Backtracking kinetics determine the force sensitivity of RNA polymerase II in a factor dependent manner
(E. A. Galburt and S. W. Grill, et al.)
**Supplemental Figures and Tables**

**Figure S1**

Force vs. time with corresponding schematics of trap and bead position during a typical experiment. (1) Fishing: a tether is formed between the streptavidin bead (SA) and the anti-dig bead (αD) by alternatively moving the two beads closer together and further apart until a finite force is detected between them. (2) Relaxation: the moveable lens element of the optical tweezers is allowed to relax until the force no longer changes with time. (3) NTP flow: activity buffer with 1 mM NTPs is flowed into the cell introducing a hydrodynamic force on the beads while the flow is present. (4) Baseline return: NTP flow is stopped once the buffer has exchanged completely. The new baseline before transcription begins is quite flat. (5) Transcription: the enzyme begins to transcribe along the DNA making the tether shorter and pulling the beads further out of their respective traps. This activity results in an increase in the force. (6) Stall: the enzyme ceases to transcribe and force remains constant. (7) Stretch: the tether is stretched to high force by changing the position of one optical trap to verify that a single tether was linking the two beads. (8) Rip: a single rip is observed which confirms that only one tether was present between the beads. Runs with multiple rips were discarded.
Diagram depicting the elongating state (E, top), backtracked state (B), and TFIIS rescued elongating state (E, bottom). The kinetic scheme discussed in the text is shown on the left. An elongating enzyme may enter a backtracked state. The enzyme may return to an elongating state via two paths. First, it can return to the original elongating state by moving forward. Alternatively, TFIIS may bind the polymerase and catalyze the endonucleolytic cleavage of the RNA transcript creating a new 3' RNA end in register with the active site of the enzyme. Both RNAP II and its bacterial homologues have been shown to backtrack during elongation. The combination of backtracking and cleavage may serve as a transcript proofreading mechanism. In addition, this backtracked state plays a role in transcriptional pausing, transcriptional arrest, and transcription-coupled DNA repair. Since backtracking involves the displacement of the enzyme along the template, it is amenable to single molecule studies and has previously been observed directly with optical tweezers in the bacterial system.
Figure S3

Backtrack distance histogram. The distribution of all observed backtrack distances is well described by an exponential (two parameter fit, $R^2 = 0.95$). Our experimental setup prohibits the detection of backtrack distances less than 3 nt. The exponential fit predicts that ~59% of all backtracking events fall within this range and are therefore not detected. Although not conclusive, the coincident observation of a backtracking event at 47% of enzyme arrests in the absence of TFIIS strongly suggests that all arrests are likely to be a result of backtracking.
An example of force-jump experiment. During active transcription, an optical trap was displaced resulting in the sudden increase in force. After one second, the trap is restored to the original position. The jumps were scored as to whether the enzyme continued transcription (1), maintained position (3), or backtracked (2, 4, 5), by looking at the positions of the enzyme immediately before and immediately after a jump (see supplementary methods). We observed a tight correlation between force-jump induced backtracking and the induction of long duration pauses and arrests.
Figure S5

(a) A further comparison of typical runs in the presence (top) and absence (bottom) of TFIIS at 100 Hz bandwidth (black) and filtered with a 3rd order Savitzky-Golay filter with a time-constant of 2.5 s (white). Note that the pause free velocity does not decrease even at the higher forces reached in the presence of TFIIS (see Figure S6). Traces are shifted in time for clarity. (b) A closer view of the end of two runs in the presence of TFIIS at 15 and 18 pN, respectively (also see Figure 3e). Once the enzymes have reached higher forces where backtracking becomes appreciable, the futile cycle of backtracking, followed by TFIIS rescue and transcription can be observed.
Pause-free transcription velocity as a function of force in the presence of TFIIS. The graph shows the normalized force-velocity curve for all polymerases that transcribed to high force in the presence of TFIIS (N=8). The pause-free velocity does not exhibit any force dependence ($R^2 = 5 \times 10^{-3}$) in the range of forces sampled (4-17 pN) indicating again that translocation is not rate limiting.
Normalized distribution of instantaneous velocity fit to a double Gaussian. The first Gaussian has a mean of 0.06 nt/s with a standard deviation of 0.8 nt/s and represents paused polymerases. The second Gaussian represents actively transcribing enzymes with a mean of 8.2 nt/s and a standard deviation of 6.6 nt/s. Due to differences in the method used to determine pause-free velocity in the main text, the value cited there is slightly higher than the mean value of the second fitted Gaussian here.
Figure S8

(a-c) Velocity as a function of force for individual enzymes. Transcription velocities between 5-25 nt/s are interrupted by pauses where the velocities drop rapidly. A velocity threshold is shown as a dotted horizontal line and can be used to identify and pick pauses. At the end of a run, the enzymes enter a pause (0 nt/s) and never recover. In some cases, negative velocities (backtracking) can be observed. In each example, the velocity drops rapidly at the end of the run, however it does so at different forces (4.5 - 12 pN) for different enzymes. This feature is maintained in the normalized F-V curve shown in Figure 2c in the main text. An un-normalized F-V curve averaged over all 33 experiments is shown for comparison in (d). Here, the gradual decrease of velocity as a function of force is merely a consequence of the different forces where the transcription ends for individual enzymes.
Table S1

<table>
<thead>
<tr>
<th>Condition</th>
<th>Force Limit (pN)</th>
<th>Velocity (nt/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-TFIIS (N=33)</td>
<td>7.4 ± 2.0</td>
<td>12.2 ± 4.5</td>
</tr>
<tr>
<td>+TFIIS low force (N=24)</td>
<td>8.5 ± 2.0</td>
<td>11.3 ± 4.1</td>
</tr>
<tr>
<td>+TFIIS high force (N=8)</td>
<td>16.5 ± 3.5</td>
<td>10.1 ± 2.6</td>
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</table>
**Supplemental Information**

**Initiation method**

This method of initiation has been extensively studied structurally via extensive footprinting assays and has been shown to be indistinguishable from elongation complexes initiated via the pre-initiation complex\(^\text{17}\). Furthermore, apart from the presence or absence of general transcription factors that might remain bound to the polymerase, elongation complexes initiated in a piecewise manner appear to have identical properties in all experiments reported to date.

**Backtrack detection**

Backtracks were detected by looking at the first 4 s of a scored pause and asking whether the enzyme moved backwards by more than 3 nt. These strict limits were determined so that we could be sure a backtrack signal was not generated by noise. In particular we minimized the probability of detecting a backtrack in DNA only control tethers, and estimate the rate of false positive backtracks to be 5% (data not shown). In particular, the concurrence of the beginning of a pause with a detected backtrack allows us to be confident of the signal.

**Normalized force-velocity curve**

For the normalized curve, the F-V curves from individual polymerases were normalized by the velocity at low force and by the force at which velocity drops by 50% and subsequently averaged. We note that the force insensitivity is not a result of the spread in individual enzyme velocities since the same trend is observed using only the fastest (or slowest) enzymes (data not shown). Additionally, enzyme velocity is uncorrelated with the largest forces reached in a run and the F-V curve is essentially unchanged if transcription velocity is calculated without removing pauses (data not shown).

**Transcription in the presence of TFIIS**

75% of the runs obtained in the presence of TFIIS (24/32) were not significantly different from runs obtained in the absence of TFIIS as they exhibited indistinguishable pause-free velocities and reached the same forces. In particular, a similar percentage of arrests correlated with observable backtracks for these 24 enzymes, suggesting that TFIIS did not bind productively in these cases.
**Supplemental Methods**

**Formation of stalled elongation complexes**

Elongation complexes were prepared as previously described\textsuperscript{17} with biotinylated RNA polymerase II\textsuperscript{18} (\textit{S. Cerivisae}, unphosphorylated C-terminal domain) using the following oligonucleotides (Oligos Etc., listed 5’-3’) via the ordered additions of RNA primer, DNA template strand, RNAP II, and DNA non-template strand.

TDS54  
CAAGGGTGTCGCTTTGGGTTGGCTTTTCGCCGTGTCCCTCTCGATGGCTGTAAGT

RNA9  
AUCGAGAGG

NDS50  
ACTTACAGCCATCGAGGGGACACGGCGAAAAGCCAACCCAAGCGACACC

The 5’-end of TDS54 created a 4-base overhang that was used to ligate the minimal EC to a 9.8 kb template DNA with pseudo-random sequence (see supplementary sequence file).

A single colony of \textit{E. Coli} transformed with the pEG2 template (see attached sequence file) was grown in Luria broth with ampicillin and the plasmid was purified (Qiagen Megaprep, 12181). The plasmid was digested with EcoRI (New England Biolabs (NEB), R0101) and end-labeled with digoxigenin-11-dUTP (Roche, 1093088) using the Klenow fragment of DNA polymerase I (NEB, M0210). After phenol extraction, ethanol precipitation with 10 M ammonium acetate, and re-suspension in TE (10 mM Tris pH 8.0, 1 mM EDTA), the template was digested with StyI (NEB, R0500) to create the proper overhang for ligation to the EC. The result of this digestion was two fragments of lengths 9.8 kb and 17 bp. After a round of phenol extraction followed by ethanol precipitation, the pellet was re-suspended in 10 mM Tris pH 7.5, 10 mM EDTA, and 400 mM NaCl. The 9.8 kb template was separated from the 17 bp fragment via precipitation with 15% poly-ethylene-glycol 8000 (0.3 volumes of 50% PEG 8K). The pellet was washed with 70% ethanol, re-suspended in TE and ligated to the minimal EC as described previously\textsuperscript{17} (T4 DNA ligase (NEB, M0202) at 12°C for 1 hour).

Polymerases were pulsed with 250 µM GTP, ATP, and CTP (Fermentas, R0481) in TB40 (40 mM KCl, 20 mM Tris, pH 7.9, 5 mM MgCl\textsubscript{2}, and 1 mM β-mercaptoethanol) for 15 minutes at 25°C. A StyI digest was performed to select for active polymerases as described in the text at 37°C for 10 minutes.
Optical trap and experimental procedure

The optical trap utilized for these experiments is based on a setup previously described 19. To reduce experimental drift, the dual-beam, single-trap setup instrument was converted to a dual-trap dual-beam machine by slightly overfilling each objective. Bead displacement was measured in both traps, and each trap was calibrated independently by the thermal noise power spectrum method 20. Trap stiffness was dependent on laser power, but typically was set to 50 pN/µm for both traps with the 2.1 µm polystyrene beads utilized (Spherotech). Bead displacements were measured in both traps and only the anticorrelated signal is analyzed further. With our 9.8 kb tether and at a bandwidth of 1 Hz, these enhancements provided us with an r.m.s. noise of 3 nt (~ 10 Å) at 5 pN load and 2 nt (~ 6.6 Å) at 10 pN load, respectively. We estimate that we are able to measure relative displacements with an uncertainty of ~3 nt. Uncertainty in velocity also varied with force, but averaged to ~1 nt/s.

The experimental procedure after assembly of a single ternary complex in between both beads is described in detail in Figure S1. To acquire data at increasing loads for individual enzymes, both traps were run without experimental feedback in passive mode. Because in this mode the distance between both trap centers remains constant, the load increased as RNAP II progressed along the DNA template and shortened the amount of DNA between the beads. The distance between both trap centers was calculated by making use of the known initial contour length of 9830 nt and utilizing the worm-like chain (WLC) model with a persistence length of P = 53 nm and a stretch modulus S = 1200 pN-nm 21. This distance was verified by video tracking. After this calibration, NTPs were added to the flow cell and changes in force measured in both traps were converted to contour lengths using the same WLC model.

Measurements of transcription velocity and stall force were verified on a separate counter-propagating dual-beam optical tweezers in constant force mode 19 (data not shown).

Force-jump experiments were performed on a third dual-trap optical-trap setup based on a 5W 1064 laser (Spectra-Physics) and a motorized stage (Queensgate) to move a telescope lens for precise and rapid manipulation of the position of one of the two traps. The experiment was performed as above, except that the moveable trap was displaced by calibrated distances for 1 s intervals during active transcription. The force jumps were of too short a duration for our filtering algorithm and the traps begin to exhibit nonlinearities above 20 pN. Therefore, to determine the transcription velocity or the distance backtracked during a jump, we compared enzyme positions immediately before and immediately after a jump.
**Supplementary Analysis**

**Lifetimes of Backtracked Pauses**

This problem is a member of the well-studied one-dimensional first passage time problems that can be found in many textbooks. For simplicity, we assume that the polymerase moves in a continuous fashion and leave an analysis of the case with discrete 1 nt steps for a future discussion. \( \psi(x,t) \) denotes the probability density of finding a particle at \( x \) at time \( t \). All particles begin at position 0 and follow Fick’s second law for one-dimensional unbiased diffusion.

\[
\frac{\partial \psi}{\partial t} = D \frac{\partial^2 \psi}{\partial x^2}
\]

The corresponding Greens function \( G(x,t) \) describes their evolution.

\[
G(x, t) = \frac{1}{\sqrt{4\pi Dt}} \exp \left( \frac{-x^2}{4Dt} \right)
\]

We want to know how many particles reach an absorbing barrier at position \( -a \) at time \( t \). First, we reason that at \( t \), all the particles between \( -a \) and \( -\infty \) must have reached \( -a \). Then, we realize that only half of the particles that reach \( -a \) end up between \( -a \) and \( -\infty \) at any given time. Therefore, the number of particles \( N \) that have been absorbed at \( -a \) at time \( t \) is give by:

\[
N(t) = 2 \int_{-\infty}^{-a} G(x, t) \, dx
\]

\[
= \int_{-\infty}^{-a} \frac{1}{\sqrt{\pi Dt}} \exp \left( \frac{-x^2}{4Dt} \right) \, dx.
\]

We change variables by substituting

\[
\frac{x^2}{4Dt} = \xi^2
\]

giving us

\[
N(t) = \frac{1}{\sqrt{\pi Dt}} \int_{-\infty}^{-a/\sqrt{4Dt}} \exp(-\xi^2) \sqrt{4Dt} \, d\xi
\]
We differentiate with respect to time since we want to know the number of particles added at time $t$. If

$$\beta = \frac{-a}{\sqrt{4Dt}},$$

then

$$\frac{\partial N}{\partial t} = \frac{\partial N}{\partial \beta} \frac{\partial \beta}{\partial t}$$

or

$$= \frac{\partial N}{\partial \left( \frac{-a}{\sqrt{4Dt}} \right)} \left[ \frac{2}{\sqrt{\pi}} \exp \left( \frac{-a^2}{4Dt} \right) \right] \left[ \frac{a}{2\sqrt{4D}} t^{-3/2} \right]$$

$$= \frac{a \exp \left( \frac{-a^2}{4Dt} \right)}{\sqrt{4\pi D}} t^{-3/2}.$$

If $a = 1$ (the position of the elongation competent state), then

$$\frac{\exp \left( \frac{-1}{4Dt} \right)}{\sqrt{4\pi D}} t^{-3/2}$$

is the number of particles that reach $-a$ as a function of time. At large $t$, this simplifies to

$$\frac{t^{-3/2}}{\sqrt{4\pi D}}.$$
Supplementary Bibliography


