Mechanisms of Bacterial Transcription Termination: All Good Things Must End

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Abstract
Transcript termination is essential for accurate gene expression and the removal of RNA polymerase (RNAP) at the ends of transcription units. In bacteria, two mechanisms are responsible for proper transcript termination: intrinsic termination and Rho-dependent termination. Intrinsic termination is mediated by signals directly encoded within the DNA template and nascent RNA, whereas Rho-dependent termination relies upon the adenosine triphosphate-dependent RNA translocase Rho, which binds nascent RNA and dissociates the elongation complex. Although significant progress has been made in understanding these pathways, fundamental details remain undetermined. Among those that remain unresolved are the existence of an inactivated intermediate in the intrinsic termination pathway, the role of Rho–RNAP interactions in Rho-dependent termination, and the mechanisms by which accessory factors and nucleoid-associated proteins affect termination. We describe current knowledge, discuss key outstanding questions, and highlight the importance of defining the structural rearrangements of RNAP that are involved in the two mechanisms of transcript termination.
**INTRODUCTION**

Understanding the mechanism and sequence determinants of transcription termination by bacterial RNA polymerases (RNAPs) is a long-standing goal in the study of gene regulation. Termination (a) prevents the inappropriate expression of downstream genes and interference from antisense transcripts, (b) defines RNA 3′ ends for precise RNA structures (e.g., small noncoding RNAs) or for regulation, (c) recycles RNAP for efficient gene expression, and (d) minimizes collisions with replication complexes that cause damaging double-strand breaks in the chromosome.

Programmed transcription termination in bacteria occurs in one of two pathways: intrinsic termination, requiring only RNAP, RNA, and DNA, or factor-dependent termination, involving proteins that dissociate the elongation complex (EC), such as Rho, or factors that target damaged ECs, such as Mfd (see the sidebar, Mfd-Dependent Termination: An Alternative Termination Paradigm) (I). We focus on the mechanisms of intrinsic and Rho-dependent termination as the major pathways that affect gene regulation, for which advances during the past several years have raised important questions for future research. Intrinsic termination occurs when an RNA secondary structure (called a terminator hairpin, T_{hp}) forms in the exit channel of an RNAP that is positioned on an unstable RNA–DNA hybrid, causing EC dissociation. Rho-dependent termination relies on the adenosine triphosphate (ATP)-dependent RNA–DNA helicase Rho,
Mfd-DEPENDENT TERMINATION: AN ALTERNATIVE TERMINATION PARADIGM

In bacteria, the superfamily 2 DNA translocase Mfd targets RNA polymerases that are stalled at sites of DNA damage to induce elongation complex (EC) dissociation via a third termination mechanism. Unlike intrinsic or Rho-dependent termination, Mfd-dependent termination of these stalled ECs is not programmed by specific nucleic acid sequences. Instead, Mfd recognizes stalled ECs and initiates the transcription-coupled repair (TCR) pathway. During TCR, stalled ECs that would otherwise block DNA replication are removed and the UvrABC repair proteins are recruited. Upon binding to both the stalled RNAP and upstream DNA, Mfd activates its adenosine triphosphate (ATP)-dependent translocase activity. The relative movement of Mfd in the direction of the stalled EC induces the hypertranslocation of RNAP, without the addition of nucleoside triphosphate (NTP) and subsequent bubble rewinding, thus inducing the release of the nascent transcript and dissociation of RNAP (127). UvrAB is then recruited directly to the damage site via Mfd’s UvrB homology domain (153). Studies into the mechanism of Mfd-dependent termination are focusing on how Mfd discriminates between arrested and paused ECs and on the fraction of TCR that depends upon Mfd action.

which binds to and translocates the nascent RNA until it encounters a paused EC and causes the release of RNAP. Rho requires a less-specific sequence at which to dissociate than intrinsic termination, which is important for its essential role in suppressing R-loops, transcription of horizontally acquired genes, and antisense transcription (2–5).

ELONGATION COMPLEX STABILITY AND ITS DESTABILIZATION BY TERMINATION

Elongation Complex Stability

To understand the dissociation of ECs at terminators, one must first understand the determinants of EC stability. RNAP has evolved to form a remarkably stable, yet dynamic, network of contacts with the nucleic acid scaffold in the EC, enabling it to transcribe more than $10^4$ nucleotides (nt) without dissociating from the template. The enzyme protects a 12–14 base pair (bp) transcription bubble, a 9–10 bp RNA–DNA hybrid, approximately 5 nt of newly transcribed single-stranded RNA (ssRNA), and approximately 18 bp of downstream duplex DNA (Figure 1) (6, 7). The major sources of EC stability are polar and van der Waals contacts between RNAP and the RNA–DNA hybrid backbone in the main channel of the enzyme, augmented by H-bonds to the ssRNA in the exit channel and long-range electrostatic and van der Waals contacts to the downstream DNA (8). Comparison of the structures of the EC versus free RNAP reveals that these contacts are established upon closure of the clamp module and a small rotation of the shelf module relative to the core of RNAP (Figure 1a) (8–12). These changes are detectable by disulfide-bond formation, using cysteine pairs strategically engineered into Escherichia coli or Thermus thermophilus RNAP (13, 14). The closed-clamp state appears to be stabilized, in part, by interactions between downstream DNA and the RNAP clamp and jaw, and, most significantly, by extensive contacts among the folded forms of three switches and the RNA–DNA hybrid and downstream DNA (7, 15–17). The switches (Sw1–5) are five highly conserved polymorphous elements at the base of the clamp that rearrange to enable the clamp to swing open 30° relative to the RNAP core, widening the main cleft (15). Compaction of the switches conversely stabilizes contacts between the closed clamp and the nucleic acids in the EC. Specifically, Sw1 makes stabilizing contacts to the template strand in the RNA–DNA hybrid; highly conserved Sw2 K334 (E. coli RNAP numbering) marks
the downstream edge of the RNA–DNA hybrid by stabilizing the 90° kink in the template strand between +1 and +2; and Sw3 forms a hydrophobic binding pocket for the first ssRNA base upstream of the RNA–DNA hybrid (Figure 1c) (8, 15, 18). The upstream edge of the hybrid is further stabilized by stacking interactions with the lid (Figures 1c and 2c) (8). This network of nonspecific interactions creates an EC that is stable up to 70°C and >0.5 M salt (19–21) but still allows for efficient translocation of DNA and RNA through RNAP.

Figure 2
The mechanism of intrinsic termination. (a) The model Arg2 terminator. The red U’s indicate highly conserved, upstream hybrid U’s that are melted upon T_hp completion; the blue underlined U’s mark the sites of termination. (b) A reaction scheme with competing steps in the termination pathway. The EC is depicted schematically in an orientation similar to that in Figure 1a, with the RNAP lid and mobile clamp module (pink), the secondary channel (light gray), RNA (red), and DNA (dark gray). The mechanism is shown in four steps: ① pausing at the 3’ end of the U-tract, ② T_hp nucleation, ③ T_hp completion and possible EC inactivation, and ④ EC dissociation. Two alternative mechanisms for T_hp completion and EC inactivation are depicted. Red arrows depict the proposed EC inactivation step, which appears to be irreversible in vitro; green arrows depict possible EC reactivation, which may occur in vivo, possibly facilitated by transcription factors. The EC subscripts n and n + 1 denote the transcript lengths. (c) Nucleic acid scaffolds at stages of transcript elongation and termination: pretranslocated EC, postranslocated EC, after T_hp nucleation, and after inactivation by hybrid-shearing or hypertranslocation. The active site is denoted by two gray ovals; the tip of the lid by a purple loop; and Sw3 by a cyan semicircle. Stacking interactions between the lid and the upstream hybrid are depicted by blue dashed lines. The position of the upstream end of RNAP relative to RNA is represented by a black dashed line. Green and blue nucleotides represent, respectively, the −1 and −10 bps. Abbreviations: bp, base pair; EC, elongation complex; NTP, nucleoside triphosphate; PP_i, pyrophosphate; RNAP, RNA polymerase; Sw3, switch 3; T_hp, terminator hairpin; U, uracil.
The challenge of transcription termination is to destabilize the highly stable EC sufficiently at specific genome locations to enable RNAP dissociation from the DNA and RNA. However, termination can occur at a given position only if RNAP enters the termination pathway faster than nucleotide addition moves the DNA template to the next position (Figure 2b, step ① versus elongation). Consequently, efficient termination is aided by factors that increase the kinetic window at the termination branch point, and it is impeded by factors that increase the elongation rate. Sequence-dependent pauses can reversibly halt ECs along the template, for durations of seconds to minutes (22, 23), thereby increasing the kinetic window for entry into the termination pathway. They may also alter the EC conformation to make it susceptible to termination. Thus, pausing is considered a necessary first step for efficient termination (Figure 2b) (20, 24): It promotes termination by stalling the ECs and either decreasing the rate of pause escape from the termination site (in intrinsic termination) (20) or allowing Rho to catch up to the EC by translocating RNA more rapidly than RNAP synthesizes RNA (in Rho-dependent termination) (25–28).

Transcriptional pausing is defined as a kinetic branching from the main elongation pathway where the EC enters a halted state that inhibits nucleotide addition. Pause escape occurs when the active site rearranges back into an active configuration and resumes nucleotide addition. An EC may isomerize into an off-pathway, paused state at any position on a template (29), but these excursions from the elongation pathway, into so-called elemental pauses, are preferentially triggered and stabilized by specific sequences in the RNA–DNA hybrid, the nontemplate strand, downstream DNA, and active site nucleotides (18, 30, 31). An analysis of the crystal structure of RNAP bound to elongation complex rearrangements of the switch regions revealed a widened main cleft, with the switch regions swung away from the nucleic acids, breaking some contacts with the RNA–DNA hybrid (18). This rearrangement partially reverses changes seen during the transition from free RNAP to the EC, as observed in previously reported crystal structures (8, 10, 15). Because complete clamp opening does not appear to occur in the elemental paused EC when probed by disulfide formation (14) (M.J. Bellecourt, D. Nayak, R.A. Mooney & R. Landick, unpublished findings), the sequences of the elemental pause likely loosen the clamp contacts sufficiently to allow transient clamp opening, which appears to become stabilized in the open position by crystal packing forces. The rearranged structure also features a kink in the active site–proximal bridge helix (Figure 1b) that obstructs the template base and nucleoside triphosphate (NTP) entry into the active site, preventing nucleotide addition.

The lifetimes of elemental pauses can be increased by the formation of RNA secondary structures in the RNA exit channel, which are called pause hairpins (in hairpin-stabilized pausing), or by backward translocation of the EC (in backtrack pausing). Hairpin-stabilized pausing occurs when complementary sequences in the nascent transcript pair to form a pause hairpin in the RNA exit channel 11 or 12 nt from the RNA 3′ end and, thus, inhibit clamp closure (13, 14). The formation of the hairpin is likely enabled by the loosening of the clamp in the elemental paused EC (18). Hairpin-stabilized pausing also requires interactions between the pause hairpin and the flap domain (Figure 1a) (32, 33), and it can be aided by binding of NusA (see, Impact of Transcription Factors on Termination, below) (34–36), which contacts both the flap tip and the pause hairpin (37–39).

Backtracking is a mechanism by which RNA and DNA reverse translocate through RNAP, such that the 3′-nascent RNA extrudes into the secondary channel (Figure 1b), consequently removing the RNA 3′ end from the active site. It can occur as a proofreading mechanism in response to misincorporated nucleotides or when the EC is positioned over a weak RNA–DNA hybrid and can gain stability by backtracking to a position where the RNA–DNA hybrid is stronger (40–42).
Backtracked pauses can be resolved by forward translocation to restore the 3’ end to the active site, by the intrinsic transcript cleavage activity of RNAP to create a new 3’-OH, or by GreA- or GreB-stimulated cleavage, in which the Gre transcription factor binds in the secondary channel and aids cleavage of the backtracked transcript in the RNAP active site.

Intrinsic terminators encode a T_{hp} that is structurally similar to a pause hairpin (Figure 2a, and see Mechanism of Intrinsic Termination, below), but whether this T_{hp} causes a hairpin-stabilized pause is still an open question. Intrinsic terminators also encode a weak, uracil (U)-rich RNA–DNA hybrid that is related to elemental pause sequences and induces pausing (20). The weak U-rich hybrid could cause backtracking in the absence of the T_{hp}. However, backtracking is an unlikely predecessor to termination, as it would inhibit T_{hp} formation. In fact, one role of the T_{hp} is thought to be the prevention of backtracking in order to lock the EC on the weak hybrid that is favorable for dissociation (21, 43). Although the precise nature of the pretermination pause is still uncertain, the U-rich hybrid at intrinsic terminators may both induce an elemental pause and instigate the initial RNAP conformational change necessary for termination by loosening protein–nucleic acid contacts in the EC and making the complexes susceptible to subsequent inactivation and dissociation.

Rho termination efficiencies have been shown to be correlated to pause strength (44). However, pause stabilization, induced either by a pause hairpin or by backtracking, impedes Rho termination (45) (see Kinetic Coupling of Rho to the Elongation Complex, below). Further characterization is required to determine whether the pauses at Rho termination sites are all elemental pauses of varying strengths or whether they are stabilized by other means. A better description of these early events in both termination pathways is vital to understanding the role of pausing in intrinsic and Rho-dependent termination.

Impact of Transcription Factors on Termination

Most mechanistic studies have been performed in cell-free environments to leverage the manipulable nature of in vitro experiments. Although this focus enables the investigation of the termination mechanism in the absence of extrinsic factors, it may miss more complex layers of regulation that occur in vivo. Multiple factors in the cell may affect termination, including transcriptional factors that can bind ECs and modulate their stability at various steps along the termination pathway.

Transcriptional factors that bind the EC can perturb termination efficiency by modulating the elongation rate, pause entry or pause lifetime, nascent RNA structure formation, or EC stability. For example, the widely distributed NusG protein has been shown to promote forward translocation—and, hence, elongation—of E. coli ECs by binding the clamp near the upstream fork junction and likely helping upstream DNA reanneal (46). In other bacterial strains, however, NusG has been shown to stimulate hairpin-stabilized pausing (in Bacillus subtilis) (47, 48) or to enhance intrinsic termination efficiency (in Mycobacterium bovis) (49), possibly by inhibiting rather than stimulating translocation as observed in E. coli (50). NusG aids Rho-dependent termination at a subset of Rho terminators (51, 52). Conversely, NusG can also facilitate transcriptional–translational coupling through an interaction with the ribosomal protein S10, which inhibits Rho-dependent termination by blocking the Rho–RNAP interaction when nascent RNA is translated (53) (see the discussion in NusG Acts as Both a Positive and Negative Regulator of Rho). The NusG paralog, RfaH, inhibits termination through a distinct mechanism; RfaH stabilizes the closed clamp by binding the clamp helices and a loop in the lobe domain, thereby impeding the clamp opening required for both pausing and termination (39, 54–56). Finally, the exit channel binding factor, NusA, can stimulate both pausing and termination at weak intrinsic terminators by stimulating hairpin formation and stabilizing the hairpin’s interaction with the flap.
domain, effectively decreasing the rate of pause escape (32, 35, 36, 38, 39, 57, 58). However, like NusG’s paradoxical roles in Rho-dependent termination, NusA can also act as an antitermination factor that inhibits intrinsic termination when complexed with other transcription and translation factors (reviewed in 59).

Gaining a complete picture of termination in vivo, therefore, requires an understanding of the abundance of various transcriptional factors in the cell and how they impact the termination mechanisms identified in purified ECs.

MECHANISM OF INTRINSIC TERMINATION

At intrinsic terminators, DNA and RNA elements at the site of termination direct a series of steps that, after RNAP transcribes kilobases (kb) of gene information, cause the enzyme to (a) terminate transcript elongation at a discrete location spanning 2–3 nt and (b) release from the chromosome and transcript. The canonical intrinsic terminator sequence (Figure 2a) consists of two main elements: (a) a guanine and cytosine (GC)-rich dyad that forms the T hp 7–8 nt from the transcript’s 3’ end, followed immediately by (b) a 7–8 nt U-rich tract, of which the first three U’s are the most highly conserved (60). In the next section, we describe the steps by which this termination signal directs EC destabilization: (a) pausing at the 3’ end of the U-tract, (b) T hp nucleation, (c) hairpin completion and possible EC inactivation, and (d) EC dissociation (Figure 2b).

Pausing at the U-Tract

As RNAP transcribes the final nucleotides of the terminator U-tract, it pauses (Figure 2b, step 1), favoring the termination pathway in the kinetic competition between elongation and termination (20, 24). Decreased elongation rates have been shown to favor termination efficiency (61), as pausing creates a window of time in which the T hp can form in the exit channel and initiate the cascade of events that cause termination (20). Mutation of the 3’-terminal U of the λR2 U-tract, which has been shown to abrogate pausing at the termination site, also eliminated termination (20); therefore, pausing at the U-tract must be crucial for the formation of the T hp. In addition to the U-tract, nucleic acid sequences in the active site, downstream DNA channel, and the RNA exit channel must have a role in establishing the pause and determining pause efficiency and duration (22, 34, 62, 63). These sequences contribute to the maximal termination efficiency by determining the proportion of complexes that enter the elemental U-tract pause and the fraction of those complexes that are able to form the T hp prior to pause escape (Figure 2b).

Terminator Hairpin Nucleation

Pausing of the EC at the end of the 7–8 nt U-tract stalls RNAP for a sufficient time to allow the T hp to form in the exit channel (Figure 2b, step 2). Two separate studies have shown that a 7 bp T hp does not nucleate until the EC transcribes 7 nt downstream from the T hp-encoding sequence (20, 64). This is consistent with evidence that RNAP protects 14 nt of RNA (approximately 9 nt in the hybrid and 5 nt in the RNA exit channel) (6), with a preference for a T hp ≥ 7 bp at canonical terminators (60, 65). Termination can also occur at low efficiency 8 nt downstream from a 6 bp T hp (35), with hairpin nucleation likely occurring 8 nt downstream from the T hp sequence. Conversely, a T hp with a longer stem or with a stabilized loop may nucleate before the EC reaches this position so that programmed pausing at the end of the U-tract becomes dispensable for T hp nucleation (66), although this relationship has not been studied systematically. However, in the
absence of a U-tract, hairpin formation does not result in efficient termination (66), verifying that both the Tₜₜ and U-tract are essential for the termination mechanism.

Upon Tₜₜ nucleation, all but the bottom 2–3 bp of the Tₜₜ rapidly pair (64), aided by the flap domain tip (Figure 1) in the case of a weak Tₜₜ (32). Whether the partially formed Tₜₜ produces a hairpin-stabilized pause remains unresolved by direct experimental evidence. However, the first step in Tₜₜ formation produces a structure remarkably similar to a pause hairpin, and likely stabilizes the pretermination pause prior to hairpin completion (64, 67).

Historically, some studies have described the effects of sequences far removed from a terminator on termination efficiency, and they have considered various explanations for the phenomenon, including the induction of persistent alternative conformations of the EC that resist termination (68, 69). However, competing RNA structures formed with the upstream transcript can inhibit Tₜₜ formation by sequestering the upstream arm of the Tₜₜ prior to hairpin nucleation (64, 70). These more recent demonstrations of the effects of alternative RNA folding highlight the importance of ensuring that comparisons of terminators are made in the context of a constant sequence to avoid the potentially complicating effects of RNA folding.

Terminator Hairpin Completion

The structure of the EC suggests that the pairing of the bottom 2–3 bp of the Tₜₜ stem would require displacement of the −10 RNA base from its Sw3 binding pocket and the unstacking of the lid from the −9 RNA–DNA bp (numbered according to the posttranslocated state; Figures 1c and 2c) (8). Consistent with this idea, Lużkowska et al. (64) found that Tₜₜ formation occurs in two steps for the λr₂ Thp. In the first step, all but the bottom 2 bp formed upon Tₜₜ nucleation 7 nt downstream from the Tₜₜ-forming sequences. In the second step, the bottom 2 bp of the Tₜₜ paired when the EC transcribed the eighth nucleotide downstream from the Tₜₜ sequence. This energetic barrier to completion of the Tₜₜ likely reflects the need to disrupt the interactions between the lid and hybrid and between Sw3 and −10 RNA that limit complete hairpin extension (Figure 2b, step ♀/♀) upon nucleation of the Tₜₜ (Figure 2b, step ♀/♀).

Termination is frequently observed 7 nt downstream from the Tₜₜ on endogenous terminators (Figure 2a) and requires that Tₜₜ nucleation and completion occur at the same template position (20, 71), which is inconsistent with the findings of the Tₜₜ-folding study described above (64). It is possible that Lużkowska et al.’s (64) use of stabilized hybrids to enable the EC to walk downstream from the Tₜₜ sequence precluded completion of the Tₜₜ until the eighth nucleotide was added (20, 21). Upstream melting of the stabilized hybrid would be disfavored in this case due to the lack of U’s (see next paragraph), thereby making the energetic barrier for hairpin completion even higher and preventing Tₜₜ completion at this position. However, the two-step folding pathway of Tₜₜ nucleation, followed by energetically expensive Tₜₜ completion, likely exists for all cases— independent of terminator structure and the exact position for each step—due to the energetic barrier created by the interactions between the lid and hybrid and Sw3 and −10 RNA.

Tₜₜ completion causes 3–4 bp of the upstream RNA–DNA hybrid to melt (Figure 2a,c) (20, 21), thus contributing to the destabilization of the EC. This hybrid melting step is essential for hairpin completion and termination and is consistent with the high degree of sequence conservation for U’s at the first three positions in intrinsic terminator U-tracts (20, 21, 60). From these observations, it appears that the length of the hairpin stem and the structure of the Tₜₜ loop dictate the kinetics and position of Tₜₜ nucleation by determining the position at which the loop and the GC-rich dyad emerge from the RNA exit channel and the amount of RNA needed to nucleate Tₜₜ formation. However, the template position at which Tₜₜ completion can occur is likely determined by the
Hybrid-shearing: slippage of the RNA past the DNA in the RNA–DNA hybrid by changes in base pairing or the generation of mismatches in the hybrid

Hypertranslocation: forward translocation of RNAP without concomitant nucleotide addition, moving single-stranded DNA into the main channel of RNAP

relative strengths of the bottom 2–3 bp of the T$_{hp}$ compared with the upstream 3–4 bp of the hybrid. These relative strengths dictate the energetic cost of melting the upstream hybrid and breaking the interactions between the lid and hybrid and Sw3 and −10 RNA, compared with the energetic gain from pairing of the bottom of the T$_{hp}$.

Upstream hybrid melting results from an incompatibility of a complete T$_{hp}$ and a 9–10 bp RNA–DNA hybrid, but the structural basis for this incompatibility is unclear from EC crystal structures. It appears entirely feasible that, in an EC with a fully open clamp, the exit channel and main cleft could accommodate a relatively continuous (albeit modestly kinked) bipartite duplex of the hairpin stem and hybrid, perhaps stabilized by a stacking interaction between the lid, the hybrid, and the T$_{hp}$ (72). Nonetheless, the demonstrated hybrid melting effect indicates that steric constraints of some sort must limit the possible nucleic acid conformations and suggests that not yet defined RNAP structural rearrangements are likely to accompany this step.

Several different models have been proposed to describe how the completed T$_{hp}$ affects the EC. These models involve (a) hypertranslocation of the EC 2–4 bp downstream from the termination point, without concomitant nucleotide addition (Figure 2) (24, 70, 73); or (b) shearing or slippage of the RNA from the RNA–DNA hybrid by a lifting or rotational wrenching of the hairpin from the exit channel (21, 70). A single-molecule force-clamp experiment revealed that both mechanisms may be possible, depending on the sequence of the terminator (70). Larson et al. (70) found that a terminator with an imperfect U-tract (a U-tract interrupted by one or more GC bp), $t_{500}$, was rendered more efficient by RNA pulling and impeded by a hindering force on DNA translocation (consistent with hypertranslocation), whereas two terminators with near-perfect U-tracks (7 or more tandem rU-dA bp and at most one rA-dT bp), $t_{his}$ and $t_{R2}$, were also aided by RNA pulling, but unaffected by force on the DNA (consistent with hybrid-shearing). Taken together, these data suggest that either mechanism can occur, with hypertranslocation favored on imperfect U-tracks and hybrid-shearing favored on weaker near-perfect U-tracks. Consistently, Peters et al. (74) have found that in a set of 100 E. coli terminators, the downstream DNA is enriched for adenine and thymine (AT)-rich sequences at positions +10 to +12 after imperfect U-tracks, but not after near-perfect U-tracks, indicating a need for the melting of downstream DNA in hypertranslocating terminators, but not in hybrid-shearing terminators. These results support a composite hybrid-shearing–hypertranslocation model in which the mechanism employed is determined by the relative energetic cost of shearing the U-tract hybrid versus melting downstream DNA (Figure 2b, step (s) (70, 75)). Extensive characterization of various terminator structures in vivo has found that the strongest terminators have both near-perfect U-tracts and AT-rich downstream DNA (65). This finding also supports the idea that both hybrid-shearing and hypertranslocation can occur and that termination efficiency is highest if both mechanisms are energetically favorable.

Thus, T$_{hp}$ completion appears to be the first step that distinguishes paused complexes from termination-competent intermediates. T$_{hp}$ completion results in nucleic acid rearrangements in the EC and likely concerted rearrangements of RNAP, crucial for efficient termination. These rearrangements may irreversibly inactivate the EC and, consequently, commit the EC to the termination pathway.

**Elongation Complex Inactivation and Commitment to Termination**

Although several studies have modeled termination efficiency as being determined by direct kinetic competition between elongation/pause escape and EC dissociation (76, 77), there is some evidence—as well as uncertainty—that an irreversible EC inactivation step occurs in the termination pathway prior to EC dissociation. In a single-molecule termination assay, Yin et al. (78) found that a terminal dwell occurred at the $t_{his}$ terminator prior to transcript dissociation.
only for complexes that terminated but not for complexes that read through the terminator. This result suggests that nucleotide addition is irreversibly halted in a step distinct from dissociation. In contrast, no terminal dwell was observed for the $t_{\text{his}}$, $\lambda_{\text{tR2}}$, or $t_{\text{500}}$ terminators in a force-clamp experiment (70). It is possible to explain this discrepancy if the assisting force used in the force-clamp study increased the rate of EC dissociation, masking a terminal dwell prior to dissociation. The terminal dwell in the Yin et al. (78) study, then, implies that ECs at the termination site can exist in two distinct states: one that is able to recover from the pretermination pause and continue elongation past the terminator, and another, inactivated state that is incapable of nucleotide addition but remains bound to the hybrid at the termination site until EC dissociation occurs at a slower rate. Notably, Nudler and coworkers (20) also reported the existence of an irreversibly trapped, or inactivated, termination complex at the $\lambda_{\text{tR2}}$ terminator prior to EC dissociation, although this interpretation has been disputed (79). The rate of transcript release in vitro, after $T_{\text{hp}}$ completion and upstream hybrid melting, occurs on the order of many minutes (21), too slow to compete with pause escape, which can occur on the order of seconds, even when stabilized by a pause hairpin (23). Given this slow rate of EC dissociation, an irreversible inactivation step seems kinetically necessary to prevent pause escape and the resumption of elongation. This step, requiring inactivation of the RNAp active site, thus represents the commitment of ECs to the termination pathway.

EC inactivation may occur through the restructuring of the active site. All proposed models for commitment posit $T_{\text{hp}}$ completion as a driving force for downstream hybrid rearrangements that result in EC inactivation (20, 21, 70). In one model, hairpin formation is proposed to be sufficient to inactivate ECs due to the substantial destabilizing effect of upstream hybrid melting (20). Here, the RNA 3’ end remains in the active site until dissociation occurs, and allosteric inhibition is proposed to be responsible for EC inactivation via invasion of the $T_{\text{hp}}$ into the main channel of RNAp (hairpin invasion) (80). However, the low-salt conditions used in these studies have been shown to allow dissociation and subsequent rebinding of the RNA in the main cleft, allowing for alternative interpretations of these results (79). Another model of inactivation of ECs is suggested by the composite hybrid-shearing–hypertranslocation model for $T_{\text{hp}}$ accommodation; both hybrid-shearing and hypertranslocation would directly inactivate the EC via removal of the RNA 3’ end from the RNAp active site (Figure 2c) (70). Further study is required to understand the mechanism of EC inactivation and whether the nucleic acid rearrangements described by any of these models are (a) sufficient to prevent nucleotide addition and (b) irreversible.

Mechanisms that invoke an irreversible commitment to termination require that all regulation of termination efficiency occurs at a step prior to the inactivation of the EC (because events occurring after irreversible inactivation cannot affect the proportion of ECs that terminate). However, a recent study has suggested that events occurring after $T_{\text{hp}}$ formation can affect termination efficiency in vivo (65). Voigt and coworkers (65) found that A-tracts immediately upstream of the $T_{\text{hp}}$ (Figure 2a) could increase termination efficiency, likely by pairing with the U-tract because complementarity of the A-tract and U-tract was required to observe the effect. A–U-tract pairing would extend the $T_{\text{hp}}$ duplex and completely eliminate the RNA–DNA hybrid; therefore, it must occur subsequent to $T_{\text{hp}}$ completion and the presumed EC inactivation. This logic implies that, in vivo, $T_{\text{hp}}$ completion must be reversible and that A–U-tract pairing can compete with EC reactivation (Figure 2b, reverse of step ☞) by accelerating the dissociation of RNA from the EC (Figure 2b, step ☞).

Interestingly, this A-tract-dependent enhancement of termination efficiency has not been observed in vitro (35). One interpretation is that in purified ECs, the rate of EC reactivation after $T_{\text{hp}}$ formation is so slow relative to the dissociation rate that it is functionally meaningless, rendering any downstream A–U-tract pairing effect irrelevant to termination efficiency. However, cellular elongation factors, such as NusA and NusG, that interact with the EC may be capable of binding
the committed, inactivated intermediate and aiding EC reactivation. This could allow competition between dissociation and EC reactivation (Figure 2b, step 2 versus the reverse of step 3), allowing the acceleration of dissociation by A–U-tract pairing to affect termination efficiency. Potential mechanisms of transcription-factor-mediated EC reactivation include (a) NusA-mediated stabilization of the flap–Thp interaction in the inactivated complex (32) or (b) NusG-mediated stabilization of the clamp–DNA interaction. Either interaction could facilitate EC reactivation by affecting nucleic acid pairing patterns in the Thp, the upstream hybrid, and the upstream DNA fork junction. In vitro testing of the effect of A-tracts on termination efficiency and EC dissociation in the presence of NusA, NusG, and other EC-stabilizing factors could help determine whether these factors sufficiently stabilize termination intermediates to make an otherwise irreversible EC inactivation step reversible.

Elongation Complex Dissociation

The final step in the termination pathway is the dissociation of RNAP from both the DNA template and the RNA transcript (Figure 2b, step 3). Several factors likely contribute to dissociation. The RNA–DNA hybrid is significantly weakened by the rU–dA bps of the terminator U-tract (81), and it is destabilized further by the shortening of the hybrid after upstream hybrid melting (20, 21). Komissarova and coworkers (21) demonstrated that a 5-bp hybrid, representative of the residual hybrid length retained by the terminator after the upstream hybrid melts (Figure 2c), almost completely recapitulates the dissociation rate of a termination complex. Thp completion must disrupt the interactions of Sw3 and −10 RNA and the lid and −9 hybrid (described above) (Figure 1c), and it likely also disrupts the H-bonds between ssRNA and the exit channel (8). It is unclear whether any of these contacts could be re-established upon hypertranslocation or hybrid-shearing (Figure 2b,c) and the consequent repositioning of the RNA with respect to RNAP.

An open-clamp conformation with loosened contacts between the RNA–DNA hybrid and switch regions also has been proposed to favor EC dissociation (11, 12, 15, 18). Consistent with this idea, results of studies using disulfide-cross-linking to probe the clamp state indicate that an open-clamp/rotated-shelf state is favored by duplex formation in the exit channel (13, 14), suggesting that Thp formation may stabilize the open clamp and weaken contacts between the nucleic acid scaffold and RNAP.

Other RNAP rearrangements have been proposed to aid EC dissociation, based on the reduced stabilities of ECs deleted for the lid, rudder, or flap modules (32, 36). However, the conformational rearrangements of RNAP in a destabilized termination complex remain poorly characterized. Further, the order of RNA and DNA release remains unknown, as does whether the order is obligate or affected by terminator sequences.

Role of RNA Polymerase Conformational Changes

A surprisingly understudied aspect of the termination mechanism is the contribution of conformational changes of RNAP to the energetics of each step in the termination pathway. Are the steps of termination governed principally by nucleic acid folding and translocation dynamics, or are conformational changes of RNAP also necessary to inactivate and dissociate the enzyme? Toulokhonov et al. (32) found that the flap tip aids nucleation of a weak Thp. Restructuring of the clamp (11–14) and lid are thought to be involved in EC dissociation (36). Do other modules also have a role in the pathway? What inactivates the EC active site at terminators? Is the removal of the RNA 3′ end from the active site (as expected in hypertranslocation and hybrid-shearing;
As discussed above, the switch regions are thought to couple clamp closure to nucleic acid scaffold binding, and, conversely, to promote efficient termination by destabilizing contacts to the scaffold upon clamp opening (15, 18). Switch rearrangements are, therefore, likely to be involved in EC dissociation. Mutational analyses of RNAP have identified several mutations in the switch regions that result in altered termination efficiencies (82, 83), which is consistent with the switch regions having a role in the termination mechanism (presumably by mediating clamp opening). Such an effect on termination efficiency in vitro implies that switch remodeling occurs upstream of EC inactivation (Figure 2b, step 1), perhaps by clamp loosening that aids both an elemental pause and T_{hp} formation (Figure 2b, steps 1 and 2) (18).

In the hairpin-invasion model (described above), Nudler and coworkers (80) suggest that folding of the active site–proximal trigger loop (Figure 1b) is also required to form a termination-competent complex, possibly by contributing to inactivation of the active site. The kinking of another active site–proximal element, the bridge helix, has been observed in the elemental pause structure (18). A similar conformation of the bridge helix in the inactivated termination complex is an attractive possibility, as it would obstruct substrate entry into the active site and elongation past the termination position (18). However, as rates of pause escape (22–23) are fast relative to the dissociation rates observed by the Gelles and Kashlev groups (21, 78), additional stabilization of the inactivated enzyme state may be required for appreciable termination efficiency.

Despite the identification of RNAP alterations that impact EC stability and termination efficiency, the state of the affected modules in termination complexes remains poorly characterized. A major future challenge will be to isolate termination intermediates so that they can be characterized. Although existing biochemical methods and X-ray crystallography have not succeeded in this task, cryo–electron microscopy (cryo-EM) methods are improving rapidly (84), and determining the structures and populations of these complexes in the termination pathway is likely to become possible in the near future.

**Rho-DEPENDENT TRANSCRIPTION TERMINATION**

In contrast to intrinsic terminators that function in response to signals directly encoded at the end of a transcription unit, Rho-dependent termination requires the RNA translocase activity of the homohexameric RecA-family helicase Rho (Figure 3), which nonspecifically recognizes and binds signals on the nascent RNA transcript. After binding to primary recognition sites, RNA is threaded through Rho’s central pore to trigger translocase activity. The dissociation of RNAP from the transcript occurs after Rho translocates all RNA upstream of the EC through the pore. In this and the following sections, we discuss recent work on Rho-dependent termination that has increased our understanding of the signals and sequences necessary for recruitment and activity of the Rho helicase, how the structure of Rho dictates its activities during termination, and how the structure and composition of the bacterial nucleoid can enhance the efficiency of Rho-dependent termination in vitro.

**Recognition of Rho-Utilization Elements**

Termination by Rho is regulated by sequences within the template DNA and in the nascent RNA transcript that can affect at least one of three steps: (a) recognition by Rho of substrate RNA, (b) translocation of RNA through the Rho motor protein, and (c) entry of the EC into a paused state on which Rho can act. Subsequent to these three steps, Rho induces termination in a fourth
step via a mechanism that involves the extraction of the nascent transcript from the RNA exit channel, followed by bubble collapse and the release of template DNA.

The first step requires the binding of Rho to the nascent RNA transcript after it emerges from the RNA exit channel of RNAP. Rho preferentially binds to C-rich, G-poor sequences that lack extensive secondary structure, with the highest affinity for poly(C) RNA sequences (85). The binding of Rho to these sequences is necessary to stimulate Rho’s RNA translocase activity. Endogenous Rho-binding elements, referred to as Rho-utilization (rut) sites (86), lie upstream of...
the site of termination and are thought to be approximately 80–90 nt long (87–89). This length is sufficient to allow for the binding of RNA to each of Rho’s six monomers: two pyrimidines tightly bound to the enzyme, interspersed by loops predicted to be at least 7–8 nt long, followed by a length of RNA that is sufficiently long to be threaded into the secondary binding site (Figure 3a) (90). The site of termination is typically within an additional 10–20 nt of the rut site (90), and it is rarely more than 100 nt downstream. The depletion of G within a natural rut site minimizes the formation of potentially interfering secondary structures, which are generally inhibitory to Rho binding (3, 91, 92). Rho has also been shown to be capable of looping out extensive secondary structures, so long as they do not sequester the primary rut RNA–Rho contacts (93–95).

A single consensus rut sequence is not thought to exist, as Rho activity can be stimulated on diverse C-rich RNAs. Indeed, termination still occurs at the model Rho terminator λR1, even after its natural rut site has been replaced with an artificial C-rich sequence (96). This lack of a single consensus sequence has hindered bioinformatic identification of rut sites, although recent efforts to identify sites of E. coli Rho activity in vivo using the Rho-specific inhibitor bicyclomycin have given new insight into Rho action on a genomic scale: More than 1,250 putative sites of Rho action were identified in this manner (2).

Biochemical studies, near-atomic resolution crystal structures, and cryo-EM have identified the sites on Rho to which rut RNA binds. Both crystallographic and cryo-EM studies have suggested that rut RNA binds the N-terminal domain (NTD) of Rho in an open-ring state (Figure 3a) (97, 98), which guides RNA into the exposed central pore of the Rho-C-terminal domain (CTD). The binding of RNA within the ring then isomerizes Rho into a closed-ring state (Figure 3b) that is competent for ATP hydrolysis and translocation (99). These structures are consistent with biochemical studies indicating that the Rho-NTD contains the primary RNA-binding site, a name accurate both in that rut RNA binds this site first and that it is the source of the high-affinity Rho–RNA interaction (97, 100). Rho binds C-rich sequences with a dissociation constant (Kd) of approximately 0.01–10 nM, an extensive range dependent upon the sequence of the RNA ligand and experimental conditions (101–106). The NTD contains a cold-shock domain-like RNA-binding domain (CSD-like RBD) whose oligonucleotide–oligosaccharide binding fold creates a narrow cleft that can only accommodate pyrimidines, with a preference for cytosine (Figure 3c).

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**Figure 3**

Structures of Rho and mechanisms of Rho termination. (a) A model of open-ring Rho bound by rut RNA at the primary binding sites and by the nonhydrolyzable ATP analog AMPNP (from coordinates provided by James Berger and derived from Protein Data Bank identifier 1PVO). The RNA dinucleotides directly bound to the primary binding sites are highlighted in yellow. (b) Structure of closed-ring Rho (Protein Data Bank identifier 3CE) bound by RNA in the secondary binding site, Mg2+, and the ATP transition state analog ADP–BeF3. The primary binding site dinucleotides were added to the structure; rut RNA loops are omitted for clarity. (c) Domain and module locations in Escherichia coli Rho (colored, where relevant, based on interaction targets in panels a and b). The NHB is shown (yellow, representing the YC-motif in the primary RNA site), as is the CSD-like RBD (yellow) and the Walker A and B ATP-binding motifs (green, ATP/ADP); also shown are the Q- and R-loops, the secondary-binding site loops (red, RNA secondary-binding site), the catalytic Glu and Arg valve (black), and the Arg finger residues (blue). Yellow and red lines indicate residues implicated in RNA binding. Dark and light purple NTD and CTD correspond to domains in panels a and b. (d) The pathway to Rho-dependent termination. Upon contact with the EC, Rho extracts the nascent transcript from the RNA exit channel by hybrid-shearing or hypertranslocation (red and gray arrows) and, possibly, RNAP rearrangement (e.g., clamp opening). (e) Possible Rho binding to RNAP throughout transcription. Such an initial state would not necessarily alter steps in Rho termination but could affect RNAP rearrangement. (f) Rho and the ribosomal small subunit protein S10 are both targets of the NusG-CTD. Binding of S10 couples transcription and translation, which sequesters rut sites in the ribosome and inhibits Rho termination. Abbreviations: ADP–BeF3, adenosine diphosphate–beryllium fluoride; Arg, arginine; ATP, adenosine triphosphate; CTD, C-terminal domain; CSD-like RBD, cold-shock domain-like RNA-binding domain; EC, elongation complex; Glu, glutamate; Mg, magnesium; NHB, N-terminal helical bundle; NTD, N-terminal domain; RNAP, RNA polymerase; rut, Rho-utilization element.
Each primary site binds a dinucleotide, for which a consensus of 5′-YC-3′ was identified. It is not yet understood whether each of Rho’s six monomers must bind the rut element or if binding by only a subset is sufficient to induce RNA entry into the pore, ring closure, and translocation activity. In addition to the primary binding site, Rho-NTD contains a positively charged N-terminal helix bundle that may assist in the binding of RNA to the enzyme via weak electrostatic interactions with the negatively charged phosphate backbone.

Rho in many bacterial phyla contain bulky sequence insertions between the N-terminal helix bundle and the CSD-like RBD that are not found in E. coli Rho. The functions of these NTD insertions, which are poorly conserved in both sequence and length, are not understood. Recent studies on M. tuberculosis Rho have demonstrated that its NTD insertion may enhance the affinity of Rho to rut RNA, while simultaneously slowing Rho motor activity. Whether all such insertions, which vary among lineages, function in this manner merits serious study.

Rho-CTD contains the RNA secondary binding site. A high-resolution image of the structure of Rho in the closed-ring conformation (Figure 3b) shows the nascent RNA transcript threaded through the central pore. This structure reveals, in agreement with biochemical evidence, that the secondary binding site is composed of the conserved Q- and R-loops, which form contacts with five nucleotides of the RNA substrate in a spiral staircase conformation. Unlike the primary site, which requires a C-rich substrate, the secondary site is capable of binding RNA substrates of any sequence. However, ATPase assays show a clear preference for poly(C). The structure also explains Rho’s specificity for RNA; whereas the primary binding sites can recognize bases from either DNA or RNA, the carbonyl group of residue V284 in the Q-loop of EcoRho forms an H-bond with a 2′-OH of the RNA ribose moiety, making binding of DNA to the secondary site unfavorable.

A major unresolved question is whether Rho binds the EC early in the transcription cycle, prior to the appearance of rut-containing nascent RNA (Figure 3e). ChIP–chip (chromatin immunoprecipitation plus DNA microarray) data show a genome-wide correlation between Rho and EC occupancy, with Rho associating with ECs early in the transcription unit without triggering termination. The precise nature of this interaction is undefined. Is this a strong, direct interaction between RNAP and Rho, or a transient interaction with minimal physiological relevance?

If Rho prebinds the EC, the local concentration of Rho would be significantly increased, poising the system for the quick capture and binding of a rut site upon its appearance in untranslated RNA. If such a Rho–RNAP interaction exists, it should be possible to identify the interaction sites on both RNAP and Rho that upon alteration, for instance by genetic mutation, would perturb Rho termination both in vivo and in vitro. Despite a long history of studying Rho and RNAP genetics, such mutations have yet to be identified; approaches using cross-link mapping and directed mutagenesis should readily enable the necessary test.

Translocation of Rho Toward the Elongation Complex

The binding of Rho to both rut RNA and ATP induces ring closure, activating Rho for catalysis (Figure 3d). Rho translocates the nascent RNA via ATP hydrolysis; although all four NTPs can support Rho translocation, ATP binds and drives translocation most efficiently, with a secondary preference for guanosine triphosphate over cytidine or uridine triphosphate. Structural and biochemical studies suggest an asymmetric model of translocation, in which the spiral staircase...
conformation of closed-ring Rho causes each Rho monomer to establish a different set of protein–RNA contacts throughout the cycle of ATP binding, hydrolysis, and pyrophosphate release. The asymmetrical model explains how Rho drives stepping of 1 nt per molecule of ATP hydrolyzed toward the site of termination (99, 119).

During translocation, rut site RNA remains stably bound to the primary binding sites, producing an RNA loop that grows in size between the primary- and secondary-binding sites (90, 119, 120). This model of Rho translocation, known as the tethered-tracking model, was proposed as an alternative to one in which these primary RNA–Rho contacts were released during translocation. Initial evidence supporting the tethered-tracking model was obtained when it was observed that active translocation by Rho extended the size of an RNA footprint during nuclease digestions (121). Further support came from single-molecule force-clamp experiments, in which force-extension curves of nascent RNA bound by active Rho were collected. Upon the addition of Rho, abrupt, discontinuous changes in extension were observed that corresponded to the displacement of Rho from RNA under a mechanical load. Importantly, these abrupt rips in extension were proportional in size to the length of the transcribed template, strongly implicating tethered tracking in translocation by Rho (90).

Translocation can be inhibited by structural roadblocks, such as RNA hairpins, ribosomes engaged in active translation, and other RNA-binding factors. Notably, it has been shown that Rho’s helicase and translocase activities are sufficient to displace streptavidin from a biotinylated RNA. Thus, the force generated by Rho may be sufficient to melt certain interfering RNA secondary structures and displace a subset of RNA-binding proteins (122).

**Kinetic Coupling of Rho to the Elongation Complex**

Rho is thought to induce termination when it contacts the RNA exit channel. Because the EC continues active elongation even as Rho engages the rut site and begins translocation, there is competition between the translocation rates of the two enzymes. This competition is referred to as kinetic coupling, and the fact that elongation-deficient or pause-susceptible RNAPs show increased Rho termination efficiencies supports the model (44). It follows that ECs composed of wild-type RNAPs will be most susceptible to Rho at long-lived pause sites. This has been verified for a wide assortment of both natural and artificial pause sites, although not all pauses support efficient termination by Rho. For example, the well-studied hairpin-stabilized his pause is resistant to Rho termination (45), likely because the interaction between the hairpin and RNAP prevents Rho from extracting the nascent RNA from the RNA–DNA hybrid. Similarly, backtrack-stabilized pauses are poorly terminated by Rho (45) because reverse displacement of the RNA 3’ end from the active site by several nucleotides would require Rho to perform more work, first to pull the RNA back into register and then to extract the transcript from the hybrid. It remains to be determined whether most sites of Rho termination are elemental pauses, stabilized neither by a pause hairpin nor through extensive backtracking. Importantly, a single rut site often precedes more than one termination site; for example, λR1 contains three major sites of termination, mapping to three pause sites from which the EC can escape before Rho can dissociate the EC (123).

**The Mechanism of RNA Release and Elongation Complex Dissociation**

If Rho’s ability to act as an RNA–DNA helicase (124) were sufficient for termination, then any EC paused downstream of a rut site should be susceptible to dissociation by Rho. In addition to bacterial RNAP, Rho can efficiently terminate T7 RNAP and eukaryotic RNAPII ECs (125, 126).
However, Rho cannot induce the dissociation of ECs formed with eukaryotic RNAPI or RNAPIII (126). Termination by Rho is likely accommodated by structural changes in both the nucleic acid scaffold and RNAP itself. ECs that fail to dissociate when acted upon by the Rho helicase likely resist all or a subset of these necessary conformational changes.

Two models for rearrangements in the nucleic acid scaffold, similar to those proposed for intrinsic termination, are feasible: hypertranslocation and hybrid-shearing. Both mechanisms rely upon Rho’s ability to exert a strong force via its motor activity (122) in addition to its helicase function. These activities are thought to supplant the role of the 6 bp during intrinsic termination, and they should proceed similarly regardless of whether Rho first binds rat RNA or RNAP.

During hypertranslocation, Rho exerts a pushing force that drives RNAP forward on template DNA without nucleotide addition, thus destabilizing the transcription bubble and driving RNA release and bubble collapse (127). Hypertranslocation is supported by the finding that Rho termination is inhibited when the upstream fork junction cannot reanneal (127), a phenomenon that generally impairs forward translocation by an EC. It has also been observed that Rho can promote forward translocation of an EC by an additional 2 bp, even in the presence of a DNA-binding protein that functions as a roadblock (127). However, actual hypertranslocation in which the 3′ end of the RNA has been displaced from the active site has yet to be directly observed.

During hybrid-shearing, Rho translocates the RNA until it reaches the RNA exit channel of the EC. Continued ATP hydrolysis by Rho exerts a pulling force on the now-taut transcript, shearing the RNA–DNA hybrid (128). Rho-driven hybrid-shearing would require a strong force because Rho termination most frequently occurs at non-U-tract pauses, where the hybrid will contain stronger base pairing than that found at the U-tract pauses of an intrinsic terminator. Although Rho can generate enough force to shear streptavidin from a biotinylated bead (122), as discussed above, it is unknown whether this is sufficiently powerful to shear a non-U-tract hybrid stabilized by interactions with RNAP.

Whereas the sequence of the RNA–DNA hybrid has been implicated in dictating which pathway to release occurs during intrinsic termination, it is unresolved whether this is the case for Rho termination. One view could be that due to the enhanced hybrid strength of the non-U-tract pause, Rho generally drives hypertranslocation of the EC and this is usually sufficient to induce termination. However, should a strong DNA roadblock prevent hypertranslocation of the EC, then Rho’s helicase activity may instead drive hybrid-shearing. It will be highly advantageous to identify DNA templates and conditions that allow detailed study of these two mechanisms.

Conformational rearrangements within RNAP itself are also thought to be necessary for efficient Rho-dependent termination (117). Many of the conformational rearrangements that have been proposed to occur during intrinsic termination would likewise destabilize the EC during Rho-dependent termination. Experiments that assay the positions of RNAP mobile elements—such as the clamp, switches, and the active-site proximal trigger loop and bridge helix—will be required to elucidate the roles of RNAP conformational changes during Rho-dependent termination.

**NusG Acts As Both A Positive and Negative Regulator of Rho**

NusG, first identified as a component of the Λ antitermination complex (129), has since been identified as the only universally conserved transcription factor. Spt4/5, its archaeal and eukaryotic paralog, is also known as DSIF in metazoans (130), and it is known to act as a positive elongation factor in yeast (130, 131). In *E. coli*, NusG associates with most ECs in vivo (116) and enhances the overall rate of elongation (132). The NusG-NTD directly binds RNAP through an interaction...
with the β′-clamp helices, near the upstream fork junction (133). The NusG-CTD, containing a
KOW domain, can target the 30S ribosomal protein S10 (also known as NusE) (53) as well as the
Rho hexamer (53, 134). These distinct and mutually exclusive targets (53) permit NusG to act as
both a negative and positive regulator of Rho-dependent termination (Figure 3f).

The NusG–S10 interaction allows for the physical coupling of transcription and translation by
the leading ribosome in bacteria (53). Coupling allows ribosomes to physically occlude the rut
site during transcription, preventing Rho binding, activation, and termination activities (135). Under
conditions of active translation, Rho can bind only to rut sites upstream of the lagging ribosome,
suppressing the activity of Rho against the EC. However, when ribosomes become stalled or
dissociate from the nascent RNA, rut sites will be exposed, allowing for Rho binding and termi-
nation. This mechanism explains transcriptional polarity, the decrease in the expression of distal
genes in an operon resulting from upstream interruptions to translation. Inefficient translation
or nonsense mutations that arise as a result of transcriptional error will inhibit transcriptional–
translational coupling and, consequently, induce the release of the ribosome from the EC by
breaking the NusG–S10 interaction. The exposed rut site can then be bound by Rho, leading to
EC dissociation.

Despite NusG’s ability to promote rut site sequestration by the ribosome, and its ability to
increase the overall elongation rate of the E. coli EC (therefore decreasing the kinetic window
during which Rho can act at any given pause site), it can also act as a positive Rho termination factor.
When NusG is present with Rho during in vitro transcription assays, termination occurs much
earlier within the transcription unit (51, 52). NusG-CTD binds Rho with a $K_d$ of approximately
12 nM (125) at a much higher affinity than the NusG–S10 interaction ($K_d$ approximately 50 μM)
(53). In fact, many Rho-dependent terminators in vivo have been shown to be NusG-dependent
(2, 51). When the nucleotide content of these NusG-dependent Rho terminators was analyzed, it
was discovered that they contained a lower ratio of C to G (2), suggesting that rut sites at NusG-
dependent Rho terminators may more often form secondary structures. The simplest hypothesis
would be that in ECs uncoupled from S10, NusG activates Rho termination by enhancing the
recruitment of Rho to rut sites that have been partially occluded by RNA secondary structure
formation due to subpar sequences. The finding that NusG does not enhance the apparent rate
of ATP hydrolysis by Rho, as measured through RNA–DNA helicase activity, appears to support
this proposal (52), although these experiments were not performed in the context of an EC.

However, this hypothesis is contradicted by several previously established biochemical results.
Richardson & Burns (136) found that NusG does not affect the half-maximal concentration of
Rho required for termination at a NusG-dependent terminator within the lacZ gene. Sen and
coworkers (134, 137) have also argued that NusG stimulates Rho at the dissociation step, not the
recruitment step. Additionally, if it can be shown that Rho is permanently associated with the EC,
even prior to the transcription and extrusion of the rut site, a role in NusG-mediated tethering of
Rho to the EC would seem unnecessary. It is possible that at NusG-dependent terminators, NusG
stimulates a step subsequent to RNA binding that depends upon the strength or structure of the rut
site in ways not yet understood (e.g., ring closure, dissociation, or even translocation). Biochemical
analyses that can monitor individual steps throughout the process of Rho termination on several
substrate RNAs will be necessary to define NusG’s exact role as a positive regulator of Rho activity.

PHYSIOLOGICAL ROLES FOR Rho

Compared with intrinsic termination, Rho has a wider variety of roles in vivo, including a role as
a sort of cellular housekeeper of transcription. In E. coli, one third of transfer RNA operons,
several small RNAs, and no more than 20% of messenger RNA-encoded transcription units are
Histone-like nucleoid structuring protein (H-NS):
binds one DNA segment in linear filaments or two DNA segments in bridged filaments

terminated by Rho (3). The number of genes terminated by Rho in the gram-positive bacterium B. subtilis, where Rho is dispensable, is believed to be substantially lower (138). Stalled ECs, which can form deleterious roadblocks to the replication fork during DNA replication, have also been implicated as targets for Rho-dependent termination (139).

Rho Acts to Prevent the Accumulation of R-Loops
A primary function of Rho may be to suppress and remove the toxic R-loops that often form upstream of active ECs (5). These three-stranded structures form when the nascent RNA transcript binds the transiently exposed DNA template strand upstream of ECs to form an RNA–DNA duplex, displacing the nontemplate strand. R-loops are not thought to be toxic on their own, but they can be processed into deleterious double-stranded breaks, giving rise to genomic instability (140, 141). Although it has long been appreciated that E. coli Rho both is necessary for cellular viability and plays a crucial part in suppressing R-loop formation, Gowrishankar and coworkers (5) recently demonstrated that ectopic expression of a phage RNA–DNA helicase can rescue Δrho lethality. This discovery implicates R-loop suppression as the essential Rho activity and opens new doors for in vivo research about Rho function.

Nucleoid-Associated Proteins Promote Rho Termination of Foreign DNA and Antisense Transcripts
Rho plays an important part in silencing foreign transcription units (i.e., cryptic prophages and other horizontally transferred genes) (3, 4). For example, toxic genes within the rac prophage are efficiently silenced via upstream termination by Rho at tmin (4). Suboptimal codon usage within exogenous DNA may prevent the efficient coupling of the EC to the ribosome, exposing rut sites for rapid binding by Rho (4). At other sites within the genome, the insertion of foreign DNA has been observed to disrupt intrinsic terminators, requiring the compensatory action of Rho (3).

Antisense transcription has also been identified as a target for silencing by Rho (2, 3). Tiling microarrays and RNA sequencing analyses have been used to map sites of Rho termination across the E. coli genome. The results suggested that a major role for Rho was the suppression of both intragenic transcription from antisense promoters within genes and intergenic transcription past the end of one gene into an oppositely oriented gene immediately downstream. Of the more than 1,250 Rho terminators identified via these methods, 88% were found to act against antisense transcription units (2).

It has long been understood that the histone-like nucleoid structuring protein (H-NS) and its closely related paralogs coat foreign genes (142), as well as sites of antisense and noncoding promoters (2, 143), via nucleation at AT-rich DNA sequences (144), a hallmark of foreign DNA in E. coli. In this way, H-NS blocks transcription initiation by physically occluding some promoters and trapping open complexes near others (143). Genetic synergy between H-NS-family proteins and Rho, however, has been recently identified and implicates H-NS in aiding Rho-dependent termination, in addition to its effects on initiation (145, 146). ChIP experiments have identified a strong correlation between Rho and regions of the bacterial nucleoid coated with H-NS (2). Of the Rho-dependent terminators shown to suppress antisense transcripts, more than 80% were correlated with H-NS, with the highest H-NS occupancy near the actual site of termination. This suggests that Rho activity is pervasive within H-NS filaments. Intrinsic terminators, however, were not found to be correlated with H-NS binding to the nucleoid (2).

H-NS forms oligomeric filaments by establishing DNA–H-NS interactions with its DNA-binding domain and H-NS–H-NS interactions with its oligomerization domain (147).
Biochemical work has established that two distinct forms of filaments can be formed in vitro (148). At high concentrations of H-NS, filaments adopt a linear conformation, in which one DNA molecule is coated. At lower concentrations, which may more closely represent physiological H-NS conditions, a bridged filament is formed in which multiple DNA molecules, or two distal segments of one molecule, are brought together, possibly through the flipping out of alternating DNA-binding domains (148). Surprisingly, ECs were found to be capable of elongating through a linear filament at nearly the same rate as on bare DNA, but backtracked pausing was preferentially enhanced under conditions that favored bridged-filament formation. The addition of Rho induced significant levels of early termination at many of these bridged H-NS filament-dependent pause sites (148). Despite backtracked pauses being atypical Rho termination sites, the long-lived nature of H-NS-dependent pauses may act to extend the kinetic window long enough for Rho to act. It will be important to determine to what extent the synergy between H-NS and Rho, now demonstrated in vitro, acts to silence the transcription of foreign DNA and antisense transcripts in vivo.

CONCLUSIONS

The sequence determinants of intrinsic and Rho-dependent termination, and the basic steps in their mechanisms, are now relatively well understood. Answering the important remaining mechanistic questions will likely require application of advanced technologies. Of particular interest are whether interactions between Sw3 and −10 RNA and the lid and −9 hybrid (8) are responsible for the energetic barrier to Tbp completion that separates intrinsic termination into two steps (64, 70), and whether the second step in which completion of the Tbp causes melting of the upstream RNA–DNA hybrid (20, 21) is accompanied by essential RNAP rearrangements that commit the EC to the termination pathway (20, 70, 78, 80). However, the effects of pairing between A-tracts upstream of Tbp and the U-tract on termination efficiency in vivo (65) are in apparent disagreement with the idea of a commitment to termination occurring upon Tbp completion. Whether the activity of dissociable transcription factors allows commitment to become reversible in vivo is an attractive possibility that needs testing. For Rho-dependent termination, the discovery of synergy between H-NS and Rho activity in vitro similarly suggests that another layer of Rho regulation may exist in vivo (2, 145, 148). Understanding differences in termination activities and mechanisms observed in vitro and in vivo will require biochemical experiments that better mimic the cellular environment, including the addition of appropriate transcription factors and nucleoid-associated proteins and better replication of cellular solutes. Most in vitro studies have used significant concentrations of Cl−, yet Cl− can compete for binding of DNA and RNA to proteins (149), and it is not representative of the cellular environment (150). The use of acetate or glutamate in place of Cl− creates a better mimic of the bacterial cytoplasm (71, 75, 149, 151), and these anions should be used in future studies of termination mechanisms, when possible. Understanding transcription mechanisms in vivo will also benefit from further extensions of high-throughput sequencing-enabled in vivo methods, such as ChIP sequencing and native elongating transcript sequencing. Finally, addressing structural questions about termination mechanisms is likely to depend heavily on exploiting recent advances in single-particle and time-resolved cryo-EM. Rapid freezing of actively terminating ECs can be used to trap intermediates in the pathway and to determine the contributions of RNAP, nucleic acid scaffold rearrangements, and dissociable transcription factors. Obtaining such information using these new methodologies has the potential to clarify the identity and stability of each intermediate in the pathway, and, thus, define the energetics of each step in the termination mechanism (152).
SUMMARY POINTS

1. The intrinsic terminator hairpin forms in two steps that are separated by an energetic barrier that slows the complete extension of the base of the hairpin. The energetic barrier appears to be created by contacts of the RNAP lid with the upstream end of the RNA–DNA hybrid and of RNAP switch 3 with the first single-stranded RNA base upstream of the hybrid.

2. In vitro, intrinsic termination appears to involve the formation of an irreversibly inactivated intermediate after terminator hairpin nucleation but before EC dissociation. The reversibility or lifetime of this intermediate may vary among experiments and terminators.

3. In vivo, the pairing of the U-tract with an A-tract upstream of the terminator hairpin can increase termination efficiency. This result appears inconsistent with the existence of an irreversibly inactivated intermediate because the pairing of the U-tract with an upstream A-tract must occur after complete formation of the terminator hairpin and, thus, after formation of the irreversibly inactivated intermediate observed in vitro. Elongation factors, such as NusA or NusG, that are present in vivo may enable the reactivation of ECs after terminator hairpin formation, and thus explain how A–U tract pairing can increase termination efficiency.

4. Recent studies have indicated that the suppression of R-loops in the genome may be a primary function of Rho in vivo.

5. The recent discovery of synergy between nucleoid-associated proteins and Rho activity in vitro suggests another layer by which Rho-dependent termination is targeted in vivo.

FUTURE ISSUES

1. How does the rearrangement of the lid–hybrid and switch 3–single-stranded RNA contacts upon completion of the terminator hairpin cause melting of the upstream end of the hybrid? Are other interactions involved in the energetic barrier to terminator hairpin completion?

2. Is irreversible EC inactivation an obligate step in the intrinsic termination pathway? How is nucleotide addition irreversibly inhibited to commit ECs to termination?

3. Do transcription factors, such as NusA or NusG, cause EC inactivation to become reversible and, thus, explain termination enhancement occurring in vivo through A–U tract pairing? Determining which factors could play this part, and characterizing their effects on the steps of termination, will provide a better understanding of the regulation of termination in vivo.

4. Multiple lines of evidence suggest that rearrangements in RNAP structural modules accompany both intrinsic and Rho-dependent termination, including clamp opening and the possible restructuring of the active site. What are these structural rearrangements and how do they facilitate termination? Are the same or different structural rearrangements involved in the two different termination mechanisms?
5. Does the direct interaction of Rho with RNAP guide the structural rearrangements of RNAP involved in Rho-dependent termination? If so, where is the Rho-binding site on RNAP?

6. Many questions remain to be answered regarding the synergy between H-NS and Rho. How does Rho overcome the backtracked EC state that is enhanced by H-NS and that ordinarily disfavors Rho termination? Are bridged states of H-NS, which seem to be required for the enhancement of Rho-dependent termination, regulated in vivo so that they differentially affect Rho in different cellular conditions?

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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LITERATURE CITED


258:9565–74


42. Komissarova N, Kashlev M. 1997. RNA polymerase switches between inactivated and activated states by translocating back and forth along the DNA and the RNA. J. Biol. Chem. 272:13329–38
81. Martin FH, Tinoco I Jr. 1980. DNA–RNA hybrid duplexes containing oligo(dArU) sequences are exceptionally unstable and may facilitate termination of transcription. Nucleic Acids Res. 8:2295–99
92. Chen CY, Galluppi GR, Richardson JP. 1986. Transcription termination at λαR1 is mediated by interaction of ρ with specific single-stranded domains near the 3′ end of cro mRNA. *Cell* 46:1023–28


127. Park JS, Roberts JW. 2006. Role of DNA bubble rewinding in enzymatic transcription termination. PNAS 103:4870–75


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