RNA polymerase (RNAP) occupies a fundamental position in the biology of living organisms. It is responsible for DNA transcription, the first step of genetic expression, and is therefore highly regulated by the cell. If transcription is not regulated, cellular processes can become unsynchronized, possibly leading to uncontrolled cell growth and cancer. Transcription is also regulated to ensure the fidelity of the RNA transcript. If RNAP incorporates incorrect bases and induces errors into the nascent RNA, defective proteins will be made that can be lethal to the cell. In addition, because the function of RNAP is so integral to life, certain antibiotics target RNAP enzymes in disease-causing bacteria. The antibiotics disrupt the enzymes’ processivity and kill the infectious bacteria.

RNAP pause types and mechanisms

The goal of this research is to study the pausing motion of RNAP as it transcribes DNA. RNAP transcription is not a continuous process; it is punctuated by temporary pauses in movement along the template DNA. During pauses, the RNAP takes 100-1000 times longer to build the RNA chain\(^1\). Pauses can therefore range from a few seconds to a few minutes duration. The cell implements different pausing mechanisms based on the conditions and nutrient requirements in the cell. For example, in Escherichia coli, pausing might couple transcription and translation by synchronizing the interaction between RNA synthesis and ribosome movement in the coding portions of mRNA. This coupling may well regulate the attenuation and termination of bacterial operons, a genetic unit of related genes and control sites\(^2\).

Specific sequences of DNA have been known to induce RNAP pausing, and different sequences have varying probabilities of RNAP pausing. It is thought that there are two mechanistically different types of sequence dependent RNAP pausing (Figure 1): Class I pauses, such as the his leader pause sites, which are dependent on the formation of a 5-basepair-stem and 8-nucleotide-loop RNA hairpin structure; and Class II pauses, such as the ops (operon polarity suppressor), which involve the RNAP backing up on the DNA and blocking the active site with nascent RNA\(^3\).

Experimental methods for studying RNAP pauses

Previously, RNAP pauses have been studied by biochemical experiments. These assays have been done in bulk solution that involve trillions of RNAP molecules transcribing simultaneously. Under these conditions, data is collected from so many molecules that fine details of motion are averaged out and cannot be distinguished. In contrast, single-molecule experiments measure motions of single molecules. These studies help to identify the uniqueness of individual enzymes and distinguish between subpopulations of RNAP molecules. Research at the

Becky Wong
Advantages of concatenated pauses studied in optical trap experiments

This research studies RNAP transcribing a DNA template of concatenated pauses, or a string of repeated and identical pause sequences. This method offers many promising insights into the polymerase’s pausing mechanisms. Previous optical trapping experiments have shown that random, stochastic RNAP pauses occur while the enzyme moves along the DNA\(^6\). The concatenated template will also allow us to study the behavior of an individual enzyme as it encounters the identical sequence of DNA multiple times. The mechanism by which a specific RNAP acts subsequent times to transcribe the same stretch of DNA has not yet been determined. This information will help elucidate the theory of molecular individuality. The idea theorizes that each molecule will act differently from another molecule even though they are identical in sequence and structure.

Using optical traps to study the pauses of RNAP gives us specific data on the transcriptional motion of the enzyme. The trap works because the focused infrared laser attracts particles toward its focal region. Thus, trapping the bead connected to the RNAP will allow us to apply a range of forces on the enzyme as it transcribes the DNA template and encounters long-lived pause sequences. The behavior of the polymerase under an applied force will provide clues regarding the mechanism of the pauses. For example, the formation of a hairpin structure in nascent RNA has been known to induce pauses in the \(\text{his} \) pause sequence\(^7\). Although it is unclear how the hairpin causes a pause, there are a few theories. One idea is that the hairpin interacts allosterically with the RNA strand thereby influencing the binding of molecules at other sites on the RNAP and disrupting transcription. Another possibility is that the hairpin structure might physically displace the polymerase from the

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**Figure 1** Model for RNAP pause states.

RNAP bound to the DNA is actively transcribing the coding strand to generate messenger RNA. From the active transcription elongation phase, RNAP enters an unactivated intermediate state and undergoes small rearrangements in the active site to form two different types of stabilized and sequence dependent pauses. The Class I \(\text{his} \) pause (left) occurs with the formation of an RNA hairpin in which the single stranded RNA bends and base pairs with itself. The hairpin in the nascent transcript interacts with the polymerase, disrupting its motion. The pause occurs before the downstream DNA. In the Class II \(\text{ops} \) pause (right), the RNAP moves backward along the RNA-DNA hybrid so that the RNA transcript is excluded from the NTP entry channel of the polymerase.

Single molecule level elucidates properties of individual polymerases, such as pause probability and pause duration. From this data, we can investigate whether particular RNAP molecules tend to pause more frequently or longer than others.

Recently, new techniques have been developed to study single RNAP molecules moving along the DNA\(^4\). These techniques have high temporal resolution and allow for the study of low probability events from single molecules which would otherwise be averaged out in bulk studies. One of the main single molecule methods is optical trapping, pioneered by Ashkin \textit{et al.}\(^5\). The technique uses a focused laser beam to manipulate microscopic particles without mechanical contact. By applying this technology in conjunction with biochemical techniques, we can study transcription with a level of detail that is impossible to achieve with conventional bulk experiments alone.
DNA and nascent RNA. The application of force on the polymerase allows us to distinguish between these two theories.

If the RNA hairpin dislodges the RNAP from the DNA and RNA transcript, the pause is mechanical and force-dependent. This should result in a greater effect of optical trapping forces on RNAP’s pausing efficiency. In the case of the ops pause sequence, the backtracking motion of the RNAP at a pause site should be highly force dependent. The optical trap experiments could either pull the RNAP forward toward the downstream DNA or pull the RNAP backwards toward the upstream DNA. This data should help us determine the translocated distance and illuminate the finer mechanisms involved with entering and exiting the pause state.

In this study, we attempted to make a concatenated template of DNA with multiple, identical stretches of DNA ligated together. Instead of tracking the motion of RNAP transcribing just one pause site, we can study transcription of eight concatenated pause sequences of DNA. Thus each run of the polymerase is effectively eight single-pause experiments in one. The concatenated templates help us characterize the pausing and velocity of RNAP-mediated transcription with resolutions approaching the order of several base pairs.

**Materials and Methods**

Ligation of pause sequences and two flanking regions of DNA

To construct the DNA template, eight separate oligonucleotides (three complementary pairs) were used. The pieces ranged in size from 73-78 base pairs. Each of the complementary oligos was annealed, creating complementary overhanging ends. The sticky ends of each of the three annealed oligos were ligated in a specific orientation of Segment A, Pause, and Segment C. Furthermore, the (A-Pause-C) sequence will be referred to as a pause unit. Each of the pause types, his and ops, were made into separate pause units, with the pause sequence flanked by the two other sets of complementary oligos. Single pause units were ligated into a cloning plasmid (pCR Blunt from Invitrogen), transformed into *Escherichia coli* Stbl2 cells (Gibco BRL), and selected on LB agar plates supplemented with Kanamycin antibiotic resistance (50 μg/ml). Preparing the pause units for cloning

The pause units were concatenated to form a repeating sequence of eight pause units using a protocol established by Carrion-Vazquez et al. The method capitalizes on the unique qualities of two restrictions sites, BamHI and BglII. In addition to being palindromic restriction sites, which ensures orientation specificity, restriction digests at BamHI and BglII sites leave complementary, compatible cohesive ends used to screen for desired ligation products. Using the iterative process of digesting, ligating and cloning, we multiplied the number of repeated pause units in a single vector. The first round of cloning yielded a 2mer (two pause units), the second round produced a 4mer, and the third round gave us the desired 8mer. The concatenation of eight repeated pause units was cloned into the pRL732 plasmid behind the strong T7A1 promoter in order to perform transcription assays.

**Constructing labeled template for optical trapping experiment**

In order to construct transcription templates for the assays in the optical trap, we needed a linearized piece of DNA with a labeled end to attach to the microscope slide. Each of the his and the ops templates were digested with ScaI restriction enzyme to linearize the plasmid. Then, a terminal transferase reaction was performed (Roche-DIG Oligonucleotide 3′-End Labeling Kit, 2nd Generation) to label both 3′ ends with a Digoxigenin-ddUTP. Since the microscope slide is coated with anti-digoxigenin, the digoxignin on the DNA attaches the template onto the surface of the slide through an antibody linkage.

Two types of experimental geometries can be produced from each of the his and ops transcriptional templates. Digestion of the DIG-labeled DNA with Eco109I restriction enzyme removes the upstream 3′ end of the DNA, leaving the downstream end of the DNA tethered to the microscope slide. Thus as the RNAP transcribes the DNA, the optical trap pulls the RNAP in the opposite direction that it would normally move as it transcribes DNA. In the hindering load regime, the DNA tether becomes shorter as it transcribes DNA (See Figure 2).

In a similar fashion, digesting the doubly-DIG-labeled DNA with ScaI leaves the downstream 3′ end label of the DNA and consequently allows for the optical trap to create an assisting force on the RNAP as it moves along the DNA template. In this case, the DNA tether becomes longer as the polymerase transcribes DNA.

**Optical Trapping**

To perform optical trapping experiments, the DNA was attached to the coverslip of a microscope slide via a digoxigenin / antibody linkage. A 500 nm polystyrene bead was attached to RNAP by a biotin molecule and avidin protein linkage, a strong noncovalent bond, and the RNAP was bound to the DNA. Once ribonucleotides (rNTPs) are added, RNAP starts transcribing and its position on the DNA template is recorded as a function of time. Using the optical trap, an effective force can be calculated from the template versus time position of the RNAP. By collecting data at varying forces and calculating the efficiency of pausing as a function of force, we should be able to distinguish between the allosteric
and mechanical models of the RNAP pausing for the Class I *his* pause and observe backtracking events with the Class II *ops* pause.

The optical trapping experiments were performed in a low vibration, temperature-regulated clean room at 21.1 ± 0.5°C. The optical trap used to collect data was fundamentally the same instrument as described in previous work from the lab. A feedback-equipped, piezoelectric stage (Physik Instrumente, Karlsruhe, Germany) was used to allow three-dimensional positioning in order to maintain constant force on the bead. Further modifications included adding a more powerful and stable Nd:YVO4 laser at 1064nm, removing the Wollaston prisms, and replacing the interferometric detection system with a position sensing diode. Custom software was written in LabView (National Instruments) to run the automated microscope stage. The instrument has allowed the collection of data with motion resolved temporally at 1/50th of a second, spatial precision of ~1nm, and accuracy within 70 nm.

**Results and Discussion**

In summary, we constructed two types of templates with different repeating pause sequences: the *his* pause and the *ops* pause. Each of the types of pause sequences has been biochemically engineered to allow the RNAP to transcribe in either the assisting load or the hindering load regime in the optical trap. These four DNA templates offer a multitude of different experiments and opportunities to probe the mechanism and force dependencies of RNAP’s pauses along the DNA.

The templates of the *his* and the *ops* concatamer of pause sequences were constructed as shown (see Figure 3). Sequence analysis confirmed that the DNA templates have eight concatenated pauses, and gel electrophoresis assays indicated the expected template lengths of 6.8 kilobases for the *his* pause template and 6.7 kilobases for the *ops* pause template.

The DNA sequence has been modified with the appropriate digoxigenin labels for the hindering load and the assisting load geometries under the optical trap. Recent data includes some partial traces of RNAP transcribing the region of DNA with the concatenated pause. The traces are considered partial because RNAP position measurements did not start precisely when the RNAP began transcribing the DNA.

We have recorded traces of RNAP transcribing the DNA sequence of concatenated *his* pauses under a 5 ± 0.8 pN hindering load from the optical trap (see Figure 4). The partial traces detect the position of the bead connected to the RNAP. The y axis indicates the template position (in base pairs) of RNAP transcribing the DNA as a function of time. The forward motion of the polymerase is indicated by the decreasing base pair position on the template over time. The RNAP moves from the end of the template toward the beginning, and the tether length of the DNA connected to the slide gets shorter. The motion is discontinuous, as transcription is interrupted by pauses. These pauses are indicated by the horizontal segments in the trace. RNAP motion is affected by the availability of free rNTPs which are incorporated into the elongating RNA transcript. Thus, these initial experiments were done under saturating 1mM concentrations of A, U, C, and G nucleotides. The high levels of rNTPs allowed for RNAP transcription to be observed.

Based on our sequence analysis, the *his* pause template should have its first pause when the RNAP has reached the template position of 2861 bp with the pause sequence repeating every 239 bp. The grey bars indicate possible locations of expected pauses. Shown in

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**Figure 2** Schematic representation of a hindering load optical trapping assay (not to scale).

The light from the focused infrared laser, known as an optical trap, exerts a force on the 500 nm polystyrene bead that grows linearly as the bead moves from the center of the trap. This force holds the bead at a predetermined position from the trap center. The RNAP is attached to the bead via a biotin/avidin linkage and is bound to the double stranded DNA and the elongating RNA (gold). The downstream end of the DNA is attached to the microscope slide surface via digoxigenin and anti-digoxigenin. The microscope slide is mounted on a piezoelectric stage. The optical trap exerts force in the opposite direction of the polymerase’s transcriptional motion; to compensate for the enzyme motion, the stage moves in the horizontal and vertical directions to maintain the bead’s position in the trap and thus exert constant force on the bead.
the figure are two separate traces of the RNAP molecules transcribing the *his* pause DNA template. Although there is a positional uncertainty error of up to 200 bp, the traces suggest regularly spaced pauses of eight concatenated sequences. Periodic pauses are observed and the two traces appear to have some correlation in terms of aligning the pauses with each other.

It is interesting to note the variation between the traces. Although both traces are from the RNAP transcribing the same sequence of the DNA, different RNAP molecules behave differently at the same pause site. For example, the RNAPs did not always pause at the expected sites. At the template position around 1600bp, there is an expected pause site. Surprisingly, however, there did not appear to be a pause in the red trace, but there was a pause in the blue trace. This discrepancy between traces supports the idea of variable pause efficiencies, in which a sequence of DNA might have only a certain probability of inducing the RNAP to pause. Another explanation for the different pausing behavior at around 1600 bp is that with the blue trace, the long pause at 1900 bp might have predisposed the molecule for more pausing later in the template. A long pause might sufficiently slow the polymerase’s transcriptional rate and allow it to enter the next pause state more easily.

The traces indicate various durations, or pause lifetimes, between different RNAPs as well as between different pause sites with the same RNAP. For example, at the template position around 1900 bp, the red trace indicates RNAP spent a few seconds in a pause, but in the blue trace, the RNAP spent orders of magnitudes longer in the pause. Other factors might have contributed to the polymerase in the blue trace displaying an exceptionally long pause at 1900bp. Previous studies in our laboratory have reported non-sequence-dependent pauses, separate from the sequence-dependent pauses studied in this project. Nucleotide

Figure 3 Diagrams of the *his* and the *ops* concatemer of eight pause sequences.

The plasmids are both over 6.5 kilobases long and have a T7A1 promoter. The pause sequences are concatenated, repeating eight times, occurring every 239 bp for the *his* pauses and every 227 bp for the *ops* pauses. Each of the eight pause sequences is flanked by two restriction sites, BamHI and BgIII.

Figure 4 Traces of RNAP transcribing the DNA template

The y-axis gives the position of a single RNAP molecule transcribing a portion of a 4 kbp DNA template recorded as a function of time. RNAP movement is observed with 1 mM rNTPs concentration and hindering force of 5pN against the direction of transcription. There is a discontinuous motion punctuated by pauses on various time scales. In the hindering load regime, the polymerase moves farther along the DNA, the template position decreases, and the DNA tether length decreases.
misincorporation events in the growing RNA transcript can induce long-lived, non-sequence dependent pauses. This type of pause induced by an error in the RNA could have contributed to the long-lived pause observed at around template position 1900bp in the blue trace. Also, non-sequence dependent pauses can occur ubiquitously throughout the template and might explain some of the early pauses occurring at template positions shorter than 1000 bp and the predicted start of our concatenated pause sequences.

Data have been collected under a limited range of conditions. Most of the data have been recorded at saturating rNTP concentrations (1mM of each A, C, G, and U nucleotides), and we have observed transcription punctuated by pauses. We plan to lower the rGTP concentrations to enhance pausing at our pause sequences. Furthermore, there will be experiments using the other geometry in which the RNAP will be subjected to an assisting load as it transcribes the DNA in the optical trap. In total, there will be four different combinations of assisting and hindering forces using two different types of pause sequences, the his and the ops.

Our findings suggest pausing at the expected pause sites, as well as random pauses between the known, predicted pause locations. There are multiple types of pauses that can be observed in any single trace of the RNAP transcription. Because each trace shows the motion of a single RNAP transcribing the pause sequences, a large number of traces need to be obtained for each combination. The pausing statistics will help to determine the probability of the RNAP entering a pause state when it encounters a pause site and the average lifetimes of the pauses. With the DNA template of identical and repeated his pause sequences, we have found that the RNAP pausing varies between successive pause sites.

References


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Becky Wong is majoring in biological sciences and pursuing a co-terminal masters degree in chemical engineering in 2004. Her love for science began in high school as a lab assistant at Mendel Biotechnology, Inc. At Stanford, she spent three years conducting single-molecule biophysics research in the laboratory of Dr. Steven Block and received a URO Major grant in 2002. The Block Lab members were instrumental in the success of her project, with special thanks to Kristina Herbert, Ravi Dalal, Dr. Keir Neuman and Professor Block. Becky would also like to thank her parents and older brother of Castro Valley, California for their continued support.