CREATION AND CHARACTERIZATION OF RPB1

MUTANTS IN RNA POLYMERASE II IN YEAST

by

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A THESIS

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RNA Polymerase II (RNAP II) is an enzyme that catalyzes the synthesis of the majority of mRNA in eukaryotic cells, making it essential for the first step of gene expression and vital for survival. The process of transcription occurs in every living cell, which makes understanding its mechansims all the more important. RNAP II consists of 12 subunits, of which Rpb1 is the largest. The Rpb1 subunit participates in RNA elongation in the active site of the enzyme. Residues located in Rpb1 have also been suggested to have a potential role in backtracking and arrest.[3] Backtracking occurs when RNAP II moves backward along the DNA and the 3' end of the RNA becomes dislodged from the active site. The backtracked RNA, which extrudes from the pore of the enzyme, binds to nearby residues creating a "backtrack site."[3] If the polymerase has not backtracked extensively, these interactions will be weak and RNAP II can spontaneously resume transcription. However, if the polymerase has backtracked extensively, forward elongation stops and RNAP II arrests.[3] This study had two primary focuses: creating mutations in budding yeast *(Saccharomyces cerevisiae)* Rpb1 residues that interact with backtracked RNA and characterizing Rpb1 mutants via sensitivity tests involving mycophenolic acid and canavanine to determine the speed and accuracy of the mutants respectively. Creating and characterizing these mutant polymerases will give insight into what happens when the backtrack site is destabilized, which in turn could elucidate why backtrack site residues have been conserved in the first place.

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Introduction

There are an estimated 37.2 trillion cells in the human body.[1] Within the nucleus of each of those cells, the process of transcription occurs to ensure that the cell can make the proteins it needs for survival. Transcription is the process of creating an RNA transcript from a DNA template. After the RNA transcript is made, another process called translation directs the synthesis of the protein, as shown in Figure 1.
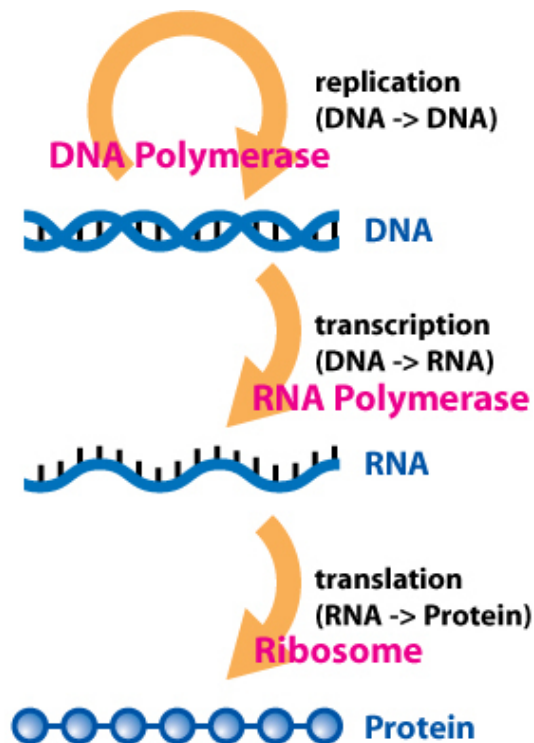
Figure 1: The central dogma of molecular biology. Image source:

https://defensedefumer.wordpress.com/2009/11/13/huh-what-what-is-molecular-biology/

RNA polymerases are the **enzymes** responsible for catalyzing the synthesis of RNA in the cell. In the nucleus of eukaryotic cells, there are three primary classes of RNA polymerases that synthesize different types of RNA.[2] RNA polymerase II (RNAP II) synthesizes messenger RNA **(mRNA)**, small nuclear RNA **(snRNA)**, and **microRNA**.

Because the majority of what RNAP II produces is mRNA, this study focused on the

transcription of mRNA and the phenomenon of backtracking in that process.

Backtracking occurs when RNAP II moves backward along the DNA template

and the 3' end of the RNA becomes dislodged from the active site.[3] To pick an

arbitrary direction, if RNAP II is moving from left to right, then backtracking occurs

when the enzyme moves from right to left. Movement of the polymerase is best

described by three possible states of the elongation complex. During active

transcription, the transcription elongation complex (TEC) can be in one of two states at

any given time: pre-translocated and post-translocated.[4] In the pre-translocated state,

RNAP II has just added a **<u>nucleotide</u>** to the RNA transcript, and in the post-translocated

state, it has moved along the DNA template and awaits the addition of another

nucleotide.[4] There is a third state called reverse translocated in which the enzyme has

moved backward along the DNA template, and this state results from backtracking. In

this state, the TEC changes conformation, and this causes the **<u>nascent</u>** RNA transcript to

extrude from the enzyme and elongation to come to a stop,[3] as shown in Figure 2.
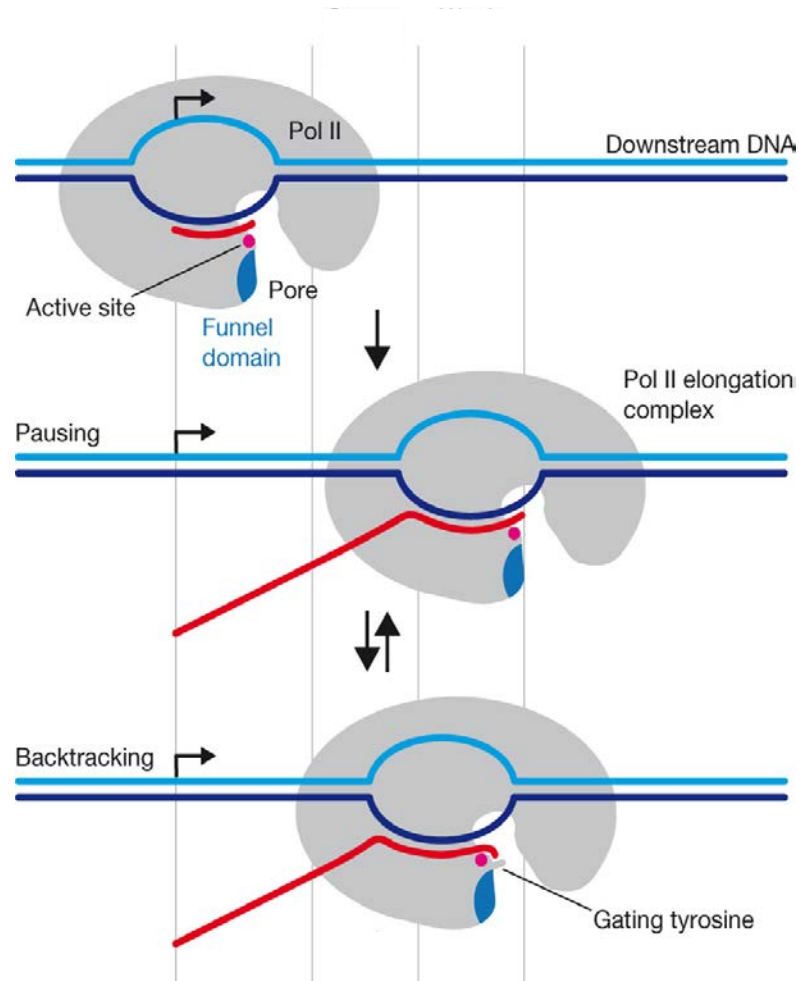
Figure 2: RNAP II in two of its possible states.[3]

The top panel depicts RNAP II in the pre-translocated state. The middle panel shows the polymerase pausing before either continuing to transcribe or backtracking. The bottom panel shows RNAP II in the reverse translocated state,[4] after it has undergone backtracking.[3]

If the polymerase has not backtracked extensively, interactions between **residues** of the polymerase and the backtracked RNA will be weak and RNAP II can spontaneously resume transcription. However, if the polymerase has backtracked extensively, forward elongation stops and RNAP II arrests.[3] More research is currently being done to understand this phenomenon and the role it plays in the transcription cycle.

This study was done in *Saccharomyces cerevisiae*, or budding yeast. Budding yeast is an ideal model organism for studying backtracking because *Saccharomyces cerevisiae* has a fast growing rate, is easily genetically manipulated, is well studied, and has many highly **conserved** sequences in the protein-encoding portions of its genome. As much as 53% **identity** has been found in yeast and human RNAP II sequences.[5] A study done by Shpakovski et al. showed that when genes encoding four subunits of human RNAP II were **cloned** into yeast, the yeast RNAP II was completely functional.[6] The conserved residues are also distributed all over the polymerase, making study in RNAP II in one organism fairly predictive of the results of the same study in another species.[5]

Existing Literature

What is the Purpose of Backtracking?

The **fidelity** with which DNA is transcribed into RNA is crucial to making functional proteins that are necessary for life. Because of the need for high accuracy, enzymes such as RNAP II have proofreading mechanisms to keep transcriptional error rates as low as 10^{-5} per nucleotide, or less than one error per protein-encoding transcript.[4] Backtracking behavior of the polymerase is thought to play a part in the proofreading activity of the enzyme. Proofreading requires that the enzyme has a mechanism for determining when an incorrect nucleotide has been added to the transcript. It is known that a related enzyme, DNA polymerase, has such a mechanism in that misincorporated nucleotides slow down the rate of addition of the next nucleotide.[7] A study done in 1998 by Thomas et al. showed that RNAP II shared a similar mechanism and that nucleotide addition after a misincorporated nucleotide occurred 15-20 times slower than after addition of the correct nucleotide.[7] The explanation of backtracking as proofreading activity seems to be logical if we only consider non-extensive backtracking (1-2 nucleotides), because in this case, the polymerase can spontaneously resume transcription.[8] The true question surrounding the purpose of backtracking arises when we consider extensive backtracking (3 or more nucleotides). With the 3' end of the RNA transcript extruding, RNAP II cannot resume transcription right away.[8] This occurs because interactions between the enzyme and the backtracked RNA stabilize the complex and lead to **transcriptional arrest**,[3] as shown in Figure 3.
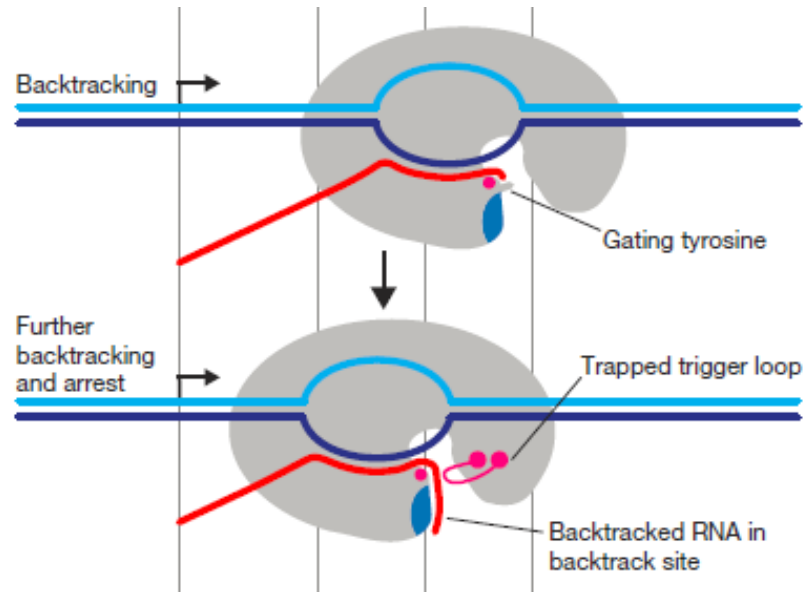
Figure 3: From the top panel down: non-extensive backtracking and extensive backtracking and arrest.[3]

Cheung and Cramer proposed that the stability of the arrested complex comes specifically from the binding of at least eight nucleotides of backtracked RNA to the Rpb1 and Rpb2 subunits of RNAP II, as shown in Figure 4. My research aims to determine what happens when the residues that are suggested to contact the backtracked RNA are mutated.
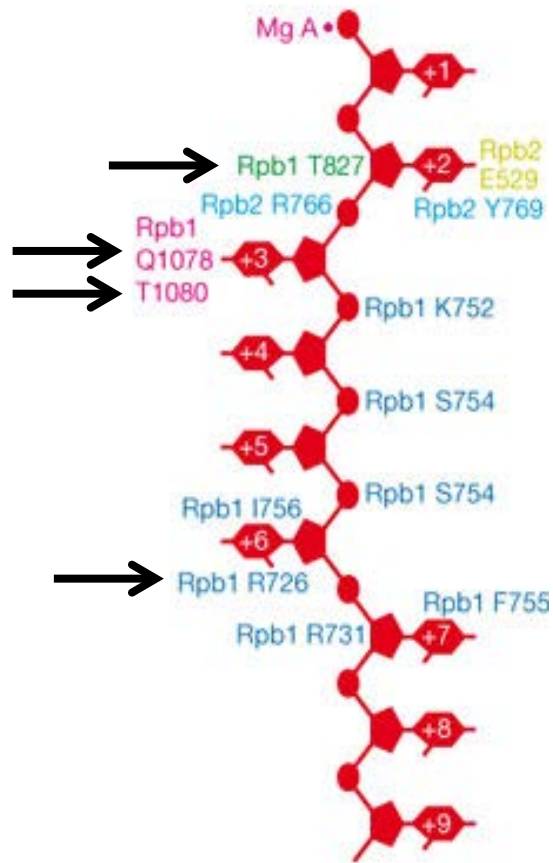
Figure 4: Backtracked RNA (shown in red) and the proposed contacts it makes with RNAP II.[3] The residues of the polymerase that I wanted to mutate are designated by black arrows.

Each of the RNAP II residues suggested to contact backtracked RNA are located in Rpb1 or Rpb2, as those subunits make up the active site of the enzyme. This is illustrated in Figure 5. We chose to mutate these specific residues because they are suggested to directly contact backtracked RNA and thus have a direct, stabilizing effect on the backtrack site.
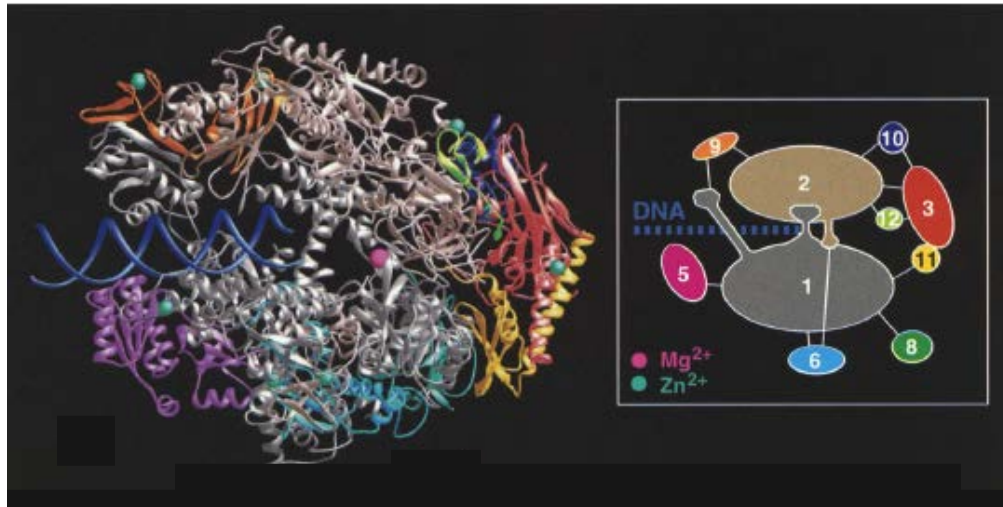
Figure 5: Structure of RNAP II.[5]

The left panel shows a ribbon diagram of the subunits of yeast RNAP II in relation to each other and to template DNA from a top view. The right panel describes the colors used to designate each subunit. Rpb1 (gray) and Rpb2 (brown) residues are suggested to contact backtracked RNA.

What Causes Backtracking?

Backtracking and transcriptional arrest have several suggested causes, including a scarcity of nucleotides, lack of RNAP II reactivation factors (in mutants), obstacles in DNA such as **nucleosomes**, DNA **lesions**,[9] and misincorporated nucleotides.[10] Several experiments were done using drugs that provoked **NTP** depletion such as 6-azauracil and mycophenolic acid in order to induce a scarcity of nucleotides. This resulted in a lower elongation rate and **processivity** of RNAP II *in vivo*, which is correlated with an increase in the frequency of arrest.[9] When these drugs were tested in combination with yeast mutants that lacked RNAP II reactivation factors, the result was even more pronounced.[9] Nucleosomes have also been shown to promote backtracking *in vitro* because they create a physical hindrance for the transcribing complex.[9] DNA damage is suggested to cause arrest that is often irreversible and can only be resolved by degradation of the polymerase.[9] Specific sequences of undamaged DNA have also

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been found to increase the incidence of backtracking and arrest. **Pyrimidine**-rich tracks

in the **non-template strand** in particular induce RNAP II arrest.[9] This was suggested

to occur because the high proportion of A-U base pairs made for a weaker DNA-RNA

hybrid, and this makes sense because there is a strong preference for pyrimidines in the

interactions between backtracked RNA and the "backtrack site."[9] Finally, experiments

have been done in which artificially induced misincorporation of nucleotides resulted in

an increase in the incidence of backtracking.[10]

How is Backtracking Resolved?

Backtracking and subsequent transcriptional arrest are not permanent or there

would be no transcription of the gene, no mature RNA from which to synthesize the

protein, and ultimately no protein made. This would lead to cell death in most cases.

The cell has developed three ways to overcome arrest: spontaneous continuation of

elongation, RNA cleavage, and degradation of the arrested RNAP II.[9] As previously

mentioned, if backtracking is not extensive RNAP II can spontaneously resume forward

elongation. Because pausing and non-extensive backtracking is thought to be a fairly

common occurrence,[9] this mechanism is likely the most utilized out of the three *in vivo*.

However, when the polymerase cannot spontaneously resume transcription, one

of the other ways it recovers from arrest is the cleavage of backtracked RNA, which

creates a new 3' end of the RNA transcript.[9] This can be achieved in a number of ways,

one of which includes the recruitment of transcription factor SII (TFIIS) to the site to

cleave the backtracked RNA, as illustrated in Figure 6.
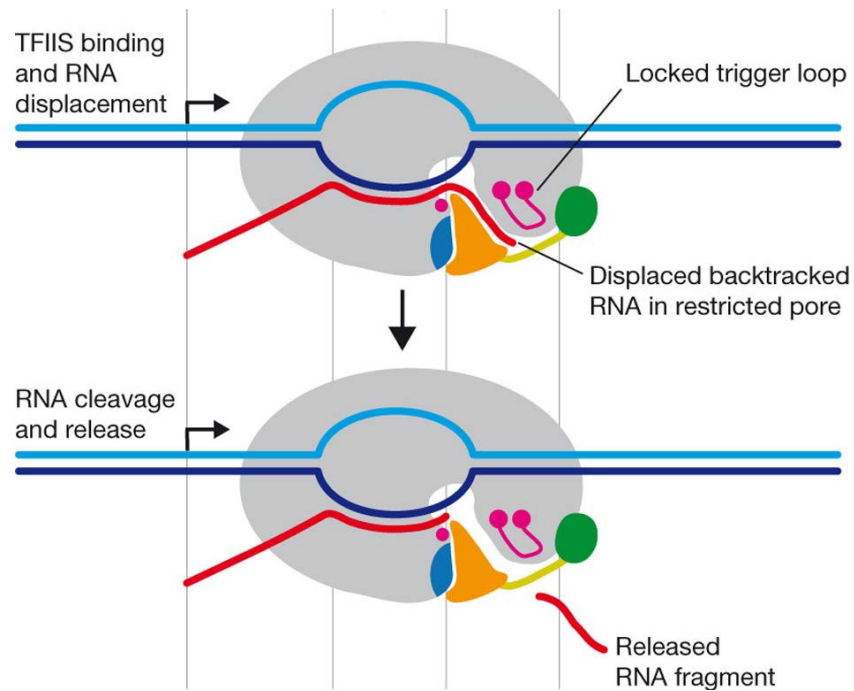
Figure 6: TFIIS cleavage of backtracked RNA.[3]

X-ray crystallography data from Cheung and Cramer has indicated that the mechanism

for this cleavage involves TFIIS weakening the interactions between RNAP II and the

backtracked RNA and subsequently displacing the backtracked RNA by competitive

binding to the backtrack site.[3] However, even in the absence of TFIIS the cell can still

induce cleavage and resume transcription. RNAP II has weak intrinsic **<u>nuclease

activity</u>** which is greatly enhanced by TFIIS.[11] Although it is still not entirely clear

which subunits of RNAP II participate in this intrinsic nuclease activity, a small subunit

called Rpb9 (see Figure 5) is thought to play a role. In the absence of a functional

Rpb9, RNAP II was less able to induce cleavage even in the presence of TFIIS and it

generated different cleavage products.[11]

An alternative mechanism for the continuation of transcription without cleavage is mediated by a protein complex called Ccr4-Not.[9] This complex has been found to localize where mRNA degradation takes place in the cell, so it could be interacting with the extruding RNA transcript to degrade it and allow the polymerase to continue to elongate.[9] If all other mechanisms of reactivation fail, the cell tags the largest subunit of RNAP II, Rpb1, for degradation[9] and this has been termed the "last resort" pathway.[12]

The process of tagging Rpb1 for degradation occurs via the **<u>ubiquitylation</u>** pathway and was originally identified as a response to DNA damage serious enough to interrupt transcription.[12] However, later studies have shown that other conditions can induce the ubiquitylation response as well. Some of these conditions include treatment with the transcriptional toxin α-amantin and specific mutations of the TFIIS gene.[12]

Research Question

      My research centered around a broad question that can be narrowed down as illustrated in Figure 7.

What is the function of extensive backtracking?

Why have residues of the backtrack site been conserved?

What happens when the backtrack site is destabilized?
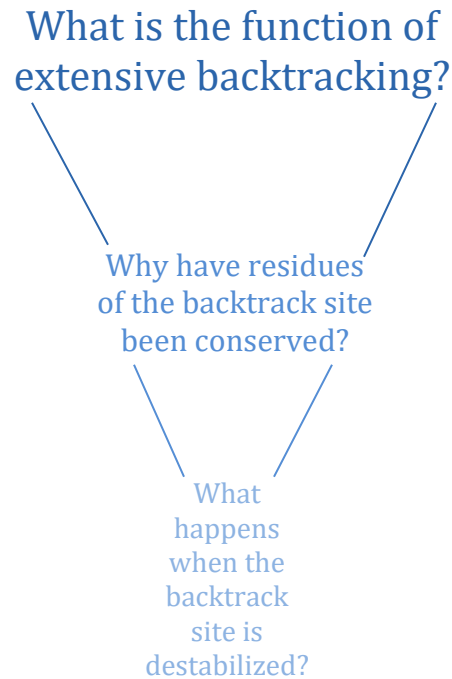
Figure 7: Guiding research questions.

      The existing literature still cannot answer what the exact function of extensive backtracking is. While there has been comprehensive work done on what this mechanism might physically look like, what causes it, and what can resolve it, its benefit to RNAP II and the cell overall is still largely unknown. The fact that these residues have been conserved and that a backtrack site exists indicates that extensive backtracking must provide a benefit to RNAP II and the cell, otherwise those residues would likely have mutated and the behavior would have disappeared. Alternatively, the backtrack site could exist in order to prevent the polymerase from moving further backwards along the DNA. In order to find out why these residues may have been conserved, it must first be determined what they are contributing to the stability of the

backtrack site and if they are necessary for viability. A common way to do this is to

mutate these residues and run **phenotypic** tests to characterize the behavior of the

mutant polymerases. This study involved the characterization of RNAP II mutants

using phenotypic tests and the creation of new mutants.

Methods

Creation of Mutants

The first backtracked nucleotide is suggested to make contact with three RNAP II residues: T827 (Rpb1), E529 (Rpb2), and Y769 (Rpb2).[3] The next backtracked nucleotide contacts Rpb1 residues Q1078 and T1080, both of which are located in a part of the enzyme called the trigger loop. Another backtracked nucleotide, located three nucleotides away, binds in a pocket, contacting two Rpb1 residues, R726 and I756 (see Figure 4).[3] This study focused on creating point mutations in four of those residues based on these proposed contacts: T827, Q1078, T1080, and R726. The point mutations would cause the DNA at these positions to code for different amino acids which should not be able to bind the same way to backtracked RNA. I will discuss the methods used to create new RNAP II mutants before I discuss the characterization of existing mutants.

The creation of mutations in the Rpb1 subunit of RNAP II consists of three phases: site-directed mutagenesis, introduction of mutation into the pRP114 vector, and recovery of the mutated yeast plasmid.[*] Site-directed mutagenesis involves **PCR** using an appropriate **TOPO** vector and mutagenic **primers,** as illustrated in Figure 8. The TOPO vector was used because it contains part of the *Rpb1* gene and is a small plasmid, making it easier to work with in PCR. If the PCR was successful, the PCR product was digested with Dpn1. The digested PCR product was then introduced into *Escherichia coli (E. coli)* via a bacterial **transformation.** If colonies grew, bacterial minipreps were

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[*] Detailed protocols can be found in Supplementary Information.

performed to extract the DNA from the bacteria and the DNA was sent for sequencing.

Once the sequencing results confirmed that the mutation was present in the TOPO

vector, it could move into the introduction into pRP114 phase.
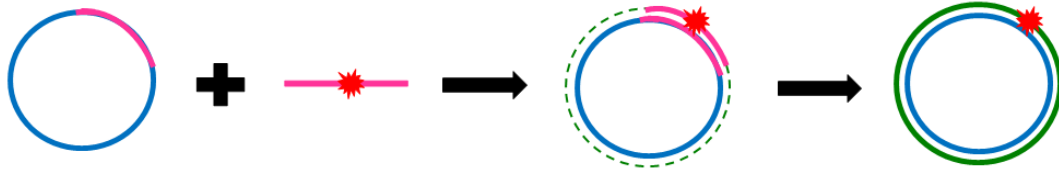
Figure 8: Site-directed mutagenesis and introduction of a mutation. From left to right: bacterial plasmid containing a portion of the *Rpb1* gene (blue with pink) and chemically synthesized oligonucleotide primer containing the desired mutation (pink) are combined in a polymerase chain reaction (PCR). The primer binds to the template DNA, and DNA polymerase extends from the primer to make a plasmid containing the mutation.

pRP114 is a yeast plasmid that is used to interchange parts of plasmids via **<u>homologous recombination</u>**.[13] pRP114 has a complete *Rpb1* gene, which allows the mutated fragment to bind where it has complementary sequence after pRP114 has been digested, and in this way the mutation can be taken out of the bacterial vector and introduced to the yeast vector, as shown in Figure 9.
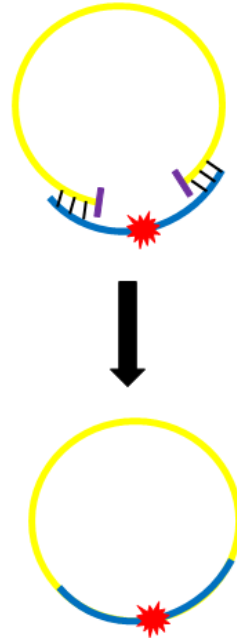
Figure 9: Homologous recombination and incorporation of mutated DNA into yeast. The TOPO vector PCR product with the desired mutation (blue) and the cut yeast plasmid (yellow) are combined in a high efficiency yeast transformation in order to facilitate homologous recombination.

The first steps in this phase include digesting pRP114 with appropriate restriction enzymes and running it on a gel to isolate the cut plasmid. Gel extraction was used to isolate the cut plasmid from the gel. Simultaneously, the TOPO vector with the desired mutation was used in another PCR reaction to **amplify** the mutation. The cut yeast plasmid and TOPO vector PCR product were then combined in a high efficiency transformation with yeast **competent** cells in order to allow homologous recombination and incorporation of the mutated fragment into the yeast plasmid. The yeast strain used for Rpb1 mutants was DHY 464. This strain of yeast has the *Rpb1* gene deleted from the chromosome so that any copies of the gene present would be on plasmids, which are much easier to keep or remove via plating on selective media. These transformations were plated on SD-Leu to select for colonies that had incorporated the mutated plasmid,

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because pRP114 contains the *Leu2* gene. As a result, only the yeast colonies that took up the mutated plasmid would survive on media without leucine.

If colonies grew from this transformation, they proceeded into the recovery phase. Colonies were streaked onto **5-FOA** plates in order to select against the *Ura3* gene, which also had a **wild type** copy of the *Rpb1* gene on the same plasmid. The loss of this plasmid meant that colonies that grew on these plates theoretically only had one copy of the *Rpb1* gene now, and it contained the mutation that was created in a previous step. The yeast DNA was isolated using a yeast miniprep protocol and transformed into *E. coli* using DH5-α competent cells. If colonies grew, bacterial minipreps were done and the DNA was sent for sequencing to confirm the presence of the mutation.

Alternative Methods

A large part of my thesis project has involved troubleshooting PCRs, and as a result it was decided that I should investigate alternative methods for making mutants. The proposed changes included introducing two new **restriction sites**, or places where an enzyme could cut the DNA. I used site-directed mutagenesis once again using the same Rpb1 TOPO vector to create two point mutations which would in turn create the restriction sites. These restriction sites were strategically placed around the mutations we wanted to make. This means that in the future, we can use the two corresponding **restriction enzymes** (DraIII and BamHI) to cut the plasmid and order an oligonucleotide with the desired mutation to incorporate into the cut plasmid. The creation of this strain with extra restriction sites will allow us to skip several steps normally involved in site-directed mutagenesis and cloning, including the time-consuming mutagenic PCR.

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Characterization of Mutants

Another focus of this project was to characterize seven existing mutants using several phenotypic tests. The seven existing RNAP II mutants characterized were R726S, A729V, V747I, T834A, D826N, M1079T, and A1076T.* These mutations were obtained in screens testing for mutant phenotypes, including the blue/white screen and mycophenolic acid sensitivity test. After the mutant phenotypes had been identified, we wanted to characterize them further using the mycophenolic acid test again as well as a canavanine sensitivity test. The results from these tests taken with the results from the blue/white screen give insight into how these mutants behave differently than wild type RNAP II in terms of their speed, accuracy, and ability to recognize termination signals. We chose to further characterize these mutants because they are in the immediate vicinity of the residues suggested to contact backtracked RNA that we hoped to mutate. These residues do not directly contact the backtracked RNA, but are likely still important for the stabilization of the backtrack site, and it would be useful to know if they share a common mutant phenotype.

Blue/white screen

The blue/white screen tests RNAP II mutants for termination phenotypes. A **reporter** plasmid, pL101Btrp, contains the *E. coli LacZ* **open reading frame** under control of the *Gal10* promoter.[14] This **promoter** is inducible in the presence of galactose. A polyadenylation signal is located in an **intron** upstream of the open

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* The naming convention for RNAP II residues is as follows: the first letter is the one-letter abbreviation for the original amino acid and the number is the position of that amino acid in the polypeptide. The second letter is only added if a mutation has been made, and it is the one-letter abbreviation of the new amino acid at that position.

reading frame of *LacZ*, which makes the time window for cleavage much shorter, because it must occur before the intron is removed by splicing.[14] If the **polyadenylation** signal is not recognized, RNAP II fails to terminate and the *LacZ* gene is transcribed.[15] Plated on media with galactose, the transcription and subsequent translation of the *LacZ* gene causes the yeast colonies to turn blue when exposed to X-Gal. If the polyadenylation signal is recognized, RNAP II will terminate and the *LacZ* gene product will not be made, leaving the colonies white. Yeast with wild type RNAP II and this reporter construct turn light blue due to random chance that splicing occurred before the polyadenylation signal was recognized.[14] This means that RNAP II mutants that have a blue phenotype (such as A729V below) have termination defects that prevent them from recognizing termination signals. Table 1 summarizes data already collected about the mutants from the blue/white screen.

Mutant	Results of Blue/White Screen
R726S	W
A729V	B
V747I	W
D826N	W
T834A	W
A1076T	wt
M1079T	wt

Table 1: Rpb1 mutations and results of the blue/white screen. B denotes a blue phenotype, W denotes a white phenotype, and wt denotes a wild type phenotype.

Mutant phenotypes in the blue/white screen are also correlated with defects in elongation speed *in-vitro*. The blue phenotype is associated with a faster-than-wild-type elongation rate, while white phenotypes are associated with both fast and slow elongating polymerases. Based on previous unpublished data collected by the lab, it

appears that a combination of the white phenotype and fast elongation rate is correlated with backtracking defects.

Mycophenolic acid

Mycophenolic acid (MPA) is a competitive inhibitor of the enzyme IMP dehydrogenase (IMPDH).[16] IMPDH is the rate-limiting enzyme in de novo guanine biosynthesis,[16] and when it is inhibited, the cell experiences a shortage of **<u>GMP.</u>** This creates an imbalance in NTPs available, which makes polymerases that already have trouble elongating especially sensitive.[21] There are two start sites for transcription of the IMPDH gene, and wild type RNAP II can shift to the downstream start site when there is a shortage of GMP.[21] The upstream start site for IMPDH is used when GMP is relatively abundant.[21] Some RNAP II mutants are unable to make this shift or prefer one start site over the other. This test is primarily used to determine whether RNAP II mutants with a white phenotype in the blue/white screen are fast or slow elongating polymerases. Both fast and slow elongating polymerases have defects relative to wild type polymerases. However, it has been hypothesized that gain-of-function mutants use the upstream start site and are associated with faster-than-wild-type elongation phenotypes while loss-of-function mutants use the downstream start site and are associated with slower-than-wild-type elongation phenotypes.[21] This hypothesis could be incorrect, because data from our lab suggests that both fast and slow polymerases are sensitive to MPA.

Canavanine

Rpb1 mutants were also characterized using a canavanine sensitivity test. This tests the accuracy of mutant polymerases. Canavanine is a compound that is structurally similar to the amino acid arginine but is toxic. The *CAN1* gene encodes an arginine transporter, and this transporter also imports canavanine into the cell, killing it. The strains in this study have a mutation in the *CAN1* gene called *CAN1-100,* which is a nonsense mutation. This mutation prevents expression of a functional arginine transporter, and as a result, cells plated on canavanine that read this gene correctly will survive. Some RNAP II mutants make errors when transcribing, which can result in the production of a functional arginine transporter and when plated on canavanine, cell death.[15] Decreased cell growth in this assay is associated with RNAP II mutants with higher rates of transcriptional infidelity.

Results and Discussion

Creation of Mutants

Of the four Rpb1 mutations, one mutant was successfully created, which was T827A. In this mutant RNAP II, the threonine side chain at position 827 was successfully mutated to code for an alanine residue (see Supplementary Information for Amino Acid Reference Table). As Table 2 shows, the troubleshooting process for the other mutations is ongoing.

PCR	Mutation	DNA Polymerase	Protocol Changes	Results
M7	R726H	KOD	5.4 µL 100:1 RPB1 TOPO 2 template DNA	low molecular weight smear
M8	R726H	KOD	Lowered Tm by 1°, 65.3° Different DMSO	low molecular weight smear
M9	T827A	KOD	Lowered Tm by 1°	successful
M10	R726H	KOD	Lowered Tm by 2°, 64.3°	low molecular weight smear
M11	Q1078H	KOD	9 µL 100:1 RPB1 TOPO 3 template DNA	low molecular weight smear
M12	T1080I	KOD	same as M11	low molecular weight smear
M13	Q1078H	KOD	1 µL undiluted template DNA	low molecular weight smear
M14	Q1078H	KOD	no DMSO	low molecular weight smear
M15	R726H	KOD	Annealing time increased to 30s Lowered Tm to 62.3° for first 7 cycles, then raised it to 66.3° for the next 13 10:1 template DNA	low molecular weight smear
M16	R726H	KOD	1 µL 10:1 template DNA Increased denaturation time to 30s Decreased Tm to 60°	low molecular weight smear
M17	Q1078H	KOD	10:1 template DNA Decreased Tm to 60°	low molecular weight smear
M18	T1080I	KOD	same as M17	low molecular weight smear

M19	T827A	KOD	Denaturation and annealing time both increased to 30s	low molecular weight smear
M20	Q1078H	Phusion	none	a long smear
M21	Q1078H	Phusion	1 µL of 100:1 RPB1 TOPO 3 Tm lowered to 54.3° for first 7 cycles and raised to 72° for last 18	a long smear
M22	Q1078H	Phusion	GC buffer instead of HF 1 µL of Phusion 3 min extension time	no true bands or smears

Table 2: PCR modifications to create Rpb1 mutants.

In polymerase chain reactions (PCRs), there are three main steps: separation of the DNA strands, primer **<u>annealing</u>** to the separated strands, and extension of the primers by a DNA polymerase to create full length DNA.[17] Theoretically the amount of DNA doubles after each cycle until later PCR cycles when there is more primer-template than there is enzyme to extend it.[17] DNA concentration plateaus at this phase and may even start to decrease. Another reason for the decrease in DNA concentration is the formation of primer-dimer, which could be what was occurring when we saw low molecular weight smears. However, it is not known whether the smear actually indicated primer dimer or unused primers. Primer-dimer occurs when two primers anneal to each other rather than the template DNA and form a duplex.[17] One study suggested that primer-dimer formation can be suppressed by adding nucleotides to the 5' ends of the primers.[17] The extra nucleotides could possibly lower the chance that the primers would anneal to each other, making binding to the template DNA even more favorable. Another technique suggested to prevent the formation of primer-dimer is increasing the temperatures used in the annealing step of the PCR.[17]   Figure 9 details other common problems and potential fixes for PCR reactions.

PCR problem	Possible reasons	Fixes
No product formed	• Problem with reagents	• Repeat the experiment, as a reagent may have been unintentionally left out
		• Check that the reagents had been fully thawed and mixed thoroughly
		• Try a new vial of dNTPs as they can be damaged by repeated freeze–thaw cycles
		• Use a different polymerase
		• Try using an additive such as DMSO or glycerol
	• Quality of the template DNA is poor	• Use a NanoDrop spectrophotometer to check the quantity and quality of template DNA
	• Too much or too little starting template	• Remake template DNA. Older stocks can degrade, particularly for genomic DNA
		• Set up a series of reactions with varying amounts of template (between 10 and 200 ng DNA)
	• Primers are not working	• Check that the primers have been diluted to the correct concentration
		• Make sure that the sequence of the primer is what you expected it to be synthesized as. Typos are common!
		• Increase primer concentration
		• Redesign the primers
	• PCR inhibitors are present in the template DNA	• Demonstrate that a control gene or another DNA sequence can be effectively amplified using the same template DNA. If it can, there is another problem with the PCR reaction
	• Problem with the settings on thermal cycler	• Check the cycler – Are the temperatures and times as you expect?
		• Change the annealing temperature. Find the optimum temperature by using a gradient cycler and test a range from the lowest primer T_m to 10 °C below the T_m
		• Try the reaction in another cycler – the calibration of the one you are using may be off
	• The template DNA is GC-rich	• Use a polymerase buffer intended for GC-rich templates. Test a full range of the additive (e.g., GC melt)

Figure 10: Possible explanations of and fixes for lack of PCR product.[18]

Site-directed mutagenesis via PCR is a complex procedure that can be influenced by a number of factors. However, the creation of the mutant with two new restriction sites, called TOPO 2 B+D, will change the way that these mutant polymerases are made in the future. With the additional DraIII and BamH1 restriction sites, the plasmid can be cut and an oligonucleotide with the desired mutations can be incorporated into the plasmid directly. The restriction sites are strategically placed around the mutations we want to make, as illustrated in Figure 11. As a result, site-directed mutagenesis via PCR can be skipped in its entirety, which will save time and allow us to move on to the next step: characterization of the Rpb1 mutants. This will also allow us to create double mutants much more easily.
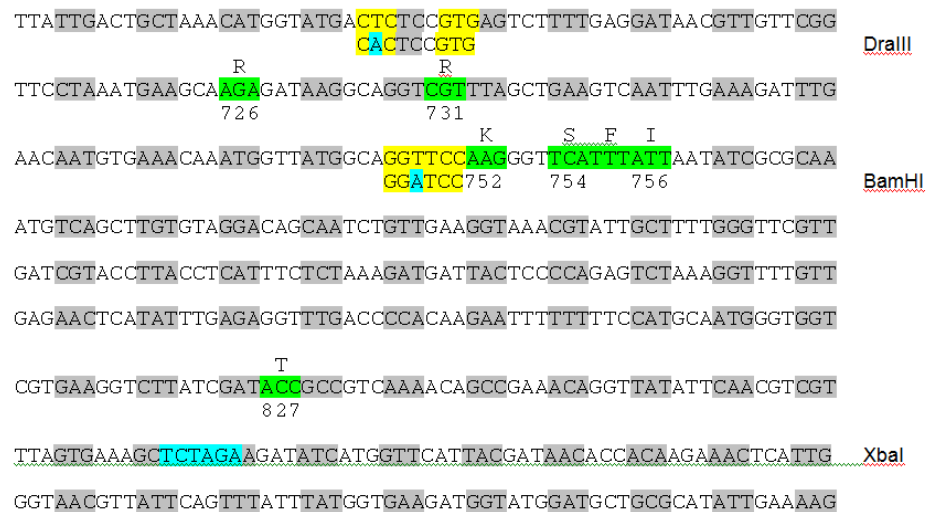
Figure 11: Restriction sites in the new TOPO 2 B+D mutant.

All of the Rpb1 residues proposed to contact the backtrack site (green), excluding the two trigger loop residues, are between the newly created DraIII site (yellow) and endogenous XbaI site (blue). The BamHI site (yellow) will allow groups of mutations in this region to be combined more easily. The blue bases in the DraIII and BamHI sites are the bases that were mutated to create those restriction sites.

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Characterization of Mutants

Mycophenolic Acid

The results indicate that T834A and A729V grew better than most of the other Rpb1 mutants, and on the other end, A1076T and R726S grew much worse. While each of these mutations is located in the same subunit of RNAP II, Rpb1, they are located in different subdomains. Some of the subdomains thought to be involved in backtracking include the pore, funnel, bridge helix, and trigger loop, some of which are shown in Figure 12.
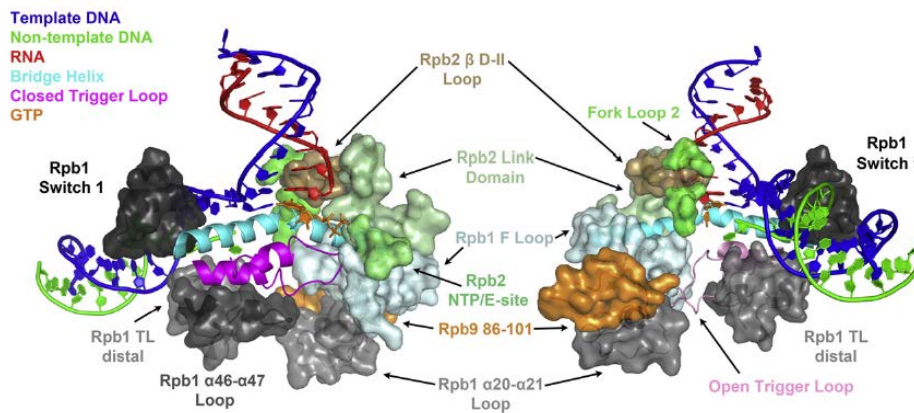
Figure 12: RNAP II trigger loop and bridge helix and other subdomains proposed to interact with them or NTPs.[19]

R726S, A729V, and V747I are located in the funnel subdomain. D826N and the new mutant, T827A (not shown above), are found in the bridge helix, and T834A, A1076T, and M1079T are in the trigger loop. This makes the results more difficult to interpret, because there is no apparent pattern associated with placement of the mutations and growth on MPA plates. Both the funnel and the trigger loop had mutations that showed very good growth and fairly poor growth. It is important to note that A729V was the only mutant which showed a blue phenotype in the blue/white screen, indicating it

could have termination defects. The other mutants tested had white phenotypes with the exception of M1079T and A1076T, which had wild-type phenotypes in that assay. The MPA test allowed us to determine the ability of the mutant polymerases to shift to the downstream start site for transcription of the IMPDH gene. Poor growth indicates less of an ability to shift to the downstream start site and is correlated with a faster-than-wild-type elongating polymerase with elongation defects. Figure 13 shows the results of the dilution series for each mutant plated on MPA.
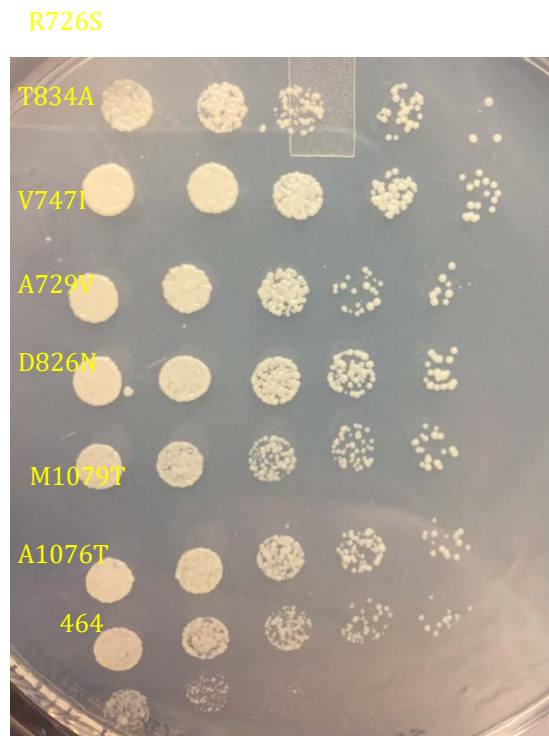

Figure 13: Results of the MPA sensitivity test. This shows that T834A > A729V > M1079T = V747I > D826N > A1076T = R726S.

These results agree with previous MPA tests done in the lab, because M1079T, A1076T, and R726S all grew fairly poorly. However, because V747I and D826N had wild type growth in previous assays, it would be worthwhile to do this test again. While

it is still unclear how mutations in different subdomains affect the ability of RNAP II to switch start sites, many studies have focused on the trigger loop in particular. It has been shown that the trigger loop regulates basic elongation rate as well as participates in NTP substrate selection and mismatch recognition.[20]

As a result, the trigger loop is a major contributor to transcriptional fidelity, and mutations in trigger loop residues can have varying effects.[19] One study suggested that RNAP II trigger loop mutants increase activity for all **substrates,** and even more so for mismatched NTPs, thereby reducing selectivity.[19] Conversely, mutations in the bridge helix have been shown to increase RNAP II activity in a way that promotes translocation or catalysis.[19] In summary, these results suggest that mutations in residues that form the backtrack site have a visible effect on RNAP II. Some mutants grew better, some mutants grew worse, but none of the mutations caused a lethal phenotype. However, the strain that grew the worst was actually the control. I also plated DHY 464, which was the parent yeast strain for these mutations. RNAP II in this strain does not have mutations in Rpb1 residues and is used for comparison against the Rpb1 mutants. Theoretically 464 should model wild-type growth, and because it didn't, this experiment should be repeated before coming to any conclusions.

Canavanine

There was no growth of any of the mutants on canavanine plates, which could suggest that the inaccuracy with which the *CAN1* gene was transcribed restored the functional mRNA, leading to a functional arginine transporter. With a functional arginine transporter, canavanine would be imported into the cell, which causes cell death. This would agree with data from previous work with these mutants, because

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R726S, A729V, T834A, and M1079T all showed decreased growth in canavanine

sensitivity tests. Each showed signs of sickness, or decreased growth, in varying levels

of severity. However, V747I, D826N, and A1076T all showed growth similar to wild-

type in previous tests, which does not agree with my results. My results could also be

confounded by the failure of the controls and/or contamination of the plates. RNAP II

mutants were plated on SD-Arg as a control, and there was no observed growth on these

plates either. This media should not have had an effect on the growth of the mutants. I

also plated DHY 464, which was the parent yeast strain for these mutations. RNAP II

in this strain does not have mutations in Rpb1 residues and is used for comparison

against the Rpb1 mutants. DHY 464 did not grow on canavanine or SD-Arg. My

results could indicate that these mutations make the yeast more "sick" than previously

thought, but these experiments should be repeated before coming to any conclusions.

Future Directions

These experiments showed that creating point mutations in various subdomains of the Rpb1 subunit of RNAP II has an effect on the ability of the polymerase to recognize termination signals, shift to downstream start sites, and the speed of elongation. The mutations may also have an effect on the accuracy with which the polymerase transcribes genes, although there is not yet enough data to come to a conclusion. In the future these experiments should be replicated in order to determine if there is any type of pattern in the phenotypes seen and the location of the mutations in Rpb1 subdomains. Next steps should include combining point mutations and effectively obliterating the backtrack site to see if it has more of an effect on the phenotype of the mutant polymerases. The ability to make double mutants will be made much easier by the creation of the TOPO 2 B+D mutant with the BamHI and DraIII restriction sites. The Rpb1 mutations should also be introduced into strains of yeast that have Rpb9 or TFIIS deletions. Rpb9 is a small subunit thought to play a role in the intrinsic nuclease activity of the polymerase, and it would be interesting to combine its deletion with the destabilization of the backtrack site. The same idea applies to the TFIIS deletion strain, because TFIIS is the transcription factor that cleaves backtracked RNA and rescues arrested polymerases. In conclusion, there is still much work to be done before we understand why backtrack site residues have been conserved and what the function of extensive backtracking truly is.

Glossary

amplify – to increase in number

anneal – the process by which nucleic acids create hydrogen bonds and form a pair of DNA molecules

cloning – the process used to assemble recombinant DNA and direct its replication in host organisms

competent – the ability of a cell to take up exogenous genetic material

conserved – a sequence in DNA or a protein that has remained essentially unchanged even between different species and throughout evolution

enzyme – proteins that are capable of catalyzing specific biochemical reactions

fidelity – accuracy

homologous recombination – exchange of genetic material between two similar molecules of DNA

identity – identical sequence

intron – an sequence of DNA that does not encode for RNA

lesions – sites of DNA damage

microRNA – small RNA that does not code for protein, functions in post-transcriptional gene regulation

mRNA – RNA that conveys genetic information to the ribosome, proteins are coded from this type of RNA

nascent – newly synthesized

non-template strand – the strand of DNA that is not being actively transcribed into RNA

NTP (nucleotide) – nucleotide triphosphate, also called nucleotides, which are essentially the building blocks for DNA and RNA (A,T, C, G, and U)

nuclease activity – enzyme activity that cleaves bonds between nucleic acids

nucleosomes – basic unit of DNA packaging in eukaryotes, consisting of DNA wrapped around a protein core

open reading frame – the portion of a gene that has the potential to be translated

PCR – a procedure used to amplify copies of DNA or introduce mutations

phenotype – the result of an organism's genotype that is observable

polyadenylation – addition of the poly(A) tail to mRNA

primer – a strand of nucleic acid that serves as a starting point for DNA synthesis

processivity – the number of nucleotides a polymerase can incorporate into the
transcript before it releases the template

promoter – a region of DNA that initiates transcription of a particular gene

pyrimidine – any of the nitrogenous bases U, C, or T, which are components of nucleic
acids

reporter – a gene that is attached to a gene of interest so that it is easily assayed

residue – a single amino acid in a protein

restriction enzyme – an enzyme that catalyzes the cleavage of DNA

restriction site – the site in DNA at which restriction enzymes cleave nucleotides by
hydrolyzing the phosphodiester bond

snRNA – small RNA that does not code for protein, functions in RNA processing

substrate – material or substance on which an enzyme acts

TOPO – a vector used for cloning

transformation – genetic alteration via direct uptake of exogenous genetic material

transcriptional arrest – when the process of transcription can no longer occur and a full
transcript will not be made

ubiquitylation – a process of protein tagging that marks other proteins for degradation

wild type – a strain, gene, or characteristic that is predominant in nature

5-FOA – 5-fluoroorotic acid, used in yeast genetics to select for absence of *Ura3* gene

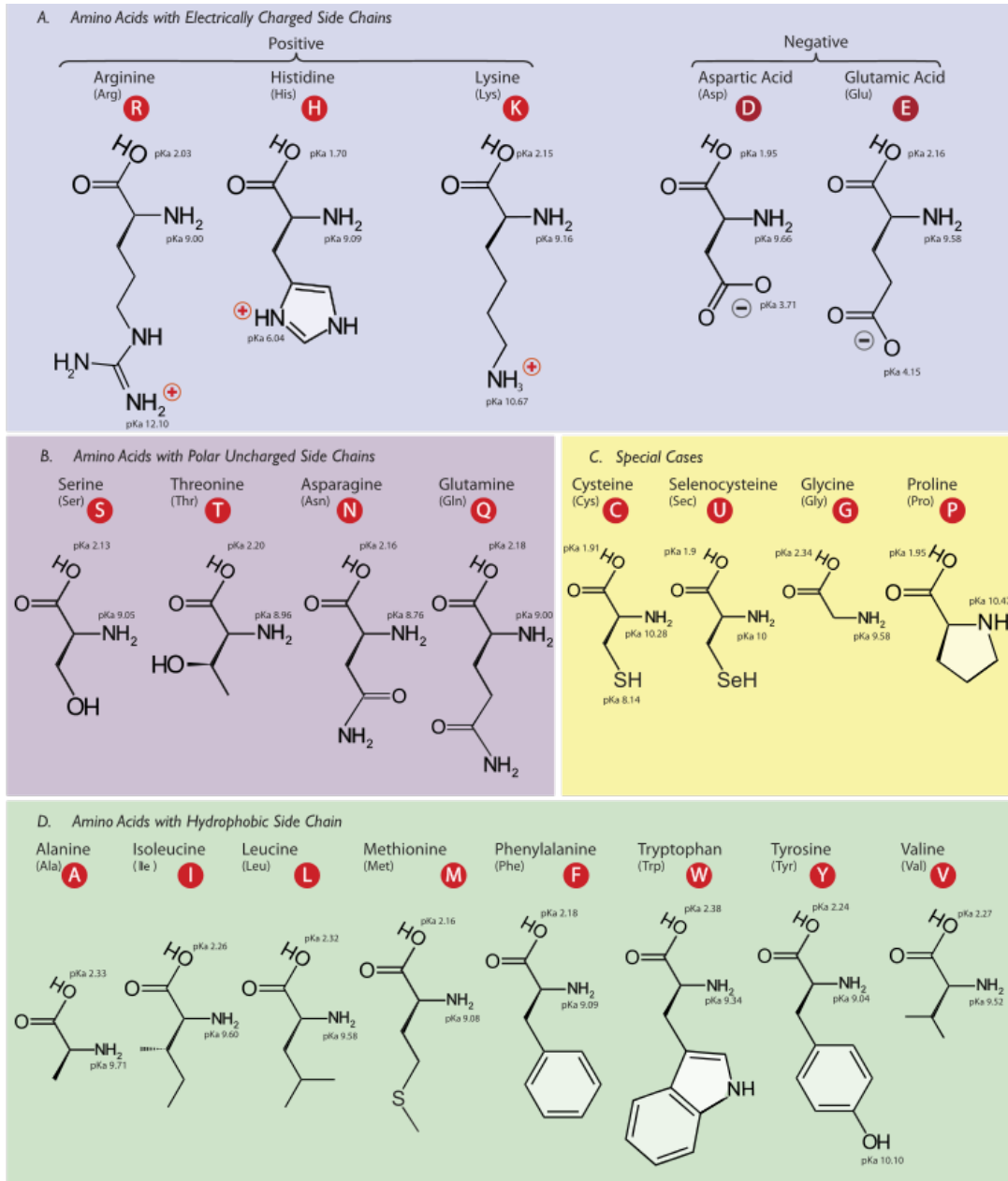# Supplementary Information

Amino Acid Reference Table

Image source: Cojocari, D. (2010). Twenty-one amino acids. [Illustration]. University

of Toronto. Retrieved from https://en.wikipedia.org/wiki/Proteinogenic_amino_acid.

Protocols

Quick-Change PCR using KOD Hot-Start Polymerase

Combine the following reagents in a PCR tube to make a 50 μL total reaction.

Reagent	Volume (μL)
H_2O	29.5
10X Buffer	5
25 mM $MgSO_4$	3
2 mM dNTPs	5
10 μM Forward primer	1.5
10 μM Reverse primer	1.5
Template DNA	1
100% DMSO	2.5
KOD Hot-Start Polymerase	1

The volume of template DNA may vary depending on the concentration of the template

DNA. 10-20 ng is sufficient for this reaction, so a dilution may be required. More

water is added if less than 1 μL of DNA is used. The reaction is then placed in the

Thermocycler, where the following protocol is used (in °C):

1) 95°, 2 min.
2) 95°, 20 s
3) T_m of the primers, 10 s
4) 70°, 3 min.
5) Repeat steps 2-4 for 20 cycles

If the PCR was successful (as determined by running the PCR product on a 1%

agarose gel), then proceed to the Dpn1 digest. For the Dpn1 digest, add 2 μL of Dpn1

to the PCR product and incubate at 37°C for 10 minutes.

Bacterial Transformation using XL2-Blue E. coli Competent Cells

This transformation was used in the creation of the T827A mutant.

Combine 20 μL of PCR product and 100 μL of XL2-Blue competent cells and set on ice

for 10 minutes. Heat shock at 45°C for 1 minute and then put on ice for 1 minute. Add

800 μL of SOC media and incubate at 37°C for 1 hour. Plate onto LB + AMP and

incubate at 37°C overnight.

Bacterial Transformation using TG1 E. coli Competent Cells

This transformation was used in the creation of TOPO 2 B+D.

Combine 100 μL of TG1 competent cells, 30 μL of sterile water, 20 μL of 5X KCM,

and 20 μL of PCR product. Incubate on ice for 30 minutes. Heat shock at 42° C for 90

seconds, and put on ice. Plate on LB + AMP and incubate at 37°C overnight.

Bacterial Miniprep

A culture must be started the day before this protocol is performed. A standard 3 mL

culture is made by combining 3 mL of LB broth with 4 μL AMP/1 mL of LB broth (for

a total of 12 μL of AMP). Incubate overnight at 37°C with shaking.

After the culture has grown to saturation, the cells can be pelleted in Eppendorf tubes

and then proceed to the following steps:

1) Resuspend in 250 μL of P1 solution and vortex

2) Add 250 μL of P2 and invert to mix

3) Add 350 μL of N3 and invert to mix

4) Centrifuge at 13,000 rpm for 10 minutes

5) Pour liquid into spin columns

6) Centrifuge for 1 minute, discard flowthrough

7) Add 500 μL of PB to column, centrifuge, and discard flowthrough

8) Add 750 µL of PE to column, centrifuge, and discard flowthrough

9) Centrifuge again and discard flowthrough

10) Transfer column to an Eppendorf tube

11) Add 50 µL of EB to center of column

12) Wait for 1 minute, centrifuge for 1 minute

13) Keep flowthrough and measure the DNA concentration on a spectrophotometer

pRP114 Enzyme Digest

This digest was specific to the yeast plasmid used in these experiments, pRP114.

Reagents	Volume (µL)
DNA	4.5
1X FD buffer	2
EagI restriction enzyme	1
XbaI restriction enzyme	1
Sterile water	11.5

Put in thermocycler and hold at 37°C for 20 minutes. Run the product on a 1% agarose gel.

Gel Extraction

Isolate the cut plasmid and cut it from the gel. Determine the mass of the gel fragments

and proceed to the following steps:

1) Use 3 equivalents of QG buffer for every equivalent of gel (1 µL/1 mg of gel)

2) Incubate ate 65°C until gel is completely dissolved, vortexing every 2.5 minutes

3) Run through gel column and centrifuge for 1 minute

High Efficiency Yeast Transformation

Five days beforehand, grow the yeast strain you wish to use on YEPD for 2-3 days at

30°C (I used DHY 464). Start a 2 mL culture from one of the colonies in YEPD the day

before and let it incubate overnight at 30°C with shaking. Add 700 μL of saturated culture to 10 mL of fresh media. Grow to OD_{600} 0.7-0.8 at 30°C. Once the sample has reached the desired OD_{600}, pellet cells at 7000 rpm. Resuspend in 300 μL of sterile water and transfer samples to Eppendorf tubes. Pellet the cells. Resuspend in 180 μL of LiAcTE media. Incubated with rotating for 20 minutes. In another tube, combine 2 μL of PCR product, 100 ng of digested yeast plasmid, and 300 μL of PEGLiAcTE solution. Add 5 μL of 2.5 mg/mL boiled ssDNA. Add 50 μL of cells from the culture to this mixture, and incubate at 30°C for 30 minutes on a rotator. Heat shock at 42°C for 20 minutes. Pellet cells and resuspend in 200 μL of sterile water. Plate onto selective media (SD-Leu in my case) and allow growth for 2-3 days at 30°C.

High Efficiency Yeast Transformation using Competent Cells

Thaw 1 aliquot of competent cells on ice for 10 minutes from the -80°C freezer. Add 10 μL of 2.5 mg/mL boiled ssDNA, 2 μL of PRC fragment product, and 100 ng of digested plasmid DNA. Add two volumes of 50% PEG_{3350} and incubate at 30°C for 30 minutes. Heat shock at 42°C for 15 minutes. Pellet cells and resuspend in 1 mL of freshly made SOS media. Incubate at 30°C for 1 hour. Pellet cells and resuspend in 200 μL of sterile water. Plate onto selective media (I used SD-Leu) and allow growth for 2-3 days at 30°C.

5-FOA Plates

Use 0.1 g 5-FOA/100 mL agar for four plates. Melt SD-bottom agar. Add 10X amino acids to agar in sterile Erlenmeyer flask. Spray stir bar with ethanol and briefly flame it. Add flamed stir bar to flask. Add 5-FOA and stir for 5 minutes. Pour plates.

Yeast Miniprep

Colonies from the 5-FOA plates should be restreaked onto SD-Leu, and a colony from the SD-Leu plate should be used to start a culture to prepare for the miniprep. Proceed to the following steps:

1) Pellet cells in Eppendorf tubes

2) Resuspend in 250 µL of zymolase, BMA, and EDTA mix and vortex

3) Incubate for 90 minutes at 37°C

4) Add 50 µL of miniprep mix, vortex

5) Incubate at 65°C for 30 minutes

6) Add 63 µL of 5M KOAc, invert to mix

7) Put on ice for 30 minutes

8) Centrifuge at 13,000 rpm for 10 minutes

9) Transfer supernatant to new tube

10) Add 720 µL of ethanol, invert to mix

11) Centrifuge for 10 minutes

12) Dry pellet using desiccator for 10-15 minutes

13) Resuspend in 50 µL of TE

14) Store at -20°C

Bacterial Transformation using DH5-α E. coli Competent Cells

Aliquots of DH5-α cells should be thawed from the -80°C freezer for 10 minutes, and proceed to the following steps:

1) Add 1 mL of cold 0.1M $CaCl_2$ to each 50 µL aliquot of DH5-α cells and mix well

2) Add 250 μL of cells to 1.5 mL of yeast miniprep DNA in a small culture tube

3) Incubate on ice for 1 hour

4) Heat shock at 37°C for 2 minutes

5) Add 1.5 mL of LB broth and incubate for 1 hour at 37°C

6) Transfer cells to Eppendorf tubes and pellet

7) Remove all but 200 μL of broth

8) Resuspend cells and plate on LB + 4X AMP

9) Incubate overnight at 37°C

Yeast Strain "Magic" Transformation

The yeast strain that you wish to use should be grown in an overnight culture in YEPD.

Once culture is saturated, proceed to the following steps:

1) Pellet 250 μL of saturated culture

2) Resuspend in 86 μL of freshly made "magic mix," 10 μL of 0.1M DTT, and 2.5 μL of 2 mg/mL boiled ssDNA

3) Add 300 ng of plasmid DNA

4) Incubate at 45°C for 30 minutes

5) Plate on selective media (I used SD-Leu) and allow growth for 2-3 days at 30°C

Mycophenolic Acid and Canavanine Sensitivity Tests

Three days beforehand, streak out the strains you wish to use on selective media and allow growth for 2-3 days at 30°C. Grow 2 mL cultures from the colonies in SD-Leu or appropriate selective media at 30°C the day before this test is performed, and proceed to the following steps:

1) Cultures must have OD_{600} between 0.6-0.8

2) Add 80 μL of water added to columns 1-8 on a 96-well plate (or as many samples as you have) and down four rows (for dilutions)

3) Using calculated value from dilution series Excel sheet, dilute to OD_{600} 0.1 in row 1

4) Do serial 1:5 dilutions starting with row 1 of each column

5) Plate 5 μL of each dilution (rows 1-5 for each sample) onto appropriate media and allow growth for 2-3 days at 30°C

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