1. In preliminary studies you've determined that your partially purified protein is stable (retains activity) between pH 5.0 and pH 7.5. On either side of that pH range the protein is no longer active. Your advisor now wants you to do a quick experiment to determine conditions for ion-exchange chromatography. He has left instructions for you. First, you're supposed to mix a bit of the crude preparation with a small amount of the ion-exchange resin DEAE-Sepharose in a series of buffer solutions that have a pH between 5.0 and 7.5. Next, you are to pellet the resin and assay the supernatant for the presence of your protein. Finally, he tells you to use this information to pick the proper pH to do the ion-exchange chromatography. You have completed the first two steps and have obtained the results shown in Figure 1. But you are a little uncertain as to how to use the information to pick the pH for the chromatography

A. At which end of the pH range is the charge on your protein more positive and at which end is it more negative? [Over this pH range the positively charged amine groups on the DEAE-Sepharose beads (Figure 1) are unaffected]
B. For the chromatography should you pick a pH at which the protein binds to the beads (pH 6.5 to 7.5) or a pH where it does not bind (pH 5.0 to 6.0)? Explain your choice.
C. Should you pick a pH close to the boundary (that is, pH 6.0 or 6.5) or far away from the boundary (that is, pH 5.0 or pH 7.5)? Explain your reasoning.
D. How will you carry out ion-exchange chromatography of your protein? What are the various steps you will use to accomplish the separation of your protein from others via ion-exchange chromatography?

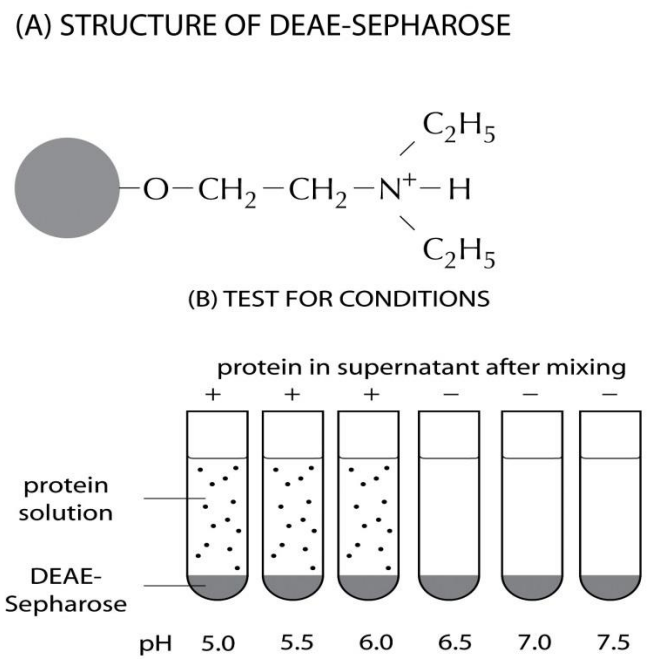

Figure 1 Preliminary test to determine conditions for ion-exchange Chromatography. (A) Structure of the charged amine groups attached to Sepharose beads. (B) Results of mixing your protein with DEAE Sepharose beads. Samples of the protein were mixed with DEAE-Sepharose beads in buffers at a range of pH values, and then the mixtures were centrifuged to pellet the beads. The presence of the protein in the supernatant is indicated by a +; its absence is indicated by a -.

2. You want to know the sensitivity for detection of immunoblotting (Western blotting), using an enzyme-linked second antibody to detect the antibody directed against your protein (Figure 2A). You are using the mouse monoclonal antibody 4G10, which is specific for phosphotyrosine residues, to detect phosphorylated proteins. You first phosphorylate the myelin basic protein in vitro using a tyrosine protein kinase that adds one phosphate per molecule. You then prepare a dilution series of the phosphorylated protein and subject the samples to SDS-PAGE. The protein is then transferred (blotted) onto a nitrocellulose filter, incubated with the 4G10 antibody and washed to remove unbound antibody. The blot is then incubated with a second goat anti-mouse antibody that carries horseradish peroxidase (HRP) conjugated to it, and any excess unbound antibody is again washed away. You place the blot in a thin plastic bag, add reagents that chemiluminesce when they react with HRP (Figure 2A), and place the bag against a sheet of x-ray film. When the film is developed you see the picture shown in Figure 2B.

A. Given the amounts of phosphorylated myelin basic protein indicated in each lane in Figure 2B, calculate the detection limit of this method in terms of molecules of protein per band.
B. Assuming that you were using monoclonal antibodies to detect proteins, would you expect that the detection limit would depend on the molecular mass of the protein? Why or why not?

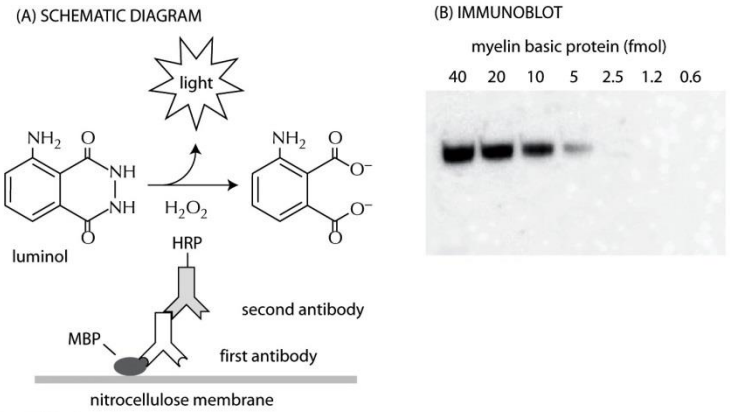

Figure 2- Sensitivity of detection of immunoblotting.
(A) Schematic diagram of experiment. MBP stands for myelin basic protein. In the presence of hydrogen peroxide, HRP converts luminol to a chemiluminescent molecule that emits light, which is detected by exposure of an x-ray film. (B) Exposed film of an immunoblot. The number of femtomoles of myelin basic protein in each band is indicated.

3- You have isolated the proteins from two adjacent spots after two-dimensional polyacrylamide-gel electrophoresis and digested them with trypsin. When the masses of the peptides were measured by MALDI-TOF mass spectrometry, the peptides from the two proteins were found to be identical except for one (Figure 3). For this peptide, the mass to charge (m/z) values differed by 79.97, a value that does not correspond to a difference in amino acid sequence. (For example, glutamic acid instead of valine at one position would give an m/z difference of around 30.) Can you suggest a possible difference between the two peptides that might account for the observed m/z difference?

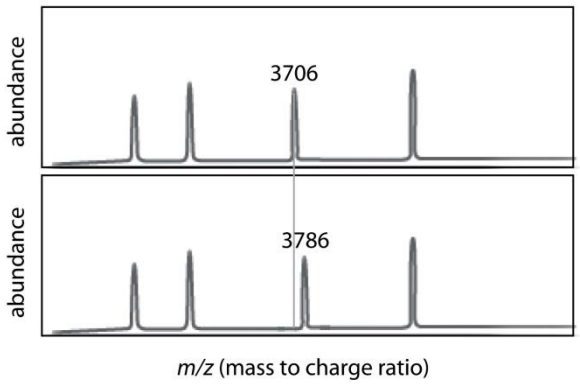

Figure 3- Masses of peptides measured by MALDI-TOF mass spectrometry.

4-You have raised four different monoclonal antibodies to *Xenopus* Orcl, which is a component of the DNA replication origin recognition complex (ORC) found in eucaryotes. You want to use the antibodies to immunopurify other members of ORC. To decide which of your monoclonal antibodies-TK1, TK15, TK37, or TK47-is best suited for this purpose, you covalently attach them to beads, incubate them with a *Xenopus* egg extract, spin the beads down and wash them carefully, and then solubilize the bound proteins with SDS. You use SDS-PAGE to separate the solubilized proteins and stain them, as shown in Figure 4.

A. From these results which bands do you think arise from proteins that are present in ORC?
B. Why do you suppose the various monoclonal antibodies give such different results?
C. Which antibody do you think is the best one to use in future studies of this kind? Why?

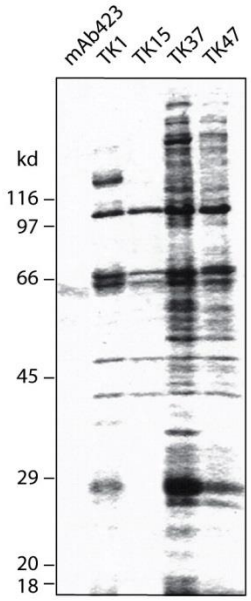

Figure 4- lmmunoaffinity purification of *Xenopus* ORC. The monoclonal antibody mAb423 is specific for an antigen not found in *Xenopus* extracts and thus serves as a control. The positions of marker proteins are shown at the *left* with their masses indicated in kilodaltons.

5. The surfaces of different varieties of cyclin A proteins all contain a hydrophobic cleft that is known to bind the sequence PSACRNLFG. This sequence is found close to the N-terminus of the cyclin-dependent kinase inhibitor p27, Looking for potential anti-cancer drugs, you decide to use phage display to hunt for peptides with very high affinity for cyclin A. You attach cyclin A to the bottoms of plastic dishes and 'pan' for phage that will bind (Figure 5). You use three different phage M13 libraries that bear randomized 7-, 12-, or 20- amino acid sequences on one of their coat proteins. You isolate phages that bind to the immobilized cyclin A and sequence the segment of their coat protein gene that encodes the peptide. Sequences 14 clones are shown in Table below. By comparing all 15 sequences. Decide which amino acid residues are likely to be the most critical for binding to cyclin A.

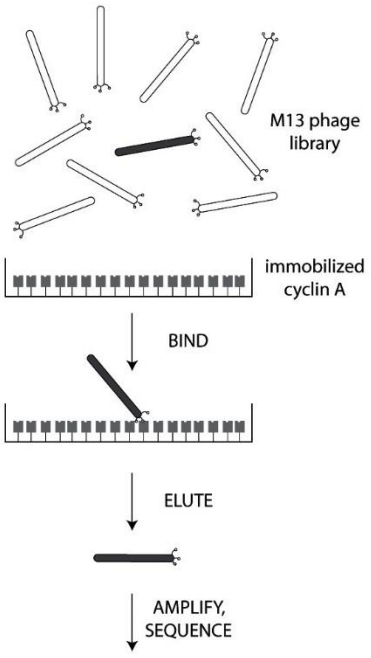

Figure 5 Panning e phage-display library for phages that bind to immobilized Cyclin A. The M13 phage library contains I randomized segment of the coat protein gene so that each phage displays one of a large number of possible amino acid sequences on its surface. The phage library is incubated with the immobilized protein in the plate, unbound phage are washed away, and bound phage are eluted and amplified by growth in *E. coli.* This cycle is repeated 2-3 mere rimes and then individual phages are isolated, amplified, and analyzed by sequencing.

CLONE	LIBRARY	PEPTIDE SEQUENCE
1	7-mer	LEPRMLF
2	7-mer	TLPRQLF
3	7-mer	LKPTKLF
4	7-mer	LIPKNLF
5	7-mer	FLPRALF
1	12-mer	NVRVELFPPTKV
2	12-mer	KSSVVRSLFVPT
3	12-mer	ERPSAQRSLVFW
4	12-mer	NLFYPRNLFPEF
5	12-mer	YPSPARNLLPMF
6	12-mer	ATIRELFPPTLP
1	20-mer	HQPESVKRSLFKPAHSALEP
2	20-mer	EVARRELFADHSLVHVGHVR
3	20-mer	EHKALPGKAVTGPKRELVFQ
p27 cyclin A-binding sequence		PSACRNLFGP

Table 1- Peptides encoded by phages that were selected by panning of a phage-display library against Immobilized cyclin A.

6. You wish to make a restriction map of a 3.0-kb BamHI restriction fragment. You digest three samples of the fragment with EcoRI, HpaII, and a mixture of EcoRI and HpaII, You then separate the fragments by gel electrophoresis and visualize the DNA bands by staining with ethidium bromide (Figure B-6). From these results, prepare a restriction map that shows the relative positions of the EcoRI and HpaII recognition sites and the distances in kilobases (kb) between them.

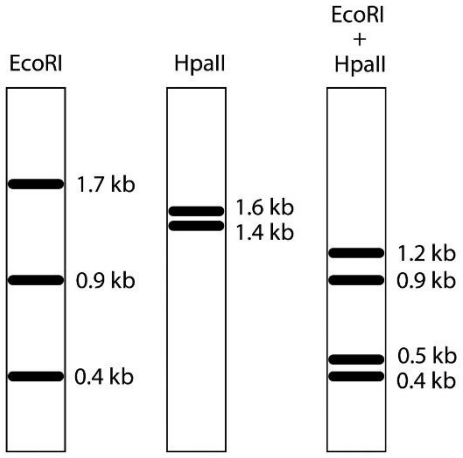

Figure 6- Sizes of DNA bands produced by digestion of a 3.0-kb fragment by EcoRI, HpaII, and a mixture of the two. Sizes of the fragments are shown in kilobases.