Transcription Termination Factor Rho Prefers Catalytically Active Elongation Complexes for Releasing RNA*

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RNA polymerase pauses at different DNA sequences during transcription elongation, and this pausing is associated with distinct conformational state(s) of the elongation complex (EC). Transcription termination by the termination factor Rho, an RNA-dependent molecular motor, requires pausing of the EC in the termination zone of Rho-dependent terminators. We hypothesized that the conformational state(s) of the EC associated with this pausing would influence the action of Rho. Analyses of the pausing behavior of the EC at the termination points of two well known Rho-dependent terminators revealed that Rho prefers actively transcribing complexes for termination. RNA release kinetics from stalled ECs showed that the rate of RNA release by Rho was reduced if the EC was irreversibly backtracked, if its RNA exit channel was modified by an RNA hairpin, or the bridge helix/trigger loop movement in its active site was perturbed. These defects were overcome significantly by enhancing the rate of ATP hydrolysis either by increasing the concentration of ATP or by using a Rho mutant with higher ATPase activity. We propose that the force generated from ATP hydrolysis of Rho is the key factor in dislodging the EC through its molecular motor action, and this process is facilitated when the EC is in a catalytically competent state, undergoing rapid “Brownian ratchet” motion.

The highly processive and stable EC3 (1–3) dissociates from template DNA only in response to transcription termination signals. There are two types of termination signals in Escherichia coli. The intrinsic terminators are characterized by a GC-rich inverted repeat followed by an oligo(dT) stretch that destabilizes the EC and the factor-dependent terminators, which depend on an essential protein, called Rho, for termination (4, 5).

Rho is a homo-hexameric RNA/DNA helicase or translocase capable of dissociating the elongating RNA polymerase from the template DNA by utilizing its RNA-dependent ATPase activity (6–8). It recognizes the Rho utilization (rut) site on the exiting mRNA through its N-terminal primary RNA binding domain (9). Binding of RNA to the primary binding site of Rho guides the 3’-side of the RNA into the central hole of the hexamer, which constitutes the secondary RNA binding domain. Q-loop and R-loop are the two major RNA binding determinants of Rho at this site. Interaction of the RNA with these two loops activates ATP hydrolysis in the nearby P-loop region (10–16).

The Rho-dependent termination region consists of a proximal 70–80-nt stretch of Cys-rich, unstructured RNA for binding of Rho (the rut site; see Refs. 17–19), followed by a distal termination zone, dispersed over a wide range, beginning about 60–90 nts downstream of the rut site (reviewed in Ref. 20). Analyses of Rho-dependent terminators revealed that the position of the rut site determines the location of the downstream termination zone (21–24). Unlike Rho-independent terminators, the termination zone of the well characterized Rho-dependent terminators, such as A trl and trp t’, do not have characteristic signatures, except that the sequences are AU-rich and the termination points are associated with pausing of the EC (25, 26). However, not all pause sites are termination points, and these include some of the strongest pauses along a terminator (27).

RNAP pauses during elongation at different sequences, which provide an adequate time window for different cis and trans factors to act upon the EC (28). Depending on the pause sequence, the active site of the EC can be in conformationally distinct states (28). Pausing at Rho-independent terminators is required for the timely formation of an RNA hairpin at the exit channel (29), although pausing in the termination zone of Rho-dependent terminators is believed to help Rho to “catch up” with the elongating EC (7).

The importance of conformational state(s) of the EC in Rho-dependent termination has not been explored. As pausing of the EC is associated with the termination points in the termination zone (25, 26), we hypothesized that the conformational state(s) associated with pausing would affect the RNA release process by Rho. Quantitative analyses of the EC paused at the termination points, and RNA release kinetics from the stalled ECs with different conformational states show that Rho prefers actively transcribing complexes for termination. These assays also reveal that an enhanced rate of ATP hydrolysis is required to release RNA from the catalytically inactive ECs. We propose that the force generated by ATP hydrolysis of Rho is the key factor in dislodging the EC through its molecular motor action.
and the process is facilitated when the EC is in a catalytically competent state undergoing rapid “Brownian ratchet” motion.

**EXPERIMENTAL PROCEDURES**

**Materials**—NTPs, poly(C), IPTG, lysozyme, dithiothreitol, and bovine serum albumin were from GE Healthcare. Radioisotopes [γ-32P]ATP (6000 Ci/mmol) and [α-32P]CTP (4500 Ci/mmol) were purchased from either GE Healthcare or Jonaki, BRIT, India. Primers for PCR were obtained either from Sigma, Applied Science. Streptavidin-coated magnetic beads were from Pierce. Streptolydigin was from Chemcon, Germany.

**Cloning, Overexpression, and Purification of Proteins**—The purification of LacR, NusA, NusG, His-tagged and nonHis-tagged Rho was performed as described previously (30, 31). His-tagged E. coli GreB was cloned in pET21b and purified using nickel-nitrotriacetic acid beads. P235H Rho (pRS630) was prepared by site-directed mutagenesis (Stratagene) of pRS630, using the primers RS345 and RS346, and was purified as described earlier (31).

**Templates for in Vitro Transcription**—T7A1-trp t’ and T7A1-λ trl templates for pausing assays, used in Fig. 1, were made by PCR amplification using RS58/RS140 primers with the plasmid pRS106 (31) and RS58/RS147 primers with plasmid pRS604, respectively. To immobilize the templates on streptavidin-coated magnetic beads (Promega), a biotinylated upstream primer RS83 was used in all cases. Primers with lac operator, ops pause, and his pause sequences were used in PCRs to incorporate these sequences in the termination zone of the trp t’ terminator. Details of the primers and plasmids are described in Table 1.

**Pausing Kinetics Assays**—All in vitro transcription assays were performed in T buffer (25 mM Tris-HCl, pH 8.0, 5 mM MgCl2, 50 mM KCl, 1 mM dithiothreitol, and 0.1 mg/ml bovine serum albumin) at 37 °C. The reactions were initiated with 10 mM DNA template, 40 nM WT RNA polymerase, 175 μM NTPs in the presence of 10 μM MgCl2 and 0.1 μM unlabeled other NTPs. Pausing assays on ops (Fig. 3B) and his (Fig. 4B) pause templates were performed essentially in the same way as described above, except that EC23 was chased with 20 μM NTPs in the presence of 10 μM rifampicin, and aliquots were removed at different time points and mixed with formamide loading dye (Fig 1, A and B). Transcripts were fractionated by electrophoresis on a 10% sequencing gel, and the gels were scanned and analyzed by Typhoon 9200 Phosphor-Imager. Pausing assays on ops (Fig. 3B) and his (Fig. 4B) pause templates were performed essentially in the same way as described above, except that EC23 was chased with 10 μM GTP and 100 μM other NTPs.

**Rho-mediated RNA Release Assays from stalled Elongation Complexes (RB; Road-blocked Complex)**—These assays were performed in T buffer using immobilized templates with lac operator sequences at different positions in the termination zone of the T7A1-trp t’ template (Figs. 2–4). The templates were immobilized before starting the reaction. Transcription
reactions were initiated as above except that 100 nM of lac repressor was added before making the EC23 complex. EC23 complex was then chased in the presence of 100 μM NTPs and rifampicin for 2 min to make the RB complexes. RB complexes were made in a similar manner on all the templates. These complexes were then immediately washed three times with 150 μl of T buffer to remove the excess NTPs and resuspended in the same buffer. The washing steps were completed within 5 min to avoid long incubation of the stalled ECs. 50 nM Rho in the presence of indicated amounts of ATP (either 25 μM or 1 mM) was then added, and aliquots of the reaction mixture were removed at different time points, and supernatant and bead fractions were separated using a magnetic stand. Half of the supernatant (“S”) was added to an equal volume of RNA loading dye (Ambion), and the rest of the reaction (half of the supernatant + pellet; “P”) was phenol-extracted and mixed with the same dye. In the experiments where antibiotics were used, the RB complexes were incubated with either 300 μM streptomycin or 60 μM tagetitoxin for 5 min before the addition of Rho.

GreB and Pyrophosphate Cleavage Assays—To assess the extent of backtracking of the stalled elongation complexes at position +124 (Fig. 2B) and at ops pause site (Fig. 3B), the respective RB complexes were prepared as described above. The backtracked complex at +124 was observed to form readily during the three washing steps, which were completed within 5 min. Then 0.5 μM of GreB was added, and the cleavage reactions were allowed to continue for 3 min. For pyrophosphate (PPi)-mediated cleavage of the RB complex at the his pause site, 1 mM sodium pyrophosphate was added, and aliquots of the reaction mixture were removed at the indicated (Fig. 4C) time points, and the reaction was stopped by mixing with RNA loading dye.

Analyses of the Pausing and RNA Release Kinetics—For the pausing assays, the amount of RNA in each pausing site (Fig. 1) was quantitated using ImageQuant software and plotted against time. The plots were fitted to the equation of exponential decay \( y = a \exp(-λt) \) to calculate the rate (λ) of escape from the paused site and efficiency of pausing (a) at that site (stated as PE in Fig. 1). The intercept of this curve on the y axis gives the value of “t½”, the pausing efficiency. The half-lives (t½) of pausing were calculated from, \( t_{1/2} = \ln2/(λn10) \).

To determine the rate of accumulation of Rho-mediated released RNA (Figs. 2–5), the band intensities were quantitated by ImageQuant software, and the fraction of released RNA \((S/S + (S + P))\) from each experiment was plotted against time. The plots were fitted to the equation of exponential rise \( y = a(1 - \exp^{-λt}) \) to calculate the rate (λ) and maximum efficiency (a) of RNA release. The error bars were calculated from the standard deviations obtained from at least three measurements. All the curve fittings were performed using Sigmaplot.

ATPase Assays—The rate of ATP hydrolysis of WT Rho and Rho P235H was measured by using RNAs containing the region of well known Rho-dependent λ trI and trp t’ terminators. DNA templates for transcribing the RNA were prepared by PCR amplification from the plasmid pRS604 using RS139/RS341 oligo pairs, and from the plasmid pRS106 using RS139/RS177 oligo pairs, to make the λ trI and trp t’ terminator sequence containing RNA fragments, respectively (Table 1). Both the RNAs were made using the Mega transcript kit from Ambion. The ATP hydrolysis was assayed by monitoring the release of P1 from ATP using polyethyleneimine TLC plates and 0.75 μM KH2PO4, pH 3.5, as a mobile phase under the following conditions. The assays were performed in T buffer (25 mM Tris-HCl, pH 8.0, 50 mM KCl, 5 mM MgCl2, 1 mM dithiothreitol, 0.1 mg/ml bovine serum albumin) at 37 °C. The rate of ATP hydrolysis at 25 μM and 1 mM of ATP together with [γ-32P]ATP (3500 Ci/mmol; BRIT, India) were measured using 25 nM and 50 nM of Rho, respectively. Reaction was started by addition of 0.2 μM of either λ trI or trp t’ RNA. Aliquots were removed and stopped with 1.5 M formic acid at various time points depending on the concentrations of ATP. Release of P1 was analyzed by exposing the TLC sheets to a PhosphorImager screen and subsequently by scanning using Typhoon 9200 (Amersham Biosciences) and quantified by ImageQuant TL software. The product formation was linear in the time range that we used for calculating the initial rate of the reaction. The initial rates of the reaction were determined by plotting the amount of P1 released versus time using linear regression methods.

RESULTS

Pausing of ECs at the Termination Points of Rho-dependent Terminators Is Associated with a Short Half-life—Earlier semi-quantitative studies with two well known Rho-dependent terminators, trp t’ and λ trI (24, 25), described the pausing of RNAP at the termination points in the termination zone of these two terminators. To further characterize the nature of these pauses, we cloned these two terminators under a strong promoter like T7A1, and we measured pausing kinetics at the major termination points (indicated by * in Fig. 1, A and B). Band intensities corresponding to the paused sites were plotted against time and fitted to an exponential decay equation to calculate the pause half-lives \((t_{1/2})\) and pause efficiencies (Fig. 1, below shown by the autoradiograms). We observed that at all the termination points, except at position 124 of trp t’, the half-lives of pausing are very short. This suggests that the nucleotide addition cycle at these points is not hindered significantly, and the ECs are in a catalytically active state. Even though the EC pauses longer at position 124 of trp t’ than the other pausing sites, it retains the competence for elongation at this point. We also observed that the pausing efficiencies at the termination points of the λ trI terminator are higher than that of trp t’ terminator. It should be noted that the difference in the RNA sequences between the two terminators may also lead to the difference in the ability of the Rho to act.

We concluded that although high efficiency pausing in the termination zone is important for termination to occur, Rho distinctly prefers catalytically active conformational states of the EC. Transient pauses are preferred over long pauses for releasing RNA by Rho.

Rationale and Design of RNA Release Assays from Stalled ECs—To gain further insight into the preference of Rho for particular conformational state(s) of the EC, we measured the rates and efficiencies of RNA release by Rho from ECs in different conformational states. We stalled elongation complexes
Elongation Complex Preferences of Rho

A) trp t' terminator

B) zR1 terminator

FIGURE 1. Analyses of pausing associated with the Rho-dependent termination points in trp t’ and λ tR1 terminators. Autoradiograms show the elongation kinetics through the termination zones of trp t' (A) and λ tR1 terminators (B). Right-most lane of each of the autoradiograms shows the terminated RNA bands in the presence of Rho. The numbering next to the autoradiogram in A denotes the positions of the terminated band with respect to the transcription start site. The numberings I–V in autoradiogram B are the positions of the terminated bands in the λ tR1 terminator. Asterisk indicates the paused bands corresponding to the positions of termination. Below each autoradiogram the pause half-lives (t1⁄2) and pausing efficiencies (PE) corresponding to each termination point (TP) are shown. RO denotes run-off transcript. C, schematic showing the design of the stalled elongation complex (RB) at different positions of the trp t’ terminator zone. The DNA template consists of a T7A1 promoter followed by rutA and rutB sites and the termination zone of trp t’ terminator. The lac operator sequence was introduced at different positions to stall the elongation complex in the presence of the lac repressor. The sequence of the termination zone is described below the schematic. To these different RBs either WT or P235H Rho were added to initiate RNA release reactions.

RNA-dependent ATPase activity of Rho is required for its transcription termination activity (40, 41). We hypothesized that change in the rate of ATP hydrolysis may have a direct consequence on the rate of RNA release by Rho from the stalled ECs. The ATP hydrolysis rate may be varied by changing the ATP concentration or by using certain Rho mutant(s) with significantly different ATPase activity. We measured the rate of ATP hydrolysis on two RNA fragments containing the λ tR1 and trp t’ terminator sequences under different conditions (Table 2, A and B). Upon increasing the concentration of ATP from 25 μM to 1 mM, the rate of ATP hydrolysis increased to ~7-fold for λ tR1 and ~9-fold for trp t’ RNA. We used a Rho mutant, P235H, reported to have a higher rate of ATP hydrolysis compared with WT (42). Consistent with this report, we observed that in the presence of 25 μM of ATP, the rate of ATP hydrolysis of this mutant is ~4-fold higher for λ tR1 and ~5-fold higher for trp t’ RNA compared with that observed for WT Rho. Therefore, to elucidate the effect of rate of ATP hydrolysis on the rate of RNA release by Rho, we compared the RNA release kinetics from the stalled ECs under conditions of low (25 μM ATP) and high (1 mM ATP/P235H Rho) rates of ATP hydrolysis.

It is believed that the free energy generated during the ATP hydrolysis is required for the translocation of Rho along the RNA (43). Therefore, it can be assumed that the translocation speed along the RNA is proportional to the rate of ATP hydrolysis. As the translocation speed of Rho remains the same in the presence of a given concentration of ATP, the release kinetics obtained from our experiments will be the measure of the rate of RNA release from the stalled EC and not the rate of translocation of Rho. The differences, if any, in the kinetics will reflect the EC conformational state dependence of Rho action.

**TABLE 2**

| ATP | Rate of ATP hydrolysis | Fold increase |
|-----|------------------------|--------------|
| Rho | [μM] | mmol/min/μg Rho |
| A. λ tR1 RNA | | |
| WT | 0.025 | 0.22 ± 0.03 | (1) |
| WT | 0.1 | 1.49 ± 0.08 | 6.7 |
| P235H | 0.025 | 0.86 ± 0.08 | 3.9 |
| P235H | 1.0 | 4.58 ± 0.57 | 20.8 |
| B. trp t’ RNA | | |
| WT | 0.025 | 0.16 ± 0.03 | (1) |
| WT | 1.0 | 1.51 ± 0.17 | 9.4 |
| P235H | 0.025 | 0.87 ± 0.01 | 5.4 |
| P235H | 1.0 | 5.95 ± 0.44 | 37.2 |
Reversible Backtracking of EC Does Not Affect Rho-dependent RNA Release—The *ops* pause sequence induces pausing because of reversible backtracking of the EC (33). We analyzed Rho-mediated RNA release from the *ops* pause site introduced within the termination zone of the *trp t* terminator (Fig. 3A). Efficient pausing was observed at the *ops* pause sequence in this context as was revealed from the elongation kinetics through this sequence (Fig. 3B). Also, the introduction of this site in the *trp t* termination zone did not affect Rho-dependent termination (Fig. 3B). ECs stalled at this pause site were sensitive to GreB cleavage but were also efficiently elongated both in the presence and absence of GreB (Fig. 3B, right panel). This suggested that the stalled EC at the *ops* pause site in the *trp t* termination zone was reversibly backtracked and was catalytically active.

Next Rho-dependent RNA release from the stalled ECs at *ops* site and at +161 were carried out in the presence of 25 μM ATP as in Fig. 2 (Fig. 3, C and D). Rho released RNA from the stalled EC at the *ops* site with comparable rate and efficiency as that from position +161. Therefore, Rho can release RNA efficiently from a reversibly backtracked EC which is catalytically active.

Catalytic Inactivation of EC by an RNA Hairpin at the Exit Channel Causes Inefficient Rho-dependent RNA Release—Pausing at the *his* pause sequence is proposed to be mediated by interaction of an RNA hairpin with the flap domain of the β-subunit of RNAP located near the RNA exit channel (33–36). The sequence at the *his* pause site codes for RNA that folds into a hairpin near the exit channel. Hairpin–EC interaction at this site was found to cause catalytic inactivation, most likely by allosterically hindering the movement of the trigger-loop (TL) structural element (44). We tested the effect of this *his* hairpin-modified EC on Rho-mediated RNA release. There were two reasons for choosing this modified EC. (i) We observed in Fig. 2 that catalytically inactive, arrested EC is a poor substrate for Rho. The *his* hairpin–EC interaction also causes catalytic inactivation. However, this inactivation is not because of irreversible backtracking of the EC, but because of altered conformations at the active site (44), which is distinct from the conformation of the EC at position +124 (Fig. 2). (ii) If the interaction of Rho with the EC is required for its action, the likely region of interaction will be the RNA exit channel. The *his* hairpin causes conformational changes in position in the presence of GreB (Fig. 2B). From the size of the cleavage product, we estimated that the EC at position +124 was backtracked by 8–9 nucleotides. This suggests that the stalled EC at +124 was arrested and irreversibly backtracked. On the other hand, stalled EC at +161 was readily elongated in the absence of GreB and was not significantly sensitive to GreB, which is indicative of its catalytic competence (Fig. 2B).

We then followed the time course of RNA release from these two ECs in the presence of either WT or P235H Rho and 1 mM ATP (Fig. 2, C–F). Rho released the RNA from the catalytically active stalled EC at position +161 with a high rate and efficiency, whereas the rate of RNA release was ~4-fold and the efficiency was ~2-fold less for the arrested stalled EC at position +124 (Fig. 2, C and E). The rate and efficiency of RNA release were drastically improved when the +124 EC was activated in the presence of GreB (Fig. 2F). A similar improvement was also observed in the presence of a mutant Rho, P235H, which has a higher rate of ATP hydrolysis (42) (Table 2; Fig. 2, D and F). These results indicate that irreversibly backtracked ECs are a poor substrate for Rho, and a higher rate of ATP hydrolysis is required to dislodge them.
Elongation Complex Preferences of Rho

A)

\[
\begin{align*}
\text{UCAUUUUCUCAGUAAUUUCCUCAG} & \quad \text{Lac-O} \\
\text{UCAUUUUCUCGCGGAGAGCGC} & \quad \text{U-Lac-O}
\end{align*}
\]

RB @ 161

161

155

B)

| Time (min) | 0.5 | 1 | 2 | 3 | 5 |
|------------|-----|---|---|---|---|
| P | P | P | P | P | P |

Ops pause

Rho (lane 2)

C)

| Time (min) | 0.5 | 1 | 2 | 3 | 5 |
|------------|-----|---|---|---|---|
| RB @ 161 | P | P | P | P | P |
| RB @ 155 | P | P | P | P | P |

D)

WT Rho + 25 \text{ M ATP}

Fraction of released RNA

Time (min)

Rate (\text{min}^{-1})

\[ \text{RB @ 161: 0.9, RB @ 155: 0.7} \]

FIGURE 3. Rho releases RNA efficiently from reversibly backtracked ECs. A, RNA sequence coded from the ops pause site (in boldface italic) introduced in the trp t’ termination zone is shown. The 3’-end of the RNA in the RB is at position +161. RNA sequences coded from the trp t’ terminator zone surrounding position +161 are also shown. Region of RNA present in the RNA: DNA hybrid is box. B, left panel shows the autoradiogram of the elongation kinetics through ops pause sequence. Ops pause site is indicated with an arrow. The right-most lane of this panel shows Rho-dependent termination from this region with the terminated bands indicated by an asterisk. The right panel shows the stalled EC (RB) at the ops pause site (lane 1). This complex is subjected to GreB induced cleavage (lane 2) or chased in the presence of IPTG and NTPs either in the absence of GreB (lane 3) or after incubating with GreB (lane 4). C, autoradiogram showing the RNA release time course from stalled ECs at ops pause site and position +161 with corresponding plots are shown in D. All the notations and calculations of the rates are same as in Fig. 2.

this part of the EC and may also block the approach path of Rho.

We introduced the his pause sequence downstream of position +161 in the termination zone of the trp t’ terminator (Fig. 4A). Elongation kinetics, using this construct, revealed an efficient pause 12 nt downstream of the his hairpin (+199), which suggested that the his pause sequence is functional in this context (Fig. 4B). We also tested the hairpin-induced catalytic inactivation of the EC at this pause site, which is indicative of the his hairpin-EC interaction at this site. We stalled the EC at +199 by adding lac repressor to the reaction and monitored the pyrophosphate cleavage potential of the EC. This is a measure of the catalytic competence of the EC when transcription elongation is blocked by the lac repressor (45). We observed very inefficient pyrophosphate cleavage (Fig. 4C), indicating that the his hairpin in our construct can efficiently cause catalytic inactivation of the EC. Therefore all the properties of his hairpin-modified EC were reproduced in our construct, and it can be used to study the effect of this modified EC on Rho-dependent RNA release.

We measured RNA release kinetics by either WT or P235H mutant Rho from the EC stalled at the his pause site in the presence of either low (25 \text{ M}) or high (1 \text{ mM}) concentrations of ATP (Fig. 4, D–G). The kinetics of RNA release at the his pause site was compared with that obtained from catalytically active EC at position +161. The rate and efficiency of RNA release were found to be reduced significantly at the his pause site as compared with that obtained at position +161 (Fig. 4E). However, the rate and efficiency of RNA release were improved under the regimen of higher rate of ATP hydrolysis either in the presence of 1 \text{ mM ATP} (Fig. 4F) or P235H Rho (Fig. 4G). Analogous to the arrested complex in Fig. 2, Rho can efficiently dislodge the catalytically inactive his hairpin-modified EC only when the rate of ATP hydrolysis is higher. A part of the energy from ATP hydrolysis may also be used by Rho to unwind the his hairpin.

Effects of the Elongation Inhibitors Tagetitoxin and Streptolydigin on Rho-dependent RNA Release—Results described above strongly suggest that the functional state(s) of the active site of the EC is important for the substrate specificity of Rho. NTP addition cycle during transcription elongation involves the movement of the two important structural elements, the bridge helix (BH) and the trigger loop (TL), located close to the Mg(II)-binding site, and their movement determines the functional state of the active site (46, 47). We hypothesized that any perturbations in the conformational state(s) of these two structural elements will affect Rho-dependent RNA release from the EC.

We modified the EC stalled at position +161 with two antibiotics, streptolydigin (STL) and tagetitoxin (TGT), which bind close to the BH and TL structural elements (Fig. 5A) (37, 47) and hinder the movement of these two elements. The STL-bound EC is capable of adding only one nucleotide (38, 39, 48), whereas NTP addition cycle is completely blocked in the TGT-bound form (37). This suggests that in the TGT-bound EC, BH and TL movement may be impermissible, whereas in the STL-bound form these two elements may have limited freedom to move. The modifications of the stalled EC at position +161 with these two antibiotics were at first confirmed from their inability to be elongated efficiently after treating the RBs with IPTG and NTPs (Fig. 5B).

We then measured RNA release kinetics by Rho from these modified stalled ECs (Fig. 5, C–G). The rate of RNA release by Rho was 2-fold lower when the EC was bound to TGT (Fig. 5, C and E). This rate, however, improved when P235H mutant instead of WT Rho was used (Fig. 5F). Therefore, when internal motion around the active site is impaired, Rho requires a higher rate of ATP hydrolysis to dislodge the EC. Interestingly, Rho was able to dislodge the STL-bound EC efficiently (Fig. 5, D and G). The STL-bound form offers a little more freedom of movement to the BH and TL elements (38, 39) compared with the
TGT-bound form (37), which might be sufficient for Rho to exert its action. As the BH/TL movement plays an important role in Rho action, it is likely that hindered TL movement in the his hairpin-modified EC (44) may have also affected the efficiency of RNA release by Rho from that complex (Fig. 4).

DISCUSSION

Pausing of the EC in the termination zone of Rho-dependent terminators is required for coupling the transcription elongation and the translocation of Rho (24, 25). ECs can exist in different conformational states at these pause sites (28). Here we present strong evidence that the rate and efficiency of RNA release by Rho are highly influenced by the conformational states of the EC in the termination zone. We showed the following: 1) pausing associated with the termination points is very short lived (Fig. 1); 2) irreversibly backtracked (Fig. 2) and his hairpin-modified ECs are poor substrates for Rho (Fig. 4); 3) RNA release by Rho is affected if the free movement of BH/TL structures in the active site of the EC is blocked completely by the inhibitor tagetitoxin (Fig. 5); and 4) increase in the rate of ATP hydrolysis by Rho (Table 2, A and B) is proportional to an increase in the rate of Rho-dependent RNA release from different stalled ECs. A higher rate of ATP hydrolysis by Rho is essential to release RNA efficiently from the catalytically inactive ECs (Figs. 2, 4, and 5). We concluded that the dislodging of the EC by Rho is facilitated only when the RNAP is engaged in active transcription.

It has been proposed that a complex Brownian ratchet motion involving the oscillation of the two structural elements, BL and TL, in the active center governs the nucleotide addition cycle during elongation (46). Structural and biochemical evidence suggests that catalytic inactivation of the ECs, modified either by the his hairpin (44) or by the antibiotic TGT (37), is because of blockage of the movement of BL and TL structural elements. The oscillation of these two structural elements is also likely to be hindered when the 3′-end of the RNA occupies the secondary channel in an arrested or irreversibly backtracked complex. We showed here that Rho-dependent RNA release is significantly affected in all these modified ECs, which suggests that conformational flexibility around the active center plays a key role in the process of termination. This suggestion is further supported by the fact that Rho can efficiently release RNA from a reversibly backtracked complex, where presumably the Brownian ratchet motion is not hindered, and from an STL-bound EC where some movement of BH and TL is allowed (38, 39).

What is the role of ATP hydrolysis during Rho-mediated RNA release from the EC? We observed that there is a direct correlation between the rate of ATP hydrolysis by Rho and its efficiency of RNA release from the stalled ECs. ATP hydrolysis by Rho is required for its transcription termination function (40, 41). Although there is no direct evidence, it is believed that the free energy derived from this ATP hydrolysis is used for the translocation as well as the motor action of Rho (43). We spec-

FIGURE 4. Catalytic inactivation of ECs caused by RNA hairpin at the exit channel affects Rho-dependent RNA release. A, RNA sequence showing the position of the his pause sequence downstream of +161 in the trp′ terminator zone. His pause hairpin-forming sequence is shown in the dotted box, and the pause site at +199 is indicated by an arrow. lac operator sequence after the pause site is also indicated. Region of RNA present in the RNA:DNA hybrid is marked by a solid box. B, autoradiogram showing the elongation kinetics through the his pause sequence. His pause site and the run-off (RO) product are indicated. C, autoradiogram showing the pyrophosphorolysis reactions (PPi cleavage) of the stalled EC (RB) at the his pause site in the presence of 1 mM sodium pyrophosphate for the indicated times. D, autoradiogram showing the time course of RNA release from stalled EC at the his pause site by WT and P235H Rho in the presence of 25 μM ATP. Representative time points are indicated. The plots corresponding to the RNA release kinetics from the RB complexes at the his pause site under different conditions are shown in E–G. Rates are indicated in the inset of each panel. All the calculations and notations are as in Fig. 2.
ulate that increase in the rate of ATP hydrolysis will also increase the translocation speed of Rho and will impart more kinetic energy to this molecular motor. This kinetic energy will be used by Rho to exert force on the elongation complex to dislodge it. A higher rate of ATP hydrolysis will ultimately be translated into a higher force that will enable Rho to act upon the poor substrates. However, this hypothesis can only be tested by a direct measurement of the force exerted by Rho using single molecule measurements. Alternatively, it may be possible that each ATP hydrolysis step by Rho is directly linked to each step of RNA release and later may involve successive steps of pulling the RNA out of the EC or pushing the EC forward. In this model, it is likely that under the regimen of a higher rate of ATP hydrolysis, the steps involved in RNA release will become faster and will outperform the NTP addition cycle of the EC. However, linking ATP hydrolysis to the steps of RNA release will also involve the utilization of the energy obtained from the hydrolysis reactions.

Why does Rho prefer catalytically competent ECs for termination? At the peak of the elongation process, the rapid Brown-
ian ratchet motion at the active center of the EC will cause breakage and formation of multiple noncovalent interactions, which may confer overall instability to the EC as compared with when it is trapped in a static conformation incapable of pursuing nucleotide addition cycles. According to our speculation, less force generated by the lower rate of ATP hydrolysis by Rho is sufficient to dissociate these relatively less stable catalytically active ECs, whereas an enhanced rate of ATP hydrolysis is required to act on the more stable inactive complexes. We propose that Rho employs a “brute force” mechanism to dislodge the EC, in which the force generated by the ATP hydrolysis sets off its molecular motor action, which in turn displaces the EC. This proposal is consistent with the recent observation that translocation of Rho can break a strong biotin-streptavidin interaction present at the end of an RNA template (49).

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