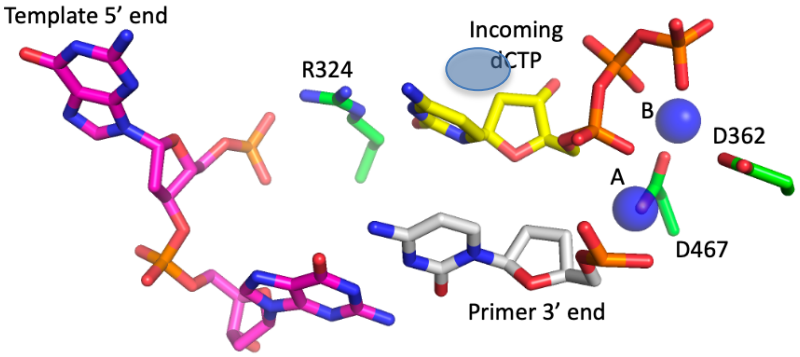
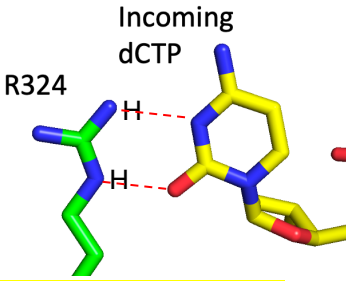
Discussion Worksheet – Winter 2024 - Week 4 (Polymerases)

Group1



Rev1 is a specialized DNA polymerase which can only incorporate a C opposite to a G (it does not incorporate G, A or T in front of a G, and does not incorporate any nucleotides opposite to an A, T or C). The crystal structure of Rev1 in complex with a primer-template duplex and with dCTP has been obtained.
The template contains a G at its 5'end, and the primer is only one nucleotide smaller than the template. A view of the active site is shown on the top. P atoms are colored in purple.A and B are two divalent cations A more detailed view of the molecular environment surrounding the "incoming dCTP" is also shown on the right.

A- What prevents the substrates from being used by the enzyme?



The primer lacks a 3'-hydroxyl which is the nucleophile for the reaction. Therefore the enzyme can bind the substrates but cannot promote catalysis.

B- What aspect of the active site of the Rev1 DNA polymerase is similar to other DNA polymerases?
There are two divalent cations positioned at similar positions relative to the substrates (one close to where the 3'-OH of the primer strand would be located; the other near the beta and gamma phosphates of the incoming dCTP). These cations are positioned by two aspartate residues, as seen in other DNA polymerases. Thus Rev1 probably uses a two metal ion catalytic mechanism similar to other DNA polymerases.

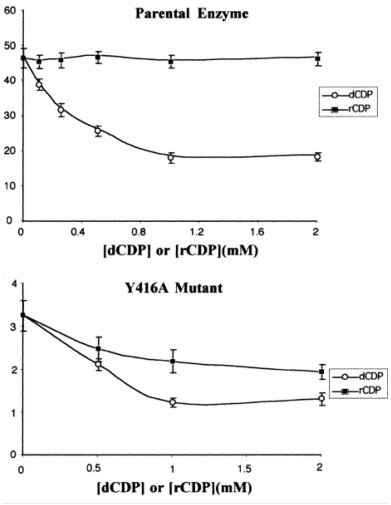
C- Assuming Rev1 uses a steric gate mechanism, where would the amino acid involved as a steric gate be located?
Taking the space of what would be a 2'-hydroxyl in the incoming dCTP – see blue oval.

D-Using the two pictures of the active site, explain the specificity of Rev1 for dCTP and compare the base selection mechanism to the mechanisms used by the DNA polymerase studied in class.
As opposed to other DNA polymerases, the base of the incoming dCTP is not base paired to the G template base. Instead, an Arginine residue is inserted opposite to the Watson-Crick edge of the incoming dCTP. The arginine donates two H-bonds (marked in red on the figure) to the cytosine base of the incoming dCTP. This allows the specific selection of C as this interaction is only possible for a C. This mechanism of selection of the incoming dCTP by pseudo-watson crick interaction between a base and an amino acid is very different from the mechanism observed for other DNA polymerases, which

select incoming dNTPs based on their ability to reconstitute a Watson-Crick base pair shape in their active site between the template DNA and the incoming dNTP.

Group2

The polymerization activity of a DNA polymerase (wild-type version = Parental enzyme) or a version carrying a Tyrosine to Alanine mutation at position 416 (Y416A) is measured in the presence of a DNA template, a primer and all 4 dNTPs (y-axis, x-origin). Numbers represent arbitrary polymerization activity. In separate experiments, the enzymes are also incubated with the same components, but also increasing concentrations of either dCDP or rCDP nucleotides and the dNTP polymerization rates measured and plotted on the y-axis as a function of the concentration of dCDP or rCDP.



A – Describe and explain the impact of increasing dCDP or rCDP on the activities of the parental enzyme.
Describe: The polymerization activity of the parental enzyme is unaffected by the addition of rCDP regardless of the concentration. However, it is gradually inhibited by adding dCDP at increasing concentrations.
Explain: dCDP cannot be used as a substate by the enzyme which requires dNTPs. However it can compete with other dNTPs for entry into the active site, and inhibits polymerization, likely by acting as a competitive inhibitor of dNTPs. rCDP does not have this effect because DNA polymerases possess a mechanism to prevent entry of rNTPs in their active sites.

B – Describe and explain the impact of increasing dCDP or rCDP on the activities of the Y416A enzyme.
Describe: The polymerization activity of the pY416A is also gradually inhibited by adding dCDP at increasing concentrations like what we saw for the parental enzyme. However as opposed to what we saw above, the Y416A enzyme is also inhibited by adding rCDPs. Note that the overall activity of this mutant is also lower than the parental enzyme based on the y-axis.
Explain: As opposed to what we saw for the parental enzyme, both rCDP and dCDP can act as competitive inhibitors. This means that the Y416A mutation prevented the enzyme from discriminating against rNTPs and allowed rCDP to enter the active site and inhibit the enzyme.

C – based on these results propose a function for residue 416 in the mechanism of the enzyme – your answer should fit into 1-2 sentences

Changing a large amino acid (tyrosine) to a shorter one (alanine) resulted in a loss of discrimination against rNTPs based on the competition experiment presented above. Based on the size change and on the effect observed, residue 416 likely acted as a steric gate to prevent or limit entry of rNTPs in the active site of the DNA polymerase.

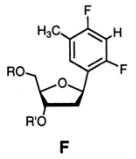


Figure 1. Structure of F.

Running start substrate

5'-TAATACGACTCACTATAG
3'-ATTATGCTGAGTGATATCCCTCTNGTCA

Standing start substrate

5'-TAATACGACTCACTATAGGGAGA
3'-ATTATGCTGAGTGATATCCCTCTNGTCA

Figure 2. The two starting substrates.

An experiment was conducted to compare the abilities of two different enzymes, Klenow fragment and Pol η, to synthesize DNA past a DNA template.

Two different substrates were tested, shown in Figure 2: the running start (Lanes 1-3) and the standing start (Lanes 4-6). The "N" in the sequence indicates the position where either a T nucleotide (lanes labeled T) or difluorotoluene nucleotide (lanes labeled F, structure shown in Figure 1.) is present in the starting substrate. These starting substrates were incubated with T, A, G, and C dNTPs as well as either Klenow fragment (shown on the left in Figure 3.), Pol η (shown on the right in Figure 3.), or no enzyme, which acts as a control (Lanes 1 and 4 on both templates).

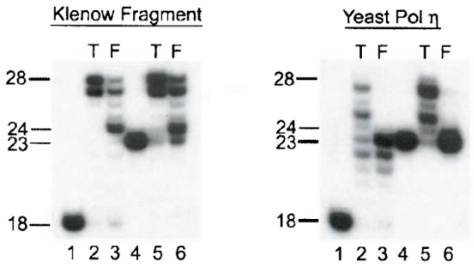


Figure 3. Experimental data.

A – What is the purpose of using F in this experiment?

F is identical to a thymidine, but a molecule with the same shape as a thymine base but lacking the ability to form H-bonds has been added at position C1'. This allows researchers to test the important of base shape vs. ability to form H-bonds during DNA polymerization.

B – For Klenow fragment acting as the enzyme: How well does the F template work compared to T with the running start substrate, and how well does each work with the standing start substrate?

Klenow is able to synthesize DNA past a DNA template for both F and T in both the running start and the standing substrate, but the F template causes Klenow to be less processive – while the T template consistently synthesized to 27 or 28 nucleotides, F has multiple lines indicating shorter strands were synthesized with lengths ranging from 24-28 base pairs.

C – For Pol η acting as the enzyme: How well does the F template work compared to T with the running start substrate, and how well does each work with the standing start substrate?

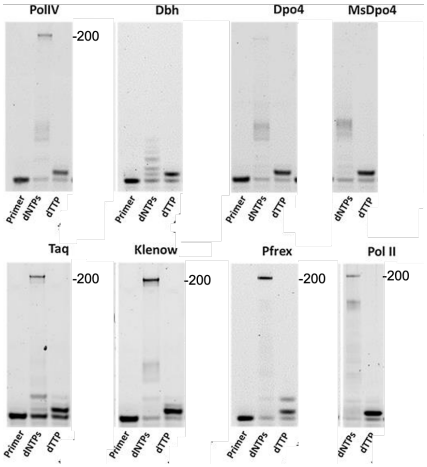
In both the standing and starting substrate, Pol η was unable to synthesize DNA past the target nucleotide (24th in the strand) in the F templates, which indicates Pol η was unable to insert a nucleotide opposite the F residue. Pol η was able to incorporate nucleotides opposite T residues in both the standing start and running start substrates, but it did not always synthesize to the end of the 28-nucleotide sequence.

D – Is Klenow or Pol η more processive on the running start substrate? Which lanes did you compare to make this conclusion?

Klenow is a much more processive enzyme than Pol η. This can be deduced by looking at the several shorter DNA strands created by Pol η, indicated by several different lines on the gel at a range of distances from the top of the gel, in contrast to the consistently larger strands created by Klenow, indicated by fewer, thicker lines closer to the top of the gel.

Group 4.

In this experiment, researchers analyze the extension of a primer annealed to a 200 nucleotide long template by different DNA polymerases. The first position in the template after the primer is an A. For each DNA polymerase, they incubate the polymerase and the template-primer duplex with either dTTP alone, or with all dNTPs for a short time (1 minute). The migration of the unextended primer is also shown for most polymerases. Only the primer is visible and the intensity of the signal is proportional to the amount of product obtained.



1- Describe the pattern of extension generated by each of these DNA polymerases in the presence of all dNTPs and compare them to each other. What information does this experiment provides regarding the properties of each of these polymerases?

Describe: Pol.IV, Taq, Klenow and Pfrex can polymerize full length extension products with very little intermediate bands. Dbh, Dpo4 and MsDpo4 cannot generate full length extension products, and generate only incomplete extension at different sizes. Pol.II has an intermediate behavior, a little bit of full-length, and many incomplete products.

This means that the most efficient enzymes (Pol.IV, Taq, Klenow and Pfrex) are either much faster or more processive than the least efficient ones (Dbh, Dpo4 and MsDpo4). In the absence of other data it is difficult to say if speed or processivity (or lack thereof) is the main problem for the least efficient enzymes.

2- Describe the pattern of extension generated by each of these DNA polymerases in the presence of only dTTP and compare them to each other. Why do you think it was important to perform this experiment considering the results obtained previously?

All of these enzymes can extend one single nucleotide efficiently and at somewhat similar leves. Therefore the low activity detected for Dbh, Dpo4 and MsDpo4 with all dNTPs cannot be attributed to the fact that the protein preparation lack activity.(ignore additional band for Pfrex)

3 – Assume Dbh is a bacterial DNA polymerase. Propose two modifications in the conditions in which the experiment performed with Dbh is done, which would result in a pattern more similar to the one observed for Taq or Pfrex.

Either incubate for a longer time, or add a processivity factor such as beta clamps. If incubating for a longer time results in a pattern similar to Taq, it means that speed was the issue. If longer intermediate bands are detected, it means that it's a processivity issue. If adding beta clamps results in a pattern similar to Taq, it means that processivity was the problem.