Trigger loop dynamics can explain stimulation of intrinsic termination by bacterial RNA polymerase without terminator hairpin contact

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In bacteria, intrinsic termination signals cause disassembly of the highly stable elongating transcription complex (EC) over windows of two to three nucleotides after kilobases of RNA synthesis. Intrinsic termination is caused by the formation of a nascent RNA hairpin adjacent to a weak RNA–DNA hybrid within RNA polymerase (RNAP). Although the contributions of RNA and DNA sequences to termination are largely understood, the roles of conformational changes in RNAP are less well described. The polymorphous trigger loop (TL), which folds into the trigger helices to promote nucleotide addition, is also proposed to drive termination by folding into the trigger helices and contacting the terminator hairpin after invasion of the hairpin in the RNAP main clef [Epshtein V, Cardinale CJ, Runckenstein AE, Borukhov S, Nucler E (2007) \textit{Mol Cell} 28:991–1001]. To investigate the contribution of the TL to intrinsic termination, we developed a kinetic assay that distinguishes effects of TL alterations on the rate at which ECs terminate from effects of the TL on the nucleotide addition rate that indirectly affect termination efficiency by altering the time window in which termination can occur. We confirmed that the TL stimulates termination rate, but found that stabilizing either the folded or unfolded TL conformation decreased termination rate. We propose that conformational fluctuations of the TL (TL dynamics), not TL-hairpin contact, aid termination by increasing EC conformational diversity and thus access to favorable termination pathways. We also report that the TL and the TL sequence insertion (SI3) increase overall termination efficiency by stimulating pausing, which increases the flux of ECs into the termination pathway.

\textbf{Significance}

RNA polymerase (RNAP), like many cellular processors of information in DNA and RNA, is a complex macromolecular machine whose multiple structural modules and domains undergo poorly understood conformational changes that mediate information processing. We investigated the role of one such mobile module, the polymorphous trigger loop (TL) of RNAP, in intrinsic transcription termination by bacterial RNAP. The TL folds into a helical hairpin to promote RNA synthesis, but also is proposed to aid termination. By separating effects of the TL and of TL variants on termination from effects on RNA synthesis, we established that TL flexibility, not the helical hairpin conformation, facilitates rearrangements of RNAP leading to termination. Our results illustrate how kinetic assays can help dissect complex macromolecular machines.

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Fig. 1. Intrinsic transcription termination and the polymorphous TL. (A) (Left) The main elements of a canonical intrinsic terminator are shown: a U-rich tract, immediately preceded by a GC-rich T<sub>hp</sub> structure. (Right) The T<sub>hp</sub> can be mimicked by annealing an asRNA (antisense RNA; dark red) complementary to the nascent RNA transcript (light red) to create a GC-rich duplex. (B) The thermodynamic model for termination (4–6), in which the relative free energy barriers to elongation (blue) versus termination (red) determine the probability of each event. The stabilities of the RNA–DNA hybrid (pink) and RNAP–NA interactions (red) are proportional to the arrow height. (C) The steps of intrinsic termination. Steps that lead to termination are indicated by red arrows; steps that lead to terminator bypass are indicated by blue arrows. RNA, red; DNA, black; RNAP, gray. (D) Structure of an EC and relevant elements generated as described in ref. 37. RNAP is shown as a white surface with modules of interest shown as Ca backbone traces. The alternative structures of the folded TH (orange) and the unfolded TL (green) structures are both shown. For clarity, the TL insertion (SI3, pink) is shown connected to the unfolded TL conformation. (E) The TH and unfolded TL conformations. Blue and red spheres indicate residues mutated in the folded TL stabilizing (Ala<sub>θ</sub>) and folded TL destabilizing (LTPP) RNAP mutants, respectively.

hybrid melting may occur in alternative pathways, depending on hybrid sequence (24, 25): (i) In hybrid-shearing, the extending T<sub>hp</sub> pulls the RNA out of register from the DNA by shearing a perfect or near-perfect rU–dA hybrid (8, 24), or (ii) in hypertranslocation when shearing is unfavorable, T<sub>hp</sub> extension pulls the entire hybrid upstream by melting upstream RNA–DNA base pairs and downstream DNA duplex base pairs without complementary ribonucleotide addition (24–26). By either route, the remaining partial hybrid cannot stabilize the EC sufficiently and EC dissociation becomes favorable (8).

In contrast to the role of nucleic acid (NA) base pairing, the contributions of structural changes in RNAP modules and domains to termination remain incompletely described (25, 27). A complexity of multiple moving domains and refolding modules is a common feature of macromolecular machines that process information in RNA and DNA. In RNAP, a hinged clamp domain forms stabilizing contacts to both RNA and DNA and may need to open for EC dissociation (17, 28–31). In addition, a highly conserved, polymorphous, ~47-aa trigger loop (TL; Fig. 1D and E) (32–34) undergoes random coil to helical hairpin transitions during catalysis of nucleotide addition (32, 34). At intrinsic terminators, the T<sub>hp</sub> is proposed to invade the RNAP main cleft and to stimulate termination via a direct contact to the TL in its helical hairpin conformation, also called the trigger helix (TH) (28). A TH–T<sub>hp</sub> contact has yet to be observed in EC structures, and this TH–T<sub>hp</sub> contact model of termination has been disputed (35). Thus, understanding the role of the TL in termination provides a useful case study for understanding roles of dynamic modules in macromolecular machines generally.

To investigate the role of the TL in termination, we developed an experimental approach that can separate effects on termination rate from effects on elongation rate, which are known to be significant for TL alterations (36–38). Whereas traditional TE measurements report an aggregate of effects on termination and elongation, isolated measurement of termination rate in our assay allowed us to determine the effects of TL mutations specifically on the termination mechanism. Using RNAP variants that (i) bias the TL toward either the folded or unfolded states, (ii) remove the sequence insertion 3 (SI3) from the TL, or (iii) delete the polymorphous arms of the TL, we were able to test the contribution of the TL to termination rate, TE, and the pausing steps leading into the termination pathway.

Results

Measurement of Termination by Elongation-Compromised RNAP Mutants. To investigate the effect of the TL on intrinsic termination, we needed to separate effects of EC rearrangements on the termination pathway, in which TH–T<sub>hp</sub> contact is proposed to play a role, from effects on the rate of nucleotide addition, which affects TE by competing with the termination pathway and is known to depend on TL folding.

We first investigated a strategy to study termination by measuring dissociation rates of static ECs stalled by NTP deprivation. We triggered formation of a T<sub>hp</sub> mimic by addition of antisense oligonucleotides (oligos) to the static ECs to stimulate termination (8, 18). However, we found that stalled ECs terminated more slowly than active ECs and were largely insensitive to TL alterations, contrary to demonstrated TL effects on termination (28) (SI Appendix, Supplementary Discussion and Fig. S1). This result may reflect the fact that stalled ECs can isomerize into alternative conformations before termination is triggered by addition of the antisense oligo. To avoid artifactual effects in static ECs, we instead developed an active EC termination assay in which termination was triggered in actively elongating ECs by antisense oligo addition.

To develop the active-EC termination assay, we modified the hairpin-stabilized his pause sequence to resemble an intrinsic terminator by adding rU–dA base pairs to create a terminator U-tract (t<sub>his2</sub>; Fig. 24). We also removed the upstream arm of the pause hairpin, which allowed assembly of stable ECs at G17 and measurement of rates of elongation and pausing at C18 and U19 in the absence of a complete termination signal. Antisense oligos that pair to the exiting RNA 8 nt to 10 nt from the U19 RNA 3′ end could then be added to create a T<sub>hp</sub> mimic (20) for measurement of the termination rate at U19, corresponding to
RNA before addition of the antisense oligo, and (iii) inefficient or slow termination.

Using this design, we first tested transcription of \( t_{\text{hlz2}} \) by wild-type (WT) and TL-deletion (\( \Delta TL \)) (SI Appendix, Table S1) RNAPs in the absence of the \( T_{\text{hp}} \) (Fig. 2A). ECs were reconstituted at G17, radiolabeled by incorporation of \([\alpha-32P]CTP\), and then elongated through U19 by incubation with UTP and ATP (Fig. 2B and C). Although \( \Delta TL \) ECs required high (10 mM) UTP and ATP concentrations, we observed near-complete elongation past the termination site for both WT and \( \Delta TL \) ECs. We next examined the effect of complementary (antisense) and control noncomplementary DNA and RNA oligos added with the UTP and ATP on transcription of \( t_{\text{hlz2}} \) by WT RNAP. RNAP was immobilized on \( Co^{3+} \) beads via a His\(_{10}\) tag to enable the detection of RNA release. As observed previously (20, 26, 40, 41), only the complementary oligos stopped RNAP at C18 and U19 (Fig. 2D) and caused release of the C18 and U19 RNAs into the supernatant, verifying that they were terminated. Because antisense RNA (asRNA) stimulated more termination than antisense DNA (asDNA) and generates a duplex similar to a natural \( T_{\text{hp}} \), we used asRNAs for all subsequent experiments.

For our measurements, we focused on the U19 position where all ECs were actively elongating. We used two asRNAs that either disrupted the upstream-most hybrid base pairs and gave higher TE (\(-8\) asRNA) or destabilized hybrid base pairs only indirectly and gave lower TE (\(-10\) asRNA) (Fig. 2E). Because reliable calculation of termination rate in our assay required a TE between 15% and 85% and because some RNAPs gave TES outside this range, only the \(-8\) or \(-10\) asRNA worked well for some RNAP variants.

**Deletion of the TL Increases TE.** We first tested whether deletion of the TL would affect termination, as predicted (28). Radiolabeled WT and \( \Delta TL \) C18 ECs were assembled as described in Measurement of Termination by Elongation-Compromised RNAP Mutants, and then elongated through U19 with UTP and ATP in the presence of \(-8\) asRNA (Fig. 2A–C). The addition of asRNA to \( \Delta TL \) (but not WT) ECs was delayed to allow U19 ECs to accumulate. The TE at U19 was greater for \( \Delta TL \) ECs (\(-85\%\)) than for WT ECs (\(-44\%\)), even at the high NTP concentrations used for \( \Delta TL \) ECs (Fig. 2C and Table 1). Thus, our results verified that the TL affects TE. However, to test the TH–\( T_{\text{hp}} \) contact model (28) unambiguously, we needed to measure the effect of the TL on termination rate (rather than the composite TE); the model predicts termination rate will be slowed when the TH–\( T_{\text{hp}} \) contact is removed. Therefore, we next sought to measure elongation rate and termination rate at \( t_{\text{hlz2}} \) separately.

**The TL Strongly Stimulates the Rate of Intrinsic Termination.** To separate elongation and termination rates, we next measured transcription of \( t_{\text{hlz2}} \) by WT and \( \Delta TL \) ECs using time-resolved assays, which required use of a quench-flow apparatus for WT ECs (Methods). By first omitting asRNA, we could obtain elongation rates and pause rates, which were then used as constants to derive termination rates from best-fit numerical integration of kinetic models of transcription in the presence of asRNA.

Radiolabeled C18 WT and \( \Delta TL \) ECs were assembled and elongated through U19 with or without asRNA as described in Deletion of the TL Increases TE, except that samples were removed and quenched at time points during the reactions (Fig. 3A–C and SI Appendix, Fig. S2A and B). The WT and \( \Delta TL \) EC elongation and pause rates were determined by fitting the proportion of C18, U19, and \( \geq A20 \) (A20\(^{+}\)) complexes present at each time point in the absence of asRNA to kinetic models that included C18-to-U19, U19-to-A20, and elemental pausing steps at C18 or U19 as necessary by numerical integration using Kintek Explorer (42) (Methods, Fig. 4, and SI Appendix, Fig. S3). We tested kinetic models for least-squares fit to the observed data using the corrected Akaike’s Information Criterion (43), and the
Table 1. Effects of TL mutations on the termination rate and efficiency at U19

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<td>$k_{term} \times 10^{-4}$ s⁻¹</td>
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<td></td>
<td>−8</td>
<td>42 ± 3</td>
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<td></td>
<td>−10</td>
<td>17 ± 1</td>
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<td>∆TL</td>
<td>−8</td>
<td>87 ± 7</td>
<td>N.D.</td>
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<td>−10</td>
<td>26 ± 2</td>
<td>3.5 ± 0.2</td>
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<td>∆S1</td>
<td>−8</td>
<td>34 ± 7</td>
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<td>−45</td>
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<td>LTP</td>
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<td>86 ± 1</td>
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<td>47 ± 7</td>
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<td>LTPpΔS1</td>
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<td>99 ± 2</td>
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<td>−10</td>
<td>53 ± 2</td>
<td>14 ± 2</td>
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<tr>
<td>AlaXΔS1</td>
<td>−8</td>
<td>−865</td>
<td>N.D.</td>
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<td></td>
<td>−10</td>
<td>44 ± 9</td>
<td>14 ± 3</td>
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N.D., not determined because the TE was outside the useful range of the assay (15–85% TE). Errors are SD of values calculated from ≥3 independent experimental replicates.

*TE = U19/(U19 + A20) × 100, where A20 includes RNA products A20 or larger; TE calculations were adjusted for conditions with delayed asRNA addition by determining the percent of U19 RNA present at the time of asRNA addition that failed to extend to A20.

‡Here $k_{term}$ ratio = $k_{term$(WT)}/k_{term$(mutant)$ for a given asRNA.

§Here $k_{term}$ ratio = $k_{term$(WT with −8 asRNA)/$k_{term$(WT with −10 asRNA)$.

Value reported is the average of two replicates.

The simplest kinetic model with statistically significant improvement relative to alternatives was chosen (Methods and SI Appendix, Fig. S3). A significant fraction of WT ECs paused at C18 and U19 in response to the U-tract sequence (Fig. 4 and Table 2). However, ∆TL ECs elongated through C18 and U19 with simple pseudo first-order kinetics, consistent with the previous finding that ∆TL ECs do not exhibit pausing behavior (22). Initially we compared WT and ∆TL RNAPs using the −8 asRNA (SI Appendix, Fig. S4A–D), but found that the TE for ∆TL RNAP was too high to allow reliable calculation of the termination rate (Table 1). Shifting the asRNA to extend only to −9 (relative to U19 at −1) also gave near 100% TE for ∆TL RNAP (SI Appendix, Fig. S4 E and F). Therefore, we chose the −10 asRNA to enable accurate comparison of termination rates of WT and ∆TL RNAPs.

The rates of termination were calculated by fitting the relative populations of C18, U19, and A20 RNA species observed in the presence of −10 asRNA (Fig. 3D and E and SI Appendix, Fig. S2 C and D) using the best-fit kinetic model of elongation and pausing with fixed rates of elongation, pause escape, and unconstrained rates of termination for positions C18 and U19 (Fig. 5). In addition, inclusion of a fixed rate of asRNA pairing, which was measured separately using a previously described assay (44) (SI Appendix, Fig. S5), and of unconstrained rates of nucleotide addition for the RNA duplex-containing EC states were required to give good fits to the data (Methods and SI Appendix, Fig. S6). Thus, in our calculations of termination rates, only four parameters were allowed to vary: the rates of elongation and termination by −10 asRNA-bound C18 and U19 ECs (black arrows in Fig. 5).

To avoid potential artifacts from comparing the behaviors of halted C18 ECs (SI Appendix, Supplementary Discussion), we compared WT and ∆TL termination rates for U19 ECs ($k_{term}$; Fig. 5). Despite the increased TE observed for ∆TL ECs (Fig. 2C and Table 1), $k_{term}$ was decreased by a factor of ~75 in ECs lacking a TL [(3.5 ± 0.2) × 10⁻⁴ s⁻¹ for WT vs. (260 ± 20) × 10⁻⁴ s⁻¹ for WT; Table 1]. Although deleting the TL slows termination rate dramatically, overall TE changes much less (Table 1) because the TL deletion also decreases nucleotide addition rate (Fig. 4 and Table 2). This masking of a large effect on termination rate when considering only the aggregate TE illustrates

Fig. 3. Limited hairpin extension enabled termination rate measurements for slow ∆TL RNAP mutant. (A) Experimental scheme for measurement of the elongation and termination rates of WT and ∆TL ECs. (B) Representative denaturing RNA gel image and (C) reaction progress curves showing the conversion of RNAs from C18 to U19 to A20 for WT ECs in the absence of asRNA. The ∆TL gel image and reaction progress curves are shown in SI Appendix, Fig. S2 A and B. (D and E) Reaction progress curves for (D) WT ECs with −10 asRNA and (E) ∆TL ECs with −10 asRNA; the corresponding gel images are shown in SI Appendix, Fig. S2 C and D, respectively. The time of asRNA addition is indicated with arrows. Error bars represent SD from three or more independent experimental replicates; error bars are smaller than the data markers in some cases.
the importance of measuring the termination rate to study the mechanism of termination. We conclude that the TL plays a crucial role in intrinsic termination by significantly accelerating the rate-limiting step in the termination pathway.

We note that −8 asRNA accelerated termination rate ~fourfold relative to −10 asRNA ([1,000 ± 300] × 10^{-4} s^{-1} for −8 asRNA vs. [260 ± 20] × 10^{-4} s^{-1} for −10 asRNA; Table 1), consistent with the idea that T_{hp} extension is at least partially rate-determining for EC inactivation.

The TL also Aids Termination by Favoring Pausing at the Termination Sites. Our kinetic analyses also yielded estimates for the rates of pause entry and escape for WT and ΔTL ECs. One-third (33%) of WT U19 ECs entered a paused state in which nucleotide addition was slowed by a factor of ~120 in the absence of asRNA, or by a factor of ~16 after asRNA binding (Fig. 4A and Table 2). However, the absence of the TL completely abrogated pausing at U19 in response to the U-tract (Fig. 4B and Table 2). The presence of the TL thus favors entry into the elemental paused state in WT ECs, which would extend the kinetic window for T_{hp} formation. The binding of asRNA also had minimal effect on nucleotide addition rate by ΔTL RNAP (Table 2), consistent with previous reports that the TL participates in the hairpin-stabilized paused state (22, 37, 45). We conclude that, in addition to stimulating termination, the TL helps establish and inhibits escape from paused states at terminators, thereby increasing flux of ECs into the termination pathway.

SI3 Increases Pausing and TE, but Not Termination Rate. The Escherichia coli TL contains a large (188-aa) insertion called SI3 (Fig. 1D and E), present in many Gram-negative bacterial lineages (46, 47). SI3 affects TL function in elongation, pausing, and intrinsic cleavage (37, 48, 49). A monoclonal antibody (mAb) that binds SI3 was shown to decrease dissociation of ECs stalled at a termination site (28). Thus, the effect of TL deletion on termination observed in our assay could reflect effects of the TL itself or of SI3, which is also deleted in ΔTL RNAP.

To test for SI3 effects on termination, we determined elongation, pause, and termination rates of an SI3 deletion mutant (ΔSI3; SI Appendix, Table S1) on the th3α2 scaffold. Termination was almost undetectable for ΔSI3 ECs with −10 asRNA, suggesting that SI3 either aids termination rate or increases the formation or dwell time of paused states leading to termination. The −8 asRNA caused ~35% TE for ΔSI3 RNAP and enabled direct comparison of termination rate to WT (Table 1 and SI Appendix, Fig. S7). Interestingly, deletion of SI3 had little effect on termination rate (Table 1). Therefore, the strong effect of the TL deletion on termination rate reflects a role of the TH-forming parts of the TL and not an effect of SI3 on termination rate.

Although SI3 did not affect termination rate, it did increase pausing significantly, with the indirect effect of increasing TE by increasing flux of ECs into the termination pathway. Deletion of SI3 decreased relative pause lifetime at U19 by a factor of ~15 ([8 ± 3] for ΔSI3 vs. [120 ± 30] for WT; Table 2), and decreased the fraction of U19 ECs that entered the paused states (~17% for ΔSI3 vs. ~33% for WT). However, SI3 had less effect on the rate of A20 addition after asRNA binding (Table 2). Although an approximately threefold effect of SI3 on pausing stabilized by a hairpin extending to −12 has been observed previously (37, 49), the closer approach of the duplex stem to the RNA 3′ end may obviate this effect of SI3 at terminators (50). As a consequence of less pausing overall, the TE for ΔSI3 ECs was ~34% vs. ~42% for WT (Table 1), indicating that SI3-mediated pause stimulation increased TE by increasing flux of ECs into the termination pathway, even though the termination rate per se was minimally affected.

Reduced TL Conformational Flexibility Inhibits Termination Rate. Our results established that the TL increases termination rate up to ~75-fold. However, it was unclear if a particular TL state (e.g., the TH as predicted by the TH–T_{hp} contact model) favors termination, if some other conformation favors termination, or if the conformational flexibility of the TL might be more important than any single conformation. To address these questions, we sought to test termination at T_{hp2} by previously characterized TL variants that either stabilize the folded TL conformation (Ala_d) or prevent folding and favor unfolded conformations (LTPP; Fig. 1E) (36, 37). For the TH destabilizing LTPP substitution, we could make a direct comparison between the mutant RNAP and WT using the −10 asRNA (SI Appendix, Fig. S8). The LTPP substitutions decreased termination rate by a factor of ~40, similar to the effect seen for ΔTL using the −10 asRNA (Table 1).
A Model for Termination Aided by TL Conformational Dynamics. The thermodynamic model of intrinsic termination posits that the relative free energy barriers to elongation and termination determine the fraction of complexes that terminate via kinetic competition between the two pathways (4, 5) (Fig. 1B). This model, with single elongation and termination pathways and simple changes to RNAP–NA contacts, was sufficient before knowledge that modules like the clamp, bridge helix, and TL occupy distinct, interconnected conformational states (17, 29–31, 44, 51, 52). Incorporation of these states and the known alternatives of hybrid shearing or hypertranslocation (24) suggests an extension of the thermodynamic model to a multistate multipath (MS-MP) model, in which different interconverting EC conformations explore multiple, alternative termination pathways (Fig. 6).

The MS-MP termination model posits that the terminating EC exists in a family of conformational states with fluctuating RNAP–NA contacts that are weaker in some states than in others. These conformational fluctuations would result in variations in EC stability (Fig. 6B, Inset) and create multiple paths to termination with different free energy barriers (Fig. 6B). Variability in free energy barriers caused by multiple molecular states has preceded in studies of ligand binding and catalysis, where differences between protein conformational substates and their relative stabilities are thought to cause variations in the activation energy of different pathways (53–58). The idea of multiple parallel routes for a single reaction is analogous to the well-documented concept of multiple protein unfolding pathways (55, 56, 59). The hybrid-shearing and hypertranslocation termination models (8, 24, 26) are examples of such different pathways in the case of intrinsic termination. The MS-MP model is similar to the original thermodynamic termination model developed by von Hippel and coworkers (4–6), but adds the dimension of protein conformational states that were not well defined when the model was developed.

A principal rationale for the MS-MP termination model is in its ability to provide a parsimonious explanation for both the ~75-fold stimulatory effect of the TL on termination rate and the reduction in termination rate when either the folded or unfolded TL conformation is stabilized (Table 1). If terminal ECs occupy multiple states with multiple possible paths to termination as proposed by the MS-MP model, conformational changes in the mobile TL that increase the conformational diversity of terminating ECs would enable the ECs to access pathways with lower free energy barriers to termination (Fig. 6B). The MS-MP model thus predicts that, when the TL is deleted or its conformations restricted by alterations (as in AlaS and LTTP RNAPs), the number of accessible conformational states of the terminating EC will be reduced (SI Appendix, Fig. S11, Inset). Restricting conformational states will inhibit access to lower-energy paths of termination (SI Appendix, Fig. S11, highlighted areas) and decrease overall termination rate, consistent with the effects of TL alterations reported here (Table 1). Conversely, a dynamic TL

| Table 2. Effects of TL mutations on pausing on the termination pathway at U19 |
|--------------------------|----------------|----------------|----------------|----------------|----------------|
| RNAP | asRNA [ATP] | \( k_p \times 10^{4} \text{s}^{-1} \) | PE* | \( k_p \times 10^{4} \text{s}^{-1} \) | \( k_{p,\text{total}} \times 10^{4} \text{s}^{-1} \) | \( k_{p,\text{total}} \times 10^{4} \text{s}^{-1} \) |
| WT | –8 | 10 μM | 15,700 ± 2,500 | 33 ± 2 | 130 ± 20 | 120 ± 30 | 980 ± 400 | 16 ± 7 |
| ΔTL | –10 | 10 μM | 30 ± 3 | N.A. | N.A. | N.A. | 20.3 ± 0.2 | 1.5 ± 0.2 |
| ΔS3 | –8 | 10 μM | 14,200 ± 1,000 | 17 ± 2 | 1,800 ± 600 | 8 ± 3 | 1,060 ± 300 | 13 ± 4 |
| LTTP | –10 | 10 mM | 42 ± 1 | N.A. | N.A. | N.A. | 13.4 ± 0.3 | 3.1 ± 0.1 |
| LTTPS3 | –10 | 10 mM | 62 ± 2 | N.A. | N.A. | N.A. | 21 ± 3 | 3 ± 0.4 |
| AlaSΔS3 | –10 | 10 μM | 83 ± 4 | N.A. | N.A. | N.A. | 19 ± 3 | 4.4 ± 0.7 |

Errors are SD of values calculated from three or more independent experimental replicates. N.A., not applicable.

*PE (pause efficiency), percent of complexes that enter the U-tract paused state, UTp.
that fluctuates among various conformations will enable the EC to explore multiple routes of termination and increase the overall termination rate. In this view, the observed termination rates are aggregates of multiple termination paths, and lessening the conformational flexibility of RNAP reduces the probability of termination.

Inhibition of Termination by TH Stabilization Contradicts the TH–T_{hp} Contact Model. The TH–T_{hp} contact model posits that formation of the TH favors termination by contacting the T_{hp} (28). However, stabilization of the TH by Ala substitutions in the T/L/TH hinge regions inhibited termination (Table 1, Ala_{G1136S}). This finding, coupled with alternative explanations for the main findings based on which the TH–T_{hp} contact model was proposed (35), favor the MS-MP termination model.

The TH–T_{hp} contact model is based, in part, on the observation of UV-induced crosslinks between the upper stem and loop of the T_{hp} and the TL, leading to the suggestion that the TH invades the main cleft of RNAP and contacts the TH in a terminating complex (18, 28). However, the low-salt conditions in which this crosslink can be detected cause the formation of binary RNA–RNAP complexes after EC dissociation (60); these posttermination binary complexes are the likely source of the crosslink (35). Moreover, a TL substitution found to inhibit termination (G1136S) (28) likely favors the TH conformation (51), whereas binding of a mAb to SI3 that stabilizes an unfolded TL also inhibits termination (28). The similar inhibitory effects of biasing the TL in opposing orientations are at odds with the TH–T_{hp} contact model but consistent with the MS-MP model.

Finally, based on inhibition of termination by the TH-stabilizing ligand tagetitoxin, it also has been argued that the unfolded TL rather than the folded TH favors Rho-dependent termination (61). It is improbable that Rho-dependent and Rho-independent (intrinsinc) termination would be favored by opposite TL conformations, putting the TH–T_{hp} contact model at odds with the proposed role of the TL in Rho-dependent termination. However, these effects are all readily explained by the MS-MP model, where different means of restricting TL conformational fluctuations all result in decreased termination rate.

The TL and SI3 also Promote Intrinsic Termination by Stimulating Transcriptional Pausing. Prior studies suggest that pausing plays a key role in termination, by allowing time for the T_{hp} to form and then to invade and destabilize the EC (18, 20, 23, 50, 62). Pausing is thought to be mediated, in part, by inhibition of TL folding aided by SI3 (22, 37, 45). Our results confirm these hypotheses and provide quantification of the effects of pausing and of the TL during termination at an active terminator, whereas earlier studies inferred roles of pausing from studies in which the T_{hp} was unable to form completely.

For WT RNAP, we could resolve two distinct paused states at U19. One paused state existed before (or in the absence of) asRNA binding with a nominal dwell time ~100 times slower than the elongation pathway (Table 2). This pause was consistent with prior descriptions of pausing in the terminator U-tract and with the properties of an elemental pause (18, 20–22). A distinct paused state existed after asRNA binding, in which the paused ECs were still capable of escape by nucleotide addition but at a rate ~20 times slower than the elongation pathway. The shorter lifetime of the RNA duplex-containing paused ECs (relative to the pause observed when the duplex was absent) is consistent with prior studies suggesting that extension of an exit channel duplex to 11 nt or less from the RNA 3′ end eliminates most of the pause-prolonging effects of exit-channel duplexes that extend to ~12 or ~13 (50).

Our results also establish that both the TL and SI3 significantly contribute to the formation or lifetime of both the elemental and RNA duplex-containing paused states. When the TL and SI3 were deleted, U19 ECs elongated at a single rate, suggesting that ΔTL ECs do not partition into a paused state distinct from the elongation pathway (Table 2). Even after exit-channel duplex formation, ΔTL ECs continue elongation at essentially the same rate. These findings are consistent with previous reports
that the TL significantly contributes to pausing (22), and that altering the TL can decrease both pause efficiency and duration (37, 45).

Of particular interest, we observed that SI3 greatly prolongs the U-tract elemental pause. The presence of SI3 not only increased pause duration ~15-fold, it also increased the fraction of ECs entering the pause from ~17 to ~33% (Table 2). Indeed, the major effect of SI3 on termination is to increase flux toward the termination pathway by increasing pausing, since SI3 had little effect on the rate of termination. Of note, SI3 is surface-exposed, and mAbs that bind SI3 can modulate TL function (28, 48). SI3 may thus present a target for extrinsic regulators that could modulate the flux of ECs into the termination pathway.

Overall, our results suggest that the TL stimulates termination not only by allowing access to lower-energy termination pathways but also, aided by SI3, by increasing entry into and retention of paused EC states that provide time for the T\textsubscript{hp} to destabilize the EC (Fig. 6).

**Conclusion**

We suggest that, in addition to NA rearrangements, RNAP dynamics play an important role in intrinsic termination based on the contributions of the TL to multiple decision points in the termination pathway. The TL is a universally conserved polymorphous module in all multisubunit RNAPs (32, 34, 37) whose conformational fluctuations are known to aid steps in the nucleotide addition cycle. Based on our results suggesting a role of TL dynamics in intrinsic termination, we propose an MS-MP model of intrinsic termination that links TL conformational changes to stability of the EC more generally.

Other highly dynamic or mobile domains of RNAP such as the clamp, switch regions, bridge helix, and flap also likely play important roles in both the termination pathway and RNAP dynamics. Our assay provides an easily adaptable method to test their contributions to each step of the termination pathway, and to characterize more completely the structural mechanism by which intrinsic termination disassembles the extraordinarily stable EC.

It is unclear if similar dynamics affect termination by eukaryotic RNAPII, but RNA secondary structures have also been proposed to aid RNAIII termination (63, 64), including effects of bacterial intrinsic terminators in yeast RNAIII (8) and the HIV-1 TAR hairpin on human RNAIII (65). Alterations of the TL in yeast RNAIII also affect TE (66), and the presence of the TL in archaeal RNAP prevents aberrant termination (67). These findings raise the possibility that the MS-MP termination model may also be relevant to eukaryotic and archaeal RNAPs. Further study of the role of TL dynamics and RNA-structure-mediated termination in eukaryotes and archaea is merited.

**Materials and Methods**

Sources of materials and proteins are described in *SI Appendix, Supplementary Methods*.

**In Vitro EC Reconstitution.** The NA scaffold for EC reconstitution was formed by mixing 5 \(\mu\)M G17 RNA and 10 \(\mu\)M T-DNA (template-DNA; #10002 and #8451, respectively; *SI Appendix, Table S2*) in reconstitution buffer (10 mM Tris·HCl, pH 7.9, 40 mM KCl, 5 mM MgCl\(_2\)), heating to 95 °C for 2 min, cooling rapidly to 45 °C, and then cooling to 25 °C in 2 °C increments for 2 min each, as described previously (68). The 17-nt RNA was designed to have 8 nt of complementarity to the T-DNA at the site of reconstitution to prevent base pairing of upstream T-DNA and RNA, and backtracking of ECs at the termination site.

ECs were reconstituted by incubating 2.5 \(\mu\)M core *E. coli* RNAPs with 0.5 \(\mu\)M NA scaffold in transcription buffer (TB; 20 mM Tris·OAc, pH 8.0, 75 mM NaOAc, 1 mM Mg(OAc)\(_2\)\(_6\)), 1 mM DTT, 0.1 mM EDTA, 2.5% glycerol, and 25 \(\mu\)g of acetylated BSA/mL) for 15 min at 37 °C. NT-DNA (nontemplate-DNA; #8450; *SI Appendix, Table S2*) was then added at 1.5 \(\mu\)M and incubated for another 15 min at 37 °C. Fully complementary NT-DNA was used to allow
the energy of DNA reannealing upon termination and bubble collapse to contribute to the termination energetics.

**In Vitro Transcription.** ECs were diluted in TB to 100 mM ECs and radiolabeled by incubation with 0.1 μM [α-32P]CTP and 0.9 μM CTP for 5 min (for WT, δS3, and ΔAlaδS3 ECs) or 0.1 μM [α-32P]CTP and 2.5 μM CTP for 30 min (for ATL, LTPP, and LTPPΔS3 ECs) to form C18 ECs. Transcription was restarted by mixing the C18 ECs with an equal volume of TB containing UTP and ATP (to the final concentrations indicated in the reaction schemes of Fig. 3 and SI Appendix, Figs. S7, S8, and S10) with or without 50 μM asRNA (100 μM asRNA for ΔAlaδS3 ECs). All NTP mixtures were supplemented with Mg(OAc)2 at a concentration equivalent to the NTP concentration to avoid Mg2+ sequestration effects. Samples incubated for 4 s or longer were stopped manually with an equal volume of 2× stop buffer (10 M urea, 50 mM EDTA, 90 mM Tris-borate buffer, pH 8.0, 0.02% bromophenol blue and 0.02% xylene cyanol). Samples incubated for 8 s or shorter were stopped using a rapid mixing quench flow apparatus (RQF-3; KinTek Corporation) by injecting 100 mM C18 ECs in one sample loop, and UTP and ATP in TB in the presence or absence of 100 μM asRNAs in the other sample loop. Samples obtained at the same times (4 s and 8 s) in the manual and quench flow reactions were similar, verifying that results from these two methods could be merged. Quench flow reactions were stopped with 2 M HCl and neutralized immediately before addition of an equal volume of 3× Tris base. RNA products were purified by phenol-chloroform extraction followed by ethahol precipitation, and resuspended in 1× stop buffer. RNA products from all timed samples were resolved by 8 M urea denaturing PAGE. Gels were exposed to phosphorimager screens, scanned using the Typhoon Phosphor-imager and quantified using ImageQuant software (GE Healthcare).

For dissociation assays (Fig. 2D), co-transcribed G17 ECs were diluted to 100 mM and mixed to 3× with 50 mM Tris-borate, pH 8.0, 0.02% bromophenol blue and 0.02% xylene cyanol by mixing quench flow apparatus (RQF-3; KinTek Corporation) by injecting the H150 tag on RNAP by incubating with the beads for 10 min at room temperature, followed by 10 min at 37 °C. ECs were radio-labeled by incubation with 0.1 μM [α-32P]CTP and 0.9 μM CTP for 5 min, then beads were washed three times with an equal volume of TB to remove unincorporated CTP and unbound RNAP and nas. Reactions were restarted by UTP and ATP addition in the presence or absence of 50 μM asRNA. Supernatant samples were taken by magnetic partitioning, and supernatant and whole reaction samples were stopped with an equal volume of 2× stop buffer. RNA products were separated by 8 M urea denaturing PAGE, and visualized by phosphorimaging, as described above.

**Kinetic Fitting of Transcription Assay Reaction Progress Plots.** Reaction progress curves for C18, U19, and A20* were generated by calculating the radiolabeled signal for each RNA species as a percent of total signal from these RNAs in each sample. A20 and all RNAs longer than A20 were combined to give the A20* fraction. The reaction progress curves were fit by comparison with numerical integration of kinetic models (e.g., Fig. 4A) using KinTek Explorer (KinTek Corporation) (42). All replicates for a given condition and RNAP variant were fit simultaneously (global fitting) to minimize effects of experimental error. Each replicate was fit individually, and the SDs in the best-fit kinetic parameters were calculated to estimate errors in the rate constants obtained from the global fits.

To determine the elongation kinetics (and pausing kinetics, where applicable) at the termination site in the absence of termination, reactions performed in the absence of asRNA were used. Data were first fit to the simplest model, C18* → U19* → A20*.

The KinTek Explorer fits were seeded with initial rate constants for these two reactions obtained from algebraic, single-exponential fits for C18 disappearance and A20* appearance using Igor Pro (WaveMetrics). All elongation kinetic models required inclusion of a termination step to fit the data adequately (e.g., Fig. 4A and SI Appendix, Fig. S3) due to weak termination that occurred at U19 even in the absence of asRNA. If the simple model failed to fit to the data adequately (as evidenced by high residuals with systematic error), pause steps for C18, U19, or both were added to the model to enable a good fit to the data (e.g., Fig. 4A and SI Appendix, Fig. S10C). In these cases, initial rate estimates from double exponential fits of the relevant reactions obtained with Igor Pro were used to seed the KinTek Explorer fits, with the faster rates corresponding to elongation and the slower rates corresponding to pause escape (e.g., C18* → U19* in Fig. 4A). For conditions exhibiting biphasic extension of C18 to U19, reaction kinetics were modeled using two U19 populations with different UMP incorporation rates. For computational simplicity, we fit the two U19 populations using a variable rapid rate to generate the second slow state (e.g., Fig. 4A). However, the method of modeling C18 extension kinetics does not impact U19 kinetics (SI Appendix, Fig. S12), which were used for all comparisons in this work. We include C18 ECs in the kinetic models only because it is necessary to analyze the kinetic behavior of U19 ECs. We restrict our conclusions about termination to the behavior of the active U19 ECs. Although the halted C18 ECs partitioned into different states with different elongation rates, as shown by Pasman and von Hippel (39), ECs revert to a naive conformational state after each round of nucleotide addition. Thus, the U19 ECs are expected to be active ECs independent of the rate or path by which they form from C18. Steps with unconstrained rates were added to the kinetic models only if they were justifiable based on known pause or dissociation propensities of the RNAP variants in question, and if the probability that the more complex model gave a better fit was greater than 0.95. The relative probabilities of the selected kinetic models were evaluated against simpler models with fewer unconstrained rates using the corrected Akaike’s information Criterion (AICc) (43).

\[
\Delta AICc = AICc(\text{selected model}) - AICc(\text{alternate model})
\]

where \(AICc\) is AICc(selected model) – AICc(alternate model) (SI Appendix, Figs. S3 and S6).

In conditions with asRNA, elongation and pause kinetics were assumed to be unchanged, since these occur before asRNA binding (e.g., compare Figs. 4B and 5B), and the relevant rate constants were thus constrained in these fits. Binding and off rates (\(k_{\text{on}}\) and \(k_{\text{off}}\)) for asRNA, asRNA concentration, and time of asRNA addition were input into the models and also constrained. Since we observed some amount of terminator escape from C18 and U19, as well as some termination (evidenced by plateaus formed by C18 and U19 with a percent occupancy of >0%), Fig. 5), four steps were added to these conditions: termination at C18, termination at U19, elongation of asRNA-bound C18, and elongation of asRNA-bound U19 (e.g., black arrows in Fig. 5B). The only free parameters in these fits were thus the rate constants for these four reactions, obtained by fitting the relevant kinetic models to their datasets by global and individual fitting (see also SI Appendix, Fig. S5).

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