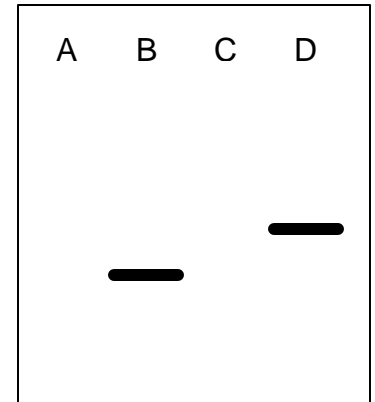
Construct D – **cap independent.** This construct is circular and lacks a 5' methyl cap. Lane D shows a translation product, and it appears that the construct has an active IRES.



b) In panel F, draw your expected results if you expressed each construct in tissue culture cells grown under oxidative stress conditions. Explain your response. (7 points)

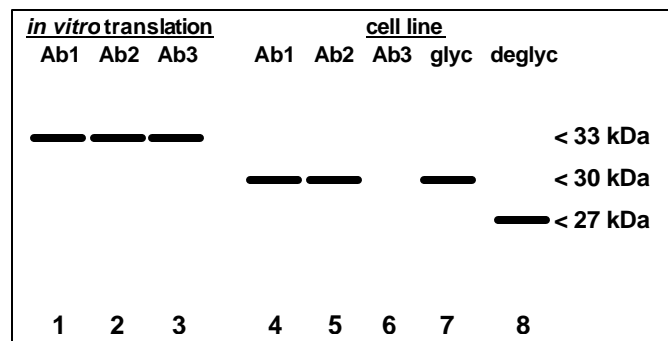
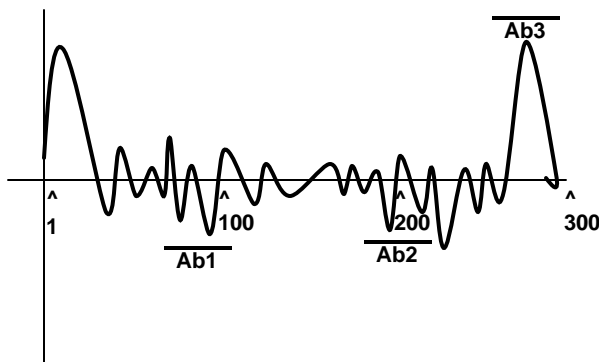
Panel F:

Your findings above suggest that the IRES segment is functional for initiation of protein translation from these RNA constructs. Since cap-independent translation relies on IRES sequences, we predict that only lanes A and B will contain the expected translation products.



MG3. You have been studying a 30 kDa glycoprotein (protein X) that is expressed on the cell surface. When you deglycosylate protein X, its apparent molecular weight is 27,000 Da on your protein gels. The average mass of an amino acid is 110 Da, and thus you estimate about 245 amino acids in length for deglycosylated protein X.

You recently isolated a cDNA that encodes protein X and identified an open reading frame (ORF) in your clone. Surprisingly, it encodes 295 amino acids – a difference of 50 amino acids from your prediction. You analyze your sequence and obtain a hydrophobicity plot showing two significant hydrophobic domains at the N- and C-terminus of protein X – each is about 25 amino acids in length.



a) What role does the large hydrophobic domain at the N-terminus of the protein likely play? (5 points)

The N-terminal segment is likely a signal sequence that facilitates translocation of the nascent peptide chain to the ER membrane surface where it can transit into the ER lumen. The signal peptidase associated with the translocon complex will cleave this signal peptide off.

b) You must assume that protein X runs true to size in your protein gels relative to your size markers. Propose a reason for the discrepancy between the predicted size from the open reading frame (~32,500 Da; 295 amino acids; see lanes 1-3) and that of the deglycosylated protein seen on your protein gels (27,000 Da; estimated 245 amino acids; see lane 8). (5 points)

The information provided suggests the loss of ~ 50 amino acids from the mature peptide. The *in vitro* translation product (~33 kDa) will lack the carbohydrate moiety. So the difference between this product and the deglycosylated protein (~27 kDa) expressed in eukaryotic cells is ~6 kDa. Loss of the signal peptide explains ~3 kDa. The hydrophobic C-terminal region suggests that this glycoprotein may be a GPI-anchored protein. GPI-linked proteins are formed when the C-terminal domain of the protein is removed and a glycolipid anchor is transferred to the main body of the protein at the C-terminus. Loss of this C-terminal segment accounts for ~3 kDa. Thus cleavage of the N- and C-terminal domains would explain the difference in mass between the *in vitro* translation product and the deglycosylated protein, and it would be consistent with your sequence analysis.

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b) You have antibodies (Ab1, Ab2, Ab3) that specifically recognize domains in three distinct regions of your protein (see above). When you test your cDNA in an *in vitro* transcription/translation reaction, all three Abs work and each identifies the 32.5 kDa protein (lanes 1-3). When you express your cDNA in eukaryotic cells, the 30 kDa glycoprotein is observed on your immunoblots (lanes 4, 5, and 7); however, only Ab1 and Ab2 identify protein X (lanes 4, 5). Ab3 does not detect protein X (lane 6). Why? How is this protein anchored in the plasma membrane. (5 points)

The *in vitro* translation product would retain both the N-terminal signal sequence and the C-terminal hydrophobic segment, and thus all 3 Abs identify the different epitope regions on the protein. The epitopes recognized by Ab1 and Ab2 are not affected. The epitope recognized by Ab3 is lost following cleavage of this domain from the protein, and the cleavage product is degraded. The glycoprotein is anchored into the plasma membrane via the GPI linkage.

Many responses suggested that the Ab3 did not work because the epitope was sequestered in the cell membrane and not accessible to the Ab. This idea would be relevant for immunomicroscopy; however, the question specified that you were using immunoblots in this study. In this situation, the cells are lysed and membranes are no longer intact. Partial credit was still given.