

**7.28 PROBLEM SET#1 2005**

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**Question 1**

You are studying the mechanism of DNA polymerase loading at eukaryotic DNA replication origins. Unlike the situation in bacteria, the three different eukaryotic DNA polymerases are not tightly associated with one another and you are interested in how they are all brought to the origin to assemble the replication fork.

Your ability to address this question is greatly facilitated by the recent development of a completely purified set of proteins that will initiate DNA replication from an *S. cerevisiae* (Baker's yeast) origin of replication.

Your first goal is to determine the order of assembly of the 3 DNA polymerases during initiation.

**(A)** Describe an assay you would use to monitor the assembly of the DNA polymerases at the origin. You have access to all the purified proteins required for replication initiation including the three purified DNA polymerases in labeled and unlabeled forms.

Using your assay, you determine what effect leaving out one polymerase has on the assembly of the other two DNA polymerases at the origin. You obtain the results shown on the next page.

DNA Polymerases Added

Pol  $\alpha$ /Primase, Pol  $\epsilon$   
 Pol  $\alpha$ /Primase, Pol  $\delta$   
 Pol  $\delta$ , Pol  $\epsilon$   
 Pol  $\alpha$ /Primase, Pol  $\delta$ , Pol  $\epsilon$

DNA Polymerases Assembled

Pol  $\epsilon$   
 Pol  $\delta$   
 Pol  $\delta$ , Pol  $\epsilon$   
 Pol  $\alpha$ /Primase, Pol  $\delta$ , Pol  $\epsilon$

(B) What can you conclude about the order of loading of the three DNA polymerases based on these data? Briefly explain the logic behind your conclusions.

You find that if you add 300 mM NaCl to your binding assays you obtain a different set of results.

DNA Polymerases Added

Pol  $\alpha$ /Primase, Pol  $\epsilon$   
 Pol  $\alpha$ /Primase, Pol  $\delta$   
 Pol  $\delta$ , Pol  $\epsilon$   
 Pol  $\alpha$ /Primase, Pol  $\delta$ , Pol  $\epsilon$

DNA Polymerases Assembled

	<u>50 mM NaCl</u>	<u>300 mM NaCl</u>
Pol $\alpha$ /Primase, Pol $\epsilon$	Pol $\epsilon$	none
Pol $\alpha$ /Primase, Pol $\delta$	Pol $\delta$	none
Pol $\delta$ , Pol $\epsilon$	Pol $\delta$ , Pol $\epsilon$	none
Pol $\alpha$ /Primase, Pol $\delta$ , Pol $\epsilon$	Pol $\alpha$ /Prim, Pol $\delta$ , Pol $\epsilon$	Pol $\alpha$ /Prim, Pol $\delta$ , Pol $\epsilon$

(C) Propose a model that explains the effect of NaCl on your assay.

You want to determine whether DNA synthesis activity is required to assemble the DNA polymerases onto the DNA. To address this question, you take advantage of the fact that your assay monitors association with the DNA template and not DNA synthesis.

You perform your assays again using mutant DNA polymerases that have a single point mutation that prevents DNA synthesis by altering binding to a catalytic  $Mg^{+2}$  but does not alter the binding to substrate. At the same time you also produce a Pol  $\alpha$ /Primase that has a similar point mutation in its Primase subunit.

You assay these mutant proteins in your template association assay and get the following results. Mutant subunits are indicated in *italics* and with an asterisk (\*).

**DNA Polymerases Added**

Pol  $\alpha$ /Primase, Pol  $\delta^*$ , Pol  $\epsilon$   
 Pol  $\alpha$ /Primase, Pol  $\delta$ , Pol  $\epsilon^*$   
 Pol  $\alpha^*$ /Primase, Pol  $\delta$ , Pol  $\epsilon$   
 Pol  $\alpha$ /Primase<sup>\*</sup>, Pol  $\delta$ , Pol  $\epsilon$

**DNA Polymerases Assembled**

**50 mM NaCl**

Pol  $\alpha$ /Prim, Pol  $\delta^*$ , Pol  $\epsilon$   
 Pol  $\alpha$ /Prim, Pol  $\delta$ , Pol  $\epsilon^*$   
 Pol  $\alpha^*$ /Prim, Pol  $\delta$ , Pol  $\epsilon$   
 Pol  $\alpha$ /Prim<sup>\*</sup>, Pol  $\delta$ , Pol  $\epsilon$

**300 mM NaCl**

Pol  $\alpha$ /Prim, Pol  $\delta^*$ , Pol  $\epsilon$   
 Pol  $\alpha$ /Prim, Pol  $\delta$ , Pol  $\epsilon^*$   
 Pol  $\alpha^*$ /Prim, Pol  $\delta$ , Pol  $\epsilon$   
 none

(D) How can you explain the requirement of primase activity for DNA polymerase  $\delta$  and  $\epsilon$  association with the origin at high salt? Why do you see a different result with the Pol  $\alpha$  mutant?

(E) Based on your hypothesis and your knowledge of DNA polymerases, what other factors would you expect to be **specifically required** for the primase-dependent, salt-resistant binding of the DNA polymerases  $\delta$  and  $\epsilon$  but not for the low-salt binding? Explain your choice.

**Question 2**

You have discovered a new single-cell, eukaryotic organism (which you decide to name *Redsoxus rutilus*) and you want to figure out what proteins are involved in DNA replication. You decide to do a screen for temperature sensitive replication mutants (you're really looking forward to that month off in the Bahamas) so you mutagenize your *Redsoxus rutilus*, grow at the non-permissive temperature with  $^3\text{H}$ -thymidine, wash away the unincorporated  $^3\text{H}$ -thymidine, put the cells in the freezer for a month, and then take them out and plate the cells at the permissive temperature.

**(A)** When looking for DNA replication mutants, why do you look for temperature sensitive mutants rather than gene deletions?

You pick a mutant that grows to analyze further (you name your mutant *Schilling1*, abbreviated *Sch1*). First, you want to test for DNA replication at the non-permissive temperature.

**(B)** You incubate your *Sch1* mutant cells at the non-permissive temperature for two generations and then test for DNA synthesis. What type of assay would you use? Why?

**(C)** You find that no DNA synthesis occurs in your assay at the non-permissive temperature. You want to ask next whether your mutant is affecting the initiation or

elongation steps of DNA replication. Your lab mate has developed a method to arrest this organism at two points in the cell cycle: in G1-phase and in the middle of S-phase.

How could you take advantage of the ability to arrest your *Schl* cells at these two stages of the cell cycle to determine what stage of replication your mutant is required for? Describe the results you would get if your mutant were defective in the initiation of DNA replication.

**(D)** Your results indicate that your mutation is only involved in the initiation of replication. Your next goal is to use the *Schl* mutant cells to purify the protein whose activity is lost in the mutant extract. How would you do this? Be sure to describe the controls that you would need to include to ensure that your approach will work.

**(E)** After many rounds of column chromatography you have a column fraction that you are convinced is fully purified. When you separate the proteins in the fraction on an SDS-polyacrylamide gel, you find that there are two proteins in 1:1 ratio. Explain how you could be convinced that the activity is pure despite more than one protein being present in the purified fractions?

**(F)** After you finish your purification, you test if your purified protein has origin DNA binding, DNA helicase, or DNA unwinding activity. In each case you find no activity.

You discuss this result with your advisor and she reminds you that one of the chromatography steps that you performed during the end of the purification separated the two proteins in your final fraction. You did not pursue this fractionation method further, however, because neither of the separated proteins complemented the mutant extract. Nevertheless, your advisor suggests that you test each of the separated proteins for the same activities.

You are surprised to find that fractions containing the larger of the two proteins have a robust helicase activity. How can you explain this result?

**(G)** You are curious to determine which of the two proteins in your purified fraction is altered in the original mutant strain. Because you had to do all your biochemical complementation assays at the non-permissive temperature, you anticipate that the purified protein is also temperature sensitive. Indeed, when you test the fractions you are purifying from the mutant cells you find that they can only complement the extracts derived from the mutant cells at 30°C but not at 42°C.

After you finish purifying the activity you find the same two proteins in the final fraction. On a hunch you test this fraction for helicase activity at 30°C and 42°C. You are excited to find that you only see helicase activity at 42°C.

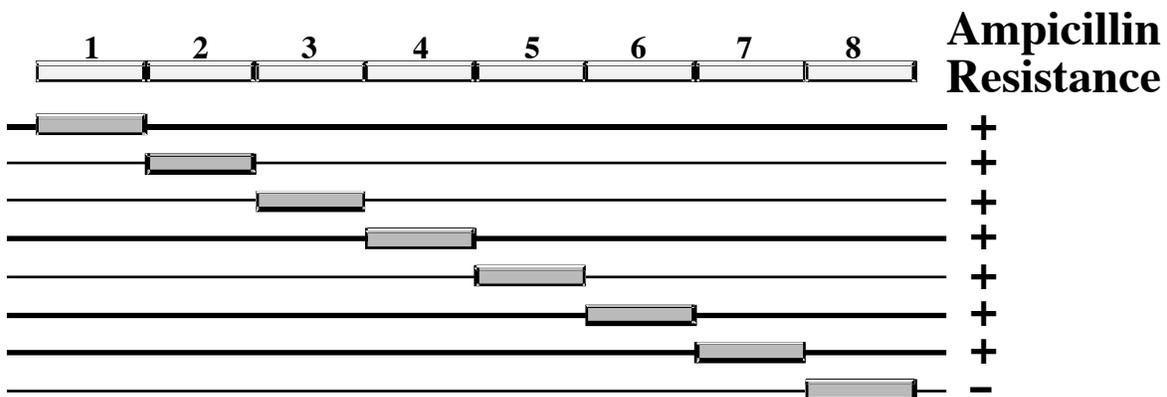
How can you explain this finding? Based on your understanding of DNA replication, propose a function for the proteins in your purified fraction. Be sure to explain how these functions explain the results of your studies of 3H-thymidine incorporation by the mutant strain.

**Question 3**

You are studying the chromosomal replication of a new strain of archaeobacteria isolated from deep sea sulphur vents called *Stinkus aquaticus*. As a first step in dissecting the replication process you want to identify the origin of replication for this organism.

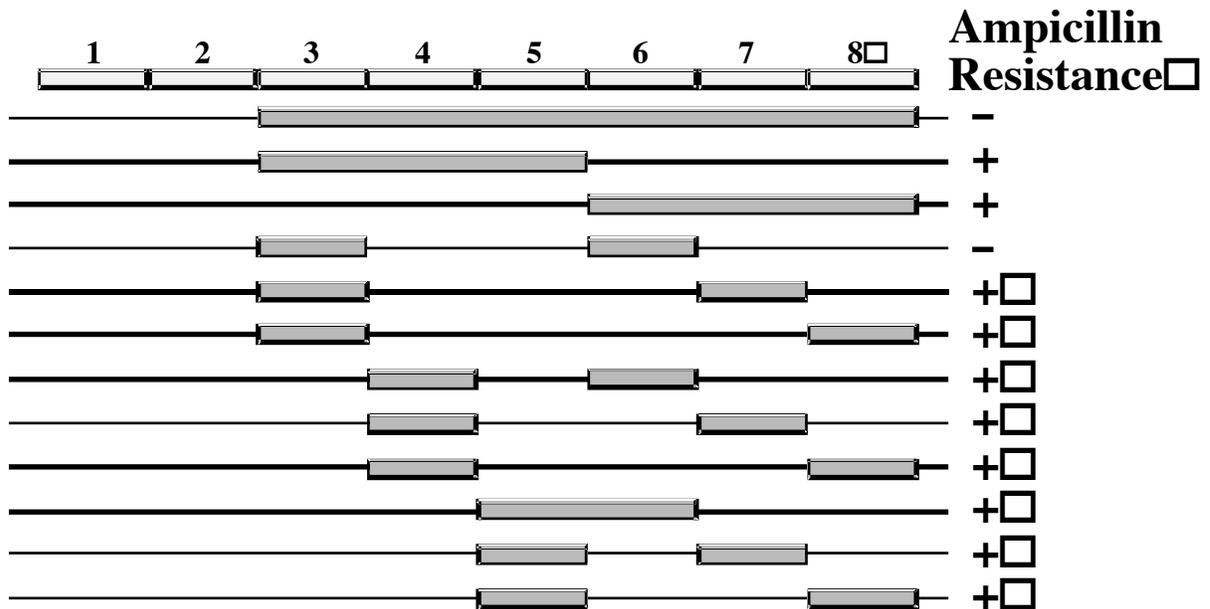
Because it is a unicellular organism you use the plasmid transformation technique to look for the *Stinkus* replicator. You find one chromosomal DNA fragment that consistently confers the ability to transform *Stinkus* to an ampicillin resistant state when ligated into a plasmid that otherwise cannot confer ampicillin resistance.

To map the sequences involved in origin function more carefully, you make a series of substitution mutants across the region and obtain the following results.



(A) What can you conclude about the DNA elements involved in the *Stinkus* chromosomal origin of replication? In particular, indicate what part of the DNA is necessary and what part is sufficient for replicator function.

Surprised at the minimal region that the initial analysis revealed, you construct a number of other substitution mutations to investigate the region to the right of element 2. You obtain the following results.



**(B)** What additional elements of the replicator does this analysis uncover? Briefly explain why these elements were not observed in the first analysis.

You have identified a *Stinkus* protein that is related to Dna. You suspect that this protein might be the *Stinkus* initiator protein and want to test your hypothesis. As a first step you perform a gel shift experiment and find that the *Stinkus* DnaA protein binds to the fragment of DNA you identified in the transformation assay.

**(C)** Describe an experiment that would determine the region of the *Stinkus* replicator the DnaA-related protein recognizes. You have access to any DNA molecule that you need as well as a standard array of restriction enzymes and other nucleases. Describe all DNA and protein molecules used in your assay.

You find that the *Stinkus* DnaA protein binds to element 2 that you identified in your mutagenesis experiment (part **A**). Having identified a function for element 2, you want to determine the function for the remainder of the origin.

**(D)** What function would you consider a likely possibility for the other regions of the origin?

(E) Briefly describe an experiment to test your hypothesis?

#### Question 4

Your introductory lab course has set up an Ames Test experiment for you to conduct. You are given two strains to study. Strain A has a single base substitution, while Strain B has two frameshift mutations. Both strains' mutations are in genes required for synthesizing histidine. Therefore, mutant strains do not grow on media lacking this amino acid. You plate cells from each strain onto minimal media plates lacking histidine and then expose them to the indicated mutagens.

Mutagen	average colonies per plate	
	Strain A	Strain B
Control (no mutagen)	35	~5
UV (1 sec)	45	30
UV (5 sec)	500	2000
UV (10 sec)	1000	2000+
MMS	350	12
9-aminoacridine	40	150

(A) In this experiment, you are exposing the cells to mutagens, yet the assay counts colonies that grow, and in order to grow the cells cannot be mutant. Please explain this apparent contradiction.

(B) Why do you see colonies appearing on the control plates? Propose an explanation for the difference in the control frequencies observed between Strains A and B.

(C) A chemical is classified as a mutagen if it produces twice the number of mutants that occur spontaneously. From the data above, suggest whether UV light, MMS, and 9-aminoacridine act as effective mutagens for each strain. What do these results indicate about the types of mutations induced by each of these treatments?

(D) Your lab partner asks you why you didn't just start with wild type (His<sup>+</sup>) cells, mutagenize them, and look for His<sup>-</sup> cells. How do you justify using the mutant reversion studies instead of his suggested approach? Identify a couple of specific advantages to the reversion analysis approach. What are the limitations of determining mutagenic specificities (i.e. of deciding whether something is or is not a mutagen) by reversion studies?

### Question 5

To identify proteins involved in DNA repair in a newly identified bacterial strain, you decide to isolate mutant versions of this strain that have a mutator phenotype. In the context of this analysis, you define mutant strain as a mutator strain if it has at least a 10-fold higher frequency of mutations than the starting strain, as determined from an *in vivo* reversion assay, similar to that used in the Ames test. The starting test strain you use for the reversion assay carries a missense mutation in a gene required for arginine biosynthesis; as a result, this strain is an arginine auxotroph.

(A) Name **four** genes (or the protein encoded by these genes) (use *E. coli* nomenclature) that are likely to give the **most** elevated frequency of mutations (e.g. >100-fold increase) by this assay. Briefly justify your answers.

As a result of this genetic screen, you isolate cells that constitutively express proteins required for trans-lesion DNA synthesis (TLS). These cells express the proteins in the absence of induction by a DNA damaging agent. The TLS proteins themselves are, however, perfectly normal in this strain.

**(B)** Why do these cells have a mutator phenotype? Explain your answer.

In the process of characterizing the strain that constitutively expresses the TLS proteins (used in part b), you notice that one clone derived from this strain has an even HIGHER mutation rate than the parent strain. You observe this high mutation frequency in many different types of *in vivo* assays, regardless of the nature of the “tester” allele used in experiment. Further analysis of this hyper-mutable strain reveals that it carries a mutation in the *umuC* gene.

**(C)** What characteristic of the UmuC (polymerase V) protein could be altered in the mutant version to explain the phenotype? Briefly explain your answer.

**(D)** Design an experiment to test your hypothesis for the nature of the defect in the UmuC protein. Explain specifically what results you would expect in your experiment with the mutant protein if your hypothesis was correct. Compare these data with those you find in control experiments using the wild-type UmuC protein.

**Question 6**

You are a UROP in a lab that studies mismatch repair. You are working with a grad student whose project is to identify the proteins required for mismatch repair in a recently identified prokaryote collected off the coast of Cape Cod. The grad student has already fractionated the cell extract and identified several fractions (A through D) that when combined can successfully repair a mismatched DNA substrate.

(A) Briefly describe 2 types of columns that can be used to fractionate cell extracts and explain how they work.

While the grad student is away on vacation, she wants you to determine what kind of proteins are in each fraction. You decide to do an *in vitro* DNA repair assay using the following substrate.

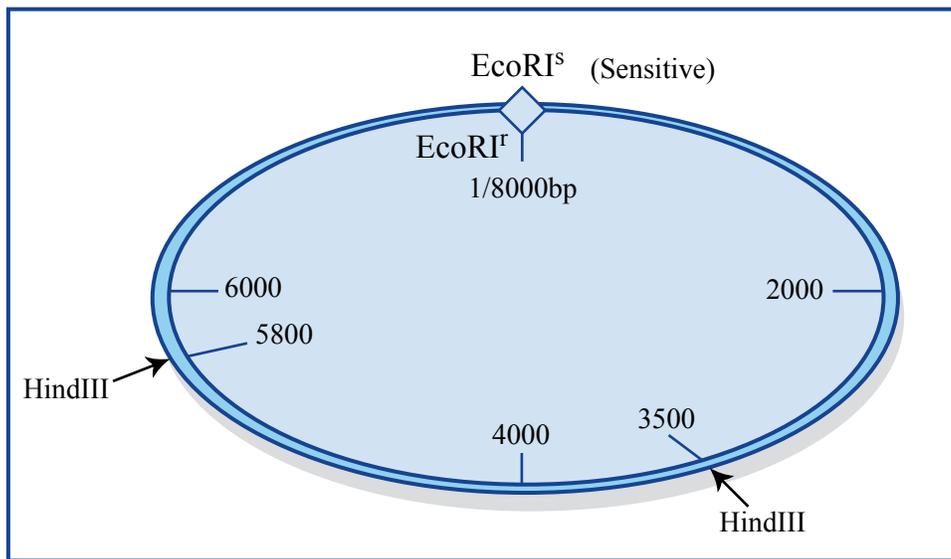
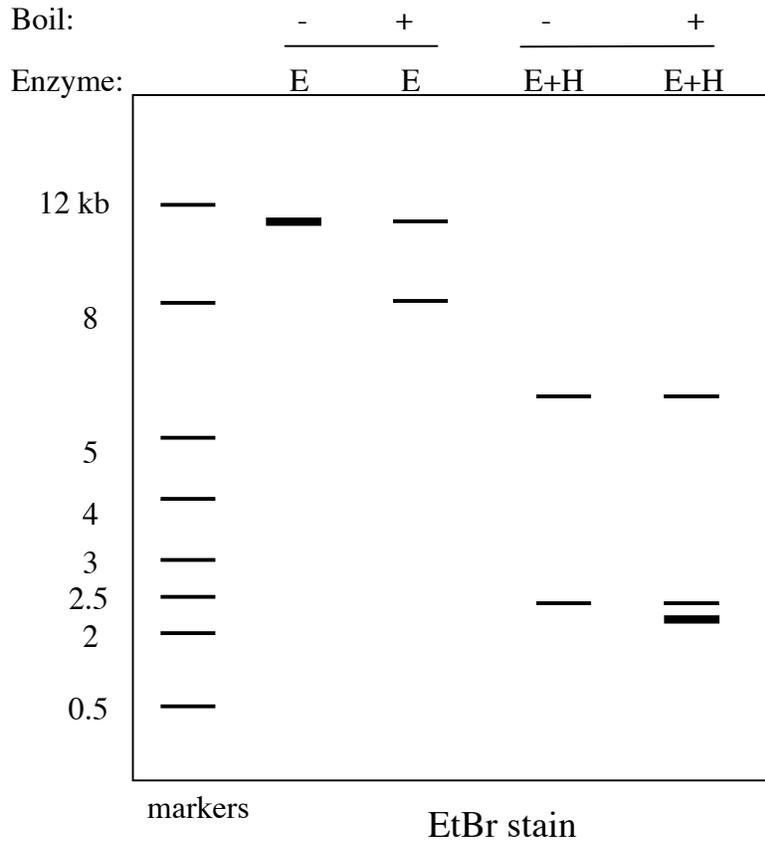


Figure by MIT OCW.

You incubate the repair substrate with each of the different fraction combinations in the presence of ATP and the four dNTPs. After the incubation, you recover the DNA and digest each sample with EcoRI (lanes marked E) or EcoRI and HindIII (lanes marked E+H) and run the resulting cleaved DNA molecules on an agarose gel. A picture of this agarose gel is shown below. The lanes are labeled at the bottom of the gel. The lane marked "Markers" contains DNA fragment size markers, and the lane marked "Substrate" contains the uncut substrate DNA (which migrates faster than 8 kb because it is supercoiled).



You are confused by the results of the A+C+D fractions. To better understand what is going on you boil each sample after the digestion reaction. You see the following results for Fractions A+C+D.



(D) Based on this data, what protein might be contained in Fraction B? Explain.

(E) To distinguish between the possible proteins in Fraction B, you decide to do a simple DNA incorporation assay with Fraction B to determine what kind of protein it contains. What assay would you do and what results would you expect for the possible proteins in Fraction B?