1. In order to please your demanding thesis advisor, you've completed an extensive fractionation and biochemical purification of proteins localized to the mitochondria, the chloroplasts, the peroxisomes, and, because you've gotten so good at purifying proteins, the nucleus, too. Everything was going fine, and you have tubes of protein extract from each of the organelles fresh out of the centrifuge and ready to be labelled (you're so good at the purification that you never label tubes until you are ready to put them in the freezer, but simply rely on memory to tell which is which while you are performing the purification). But on the way back from the centrifuge, the new (and somewhat clumsy) post-doc in the lab bumps into you and tubes go flying. You groan and say to yourself, "Great now I have to start the purification all over again, just because I didn't keep my tubes labelled. How can I ever sort this mess out?" The new post-doc, eager to redeem himself, suggests that you run a little of each extract out on an SDS-PAGE gel and sequence some of the proteins from each tube. That way you'll be able to tell relatively easily which tubes contain proteins from which organelles (and maybe finally learn the lesson of why it's important to ALWAYS LABEL YOUR TUBES!).

a. Assuming that the new post-doc is correct and this approach is possible, why would this type of experiment help you to sort out what type of proteins are in each tube?

b. You try the approach and obtain the following partial polypeptide sequences from 4 specific proteins you sequenced (listed using 1 letter amino acid code from N to C termini, sequence may be from either N or C terminus). Using these sequences, tell to which organelle the proteins would be targeted and give a short explanation of how you determined this.

1) MSTSVASSTVTASTVGPCIPVNQFWQNKMARHKCGHE--

2) MSKRTKPRLNGQPCSVMLFYGPSCFGHESTPYWFQ--

3) MSLQFWGQNDVILAQNTGPCKHRKGYWPCFMLDESTLLLLL--

4) SGRTWFGNEHWTRKLIVQLVCCGLQNAWTPLGSKL--

c. Do all of the proteins you've sequenced enter their target organelles in the folded state? If not, which ones enter in an unfolded state and how is accomplished?
d. What forms of energy do each of these proteins that you've sequenced require to be imported into their respective organelles?

2. You discovered 2 new proteins (Y and Z) that are encoded by nuclear genes, translated in the cytosol and then may be imported into the mitochondria. You have the cDNAs that encode these proteins. You want to know if these proteins are taken up by the mitochondria and where in the mitochondria these proteins would be targeted.
   a. How could you determine if the proteins were imported by the mitochondria? Design an experiment and don't forget the controls!
   b. It is formally possible that your proteins are normally targeted to the mitochondria, but that in this assay, they may not be imported. Why might that be and how could you test this?

c. You tried a very simple pilot experiment where you had protein X alone and then protein X added to mitochondria, and you observed the following result for protein X:

   ![Image of gel with lanes 1, 2, 3, and 4]

   Lane 1: protein X alone
   Lane 2: protein X with mitochondria (not purified from supernatant)

   Why does the lower band appear in lane 2?

d. Being a good scientist, you repeated the simple experiment, but realized afterward that the yeast (from which the mitochondria for the experiment were taken) were treated with Dinitrophenol (DNP), an agent which uncouples electron transport from proton movement. What result did you see in this experiment and why? Fill in lanes 3 and 4 on the gel with the expected bands (using lanes 1 and 2 as a guide).

e. You've heard from a reliable source (MCB, 4th ed.) that even though a mitochondrial protein can't be imported when there is no proton
motive force, it can still bind to the receptors of the outer mitochondrial membrane. Design an experiment to show that this is the case.

3. Predict the arrangement of each of the following proteins with respect to the ER membrane and lumen.

4. You are studying a human secreted protein that is 200 amino acids in length that has the following properties:
   - An arginine residue at amino acid 47 and a lysine residue at position 74
   - No other arginine or lysine residues
   - Only 3 cysteine residues at positions 36, 58, and 143
   - Only 3 asparagine residues at positions 30, 52, and 110
   - There are no O-linked sugars in this protein
   - A signal sequence at the N-terminus for insertion into the ER followed by a cleavage site at amino acid residue 23

You have the cDNA that encodes for the protein, so you use it to in-vitro translate the protein in a cell-free system without microsomes present. In addition, you use recombinant DNA technology to express the protein in cultured CHO cells, which do not normally express the protein. You then immunoprecipitate the secreted protein from the extracellular medium and incubate the protein as indicated for each lane below. After incubation, you run the protein on a non-reducing SDS-PAGE gel (i.e. no
reducing agent added) and silver stain to visualize the bands. (Notes: Trypsin is a protease that cleaves proteins after arginine and lysine residues; BME= β-Mercaptoethanol which is a reducing agent; PNGase F is an endoglycosidase that cleaves all Asn-linked sugars; the average molecular weight of an amino acid is 110 Daltons (Da)). Shown below is the gel:

Lanes 1-2: in-vitro translated proteins
   Lane 1: no trypsin + BME
   Lane 2: with trypsin + BME

Lanes 3-7: protein immunoprecipitated from extra-cellular medium of wild type cells
   Lane 3: no trypsin
   Lane 4: with trypsin
   Lane 5: with trypsin + BME
   Lane 6: with trypsin + PNGase F
   Lane 7: with trypsin + BME + PNGase F

Lanes 8-12: protein immunoprecipitated from the extracellular medium of cells lacking protein disulfide isomerase (PDI-)
   Lane 8: no trypsin
   Lane 9: with trypsin
   Lane 10: with trypsin + BME
   Lane 11: with trypsin + PNGase F
   Lane 12: with trypsin + BME + PNGase F

Using lanes 1-7, answer the following questions:

a) Is there any disulfide linkage in the secreted protein? If so, which cysteines are involved? Explain your answer.

b) Is the protein a glycoprotein? If so, which residue(s) are glycosylated? Explain your answer.
You now express your protein in cultured CHO cells that are mutant for protein disulfide isomerase (PDI) (i.e. PDI does not function in these cells). You discover that your protein is not secreted from these mutant CHO cells.

c) In which cellular compartment of the PDI- CHO cells would you find your protein? Why?

You repeat your immunoprecipitation from total cell lysate made from the PDI- mutant CHO cells and incubate your immunoprecipitated protein as indicated. The results of your experiments are shown in lanes 8-12.

Using lanes 8-12, answer the following questions:

d) Is there any disulfide linkage in the protein? If so, which cysteines are involved? Explain.

e) Is the protein glycosylated? If so, which residue(s) are involved?

5. You are evaluating the early steps in translocation and processing of the secretory protein prolactin. By a new experimental approach, you can use truncated prolactin mRNAs to control the length of the nascent prolactin polypeptides that are synthesized. When prolactin mRNA that lacks a chain-termination (stop) codon is translated in vitro, the newly synthesized polypeptide ending with the last codon included on the mRNA will remain attached to the ribosome, thus allowing a polypeptide of defined length to extend from the ribosome.

You have generated a set of mRNAs that encode segments of the N-terminus of prolactin of increasing length, and each mRNA can be translated in vitro by a cytosolic translation extract containing ribosomes, tRNAs, aminoacyl-tRNA synthetases, GTP, and translation initiation and elongation factors. When radio-labeled amino acids are included in the translation mixture, only the polypeptide encoded by the added mRNA will be labeled. After completion of translation, each reaction mixture was resolved by SDS-PAGE, and the labeled polypeptides were identified by autoradiography.

The autoradiogram depicted below shows the results of an experiment in which each translation reaction was carried out either in the presence (+) or absence (-) of microsomal membranes.
a) Based on the gel mobility of peptides synthesized in the presence or absence of microsomes, deduce how long the prolactin nascent chain must be in order for the prolactin signal peptide to enter the ER lumen and to be cleaved by signal peptidase. (Note that microsomes carry significant quantities of SRP weakly bound to the membranes.)

b) Given this length, what can you conclude about the conformational state of the nascent prolactin polypeptide when it is cleaved by signal peptidase? The following lengths will be useful for your calculation: the prolactin signal sequence is cleaved after amino acid 31; the channel within the ribosome occupied by a nascent polypeptide is about 150 angstroms (Å) long; a membrane bilayer is about 50 Å thick; in polypeptides with an \( \alpha \)-helical conformation, one residue extends 1.5 Å, whereas in fully extended polypeptides, one residue extends about 3.5 Å.

The experiment described in part (a) is carried out in an identical manner except that microsomal membranes are not present during the translation but are added after translation is complete. In this case, none of the samples shows a difference in mobility in the presence or absence of microsomes.

c) What can you conclude about whether prolactin can be translocated into isolated microsomes post-translationally?

In another experiment, each translation reaction was carried out in the presence of microsomes, and then the microsomal membranes and bound ribosomes were separated from free ribosomes and soluble proteins by centrifugation. For each translation reaction, both the total reaction (T) and the membrane fraction (M) were resolved in neighboring gel lanes. The autoradiogram is shown below.
d) Based on the amounts of labeled polypeptide in the membrane fractions in the autoradiogram, deduce how long the prolactin nascent chain must be in order for ribosomes engaged in translation to engage the SRP and thereby become bound to microsomal membranes.

6. You identify a novel human protein and you have no idea what its function is. However, you have made an antibody against it, and upon performing an immunofluorescence experiment in cultured HeLa cells, you observe that the protein is present in the nucleus during G1, but is present in the cytoplasm throughout the rest of the cell cycle. You would like to know whether the shuttling of the protein in and out of the nucleus is important for its function.

You reason that the protein must have an NES (nuclear export signal) and you want to mutate the signal and examine the consequences.

In order to identify the NES sequence in your protein, you perform a heterokaryon experiment. You subdivide your protein into four segments, A, B, C, and D, and fuse each segment individually onto the C-terminus of the human nucleoplasmin protein using recombinant DNA technology. The wild type nucleoplasmin protein localizes to the nucleus at all times during the cell cycle. You express each fusion protein in HeLa cells, and then fuse the transgenic HeLa cells with Xenopus cells, creating a heterokaryon where both the HeLa cell and Xenopus nuclei are in a common cytoplasm. Using an antibody to the human nucleoplasmin protein (which does not recognize its Xenopus homolog) you examine the localization of each fusion protein. You obtain the following data:

<table>
<thead>
<tr>
<th>Fusion protein</th>
<th>Detected in HeLa cell nucleus</th>
<th>Detected in Xenopus cell nucleus</th>
</tr>
</thead>
<tbody>
<tr>
<td>h-nucleoplasmin only</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>h-nucleoplasmin-A</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>h-nucleoplasmin-B</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>------------</td>
<td>--------------</td>
<td>---------</td>
</tr>
<tr>
<td>h-nucleolusmin-C</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>h-nucleolusmin-D</td>
<td>yes</td>
<td>no</td>
</tr>
</tbody>
</table>

a) Which fragment(s), A, B, C, or D, contains the NES and how do you know?

b) If you now wanted to identify the NLS in your protein and you did not have the protein’s sequence, how could you do so?