

7.28 Spring 2005

Exam Two

Name \_\_\_\_\_

Question 1     \_\_\_\_\_/ 30 points

Question 2     \_\_\_\_\_/ 20 points

Question 3     \_\_\_\_\_/ 26 points

Question 4     \_\_\_\_\_/ 24 points

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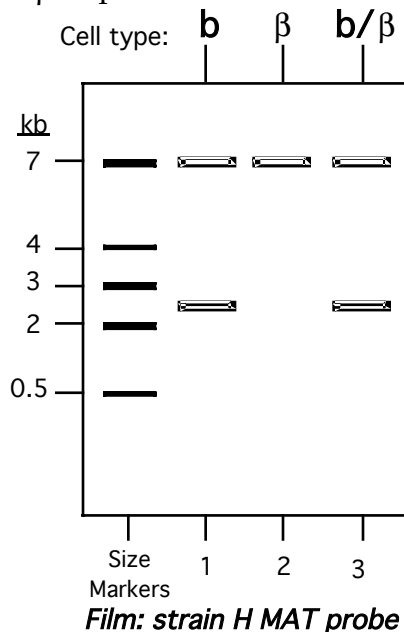
Total            \_\_\_\_\_/ 100 points

Name \_\_\_\_\_

**Question 1 (30 Points total).**

You have discovered a new species of yeast (*M. cathecus*) that is able to switch mating types in a manner similar to the normal budding yeast, *S. cerevisiae*. *M. cathecus* cells exist in three forms: haploids with the **b** mating type, haploids with the  $\beta$  mating type, and **b**/ $\beta$  diploids. The genome sequence of *M. cathecus* is not yet known.

You succeed in cloning the *MAT* locus from one specific isolate of *M. cathecus*, called strain H. Unfortunately, strain H grows poorly in the lab, and its mating type is unknown. Using this cloned DNA, you make a radioactive probe and use this DNA to probe a genomic Southern blot of total *M. cathecus* DNA that has been digested with the restriction enzyme EcoRI. The DNA from three different cultures of a strain well-adapted to lab growth conditions is present on the blot. The cultures contained the following types of cells: (1) **b** cells, (2)  $\beta$  cells and (3) **b**/ $\beta$  diploid cells.



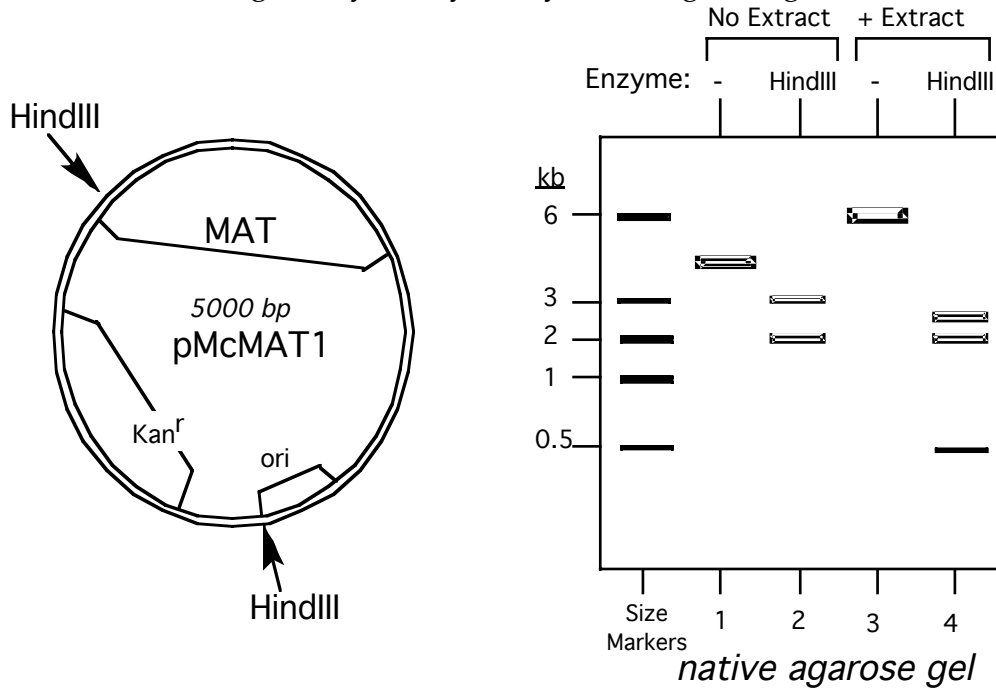
**1a (6 points)** Based on these data, what size EcoRI fragment carries the *MAT* locus? Also, which mating type was strain H? Briefly explain your answers.

**The 2.5 kB fragment carries the *MAT* locus. Strains of mating types **b** and  $\beta$  should be different at their *MAT* locus since this contains the genes that determine mating type. The 2.5 kB fragment is present in **b** strain but not in  $\beta$  strain indicating that this fragment most likely contains the *MAT* locus.**

**Strain H is **b** type. The probe made from its *MAT* locus recognizes two bands in the **b** strain-one copy at the *MAT* locus and one copy in the HML/HMR-like silent locus. The probe only recognizes one band in the  $\beta$  strain-the one copy in its HML/HMR-like silent locus.**

Name \_\_\_\_\_

As is true in budding yeast, *M. cathecus* encodes a protein that introduces a double-stranded break (DSB) at the *MAT* locus. Using your cloned *MAT* DNA as a substrate, you are able to observe this DNA cleavage in *M. cathecus* cell extracts. A schematic map of the *MAT* clone is shown below, as are the results of some *in vitro* DNA cleavage assays analyzed by native agarose gel.



**1b (5 points)** Based on these data, draw on the schematic of the *MAT* clone where the *M. cathecus* nuclease cleaves the DNA.

**The 0.5 kB band in Lane 4 indicates that the nuclease cleaves the DNA 0.5 kB to the right of the HindIII site in the *MAT* locus on the plasmid. Some people put the site near the *ori*, but this is wrong since we know the nuclease makes a DSB within the *MAT* locus.**

**1 c (5 points)** If the genome size of *M. cathecus* is ~ 20 megabases, what is the minimum length the recognition sequence for the *MAT*-cleaving nuclease is likely to be? Briefly explain your logic. (If you don't have a calculator, set up the equation you would use to calculate this.)

**There should be only one recognition site for the *MAT*-cleaving nuclease in the entire genome since this cleavage event needs to be tightly regulated and only occurs at the *MAT* locus. To determine what the minimum length of that sequence is, solve for *x* in the following equation:**

$$(1/4)^x = 1/20,000,000$$

**1/4 is the probability of getting a specific base in any one position, and 20,000,000 is the size of the genome.**

**Note:** Technically, there are actually three sites in the genome that have this nuclease recognition sequence - at the *MAT* locus, *HMR* and *HML*. The cleavage sites in *HMR* and *HML* are inaccessible to the endonuclease due to chromatin structure. This point was not required for full credit because we did not discuss this in class.

**1d (6 points)** You notice that when you add ATP to the extract assays for *MAT* cleavage (as in part b) that the DNA is further digested, suggesting that processing at the DSB is occurring *in vitro*. Therefore, you decide to investigate how the DSB at the *MAT* locus is processed prior to assembly of RecA-like proteins on this DNA. Design a simple experiment to ask if this processing occurs using a mechanism similar to RecBCD or similar to the MRX complex. Briefly explain how your experiment distinguishes between these two pathways. (*Hint: it may be helpful to use material introduced earlier in this question.*)

**RecBCD degrades DNA strands in both directions: 5' → 3' and 3' → 5'. The MRX complex only degrades in the 5' → 3' direction. The best way to distinguish between these two mechanisms is to do a Southern blot.**

- 1. Add the cloned *MAT* DNA to *M. cathecus* cell extract +/- ATP. The -ATP extract is a negative control.**
- 2. Incubate to allow time for DSB formation and DNA processing.**
- 3. Isolate DNA from extracts and cut with HindIII.**
- 4. Run on a denaturing gel. Transfer DNA to a positively-charged membrane.**
- 5. Probe membrane with a probe specific to the 5' → 3' 0.5 kb fragment HindIII site-5'----- 0.5 kb fragment ----- 3'. Make the probe specific to part of the strand near the Hind III site.**

**If the *M. cathecus* machinery is like RecBCD, you should see a band that is less than 0.5 kb because the 3' end has been degraded. If the *M. cathecus* machinery is like MRX, you should see a band that is 0.5 kb because the 3' end has not been degraded.**

**(You could do the same experiment using a probe for the 2.5 kb fragment containing the other side of the *MAT* locus.)**

**1e (8 points)** You purify the Rad51 protein (McRad51) and a BRCA2 homolog from *M. cathecus*. DNA binding experiments reveal that McRad51 binds DNA in a highly cooperative manner and that BRAC2 can load McRad51 on an ssDNA-dsDNA junction. Binding of McRad51 is more cooperative than observed with the *S. cerevisiae* Rad51 (ScRad51). You reason that the functional Rad51 filaments assembled by McRad51 therefore may be longer than those made by the same concentration of ScRad51. Design an *in vitro* experiment to determine if this hypothesis is correct (you also have purified *S. cerevisiae* recombination proteins). Be sure to include a description of the expected results of this experiment if the hypothesis proves true.

**In order to test whether the functional filaments made by McRad51 are longer than those made by ScRad51, you need to do a strand exchange assay like the one used in Yang *et al.***

- 1. Make a gDNA substrate with a ss-ds DNA junction. Add RPA, then McBRCA2, then McRad51 or ScRad51 or a buffer control.**
- 2. In separate experiments, add labeled ds DNA that is homologous to the gDNA substrate—begin with a region next to the ss-ds junction and proceed along the substrate at increasing distances from the junction. A good negative control for this experiment is to use labeled dsDNA that is not homologous to any region on the gDNA substrate.**
- 3. Run samples on a native gel—labeled DNA that was not exchanged will run near the bottom of the gel, labeled DNA that was successfully exchanged will be shifted up to the size of the gDNA substrate.**

**If the functional filaments made by McRad51 are longer than those made by ScRad51, you will see strand exchange occurring at longer distances from the junction for McRad51 compared to ScRad51.**

**NOTE: Gel shift, DNaseI protection, or cooperative binding assays were not correct answers because the question asked for a functional analysis. In addition, the question stated that binding of McRad51 had already been determined to be more highly cooperative than that of ScRad51.**

Name \_\_\_\_\_

**Question 2 (20 points total)**

**2A (6 points).** You have isolated spontaneous mutations in the LEU6 gene of a haploid strain of the yeast *S. cerevisiae*. Some of your mutations result in a complete loss of LEU6 gene function. You reason that this phenotype may be due to insertion of a mobile DNA element into the gene.

To test this idea, you sequence the LEU6 region from several of your mutants and compare the DNA to the previously determined sequence for LEU6 in the parental strain. Numerous strains do in fact carry insertions, and a quick analysis indicates that several different types of mobile DNA elements are represented in the group of mutants.

You wish to determine if the insertion mutations are caused by: (1) a cut and paste transposon; (2) a retrovirus, or (3) a DNA virus that inserts by conservative site-specific recombination.

**2a (6 points).** Below is the sequence of one of the insertion mutations (Some sequences from the middle of the element deleted at the // symbol.) The sequence of the parental DNA at the insertion site is also shown. Based on these data, which class or classes of elements are most likely responsible for the mutation in this isolate? Briefly explain.

Chromosomal carrying insertion (inserted DNA sequences are in capital letters):

5'tcgcagcgcacgccttGAGCGGGACTCTGGGTATACGGATCCC//GTTTCGT  
ATACCCAGAGTCCCGCTCgccttctatgccttcttgacgagttcttct□

Chromosomal site:

5'tcgcagcgcacgccttctatgccttcttgacgagttcttct□

**In this mutant you can see target site duplication and simple inverted repeats so the mutation is most likely caused by (1) cut and paste transposition. Many students thought it might be retroviral insertion, however, the LTRs common to retroviruses are different from the inverted repeats common to transposition. 2pts for noticing the inverted repeats, two pts for noticing the target site duplication and 2 pts for getting the correct answer.**

Name \_\_\_\_\_

**2B (6 points).** One particular insertion allele shows a relatively high frequency (~1 in a 100 cells) of spontaneous reversion to the Leu<sup>+</sup> phenotype. Of the three classes of elements you are considering, which type of element is most likely to be responsible for this “unstable” allele. Explain your choice.

**This mutation is most likely caused by (3), the DNA virus that inserts by CSSR. The mechanism by which these viruses leave the host DNA both preserves the original sequence and does not need much energy leading to a high reversion rate, reversion to wildtype sequence and hence wildtype phenotype. Many students thought that cut and paste transposition might have just as high a reversion rate (which theoretically could be true), however, cut and paste transposition usually leaves extra DNA sequence behind, meaning that the leu gene would not revert to wildtype sequence and hence the cells would not be Leu+. 2pts for noting the conservation of energy of CSSR, 2pts for noting that it does not change the sequence upon looping out and 2 pts for the correct answer.**

**2C (8 points).** To identify strains that carry retrovirus insertions you decide to take a biochemical approach. You reason that cells harboring a retrovirus should contain an enzyme that is not normally present in uninfected cells and that you should be able to detect this enzyme in cell extracts. What is the enzyme you will look for? Briefly describe how you would set up a sensitive, functional assay for this enzyme.

**Retrovirally infected cells would contain the enzyme reverse transcriptase. There were many variations of functional assays for this enzyme and points were given depending on whether these assays would work or not. Here is one possible answer: A sensitive functional assay using cell extracts would involve testing for the presence of some cDNA using radiolabeled dNTPs. One would probably want to add exogenous RNA template of known sequence with a primer (RT requires a primer) and then look for the cDNA version of that RNA template (southern, gel, filter binding to look for general dNTP incorporation). Good controls were definitely required for this experiment – radiolabeled dNTPs could be incorporated into DNA by the endogenous DNA pols or DNA repair enzymes so a filter binding probably wouldn't be the best means for detecting label. Some students wanted to rid the extract of DNA and RNA, which would cut down on background quite a bit.**

**Points were given for knowing that the enzyme to test for was RT (2 pts), for an assay that at least made theoretical sense, for a correct template (RNA + primer) and for good controls.**

**Question 3 (26 points total).** You and your labmate are studying RNA Pol II transcription. She has developed a clever genetic screen to isolate mutants in TFIID. Unfortunately for your colleague, the method that she used makes it very difficult to clone the mutations but relatively easy to purify the mutant TFIID complexes (called M1, M2, M3). As the lab biochemist, you offer to purify the mutant TFIID complexes and determine their defects.

**3A (6 points).** You first want to determine whether the three mutant TFIID complexes are capable of assembling into a RNA Pol II transcription pre-initiation complex. Describe the assay you would use. You have access to purified RNA Pol II and its auxiliary transcription factors and any DNA that you need.

**3A. A simple gel shift should work to look at binding of TFIID to the RNA Pol II complex. Many students wanted to use antibodies to TFIID to do a supershift assay, but this probably wasn't needed due to the fact that we already knew TFIID should bind to the complex. The supershift assay might not be a bad control, but these reagents weren't included in the list given in the question.**

**The gel shift assay would require labeled promoter DNA, with RNA Pol II plus aux. TFs, adding the mutant TFIIDs. Controls would be no TFIID (lesser shift) and wildtype TFIID (should see more shift). Run on non-denaturing gel and do Xray to detect labeled DNA.**

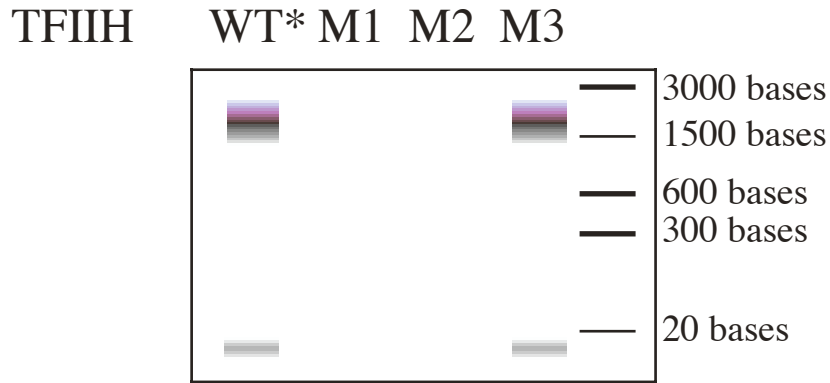
You find that the M2 and M3 mutant proteins are both capable of assembling into the RNA Pol II transcription complex.

You next perform an incorporation assay using a simple CORE promoter that contains only a TATA and BRE element (CORE). To assay the function of the TFIID proteins, you make a crude cell extract and specifically deplete the endogenous TFIID (with a TFIID antibody). You add the promoter to the extract in the presence of a wild-type (WT) TFIID, or the various purified mutant TFIID complexes.

You separate the products on an agarose gel and expose it to X-ray film.



Name \_\_\_\_\_



Denaturing Agarose Gel  
(exposed to X-ray film)

**3B (6 points).** Based on your findings thus far and your knowledge of TFIIH, what function is most likely defective in the M2 mutant. Briefly describe the assay you would use to test your hypothesis.

**We see that the M2 mutant is capable of assembling into the RNA Pol II txn complex but no transcript is made (not even abortive transcript). This suggests that the mutant TFIIH can't perform its helicase activity. Test this by performing a DNA unwinding assay:**

1. Set up by adding RNA Pol II, plus transcription factors to the promoter DNA
2. Add ssDNA cleaving reagent (KMnO<sub>4</sub>)
3. Anneal labeled primer near promoter (facing promoter)
4. Add DNA polymerase to extend primer
5. Run on denaturing gel and expose to film

**Do controls: +/- wt TFIIH**

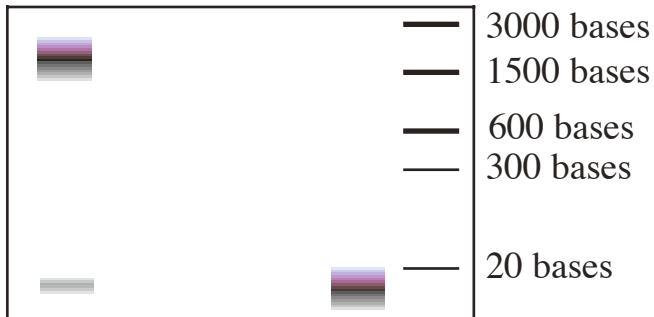
**3C (4 points).** When you perform the assay to test your hypothesis, you are surprised to find that even your wild type protein shows no activity. You talk to your advisor and she suggests that you should add ATP to your assay. You immediately realize why your assay didn't work. Briefly explain why you needed to add ATP.

**The helicase activity of TFIIH requires ATP (TFIIH is an ATPase).**

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You are surprised by the strong transcription you observe with the M3 mutant. You wonder if it might show more of a defect in the presence of a promoter that is activated. To test this you use a different model promoter (called CORE+Act) that has several binding sites for an activator that you know works by binding to the mediator. You repeat the previous transcription assays with this new promoter and observe the following results.

WT\* M1 M2 M3



Denaturing Agarose Gel  
(exposed to X-ray film)

**3D (4 Points).** What aspect of TFIIF function do you think is defective in the M3 mutant? Explain the different products you observe when the Core vs. Core+Act promoters are used.

**The M3 TFIIF mutant can assemble into the RNA Pol II txn complex, and we can see full size transcript with the CORE promoter, but only abortive transcript with the CORE+ACT promoter. The CORE+ACT promoter recruits the Mediator to the promoter. The Mediator binds the CTD of RNA Pol II when it is unphosphorylated and releases RNA Pol II when the CTD becomes phosphorylated (by TFIIF). Most likely, the M3 TFIIF mutant can no longer phosphorylate the CTD of RNA Pol II. Without phosphorylation of the CTD, Mediator never releases RNA Pol II and promoter clearance can't occur (thus the abundance of the abortive transcripts). In the case of the CORE promoter, there is no Mediator binding and thus the RNA Pol II is not dependent on TFIIF phosphorylation of the CTD to undergo promoter clearance.**

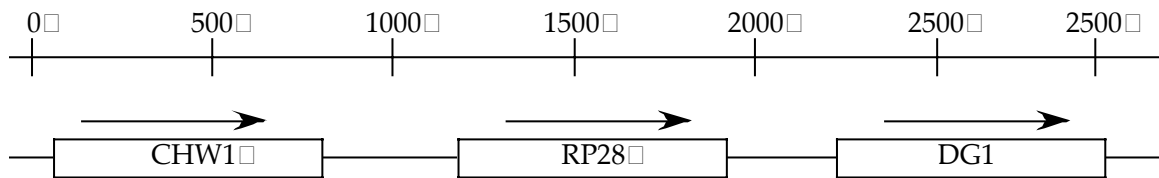
Name \_\_\_\_\_

You repeat the experiment one more time using a third promoter that has binding sites for a different activator that functions by recruiting TFIID to its binding site on the promoter.

**3E (6 Points).** Based on your proposed defect in the M3 mutant, draw the pattern of transcription products you would expect with this new promoter (show both WT and the M3 mutant). Provide a brief explanation of the logic behind the pattern that you have drawn.

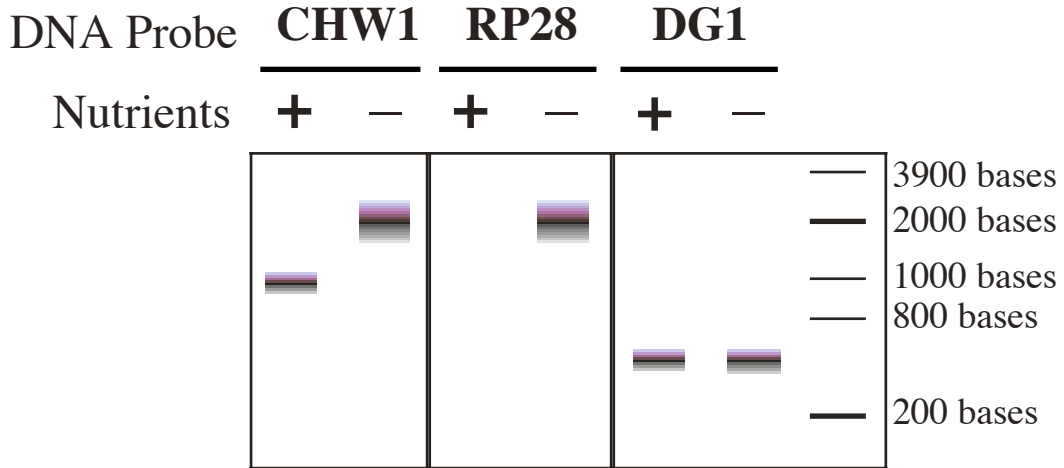
**The pattern of transcripts would most likely look like wildtype. In this case, the Mediator complex is not being recruited by the activator, rather TFIID is, so phosphorylation of the CTD is not as important for release of RNA Pol II from the promoter.**

**Question 4 (24 points).** You are studying the expression of an *E. coli* ribosomal protein (RP28) that inhibits protein synthesis by binding a short RNA hairpin (5'-GAUAGNNNCUAUC-3') found in one of the ribosomal RNAs. This protein is only expressed when there are very low nutrients to reduce protein synthesis and conserve resources. The location of the RP28 gene and its two neighbors is illustrated below (the arrows indicate the direction of transcription).



As a first step, you perform a northern blot under high and low nutrient conditions and probe the blot with a DNA probe that will hybridize to the RP28 mRNA. You are surprised to see only one transcript that is much longer than you expected. To determine whether the rest of the transcript overlaps the CHW1 or the DG1 gene you use DNA probes for each of these RNAs. The results of all three northern blots are shown below.

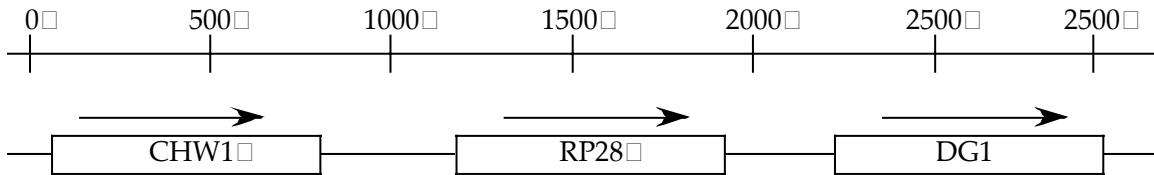
### Northern Blot



**4A (4 points).** Based on the results of the northern blots, how many different mRNAs are synthesized that overlap the DG1, RP28 and/or CHW1 genes?

**3 different mRNAs were made- 1 ~ 600 bases, 1 ~ 1000 bases, and 1 ~ 2000 bases. This question was asking for the total number of unique mRNAs based on the Northern blot data.**

**4B (6 points).** Draw the approximate location of each transcript on the map below.



**Transcript 1 overlaps CHW1 and is ~ 1000 bases long. Transcript 2 overlaps CHW1 and RP28 and is ~ 2000 bases long. Transcript 3 overlaps DG1 and is ~ 600 bases long.**

Based on your map, you suspect that transcription termination plays an important role in the control of RP28 expression. Because of this you decide to precisely map the 3' ends of each of the mRNAs that are detected by the CHW1 and RP28 probes.

**4C (6 points).** Describe the assay you would use including the location of any DNA probes you would need and how they would be labeled.

**Use an S1 protection assay to map the 3' ends of these genes.**

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1. Isolate RNA from + and – nutrient cells.
2. Make 2 ss DNA probes labeled on the 3' end or along entire length.  
Probe#1 should overlap the end of the 1000 base mRNA(CHW1), and  
Probe#2 should overlap the end of the 2000 base mRNA(CHW1 + RP28).
3. Hybridize Probe#1 to the RNA from + nutrient cells. Hybridize Probe#2 to the RNA from – nutrient cells.
4. Treat with S1 nuclease to cleave ssDNA.
5. Separate DNA on a high resolution denaturing gel to distinguish the 3' ends of the RNAs.

You find that there is an intrinsic terminator very near the 3' end of the ~2000 base transcript. In contrast, you do not find an intrinsic terminator near the end of the shorter ~1000 base transcript. Instead you find a C-rich region approximately 200 bases before the end of the transcript.

Interestingly you also find 12 copies of the RP28 RNA hairpin binding site between the C-rich region and the termination site that you mapped for the short transcript.

**4D (8 points).** Using your understanding of the function of the Rho termination factor and the results of your transcript mapping experiments, propose a model that explains the presence of the larger transcript in the presence of low nutrients.

**In high nutrients, Rho binds the C-rich region, moves in a 5' -> 3' direction toward RNAP, and pulls the RNA out of the active site to terminate transcription at 1000 bases.**

**In low nutrients, Rho must be downregulated—there may be less ATP available in starved cells, or Rho transcription or translation may be inhibited. As Rho is being downregulated, RNAP is able to read through the RP28 template and terminate at 2000 bases at the intrinsic terminator. The small amount of RP28 protein that is initially made can bind to the RP28 RNA hairpin binding site after the C-rich region. This binding event blocks any available Rho from “catching up” with RNAP near the 1000 base termination site. A positive feedback loop is created—as more RP28 is transcribed and translated, fewer 1000 base transcripts can be made as Rho is inhibited.**