Binding and Translocation of Termination Factor Rho Studied at the Single-Molecule Level

Daniel J. Koslover†, Furqan M. Fazal†, Rachel A. Mooney, Robert Landick and Steven M. Block

1Biophysics Program, Stanford University, Stanford, CA 94305, USA
2Department of Applied Physics, Stanford University, Stanford, CA 94305, USA
3Department of Biochemistry, University of Wisconsin-Madison, Madison, WI 53706, USA
4Department of Biology, Stanford University, Stanford, CA 94305, USA

Received 20 April 2012; received in revised form 13 July 2012; accepted 27 July 2012
Available online 9 August 2012

Edited by J. Berger

Keywords: RNA polymerase; transcription; optical trap; single-molecule biophysics; force spectroscopy

Rho termination factor is an essential hexameric helicase responsible for terminating 20–50% of all mRNA synthesis in Escherichia coli. We used single-molecule force spectroscopy to investigate Rho–RNA binding interactions at the Rho utilization site of the λ rI terminator. Our results are consistent with Rho complexes adopting two states: one that binds 57±2nt of RNA across all six of the Rho primary binding sites, and another that binds 85±2nt at the six primary sites plus a single secondary site situated at the center of the hexamer. The single-molecule data serve to establish that Rho translocates 5′→3′ toward RNA polymerase (RNAP) by a tethered-tracking mechanism, looping out the intervening RNA between the Rho utilization site and RNAP. These findings lead to a general model for Rho binding and translocation and establish a novel experimental approach that should facilitate additional single-molecule studies of RNA-binding proteins.

© 2012 Elsevier Ltd. Open access under CC BY-NC-ND license.

Introduction

Transcription by RNA polymerase (RNAP) must be terminated as the transcription elongation complex (EC) reaches the end of a coding region in order to ensure proper gene expression. Intrinsic termination, one of two bacterial termination mechanisms, relies upon the destabilization of the EC at specific DNA terminator sequences and does not require any accessory proteins.1,2 The other mechanism, Rho-dependent termination, relies upon the action of Rho ATPase, which translocates 5′→3′ along the nascent RNA and displaces RNAP from the DNA template.3,4 Because the efficiency of termination is modulated by regulatory cofactors and antitermination signals,5 termination constitutes a fundamental mechanism for controlling the level of gene expression.

Rho, a highly conserved termination factor, is an essential protein in Escherichia coli6,7 that is present in nearly all prokaryotes.8 In addition to its role in terminating the transcription of native mRNA, Rho may modulate antisense transcription,9 influence the synthesis of tRNAs and small regulatory RNAs,10 silence foreign DNA,11 and prevent the formation of R loops during transcription.11 In E. coli, each 47-kDa Rho protomer is composed of 419 amino acids that fold into two major domains. The 130-amino-acid N-terminal domain constitutes the primary RNA-binding region. The C-terminal domain is homologous to F1-ATPase12 and hydrolyzes ATP via residues found at the interface between adjacent
protomers. The six C-terminal domains collectively form a secondary RNA binding site in the central channel of the hexamer that interacts with RNA via a “spiral staircase” of loops. Thomsen and Berger have proposed a model for translocation in which RNA is pulled through the center of the hexamer by conformational changes in these loops that are, in turn, driven by a rotary mechanism of ATP hydrolysis.

During Rho-dependent termination, Rho loads onto the transcript at a Rho utilization (rut) site, a cytidine-rich 5’-element of approximately 70–80 nt. Once Rho binds, RNA must pass through the center of the hexamer ring for ATP hydrolysis to occur, a topological constraint that is accommodated by Rho initially adopting an open, “lock-washer” state. However, the precise manner in which Rho initially associates with RNA is presently unknown. Kim and Patel have proposed that Rho first interacts with RNA via its six primary binding sites then binds via its central secondary site and, finally, transitions to a closed, hexameric conformation. Their kinetic analysis is consistent with the fact that RNA binds to Rho secondary sites with an affinity proportional to the ATP concentration, whereas binding to the primary sites occurs in an ATP-independent fashion.

After binding to a rut site, Rho actively translocates 5’→3’ downstream toward RNAP. Although the precise mechanism by which it tracks along mRNA is not well established, Rho motion is likely to influence how other transcription factors interact in vivo, how ribosomes behave at intragenic terminators, and whether additional Rho complexes are able to bind in succession to a single rut site. Over the last two decades, several models have been advanced to describe Rho interactions with RNA during active translocation. One model, called “pure tracking”, proposes that each N-terminal site alternates between a low-affinity binding state and a high-affinity binding state as a result of ATP hydrolysis, producing a periodic separation from, and reattachment to, the transcript. Such a mechanism could, in principle, allow Rho to advance via a biased random walk. However, the original pure tracking model is excluded by the requirement that RNA must bind to the Rho secondary binding site (Fig. 1a) during active translocation and by structural evidence suggesting that ATP hydrolysis is closely coupled to conformational changes in the Q and R loops of the secondary site. A second model (Fig. 1b) proposes that Rho binds continuously to the rut site via its primary binding domains while simultaneously pulling the downstream RNA through the center of the hexamer. In this model, called “tethered tracking”, Rho advances toward RNAP by looping out the intervening mRNA. However, the results supporting tethered tracking are controversial, and the data have alternatively been interpreted to be the consequence of multiple Rho complexes binding to an individual RNA transcript. Additionally, tethered tracking has proved to be difficult to distinguish experimentally from a third possibility, one we refer to as “rut-free tracking”, where Rho factor detaches from the rut site at the start of translocation but continues to associate with mRNA via its secondary binding site (Fig. 1c).

Fig. 1. Proposed models of Rho translocation. (a) A Rho hexamer (left; light and dark blue) binds RNA (red) at a rut site. It subsequently translocates toward a downstream molecule of RNAP (right; green). Rho binds via six N-terminal primary domains (light blue) and a single secondary binding site (center). (b) The tethered-tracking mechanism. Rho maintains binding interactions with the rut site via its primary binding domains while moving 5’→3’, threading the downstream nucleotides through the secondary site, and consequently forming a growing loop of RNA, until it reaches the position of RNAP. (c) The rut-free tracking mechanism. The primary binding domains release the rut site once translocation begins, and Rho interacts with RNA exclusively via its secondary site. No loop is formed in the RNA as Rho moves toward RNAP.

Single-molecule optical trapping assays have previously been shown to be capable of inducing
the mechanical release of DNA-binding proteins via the application of longitudinal loads that stretch the DNA substrate. Proteins that have been studied in this fashion include nucleosomes,25,26 histone-like nucleoid structuring proteins (H-N5),27 and MutS family proteins.28 In particular, Hall et al. mapped histone–DNA interactions to reveal regions of strong binding with near-base-pair accuracy.26 Here, we extend this general approach to studying a protein bound to RNA, examining Rho binding and translocation, and determining the binding footprint of Rho with near-nucleotide resolution. A longitudinal load, applied by two optical traps to the opposite ends of a nascent transcript, was used to displace individual complexes of Rho bound to an RNA substrate transcribed by a single molecule of RNAP. The data for substrates of different length indicate that Rho moves via a tethered-tracking mechanism. We present evidence for the existence of two RNA binding states, with one of these states binding via the secondary site.

Results

Single-molecule dumbbell assay

We employed a “dumbbell” assay to apply tension between the 5′ end of a nascent mRNA molecule transcribed in situ and its 3′ end, which remains associated with a molecule of RNAP that is transcriptionally stalled at a biotin-streptavidin roadblock.32 (Fig. 2a). The dumbbell was composed of two beads, held in separate optical traps, with one bead attached to a 3,057-bp double-stranded DNA (dsDNA) “handle” hybridized to the 5′ end of the nascent RNA via a 25-nt complementary overhang.33 The opposite bead was bound directly to RNAP via a biotin-avidin linkage.

The RNA substrates employed in these experiments were encoded by DNA templates composed of three segments. The first segment of each template consisted of a short (11bp) spacer placed adjacent to the 25-nt hybridization region used to attach the DNA handle. The second segment consisted of a 68-bp element derived from a sequence immediately downstream of the cro gene of bacteriophage λ, which contains the rut site of the λtR1 terminator but is missing the remainder of the terminator region.29,30 The third segment was derived from sequences of the pALB3 plasmid32 and had a length that varied with the experiment consisting of 30bp, 75bp, or 150bp, with a biotinylated nucleotide at the 3′ end used to bind streptavidin: these templates were named RB30, RB75, and RB150. The final ~25-nt segment of each template was not transcribed or generated RNA that was inaccessible to Rho binding due to the footprint of RNAP.34,35 Transcriptional runoff assays using polyacrylamide gel electrophoresis were used to confirm that Rho-dependent termination did not take place on a longer template that carried only the rut element but could be restored to a frequency of 45% on a variant of this same template carrying the full-length λtR1 terminator (data not shown). A termination efficiency of 60% was observed in a single-molecule assay of this same template (see Supplementary Data).
Dumbbells were assembled and transcription was initiated in vitro by the introduction of NTPs, whereupon elongation continued until RNAP reached the roadblock position. We thereafter collected force–extension curves (FECs) for the RNA transcripts in the presence of 20 nM Rho protein. Records were acquired by moving the optically trapped beads apart at constant velocity until a preset (maximal) force was attained, and such measurements were repeated once every ~30 s to allow sufficient time for the RNA to relax and for any translocation events to occur. FECs provide information about the displacements and energetics associated with the underlying transitions. Similar force spectroscopy has been used to probe structural changes in a variety of nucleic acids, where “rips”—abrupt, discontinuous changes in the extension—correspond to structural features, such as unfolding or unbinding. \(^{2,36,57}\) We calculated the associated change in extension for rips by fitting the pre-rip and post-rip segments of FECs to a worm-like chain (WLC) model (see Materials and Methods). In the absence of Rho, we consistently observed a small rip in FECs (Fig. 2b), corresponding to a release in the tether length of 10 ± 1 nm \((N = 207; 14 \text{ molecules})\). We attributed this rip to the unfolding of the 15-nt boxB hairpin located within the \(\text{rut}\) site because the measured size is consistent with a release of 16 ± 1 nt of RNA, assuming a rise per base of 0.59 nm. \(^{38}\) In the presence of Rho, the majority of FECs (~95%) were identical with those observed in its absence; however, we also obtained a number of FECs (observed in 26% of transcripts) displaying more prominent rips, characterized by larger changes in tether extension (>18 nm) at high force (blue traces; Fig. 2b). Based on their characteristics, we concluded that these larger, Rho-dependent rips corresponded to the displacement of Rho from the RNA under mechanical load.

**Two initial Rho binding states**

Rho must first bind to RNA to achieve termination. This loading process, which does not require ATP hydrolysis, \(^{39,40}\) is proposed to occur in a minimum of two steps. \(^{20,21}\) In the first step, Rho binds RNA with high affinity \((K_s \sim 10^{10} \text{ M}^{-1})\) at the six primary sites. In the second step, Rho binds RNA at the secondary site with a weaker affinity \((K_s \sim 4 \times 10^6 \text{ M}^{-1})\) at saturating ATP) and undergoes a conformational change. This change is thought to correspond to a transition from an open-ring, lock-washer configuration state to a closed state, forming a catalytically active ATPase poised to translocate. \(^{35}\)

To investigate the initial binding states, we collected FECs in the presence of the non-hydrolyzable ATP analog adenosine 5’-(\(\beta,\gamma\)-imido)triphosphate (AMP-PNP). We began by examining Rho interaction with RB30, a short template carrying a \(\text{rut}\) site plus a few nucleotides of exposed upstream and downstream sequences (Fig. 3a). We observed two well-separated populations of Rho-dependent rips (Fig. 3b) centered at 29 ± 1 nm and 46 ± 1 nm. Based on their characteristics (discussed below), we refer to these populations as “primary rips” or “secondary rips”, where the first set corresponds to the release of RNA from Rho primary sites and where the second set corresponds to the release of RNA from both primary and secondary sites. Additional populations of rips were observed on longer templates; however, as we explain below, all rips scored in the presence of AMP-PNP could be interpreted in the context of these two binding states.

**Multiple Rho bind long templates**

We also examined Rho binding to a series of longer transcripts in the presence of AMP-PNP. FECs for RB75 exhibited both primary and secondary rips (as with RB30), as well as a new rip population centered at 57 ± 1 nm (Fig. 3c). On the RB150 template, we observed primary rips plus two additional rip populations centered at 55 ± 1 nm and 84 ± 1 nm (Fig. 3d). About 20% of the two new populations observed with the longer transcripts also exhibited sub-rips of roughly the same size as primary rips (28 nm). Because the sizes of the two new populations were integral multiples of 28 nm, we concluded that these likely corresponded to the release of multiple complexes of Rho from RNA. Although our templates carried a single \(\text{rut}\) site, the observation of multiple Rho-release events was not entirely unexpected, given that Rho is known to bind to RNA cooperatively. \(^{23,41,42}\) Based on the size of the RNA footprint of Rho, up to three complexes of Rho could bind to RB150 at a time, two could bind to RB75, but just one could bind to RB30 (see Discussion).

**Rho translocates via tethered tracking**

The two mechanisms for translocation considered here—\(\text{rut}\)-free tracking and tethered tracking—lead to different predictions for the sizes of rips exhibited after Rho moves from the \(\text{rut}\) site toward RNAP. If Rho moves via \(\text{rut}\)-free tracking, contacting RNA exclusively via its secondary binding site (Fig. 1c), one would expect to observe only small rips (~2 nm, estimated from Ref. 13), with sizes independent of the length of the underlying template. However, if Rho moves via tethered tracking, one would expect to observe ever larger rips, proportional to the lengths of the templates and only taking place in the presence of ATP, corresponding to the release of the tether length of RNA looped out during translocation (Fig. 1b).

We collected FECs on RB30 in the presence of ATP. Here again, we observed both primary and secondary rips (\(30 \pm 1 \text{ nm and } 46 \pm 1 \text{ nm}; \text{Fig. 3b}’\)) that matched those previously seen with AMP-PNP and...
did not resolve a distinct translocation rip (i.e., a rip corresponding to the release of Rho complexes that translocated). Rho can potentially translocate only a short distance (≤5nt) on this transcript before encountering RNAP, and therefore, some of the 46-nm rips observed could, in principle, correspond to the release of Rho complexes that translocated via tethered tracking. However, we could not distinguish these from secondary rips based on size alone. This ambiguity was resolved by examining a series of longer templates (see below).

When the RB150 template was examined, four populations of Rho-dependent rips were observed (Fig. 3d'). Three of these populations were observed in the presence of both ATP and AMP-PNP, centered at 30±1nm, 56±1nm, and 84±1nm, and were interpreted as corresponding to the release of one, two, or three Rho complexes from the template, respectively. The fourth population, observed only in the presence of ATP, was centered at 111±1nm. This rip distance is close to that expected for tethered tracking, that is, for a Rho unbinding event (45nm) accompanied by the release of nearly all the downstream RNA, for an additional ~120nt (~71nm). However, the size of the fourth rip population was also consistent, within error, with the release of a fourth Rho molecule, bound to RNA in an ATP-dependent manner. To disambiguate this result, we studied a template of different length, RB75. Based on the average rip values obtained for RB30 and RB150, the tethered-tracking mechanism yields an estimated RNA release of 70±2nm for this
template. [We note that this template was created so that the distance corresponded to a nonintegral multiple (~2.5) of the primary rip size and was therefore unlikely to be mistaken for Rho release.] We again obtained four populations of rips (Fig. 3c). Three of these populations were observed in the presence of either AMP-PNP or ATP and centered at 28±1nm, 44±1nm, and 57±1nm, respectively. The fourth population (a “translocation rip”), observed only with ATP, measured 73±1nm, and matched, within experimental error, the value predicted for tethered tracking. (The existence of this population in the presence of ATP is statistically significant at the 97% level, based on a t-test over the subset of rips >50nm.) Thus, the data support tethered tracking and disfavor rut-free and pure tracking because the latter mechanisms would not be expected to produce template length-dependent loops.

A complementary oligomer inhibits multiple Rho binding while permitting translocation

In light of observing multiple Rho-release events, we also explored experimental conditions designed to allow only a single Rho complex to bind the transcript. Rho does not bind to RNA–DNA hybrids, thus, we examined the template RB75 in the presence of a 20-nt “blocking” DNA oligomer complementary to a portion of the RNA segment located transcriptionally downstream of the rut site (Fig. 4). Because Rho is known to act in vitro as an RNA–DNA helicase and separate short (<120nt) DNA oligomers from complementary RNA, we anticipated that the use of a blocking oligomer would not significantly affect any translocation.

In the presence of excess DNA oligomer (100nM), we observed three populations of rips on RB75 (Fig. 4), corresponding to primary and secondary rips of 28±1nm and 45±1nm, respectively, as well as to a third population of 71±2nm previously assigned to a translocation rip. In these experiments, the 56-nm-long rips observed in the absence of blocking oligomer were nearly nonexistent. Because the ATP-dependent rips (71nm) persisted in the presence of complementary oligomers, these results serve as additional confirmation that such rips arise from tethered tracking, rather than multiple Rho unbinding events. We note that the fraction of Rho complexes that displayed translocation rips in the presence of blocking oligomers (11±3%) was similar to that observed in their absence (10±3%), suggesting that the presence of a DNA–RNA hybrid did not adversely affect Rho translocation.

Role of the boxB hairpin in binding Rho to the rut site

The rut site of λR1 contains a 15-nt boxB hairpin, a motif known to suppress Rho-dependent and intrinsic termination in phage λ by recruiting N protein to the transcript. The deletion of this hairpin is reported to have essentially no effect on the termination efficiency in vitro or in vivo. Although the role of the boxB hairpin is not well understood, point mutations that structurally destabilize it can dramatically reduce termination efficiency. Based on their results, Vieu and Rahmouni proposed that boxB plays a role in clamping together the high-affinity regions of the rut site for optimal binding by Rho. A structure for boxB complexed with Rho has not yet been solved, but this model implicitly requires the hairpin to remain folded when Rho is bound to the full rut site. However, our force spectroscopy data imply that boxB gets unfolded upon Rho binding because we never observed a corresponding ~15-nt rip, together with a Rho-release rip, within the same FEC. Furthermore, all rips corresponding to the release of multiple Rho complexes had sizes that were integral multiples of the primary rips, even though the RNAs to which these were bound carried only a single copy of boxB.

Assuming that the boxB hairpin unfolds when Rho binds the rut site, one would expect the removal of this hairpin to have no effect on the size or occurrence of primary rips, provided that Rho can form alternate contacts with the RNA. To test this prediction, we collected FECs for a modified RB30 template in which boxB was deleted from the rut site. The sizes of the primary rips were unchanged (27±1nm; Fig. S1). Although boxB may act to promote termination in vivo in some fashion, our results suggest that the explanation for this behavior may
be more complicated than the hairpin acting as a simple clamp.

Finally, because Rho may associate with RNA up to 11–18 nt upstream from the canonical λR1 rut site (see Discussion), and our templates coded for 18 nt of exposed upstream RNA, we considered the possibility that these templates might preclude Rho from binding the rut site in the same fashion as it does in vivo. To address this possibility, we added an additional 39 nt to RB75, bringing the total of exposed upstream nucleotides to 57. We observed no significant change in the sizes or frequencies of any unbinding rips collected on this template (Table S1).

Discussion

Rho adopts at least two stable RNA binding states

We observed two populations of Rho-dependent rips, with extensions independent of template length. Because these rips occurred under external loads in the presence of either ATP or AMP-PNP, we conclude that they correspond to the mechanical displacement of Rho from the RNA without accompanying hydrolysis or translocation. Averaging our data over all templates and conditions (Fig. 5), we obtained rip sizes of 28.0±0.6 nm and 45.1±0.9 nm (mean±standard error) [Cited values include a 2% error from uncertainties in the applied force and persistence length of RNA; Gaussian fits to these distributions returned rip sizes of 27.9±0.6 nm and 44.9±1.0 nm (mean±standard error)].

The larger rip population (45 nm) often exhibited sub-rips: an initial release of 17 nm followed by the remaining 28 nm (29±5% of all data), a characteristic indicating that any contacts formed in the 28-nm binding state are a subset of those in the 45-nm binding state. We therefore associate the 28-nm “primary rips” to RNA release from all of the six Rho primary binding sites, while the 45-nm “secondary rips” are attributable to RNA release from both the single secondary site and six primary sites (Fig. 5). These assignments are consistent with states predicted by Kim and Patel\textsuperscript{20} and Richardson\textsuperscript{21} and lead us to a model for Rho binding in which Rho binds RNA at the primary sites before associating with the secondary site (see below). This particular ordering of states was previously proposed by Kim and Patel\textsuperscript{20} using an independent approach that relied upon kinetic measurements. We note that the transition to a complex with contacts only at its primary sites is accompanied by the rotation of the entire Rho hexamer and the reorientation of the RNA released, which results in a comparatively large change in tether extension (here, 17 nm).

The relative frequencies of various rip populations varied from template to template. The secondary and translocation rips, which together correspond to events where RNA is released from the Rho secondary site, accounted for 36±6% of all rips (ATP and AMP-PNP data combined) collected on RB30, but just 22±3% of rips collected on RB75. One possible interpretation of this variability is that the Rho secondary site may be somewhat obstructed or inhibited with the longer transcript. However, when the blocking oligomer was added to RB75, the frequency of rips was restored to the level found in RB30 (41±5%). We would speculate that once Rho binds RNA at its primary sites, it may either bind to RNA at its secondary site or facilitate the cooperative binding of a second Rho molecule to the RNA, but not both. This would explain the findings because the RB30 template is too short to bind more than a single Rho molecule. The high frequency of multiple Rho-release events on the long RB150 template may likewise explain why a distinct 45-nm peak was not observed on this template.

In the presence of ATP, Rho complexes bound to RNA produced a single, low-variance population of translocation rips that depended only upon the template length. Because we did not observe translocation rips of intermediate sizes or wider variance, we conclude that the tether-looping-out process must occur rapidly under our experimental
conditions (in seconds or less). The observation of isolated secondary rips in the presence of ATP indicates that binding to the Rho secondary site is not sufficient, in and of itself, for translocation to occur. This is an indication that Rho complexes likely transition from a secondary rip state to a distinct, translocation-competent state that can hydrolyze ATP (see Supplementary Data).

**Rho binding footprints of 57 nt and 85 nt**

Although Rho is generally thought to have an RNA footprint of 70–80 nt, estimates as low as 55 nt and as high as 84 nt have been reported. Typically, such footprints have been based on RNase protection assays or the sizes of minimum templates required for termination. The number of RNA nucleotides bound to Rho, \( N \), may be estimated from the rip size, \( r \), and the width of the Rho enzyme projected onto the pulling coordinate, \( d \), according to \( N = (r + d) / 0.59 \), where the nucleotide pitch is taken to be 0.59 nm/nt. For rips involving the release of RNA from the primary sites alone, we obtained a value for \( d \) from the open crystal structure (Protein Data Bank ID: 1PVO), where the distance between dinucleotide fragments bound to subunits A and F, the two protomers separated by a gap, was taken to be 5.4 ± 0.7 nm (the uncertainty arises from the choice of atoms assumed to make contact). Based on this value, we estimate a footprint of 57 ± 2 nt for the six sites or 9–10 nt per site. For rips involving the release of RNA from both primary and secondary sites, we modeled the dinucleotide fragments into their equivalent locations in the closed crystal structure (Protein Data Bank ID: 3ICE), which has a 6 nt RNA helix at the secondary site and obtained an estimate for the distance between these dinucleotides and the 3' end of the helix, \( d = 5.0 ± 0.5 \) nm. Based on this value, we estimate that 85 ± 2 nt of RNA associate with both primary and secondary sites. This footprint size is consistent with a minimal template for termination of 97 nt, assuming that an additional 12–15 nt are sequestered inside the transcription bubble and inaccessible to Rho.

**Transient Rho unbinding intermediates**

Two populations of intermediate states were observed in ~13% of all primary rips (28 nm). One population was characterized by an initial sub-rip averaging 9.6 ± 0.3 nm (\( N = 38 \)), and the second population was characterized by a sub-rip averaging 16.0 ± 0.4 nm (\( N = 27 \)) (Fig. 6). These intermediates are likely to represent the release of RNA from a subset of the six primary sites. We therefore modeled structural pathways in which RNA unbound from one to four of the primary sites prior to complete release (Fig. S2). The size of the smaller sup-rip population is consistent with RNA release from a single primary site (7.9 ± 0.8 nm expected) followed by release from the remaining five sites (20.1 ± 0.8 nm). The larger sub-rip population is consistent with release from two primary sites (14.9 ± 0.6 nm expected) followed by release from the remaining four sites (13.1 ± 0.6 nm).

**Rho translocates 5'–3' via a tethered-tracking mechanism**

A tethered-tracking mechanism has important implications for Rho-dependent termination. Because the rut site, once bound, remains occupied during subsequent Rho translocation, additional Rho complexes are prevented from binding sequentially to this same site. If a translocating ribosome were to encounter Rho at an intragenic rut site, it must either displace it or pause until Rho dissociates. Active translation, therefore, may act to suppress termination in transcripts bound by Rho.
Rho-dependent termination might also become coupled to translation because the Rho footprint on the rut site may extend into the gene-coding region, as seems to be the case for λtR1. Adding the sizes of the Rho binding footprint (85±2 nt), the footprint of the RNAP transcription bubble (12–15 nt), and the RNAP offset from the biotin-streptavidin roadblock (10 nt) 34 to the measured translocation distance for each transcript, we can estimate the location of the 5′ boundary of the Rho-binding element. Given the sequences of the associated DNA templates, this estimate positions that boundary 11–18 nt upstream of the nominal 5′ border of the canonical λtR1 rut site. In the αtR1 terminator, the start of the rut site lies just 7 nt downstream of the end of the end of the cro gene. 29,30 Based on these data, therefore, it is possible that Rho binding to this rut site may occlude the cro stop codon and consequently affect translation (and vice versa).

Populations of intermediate states (sub-rips) were observed in 56% of records displaying translocation rips (Fig. S3). A fraction (23%) of the translocation rips themselves occurred in two steps, with a release of all but 28 nm, followed by a final, 28-nm sub-rip (Fig. S3b). These data were interpreted as Rho unbinding RNA from the secondary site, releasing the RNA loop formed by tethered tracking, followed by RNA dissociation from the primary sites. The remainder of records (33%) exhibited sub-rips of variable sizes that could not be assigned to any particular unbinding intermediate (Fig. S3c). These may reflect the formation of secondary structure formed within the looped-out RNA (Fig. S3c′). We speculate that loops formed in the process of tethered tracking may play a role in the folding of structured, noncoding RNAs that are terminated by Rho. 9

The frequency with which Rho binds via its secondary site, estimated from the ratio of secondary and translocation rips to primary rips, provides further evidence that tethered tracking is the dominant translocation mechanism. We note that the decrease in the number of secondary rips observed on RB75 in the presence of ATP (as compared to AMP-PNP) was compensated by a corresponding increase in the number of translocation rips, such that the total rip frequency remained unchanged. Instead, if Rho were to engage in rut-free tracking, then the decrease in secondary rips would not be compensated in such a fashion because the loss of this subpopulation would produce no further detectable rips. Nevertheless, on the basis of our data, one cannot formally exclude a more complicated mechanism whereby Rho first translocates via rut-free tracking but then rebinds to the upstream rut site once it has stopped or stalled. That said, we note that experiments conducted with a blocking oligomer induce the formation of a comparatively inflexible DNA–RNA hybrid region (∼6 nm), which is expected to reduce the ability of any Rho complexes engaged in rut-free tracking to rebind. However, the observed frequency of translocation rips was similar in the presence and absence of the blocking oligomer, disfavoring this modified version of rut-free tracking.

In one recent study carried out in vitro, it was proposed by Epshtein et al. that Rho associates directly with RNAP throughout elongation and that it can do so even in the absence of RNA. 49 However, this model was challenged by Kalyani et al. who argued that Rho does not bind to an EC in the absence of a rut site and found no evidence for a direct interaction between Rho and RNAP prior to termination. 50 We note that, if Rho and RNAP were initially conjoined as proposed by Epshtein et al., the subsequent association of Rho with a rut site on the RNA would form a loop that shortened—not lengthened—during Rho translocation (Fig. 7). The mechanical disruption of such loops by tension would result in rips with sizes proportional to the number of nucleotides separating Rho from the EC. In contrast to the loops formed by tethered tracking, these loops are expected to form even in the absence of ATP. However, we did not observe any rips with the required characteristics in the presence of AMP-PNP, suggesting that Rho does not associate with RNAP in our assays. It remains possible that Rho binds only very weakly to RNAP and is consistently released during the acquisition of FECs at even the lowest tensions (<3 pN). If we assume that the binding interaction distance between Rho and RNAP is no greater than 1 nm, our force measurements place an upper limit of just 3 pN·nm, or ~0.8 $k_B T$ (0.4 kcal/mol), on the equilibrium binding energy between Rho and RNAP. We note that Rho is likely to be indirectly coupled to RNAP in vivo because accessory factors such as NusA and NusG are known to interact with both enzymes. 51,52

Model for Rho binding and translocation

Based on the observed sizes, characteristics, and ATP dependence of the FECs for Rho bound to templates of different lengths, a general model for Rho binding and translocation that is quantitatively consistent with our data can be formulated (Fig. 8). Initially, Rho binds to RNA at a rut site via one or more primary domains, transiently associating as the hexamer gets loaded. After 57±2 nt of RNA are bound by its primary sites, Rho binds via its secondary site and (presumably) undergoes a conformational transition into a closed-ring form, which all together subdends a footprint of 85±2 nt. In the final step, Rho helicase is activated and it advances downstream, toward RNAP, while remaining bound via the rut site, looping out the intervening RNA sequence via a tethered-tracking mechanism. We anticipate that variations of this single-molecule assay will be particularly useful in
studies of Rho helicase, being well suited for characterizing basic motor properties, such as velocity, processivity, stall force, and load dependence. In particular, a high-resolution assay for Rho translocation may be able to resolve whether its step size is 1 nt, 7 nt, 53 or some other value. Finally, we expect single-molecule assays to shed additional light on the means by which Rho terminates transcription and on the modulatory roles of accessory factors, such as NusA and NusG.

Materials and Methods

Assay

To produce a dumbbell assay (Fig. 2a), we initiated biotinylated RNAP molecules at a T7A1 promoter and stalled them after transcribing 29 nt.54 Purified ECs were then incubated at roughly 1:1 stoichiometry with 730-nm-diameter avidin- or streptavidin-coated beads at room temperature (21.5±0.5°C). DNA handles (3057 bp) produced by autosticky PCR of the M13mp18 plasmid33 were coupled to 600-nm-diameter avidin-coated beads via biotin linkages (30 min incubation; washed twice). The handles carried 5'-single-stranded overhangs complementary to the first 25 nt of RNA transcribed, leaving 11 nt of RNA between the handle and the rut site insert. Handles and ECs were mixed and incubated for 1 h at room temperature to form dumbbell tethers.

Transcription in dumbbells was restarted inside a flow chamber (~5 μl) by introducing 1 mM ATP, GTP, CTP, and UTP in the presence or absence of Rho protein (20 nM). Where appropriate, 1 mM AMP-PNP replaced ATP. Experiments were carried out in elongation buffer [50 mM Hepes (pH 8.0), 50 mM KCl, 5 mM MgCl2, 0.1 mM ethylenediaminetetraacetic acid, and 0.1 mM DTT; 26±1°C] plus an oxygen-scavenging system [8.3 mg/ml glucose (Sigma), 46 U/ml glucose oxidase (Calbiochem), and 94 U/ml catalase (Sigma)]. Catalase and glucose oxidase were FPLC (fast protein liquid chromatography) purified (GE Healthcare) by a Superdex 200 10/300 GL column and verified to be RNase free (Ambion RNase Alert); 0.2 U/μl SUPERase·In (Ambion) was added to elongation buffer to inhibit RNase activity.

E. coli Rho protein was expressed from plasmid pCB111 by inducing a culture with an M13 derivative encoding T7 RNAP under lac control55 with IPTG and purified as previously described.56

Data collection

The optical trapping instrument was described previously.57 Uncertainties in force due to variations in bead size and systematic calibration errors were estimated at 15%. Position data were acquired at 2 kHz using custom
software (Labview), filtered at 1 kHz using an 8-pole low-pass Bessel filter, and analyzed in Igor Pro (WaveMetrics). Tether lengths and additional sources of error were estimated as previously described. FECs were collected by slewing the movable trap with an acousto-optic deflector (IntraAction, Inc.) at ~190 nm/s as the position of the bead in the stationary trap was recorded. Data points in FECs represent 1.25 ms integration time. For experiments involving blocking oligomers (introduced along with NTPs), tethers were maintained at an initial extension of 1.10 μm for ~35 s (at F=15 pN) to facilitate annealing and minimize the formation of secondary structure; this procedure was repeated every time a potential translocation rip was observed.

**Force–extension curves**

To compute rip size, we subtracted the extensions returned by fits to WLC models before and after the associated rip. The pre-rip portion of each FEC was fit to a WLC model using the modified Marko–Siggia relationship; because the pre-rip segment corresponds almost entirely to dsDNA, the elastic modulus was set to 1200 pN/nm. To ensure single-molecule behavior, we rejected any tethers exhibiting too short a persistence length (<18 nm) or an incorrect contour length from further analysis. The post-rip portion of each FEC was fit to a double-WLC model, with the parameters of the first WLC set to those returned by the pre-rip fit phase. For the additional WLC, we used a persistence length of 1.0 nm for single-stranded RNA and an elastic modulus of 1600 pN/nm. To estimate the size of the boxB rip, we assumed an A-form double-stranded RNA helix width of 2.2 nm, which we subtracted from the extension of the pre-rip portion when fitting FECs.

Our analysis excluded any small rips observed at low forces that were seen in both the presence and the absence of Rho: we imposed a minimum force requirement, such that only events above the cutoff (from 9.0 pN to 12.0 pN, depending upon the length of the template) were considered. Similarly, we excluded rips smaller than 25 nt that were Rho independent and due to the unfolding of RNA secondary structures, such as the 15-nucleotide boxB hairpin.

**References**


**Acknowledgements**

We thank J. Andreasson and E. Koslover for reading the manuscript, J. Andreasson and P. Anthony for advice on experimental procedures, and J. Gelles for helpful discussions. F.M.F. was supported by a National Science Foundation Graduate Research Fellowship. This work was supported by a grant from the National Institute of General Medical Sciences.

**Supplementary Data**

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jmb.2012.07.027


Supplementary Online Material for:

Binding and Translocation of Termination Factor Rho Studied at the Single-Molecule Level

Daniel J. Koslover¹†, Furqan M. Fazal²†, Rachel A. Mooney⁴, Robert Landick⁴, Steven M. Block²,3*

¹Biophysics Program, Stanford University, Stanford, CA 94305, USA.
²Department of Applied Physics, Stanford University, Stanford, CA 94305, USA
³Department of Biology, Stanford University, Stanford, CA 94305, USA
⁴Department of Biochemistry, University of Wisconsin-Madison, Madison, WI 53706, USA
†D.J.K and F.M.F. contributed equally to this work.
*Corresponding author. Department of Biology, Stanford University, Stanford, CA 94305, USA. E-mail address: sblock@stanford.edu

Supplementary Results and Discussion

Rho Terminates RNAP with Close to 50% Efficiency

The λtR1 terminator was inserted into the rpoB gene of plasmid pRL732,¹ downstream of the T7A1 promoter. Rho-dependent termination was assayed in vitro under both single-molecule and bulk conditions in the same elongation buffer (Experimental Procedures). We observed a termination efficiency of ~60% in a single-molecule assay (9 of 15 records terminating, 5 pN hindering load, 5 nM Rho) that was based on a previously described experimental geometry,² with RNAP attached to one bead via a biotin-avidin linkage, and the downstream end of the DNA template attached to a second bead via a digoxigenin-antidigoxigenin linkage. A termination efficiency of ~45% was observed with the same template when assayed by a polyacrylamide gel (20 nM Rho). Termination was not observed under either condition in the absence of Rho.
**Initiation of Rho Translocation May Require an Additional Step after Ring Closure**

Rho complexes studied in the presence of ATP could be separated into two distinct populations: those that generated translocation rips, which presumably moved along RNA, and those that generated only primary and secondary rips, which presumably failed to move (the latter population was also reproduced in the presence of AMP-PNP). The force required to release the RNA associated with translocations rips in complexes that moved (16.2 ± 0.8 pN; \( N = 43 \)) was the same, within error, as the force associated with secondary rips in complexes that did not move (14.7 ± 0.7 pN; \( N = 49 \)). If Rho were to first bind RNA at both primary and secondary sites, and subsequently transition from the open form to the closed form prior to translocation, one might expect the force required to release RNA from the open form to be significantly lower than from the closed form, where the RNA is threaded through the middle of the complex and therefore topologically constrained. The similarity in the release forces therefore suggests that the Rho complexes sampled in these experiments is in the same conformational states before and after translocation (both open, or both closed). The simplest explanation, which we favor, is that Rho transitions to the closed form upon binding RNA at its secondary site prior to translocation, and therefore that data acquired on secondary and translocation rips reflect the release of RNA from complexes that had already transitioned to a closed form in all instances. Because we observed a population of complexes that bound to both primary and secondary sites in the presence of ATP but did not translocate, it seems likely that Rho undergoes an additional transition following closure of the enzyme in order to become competent for translocation. The alternative possibility, which cannot be distinguished by these data, is that Rho complexes that fail to translocate remain in an open state, and complexes that successfully translocate transition back to this same open state after movement ceases.
Supplementary Experimental Procedures

Sequence elements introduced into the pALB3 template:

1. Insert with λtR1 terminator (129 nt)

ATAACCCCGCTTTACACATCCAGCCCTGAAAAAGGGCATCAAATTAAACCACACC
TATGGTGTATGCAATTTATTTGCATAATCATCAATATTTGTTATCTAAGGAAATCTTA
CATATGGTTGTGC

2. Insert with λtR1-rut site (68 nt)

ATAACCCCGCTTTACACATCCAGCCCTGAAAAAGGGCATCAAATTAAACCACACC
TATGGTGTATG

3. Insert with λtR1-rut site, boxB-deletion mutant (53 nt)

ATAACCCCGCTTTACACATCCAAATAAATCAAACCACCTATGGTGTATG

4. Insert with λtR1-rut site with additional upstream sequence derived from cro (107 nt)

ATAAAAGCCCTTCCCGAGTAAACAAAAAAAAACACAGCATAAATAACCCGCTTTACA
CATTCCAGCCCTGAAAAAGGGCATCAAATTAAACCACACCTATGGTGTATG

(Note: Nucleotides 2–39 in this sequence are from the cro gene. The first nucleotide was
mutated to A to minimize any secondary structure formation in the transcribed RNA, as
predicted by mfold.3)

Sequences were inserted 36 bp downstream of the T7A1 promoter in the pALB3-DNA
template at a BstEII digestion site. For single-molecule experiments, DNA templates were
amplified from plasmids by PCR using a downstream primer with a 3′-biotin moiety that
ultimately formed part of the biotin-streptavidin roadblock. The roadblock was constructed by
incubating the PCR product with 20-fold excess streptavidin (ProZyme) for 30 min at room
temperature (21.5 ± 0.5 °C) followed by addition of 1000-fold excess biotin to bind any residual
streptavidin. After incubating for an additional 30 min, the DNA was purified (QIAquick PCR
Purification Kit, Qiagen).
To prepare stalled elongation complexes (ECs), biotinylated RNAP was initiated at 37 C at the T7A1 promoter in the presence of 2.5 μM ATP, CTP, and GTP and allowed to transcribe the first 29 nt of the template. ECs were then purified from free nucleotides on a Sepharose column (GE Healthcare) and stored at –80 °C. Aliquots of frozen ECs were thawed for experiments and stored at 4 °C for up to 3 days. Carboxyl-functionalized polystyrene beads (0.60 μm and 0.73 μm diameter; Bangs Laboratories) were functionalized with avidin as described. Beads were washed in wash buffer (50 mM HEPES [pH 8.0], 130 mM KCl, 5 mM MgCl₂, 0.1 mM EDTA, 0.1 mM DTT) and sonicated prior to use. Rho was stored at –80 °C in 50% glycerol and 50% wash buffer at a concentration of 1.56 μM (assuming hexamers). On the day of the experiment, Rho was diluted to a final concentration of 20 nM in elongation buffer (Experimental Procedures).

To make the 3,057-bp dsDNA handle, autosticky PCR of the M13mp18 plasmid by rTth DNA Polymerase, XL (Applied Biosystems) was performed. One primer was 25-nt long and carried a 5’ biotin while the other had a 25-nt hybridization segment followed by a single abasic site and a 25-nt overhang.

All primers and oligomers were purchased (Integrated DNA Technologies) and all templates were sequenced (Sequetech). The 20-nt-blocking oligomer was complementary to nucleotides 11 through 30 of the transcript downstream of the rut site. Concentrations of DNA, glucose oxidase, and catalase were measured on a Nanodrop 2000 Spectrophotometer (Thermo Scientific). NTPs and AMP-PNP were purchased (Roche Molecular Biochemicals). All gel-based experiments were carried out at room temperature.
**Supplementary References**


**Supplementary Figure Legends**

**Figure S1. boxB Hairpin Unfolds when Rho Binds rut Site**

Histogram of the rip sizes for Rho bound to template RB30 with a boxB deletion (7 molecules; \(N = 67\)). Inset: schematic of the construct, using the same color scheme as Fig. 3a. A single population of unbinding rips is centered at 27 ± 1 nm, and has the identical size (within statistical error) as the 28 nm primary rips observed when boxB was present (Fig. 3). No secondary rips (45 nm) were observed.

**Figure S2. Predictions for Intermediate Rho Unbinding Rips**

Combining our unbinding results with structural data, we computed the sizes of sub-rips expected for four potential intermediate states during the release of RNA from the Rho complex. Here, we consider a scenario where RNA dissociates from the six primary domains sites in a two-step process, with the first step corresponding to release from one, two, three, or four sites. (An intermediate where RNA is released from five sites was not considered, since it cannot be distinguished experimentally from the complete release of RNA.) The size of the second sub-rip
was calculated using $r = 0.59N - d$, where $r$ is the sub-rip size (in nm), $N$ is the number of nucleotides bound to Rho, and $d$ is the distance (in nm) separating the initial and final attachment points of the RNA. The value of $d$ was determined using the locations of dinucleotides fragments found in the open crystal structure (PDB ID: 1PVO), and measured $6.59 \pm 0.30$ nm (a), $6.90 \pm 0.04$ nm (b), $5.70 \pm 0.15$ nm (c), and $3.02 \pm 0.04$ nm (d). To obtain $N$, we multiplied the number of nucleotides predicted to bind all primary domains in a lock-washer configuration, $56.6 \pm 1.6$, by the factors $4/5$ (a), $3/5$ (b), $2/5$ (c), and $1/5$ (d). The expected size of the first sub-rip was obtained by subtracting the computed value of the second rip from the primary rip size of 28.0 nm.

Figure S3. Intermediates in Translocation Rips

(a–c) Representative FECs, depicting examples of the different types of translocation rips. FECs without Rho-dependent rips (green) are shown for comparison.

(a) 44% of translocation rips (blue trace) occurred as a single event with no intermediate.

(b) 23% of translocation rips (red trace) displayed two sub-rips. The size of the first sub-rip was proportional to the length of the template (~44 nm for RB75, ~83 nm for RB150); the size of the second sub-rip was ~28 nm.

(c) 33% of translocation rips (black trace) displayed sub-rips of variable size that could not be associated with any specific intermediate structure.

(c') A model in which non-specific interactions formed within the loop of RNA produced during tethered tracking (e.g., RNA secondary structure formation) give rise to the variable sub-rips in experimental records. In such a scenario, sub-rips can occur only after an initial release of at least
17 nm (after RNA has been released from the Rho secondary site, but prior to release from any primary sites) and the RNA loop experiences the applied force.

**Table S1. Summary of data for all five templates studied, under the nucleotide conditions indicated**

The measured sizes of primary, secondary, and translocation rips are shown. Integers in parentheses denote the associated numbers of rip events.
Figure S2

<table>
<thead>
<tr>
<th>First rip (nm)</th>
<th>Second rip (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.9 ± 0.8</td>
<td>20.1 ± 0.8</td>
</tr>
<tr>
<td>14.9 ± 0.6</td>
<td>13.1 ± 0.6</td>
</tr>
<tr>
<td>20.3 ± 0.4</td>
<td>7.7 ± 0.4</td>
</tr>
<tr>
<td>24.3 ± 0.2</td>
<td>3.7 ± 0.2</td>
</tr>
</tbody>
</table>

(a) (b) (c) (d)
Figure S3

(a) 44%
(b) 23%
(c) 33%

Force (pN) vs. Extension (nm)
<table>
<thead>
<tr>
<th>Construct</th>
<th>Condition</th>
<th>Number of Molecules</th>
<th>1 Primary (nm)</th>
<th>1 Secondary (nm)</th>
<th>2 Primary (nm)</th>
<th>3 Primary (nm)</th>
<th>Translocation (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RB30</td>
<td>AMP-PNP</td>
<td>8 (33)</td>
<td>28.5 ± 1.0 (24)</td>
<td>46.4 ± 1.1 (9)</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>RB30</td>
<td>ATP</td>
<td>8 (37)</td>
<td>29.6 ± 1.1 (21)</td>
<td>46.3 ± 0.8 (16)</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>RB75</td>
<td>AMP-PNP</td>
<td>22 (65)</td>
<td>28.4 ± 0.6 (44)</td>
<td>44.8 ± 0.5 (14)</td>
<td>57.3 ± 1.3 (7)</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>RB75</td>
<td>ATP</td>
<td>33 (96)</td>
<td>27.8 ± 0.5 (62)</td>
<td>43.9 ± 0.5 (12)</td>
<td>56.8 ± 1.0 (12)</td>
<td>-----</td>
<td>72.7 ± 1.5 (10)</td>
</tr>
<tr>
<td>RB75</td>
<td>ATP w/ blocking oligo</td>
<td>43 (98)</td>
<td>27.7 ± 0.4 (57)</td>
<td>45.3 ± 0.7 (29)</td>
<td>57.5 (1)</td>
<td>-----</td>
<td>70.7 ± 1.5 (11)</td>
</tr>
<tr>
<td>RB150</td>
<td>AMP-PNP</td>
<td>17 (54)</td>
<td>26.8 ± 1.0 (6)</td>
<td>-----</td>
<td>54.7 ± 1.4 (25)</td>
<td>83.7 ± 1.1 (23)</td>
<td>-----</td>
</tr>
<tr>
<td>RB150</td>
<td>ATP</td>
<td>37 (114)</td>
<td>29.7 ± 0.8 (20)</td>
<td>-----</td>
<td>55.6 ± 1.0 (41)</td>
<td>83.9 ± 0.9 (35)</td>
<td>111.0 ± 1.3 (18)</td>
</tr>
<tr>
<td>RB30 boxB delete</td>
<td>ATP</td>
<td>7 (67)</td>
<td>26.5 ± 0.3 (67)</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>RB75 w/ additional 39 nt upstream</td>
<td>ATP</td>
<td>27 (60)</td>
<td>29.4 ± 0.6 (24)</td>
<td>44.1 ± 1.1 (8)</td>
<td>56.1 ± 0.5 (24)</td>
<td>-----</td>
<td>69.5 ± 2.4 (4)</td>
</tr>
</tbody>
</table>