Question 1. Your lab is studying the MRX complex in *S. pombe*, otherwise known as fission yeast. For your dissertation work, you are studying the function of Rad50, one of the subunits of this complex. RecBCD, an enzyme in *E. coli*, plays a similar role in homologous recombination to the MRX complex.

1A) What is different about the resulting resection of ends by these two complexes?

When you first joined the lab, you did a genetic screen to isolate temperature-sensitive mutants with defective spore viability. You determined that one of these mutants has a specific missense mutation in the gene encoding Rad50, and you call the mutant *rad50-ts*.

rad50-ts mutant spore viability is 0.1% of wild type levels when measured at high temperature. *rec12*$^{-}$, a mutant in the *S. pombe* Spo11 homolog, is also defective in spore viability. *rec12*$^{-}$ mutant spore viability is 20% of wild type levels. You make a double mutant strain containing both of these mutations and find that spore viability of *rad50-ts* increases 100-fold.

1B) How can you explain this result?

In further characterization of the *rad50-ts* phenotype, you find that these cells are defective in the removal of the Spo11 protein from DSB ends.

Because you know that homologous recombination is involved in some kinds of DNA repair and the DNA damage response pathway, you test the sensitivity of *rad50-ts* vegetative cells (mitotically dividing) to several different mutagens at the restrictive temperature:

Mutagen	**Percentage viability (compared to WT)**
Camptothecin*	47%
Gamma-irradiation	95%
Ethyl methane sulfonate (EMS)	98%

* CPT (Camptothecin) is a topoisomerase I inhibitor. Single-strand breaks persist because CPT prevents completion of the topoI catalyzed reaction.

1C) Why does the CPT-sensitivity make sense given what you know about the *rad50-ts* phenotype? What do you hypothesize is a crucial function of Rad50 *in vivo*?

1D) You might expect that Rad50 mutant cells would be sensitive to gamma irradiation. Why? How can you explain the results shown above for *rad50-ts*?

1E) You want to test the activity of purified Rad50 and mutant rad50-ts protein to determine whether you are correct about the function that is disrupted in the mutant. You have access to labeled linear dsDNA containing an *S. pombe* meiotic hotspot, purified Spo11/Rec12, purified Rad50 or rad50-ts. Describe the assay you would perform.

Question 2A. A fundamental principle of genetic analysis is that physical distance on DNA and genetic distance (e.g. distance on a genetic map) are **generally proportional** over the entire genome. Although some regions exhibit unusually high or low recombination frequencies, all regions of the genome can and do participate in homologous recombination. Based on your knowledge of the mechanism of homologous recombination list two properties of this process that serve to ensure that all DNA sequences can participate in recombination. Explain your answers. (Hint: Think about both how homologous recombination is initiated as well as the mechanism of homologous recombination.)

Property 1:

Property 2:

Read the next experimental section, and use these data to answer parts b-e below

Although the frequency of recombination is generally quite uniform throughout the chromosome, detailed analysis of specific chromosomal regions reveals that some regions show a higher-than-average recombination frequency, whereas other regions show a lower frequency. You decide to study this phenomenon in *E. coli* to try to understand the molecular mechanisms responsible for these "hot" and "cold" regions.

The experimental set-up is as follows. You determine the frequency of homologous recombination within 8 regions of the chromosome by generalized phage-mediated transduction. The recipient strain has multiple auxotrophic markers, meaning it has a defective gene in each of the 8 regions. The donor strain has wild-type alleles for each of these genes.

Recipient: his^-, trp^-, lac^-, ara^-, val^-, leu^-, thi^-, ura^-
Donor: his^+, trp^+, lac^+, ara^+, val^+, leu^+, thi^+, ura^+

The transducing phage is grown on the donor cells, and the phage packages, at random, 50kb segments of the donor cells chromosome. The phage are then used to infect the recipient cells, thereby introducing segments of the donor chromosomes. If

recombination between the incoming DNA and the recipient host chromosome is successful, than the recipient cells may gain a wild-type allele.

Below is a table of recombination frequencies observed in each of the marked chromosomal locations.

Table 1: Frequency of Wild-type Recombinants:

Region 1	His+	0.2 %
Region 2	Trp+	0.2 %
Region 3	Lac+	0.02 %
Region 4	Ara+	0.2 %
Region 5	Val+	0.02 %
Region 6	Leu+	0.6 %
Region 7	Thi+	0.2 %
Region 8	Ura+	0.2 %

To explore the mechanisms responsible for the higher- and lower-than-average recombination frequencies, you decide to study regions 1, 3, 5, and 6 in more detail. To do this, you repeat the same type of *in vivo* recombination assay, except this time the recipient strain carries a mutation in one of the following recombination genes: *recB*, *recD*, or *ruvC*. Below is a table of these results; in each case the frequency of wild-type recombinants is shown (e.g. His^+ for region 1) as in table 1.

Table 2: Frequency of wild-type recombinants:

	Mutation in Recipient Cells		
	RecB	*RecD*	*ruvC*
Region 1	<0.001%	0.6%	<0.001%
Region 3	<0.001%	0.12%	<0.001%
Region 5	<0.001%	0.6%	<0.001%
Region 6	<0.001%	0.6%	<0.001%

2B. Based on these findings, suggest a hypothesis for the mechanistic basis of the aberrant recombination frequency observed in table 1 for region 5. Explain the experimental support for your model.

2C. Suggest a hypothesis for the mechanistic basis of the aberrant recombination frequency observed in table 1 for region 6. Explain the experimental support for your model.

2D. To explore the mechanism further you isolate a 15 kb fragment of DNA from region 5 and a 15 kb fragment from region 6 and incubate each of these DNA fragments *in vitro* with purified RecBCD enzyme. Diagram the **DNA substrates** (marking the position of the gene and any other important features) and the **DNA products** for each of these two fragments. **Your diagram should be approximately to scale; there is no need to draw a gel---just a line drawing of the DNA.** You should include the position of bound RecA in your diagram of your final DNA products.

2E. Having completed your analysis of regions 5 and 6, you turn back to region 3. At a loss for an explanation for the poor recombination seen in region 3, you talk to your labmates to see if they have any good ideas. One smart person suggests that you examine the *E. coli* genomic sequence immediately surrounding the *lac*$^-$ mutation on the recipient chromosome. Given that the sequence is readily available, you decide this is an excellent idea. What sequence motif does your friend suggest you look for? What distribution of this motif could provide an explanation for your recombination frequency data?

Question 3. As part of your UROP project, you have identified two spontaneous mutants of your favorite yeast strain, *Saccharomyces spikus*, which have the following types of colony morphology in the haploid mutants.

1. One mutant has a bald colony phenotype instead of the normal hairy colony phenotype. (The "hairy" appearance is caused by hyphae projecting from the surface of the colony.)

2. The second mutant fails to produce the blue pigment observed in wild type colonies, so the colonies are white.

You are interested in further investigating the mutants you have identified. You have investigated the inheritance pattern of each single mutant through several different genetic crosses and have found that each mutant phenotype appears to segregate as a single genetic locus. You call these two genetic loci *HAIRY* and *BLUE*. You have decided to determine the chromosomal position of the two mutations relative to each other. Therefore, you have mated your bald mutant (of mating type 1) with your white mutant (of mating type 2.)

In *S. spikus*, like *S. cerevisiae* and other eukaryotes, DSBs are generated in the chromosomal DNA during meiosis. The MRX complex of *S. spikus* processes these DSBs to generate a substrate suitable for binding by the *S. spikus* RecA homologs, Rad51 and Dmc1. Unlike *E. coli* RecBCD, the *S. spikus* MRX complex only degrades DNA in the 5' to 3' direction. These DSBs are then repaired by recombination between non-sister homologous chromatids. Homologous recombination is required for proper segregation of chromosomes during meiosis.

In addition, *S. spikus*, like *S. cerevisiae* and some other fungi, forms spore sacs that contain all four spores generated from a single meiosis. These spore tetrads can be separated and the colonies formed by each spore can be analyzed. After your yeast mating, you separated the tetrads that were produced and analyzed morphologies of the colonies that grew from the spores. You obtained the following results:

Tetrad Class	**Types of tetrad classes observed:**	**# of tetrad class observed:**
A	2 hairy, white colonies 2 bald, blue colonies	540
B	1 hairy, white colony 1 bald, blue colony 1 hairy, blue colony 1 bald, white colony	9
C	1 hairy, white colony 2 bald, blue colonies 1 bald, white colony	6
D	2 hairy, white colonies 1 bald, blue colony 1 hairy, blue colony	5

3A. Based on this data, do you think the genes encoding the proteins required for hyphae development and blue pigment production are found on the same chromosome? Why or why not?

3B. What process or processes are responsible for generating tetrad groups B?

3C. What process or processes are responsible for generating tetrad groups C and D?

3D. Based on this data, can you predict whether DSBs are more likely to occur close to the *BLUE* or *HAIRY* gene? What is the evidence that supports this prediction?

3E. How could you test your prediction? Describe an assay you could use to identify sites of DSBs.

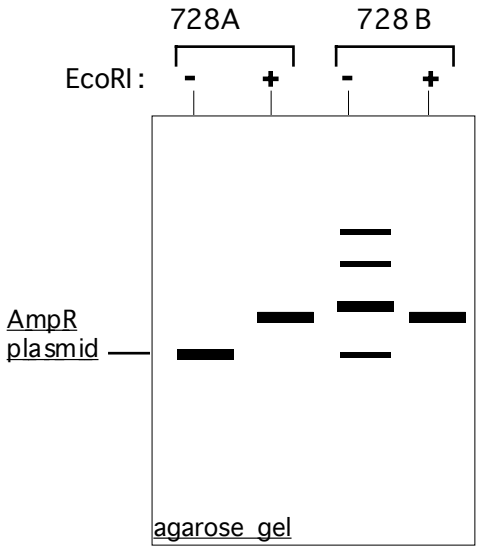
3F. Diagram how class C tetrads were generated. (Remember that the *S. spikus* MRX complex only degrades DNA in the 5' to 3' direction to generate 3' tails for Dmc1/Rad51 binding.)

3G. How would your results be different if both the bald and blue mutants that you used in your mating were also mutated in the *S. spikus* MutS homologs.

Question 4. You are studying mechanisms by which bacteria acquire and maintain genes encoding antibiotic resistance. One strain you are working with (named 728A) is highly resistant to ampicillin; 100% of the cells plated on Amp-containing media form colonies.

Knowing that both plasmids and transposons often carry genes imparting drug resistance, you analyze strain 728A and find that it carries a plasmid. **To explore the mechanisms used by the cells to maintain this plasmid, you mutagenize the strain, and isolate a variant (called 728B) which loses the ampicillin resistance phenotype at high frequency.** When a liquid culture of 728B is plated on Amp-containing media, typically only 10-15% of the cells give rise to colonies.

To investigate why 728B shows reduced inheritance of the ampicillin resistance plasmid, you purify the plasmid DNA from cultures of 728A and 728B cells and analyze the DNA on a native agarose gel. Gel analysis is done both before and after digesting the DNA with EcoRI, which cleaves the plasmid DNA once.



4A) Based on this analysis, what is a reasonable explanation for the reduced efficiency of plating seen with the 728B cells on Amp-containing med**4B)** Further analysis of the 728B cells reveals that the mutation causing poor plasmid inheritance is **on the plasmid DNA. Furthermore, the gene encodes a trans-acting factor.** Briefly describe one or two experiments that would support these conclusions.

4C) Given the results in parts a and b, you come up with a hypothesis for the type of protein that may be encoded by the gene which is mutated in the 728B cells. You decide to try to isolate the wild-type protein using a biochemical approach.
Describe:

(1) The assay you would use to follow the purification.
(2) What cells you would use as starting material; and
(3) If there are any small molecule cofactors you would be likely to need for your assay to be functional.

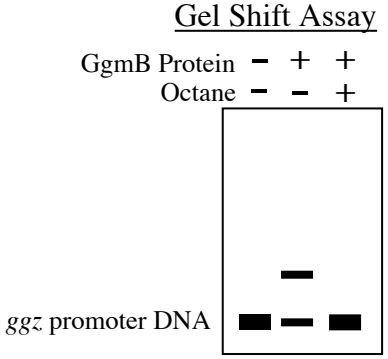
4D) While continuing with your protein purification, you also return to using genetics to understand the mechanism of plasmid maintenance. Therefore, you decide to see if you can isolate a mutation that suppresses the phenotype of the 728B strain. To do this, you start with a culture of 728B cells, mutagenize these cells, and screen for a new mutant that restores the plating efficiency on Amp-containing media to nearly 100%. You succeed in finding a strain with this phenotype, and name this strain 728C. This time the new mutation responsible for suppression maps to the cellular chromosome rather than the plasmid.

Suggest one chromosomal gene that might be mutated in the 728C cells and would cause this suppression. Explain your answer.

What is another phenotype you would expect the 728C cells to have if your hypothesis is correct?

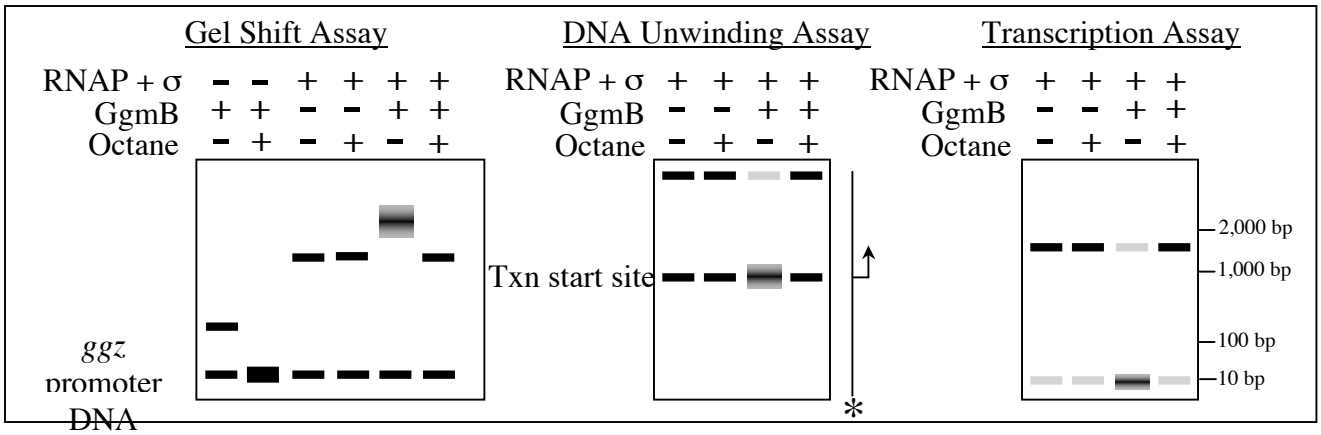
Question 5. You are studying the regulation of the *gasguzzler* (*ggz*) gene in a novel bacteria that is able to use Octane as a carbon source. *ggz* is expressed at basal levels in the absence of octane and is induced by addition of octane. You have isolated a mutant, which you call *ggmB*, which results in constitutive expression of the *ggz* operon. Based on genetic evidence, you think that the *ggmB* encodes a transcriptional repressor.

To further study how the GgmB protein represses transcription in the absence of octane, you have purified the GgmB protein. You use this purified protein to measure binding to the *ggz* promoter region *in vitro* using gel mobility shift assays. You use radiolabeled dsDNA that contains ~200 basepairs upstream of the *ggz* gene as a substrate for DNA binding. The results of your assay are shown below:



5A Based on these results, how do you think octane regulates GgmB activity?

You want to determine how GgmB represses transcription of *gasguzzler*. To this end you perform assays to look at RNAP binding, promoter unwinding, and transcription product length in an *in vitro* assay that shows octane-dependent expression of the *ggz* promoter. Your results are shown below.



5B Based on this data, describe a model for the mechanism that GgmB uses to regulate *ggz* promoter function. Include in your description the stages in the transcription process that are affected and how octane-dependence is conferred.

Question 6 You are a graduate student in a lab working on prokaryotic transcription. You decide that you are interested in creating a very strong promoter that gives very high transcription rates. As you think about how to do this, you start thinking about the conundrum that RNA polymerase holoenzyme must deal with. It must bind DNA in a site-specific manner at the promoter but then must move away from the promoter and transcribe DNA into RNA in a non-site-specific manner.

(A) How does the RNA polymerase holoenzyme accomplish these tasks?

You know that strong prokaryotic promoters often have –10 and –35 sites that have sequences that are close to the consensus sequence and that they also often have an UP element. You decide to take a strong prokaryotic promoter, whose sigma factor is called $\sigma^{7.28}$, with all of these elements and make it stronger by putting in the exact consensus sites for the –10 and –35 sites. You decide to call this promoter your *gnu* promoter.

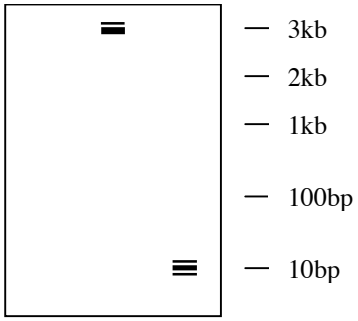
(B) How would you test your *gnu* promoter *in vivo*? Don't forget your controls!

You find that you cannot detect any transcription *in vivo* and so decide to try some *in vitro* experiments. First, you want to measure closed complex formation using Core RNAP and $\sigma^{7.28}$ (the RNAP holoenzyme).

(C) What experiment would you do to test binding and how would you test to see if this holoenzyme binds more or less tightly to your *gnu* promoter versus a wildtype promoter?

Your experiment tells you that the RNAP holoenzyme binds your *gnu* promoter even better than your wildtype promoter. You decide to do an incorporation assay to see if you can get any transcription *in vitro*. Your results are shown below.

RNAP+$\sigma^{7.28}$	+	+	+
WT promoter	-	+	-
Gnu promoter	-	-	+



(D) What step in the transcription initiation process is the *gnu* promoter defective for?

(E) You decide that you might still be able to get super duper transcription levels from this promoter but will need to make a mutation in the sigma factor. Based on your answer in part D and your knowledge of sigma factor function, what part of $\sigma^{7.28}$ would you target for mutation so that it would work well with your new promoter?

Question 7 You are studying the regulation of a eukaryotic gene involved in memory called Absent Minded. Animals with mutants in this gene have severe short-term memory loss.

You want to understand what controls the levels of Absent Minded gene expression to see if you can increase its expression in normal animals and therefore increase short-term memory. Using mutational mapping of the promoter, you identify two 10 bp regions of the promoter that when mutated strongly reduce expression of the Absent Minded promoter *in vivo*. You purify the proteins that bind these promoter elements and call the proteins ABR1 and ABR2.

To be sure that these proteins are involved in activation of the Absent Minded promoter, you fuse the Absent Minded promoter to the LacZ gene and test the levels of expression in strains that lack the ABR1 and ABR2 genes. You get the following results:

Activator	*in vivo* Transcription Units
Wild Type	2000 U
ΔABR1	50 U
ΔABR2	200 U
ΔABR1+ ΔABR2	50 U

7A Based on these data, what can you conclude about the function of ABR1 and ABR2 at the Absent Minded promoter.

Your next step is to purify the ABR1 and ABR2 proteins. You first test their ability to activate transcription *in vitro* by adding the purified activators to a plasmid containing the Absent Minded promoter. You then add purified RNA Pol II and auxiliary factors (TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH). You get the following results:

Activator	Absent Minded mRNA (pmoles/minute)
None	500
ABR1	500
ABR2	3,000
ABR1+ ABR2	3,000

7B Based on the differences between the reconstituted *in vitro* transcription experiments and the *in vivo* promoter fusion experiments, propose a function for the ABR1 regulator. Explain your reasoning.

You decide to map the transcriptional activation domain of ABR1. Since a lab mate had recently mapped the DNA binding domain of ABR2, you decide to use this DNA binding domain to map the ABR1 transcriptional activation domain.

7C Describe how you would test for the ABR1 activation domain using the ABR2 DNA binding domain.

You set up your assay but find that **none** of the constructs can activate transcription *in vivo*. Frustrated, you alter your assay to use the more traditional Gal4-DNA binding domain instead and observe the expected activation.

7D Propose a model to explain the different results for the ABR2 and Gal4 DNA binding domain fusion proteins.

7E Based on all of the observations concerning ABR1 and ABR2, propose a model that explains their synergistic function in the cell.

Question 8. Your favorite strain of yeast are a beautiful purple color, and you have identified the responsible gene, *PUR3*. You want to understand the regulation of *PUR3* so that you can enhance its expression and make the yeast an even more vibrant shade. Analysis of the promoter region of *PUR3* indicates that there are two potential TATA boxes, T_1 and T_2. You want to determine which TATA box is used to initiate transcription, and plan to do S1 nuclease protection assays using the following templates:

Template 1 (WT):

CCTTATACAAATAATGAATTGCACGCCCTATATAAA-30BASES-GATTGACATTATGAATTACTATTTCGCT

T_1 T_2 Nuclease protection probe annealing site

Template 2:

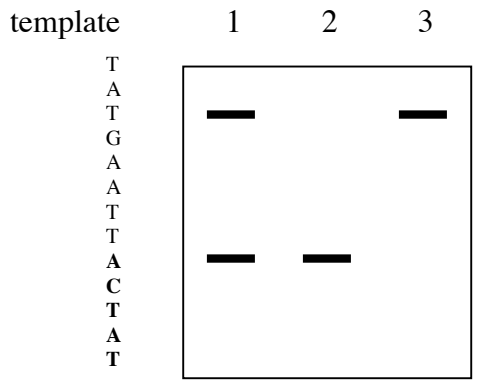
CCTTCGTCCTCTAATGAATTGCACGCCCTATATAAA-30BASES-GATTGACATTATGAATTACTATTTCGCT

T_1 T_2 Nuclease protection probe annealing site

Template 3:

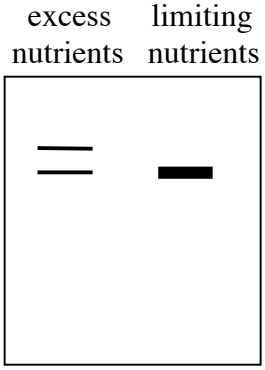
CCTTATACAAATAATGAATTGCACGCCCTCGTCCTC-30BASES-GATTGACATTATGAATTACTATTTCGCT

T_1 T_2 Nuclease protection probe annealing site



8A. What can you conclude about the use of the two TATA boxes? Identify the +1 site for each TATA box.

8B. You go on vacation for a few days and forget some yeast you left growing. They must be starving! However, you notice that the yeast on these plates are a darker, more appealing shade of purple. You decide to compare levels of *PUR3* RNA prepared from cells grown in media with excess nutrients to those grown in media with limiting nutrients using northern blots. You observe the following autoradiogram:



How can you explain the different bands seen in the two samples?

8C. You believe that a starvation-inducible gene product could activate *PUR3* transcription. Describe how you could use microarrays to identify starvation-inducible genes.

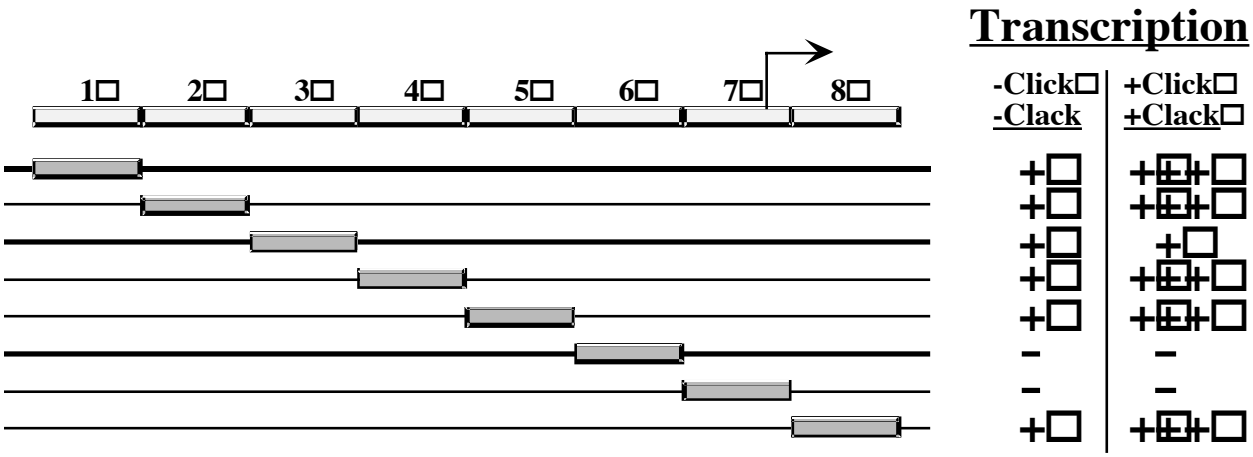
8D. Using the microarrays, you identify about a dozen starvation-inducible genes that could potentially be activators of *PUR3*. Describe either a simple genetic or biochemical assay that you could use to determine if any of these gene products activates *PUR3*?

8E. Using this approach you identify a protein (Pta1) that can activate *PUR3* transcription. You want to know the stage at which Pta1 acts to increase *PUR3* transcription. A friend down the hall has developed new methods that allow her to measure the association and dissociation of the holoenzyme complex, the transition to processive elongation, and the rate of elongation (80 bases/sec).

She reports that on average for *PUR3* transcription, it takes the holoenzyme 0.2 seconds to associate and 0.3 seconds to dissociate from the DNA. Once a closed complex is formed, it takes the holoenzyme 5 seconds to transition to processive elongation. She cannot measure the individual steps in this process, but can tell you that once the open complex is formed it takes 9 seconds to generate the 600 nucleotide long transcript. Use this information to predict the steps at which Pta1 could act.

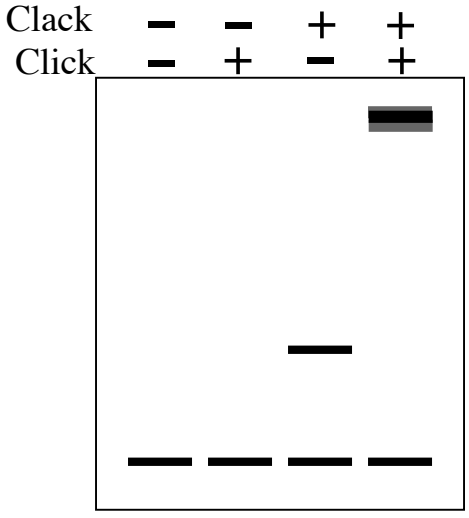
Question 9. You have developed a transcription assay that recapitulates the *in vivo* regulation of the promoter for the RNA Pol II-transcribed Ratchet gene. By fractionating this extract, you have purified and cloned two specific transcription factors that, in addition to the general RNA Pol II transcription factors, are required for correct regulation of the promoter.

As a first step to understand the function of these factors you make a series of deletion mutants of the Ratchet promoter and test their effect on transcription in the presence and absence of the Click and Clack factors. You find the following results.



9A. Based on this data, what would you predict the function of elements 3, 6, and 7 are

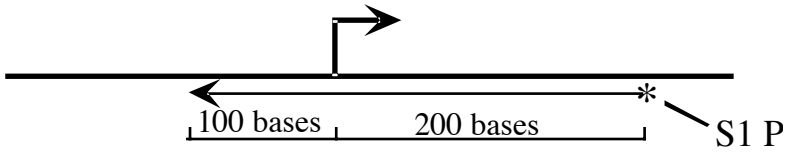
To further characterize the factors that you have identified, you perform a gel shift assay to look at the ability of the different factors to bind to the Ratchet promoter. You obtain the following results.



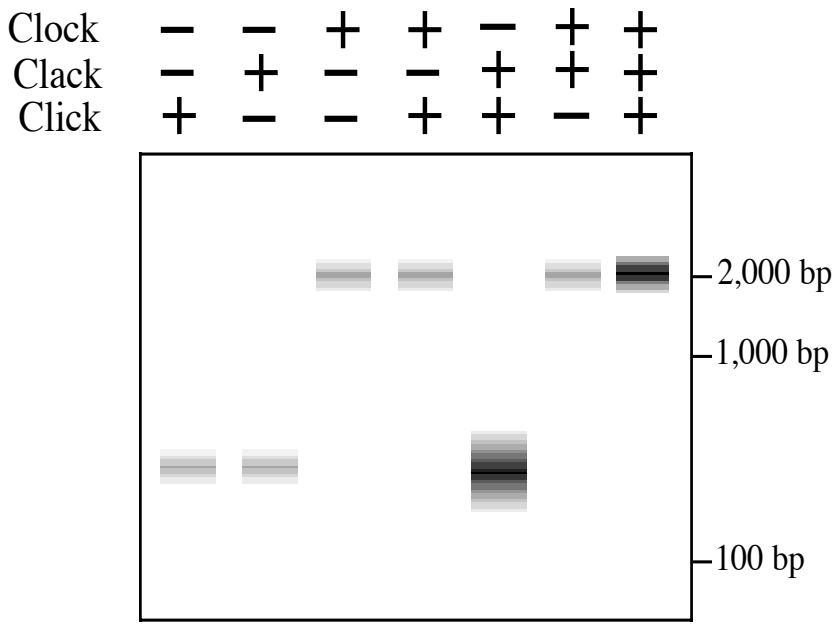
9B. Based on this data, what can you conclude concerning the function of Click and Clack? How is this finding consistent with the data obtained in part 7a?

9C. How would you determine the region of the Clack factor required for activation of the Ratchet promoter?

Throughout your analysis of the Ratchet promoter you have been frustrated by the lack of effect of a third transcription factor, **Clock**, which is known to activate this promoter *in vivo*. All of your previous assays for transcription were performed using S1 assays and a ssDNA probe of 300 bases in length that overlaps the ratchet start site by 100 bases.□



Thinking you may have missed something, you analyze the products of a set of transcription reactions performed in the presence of radiolabeled UTP on a denaturing agarose gel. To cause termination of RNA Pol II artificially, you cut the template DNA 2 kb downstream of the promoter with a restriction enzyme. You obtain the following results (hint: promoter clearance is complete after 100 bases of RNA synthesis).



9D. What aspect of RNA Pol II function is affected by addition of Clock? Why was this effect missed by the S1 assay and detected by the transcription run off assay?