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Rho-dependent Transcription Termination: More Questions than Answers

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Abstract

Escherichia coli protein Rho is required for the factor-dependent transcription termination by an RNA polymerase and is essential for the viability of the cell. It is a homohexameric protein that recognizes and binds preferably to C-rich sites in the transcribed RNA. Once bound to RNA, it utilizes RNA-dependent ATPase activity and subsequently ATPase-dependent helicase activity to unwind RNA-DNA hybrids and release RNA from a transcribing elongation complex. Studies over the past few decades have highlighted Rho as a molecule and have revealed much of its mechanistic properties. The recently solved crystal structure could explain many of its physiological functions in terms of its structure. Despite all these efforts, many of the fundamental questions pertaining to Rho recognition sites, differential ATPase activity in response to different RNAs, translocation of Rho along the nascent transcript, interactions with elongation complex and finally unwinding and release of RNA remain obscure. In the present review we have attempted to summarize ‘the knowns’ and ‘the unknowns’ of the Rho protein revealed by the recent developments in this field. An attempt has also been made to understand the physiology of Rho in the light of its phylogeny.

Keywords

Rho; transcription termination; RNA polymerase; RNA-dependent ATPase; RNA/DNA helicase

The regulated expression of genes in an organism confides on the signals in the DNA that effect every phase of the transcription process by a DNA dependent RNA polymerase. Once the process of transcription begins, the RNA polymerase makes a stable elongation complex with the DNA and the nascent RNA (Wilson and von Hippel, 1994; Mooney *et al*., 1998). The regulation of gene expression begins from appropriate recognition of the promoter site by a RNA polymerase and extends to each step during RNA chain elongation when the transcription is interrupted by a pause, an arrest or a termination signal. Thus, initiation, elongation and termination of the transcription act in concert for an orderly expression of genes. Distinguished by their mechanism and structural features there are two kinds of terminators in *E. coli* genome. (a) Intrinsic terminators characterized by a GC rich inverted repeat followed by a stretch of consecutive thymidylate that induce RNA polymerase to pause, destabilize and disengage for releasing RNA without the involvement of any auxiliary proteins and (b) the factor-dependent terminators that have a highly inconsistent sequence homology but characteristically require the essential protein factor Rho for termination (for review on transcription in prokaryotes see Richardson, 1993; Richardson and Greenblatt, 1996; Uptain *et al*., 1997).

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Rho, encoded by the gene *rho*, is a universal protein found throughout the bacterial world with a very few exceptions. Rho is a homohexamer with a protomer of 46.8 KDa which is a product of a single polypeptide of 419 residues. Since its discovery and purification by J.W. Roberts in 1969 (Roberts, 1969), Rho has become instrumental in exploring the regulatory role of auxiliary proteins in prokaryotic transcription termination by RNA polymerases.

In the cell, Rho binds to untranslated naked RNAs and terminates the synthesis of mRNA at the end of a significant number of operons. Rho is essential for the survival of most of the prokaryotes. As a molecule, Rho is a RNA/DNA helicase or translocase that dissociates RNA polymerase from DNA template to release RNA, deriving energy by hydrolyzing ATP through its RNA-dependent ATPase activity to bring about termination (Richardson, 2002; Richardson, 2003). As transcription and translation occur simultaneously in prokaryotes, when translational termination occurs within a gene, Rho can cause transcriptional termination of the downstream gene in an operon, thus preventing its expression. This effect, called translational polarity (Adhya and Gottesman, 1978; Nudler and Gottesman 2002), can be suppressed by either a mutation in *rho* or by its inhibitor (Das *et al.*, 1976; Linderoth and Calendar, 1991).

Properties of Rho is a reflection of its structure

Functional domains

Prior to the elucidation of the crystal structure of Rho complexed with nucleic acid in free and nucleotide (AMPPNP) bound states (Skordalakes and Berger, 2003), attempts were made to dissect the domain structure by cleavage protection assays, partial tryptic digestion, photo affinity labeling with nucleotides and synthetic ribo oligos and NMR and X-ray studies of N-terminal RNA binding domain (Bear *et al.*, 1985; Dolan *et al.*, 1990; Bogden *et al.*, 1999; Wei and Richardson, 2001a). These studies, along with the crystal structure and further mutational analysis have specified the following functional domains in a Rho protomer (Fig. 1): (a) Core RNA binding domain called the primary RNA binding site of Rho that can bind to a single stranded DNA molecule as well as a single stranded RNA molecule held responsible for tethering of Rho to the Rho loading or Rho utilization (*rut*) site, extends from residues 22-116 (Modrak and Richardson, 1994). (b) P-loop, associated with ATP binding and ATPase activity of Rho, spanning from 179-183 is highly conserved among RecA family of ATPase (Opperman and Richardson, 1994; Wei and Richardson, 2001a). (c) Q-loop and R-loop comprising the secondary RNA binding site of Rho. Q-loop is formed by 8-residue segment within 278-290 (Wei and Richardson, 2001a; Wei and Richardson, 2001b; Xu *et al.*, 2002; Skordalakes and Berger, 2003). R-loop, a less defined and characterized part of Rho protein, is thought to span between 322-326 amino acid residues in *E. coli* (Miwa *et al.*, 1995; Burgess and Richardson, 2001a; Skordalakes and Berger, 2003).

The Oligomeric assembly of Rho

Rho can self assemble in a solution with a variety of assembly states, the homohexamer being the most predominant (Geiselmann *et al.*, 1992a). The major factors that influence Rho subunit assembly are the ionic environment, concentration of the Rho protein and the presence of cofactors (Geiselmann *et al.*, 1992a; Geiselmann *et al.*, 1992b). Rho protomers assemble into stable dimeric or tetrameric forms under a high concentration of salt, low protein concentration and in the absence of cofactors. Almost a homogenous hexameric population of Rho can be obtained in a weak ionic environment of 50-100 mM salt and a 2-10 μM of Rho, either in presence or absence of a small RNA cofactor (Geiselmann *et al.*, 1992a). The hexameric unit of Rho in the absence of RNA can exist in either an open ‘key washer’ like pentameric form or in closed ‘ring like’ hexameric form. Both have been

argued to be the functional states of Rho and the 'open' Rho structure can be converted into closed form in the presence of a single stranded nucleic acid that is presumed to wrap around the periphery of the Rho molecule, often in presence of nucleotide cofactors (Gan and Richardson, 1999; Yu *et al.*, 2000). A dodecamer state has been observed in the presence of a RNA cofactor with a stoichometry of one Rho hexamer for every two to three RNA molecules. The dodecamer assembly is speculated to increase the 'effector concentration' of Rho at the site of action *in vivo* (Geiselmann *et al.*, 1992a). The combinatorial analysis through electron microscopic, hydrodynamic, X-ray and neutron scattering techniques, the geometry of the Rho hexamer was found to be a regular hexagon (Gogol *et al.*, 1991; Geiselmann *et al.*, 1992a; Geiselmann *et al.*, 1992b).

The crystal structure of Rho revealed its asymmetric nature. Subunit interactions occur between the connecter loop of the second and the third α-helices in the N-terminal domain of one subunit with the 'linker' region of the other subunit. The C-terminal contact between subunit is adjacent to the P-loop of the ATP binding domain. The periphery of the ring is defined by the N-terminal, while the Q-loop of each protomer together contributes to the constricted inner ring. The movement of one protomer with respect to the other by ~ 15° gives rise to displacement of 45° between the first and the last protomer of the pentameric open ring assembly. This generates a gap sufficient to bind a single molecule of single stranded nucleic acid (Skordalakes and Berger, 2003). Thus, it supports the interpretation from earlier studies that 'notched' or open ring assembly is the functional state of the protein for loading RNA into the hole of the hexamer (Yu *et al.*, 2000).

Primary nucleic acid binding site

N-terminal domains consist of three α-helices appended to five stranded β-barrel comprising the oligonucleotide or oligosaccharide-binding OB fold. Even though earlier experiments gave direct and indirect evidences of primary RNA binding domain extending from 22-116 (Modrak and Richardson, 1994), the first nucleotide base packs into the hydrophobic pocket of Tyr80, Glu108 and Tyr110 (Skordalakes and Berger, 2003). Protection assays by a homonucleotide and by the natural template *λ cro* containing *rut* site supported by X-ray crystallography evidence have revealed that there are three distinct contact regions housed in the (i) N-terminal residues 58-62 with Leu58 and Phe62 creating a hydrophobic shelf for ribose interactions (Bogden *et al.*, 1999), (ii) the loop connecting third and fourth β strands (Wei and Richardson, 2001a), (iii) residues 103-110, that include the hydrophobic enclosure of Glu108, Arg109and Try110 engaged in Vander Waals interaction with the base of cytidine (Wei and Richardson, 2001a). While this enclosure is enough to accommodate pyrimidines, it is too small for larger molecules like purines. This has been argued to be one of the reasons for the preference for 'pyrimidine'-rich sequence for Rho loading. Further X-ray crystal structure of N-terminal domain has revealed conjunction between Arg66, Glu56 and Asp78 that creates a stearic conformation to select pyrimidines over purines and it is interpreted that Watson/Crick-like-interaction (stacking of nucleotide bases on aromatic protein side chains) selects cytosine over uracil (Bogden *et al.*, 1999). This explains from the structural point of view the basis of recognition of Rho utilization site (*rut* site) that are rich in unpaired cytidylate (C) residues and lack secondary structure (Galluppi and Richardson, 1980; Richardson and Richardson, 1992; Wang and von Hippel, 1993). Interestingly the crystal structure has overcome the notion that primary binding RNA cleft residues are at the periphery of the Rho molecule. The orientation of the N-terminal modulate primary RNA binding cleft face inwards, thus making mRNA to follow a somewhat zig-zag path that extends from the periphery to the center of each protomer for binding to the Rho molecule.

Secondary nucleic acid binding site

Secondary RNA binding site of Rho is characterized by Q-loop and R-loop. While R-loop has implications in binding both ATP and RNA (Xu *et al.*, 2002), Q-loop that extends towards the center of Rho ring is strictly for RNA binding. The proximity of R-loop to ATP binding domain or P-loop suggests a possible explanation to the coupling of RNA binding to ATP hydrolysis. The residues constituting Q-loop as per the model derived from crystal structure varies from 278-292, however, protection assays show maximum protection at the position 285 by RNA (Wei and Richardson, 2001a; Wei and Richardson, 2001b).

Even though orientation of primary binding domain towards center defies mRNA path along the periphery, the orientation of primary binding and secondary binding domain in the crystal structure orients the 3'end of nascent transcript towards secondary site. This structure is consistent with the stopped flow fluorescence and the pre-steady state ATPase kinetic studies where it is shown that the RNA binding to primary binding site is followed by a state when RNA reaches the secondary binding through the hole in the Rho-ring and leads to the formation of ATP hydrolyzable intermediate leading to translocation (Kim and Patel, 2001).

The ATP binding domain

The ATP binding domain is located at the interface of C-terminal between adjacent subunits. Even though equal electron densities for bound nucleotide in all the six ATP binding pockets were observed in the crystal structure, earlier studies have reported that the six subunits of Rho are functionally different with respect to the ATP binding and hydrolysis (Stitt, 1988; Geiselmann and von Hippel, 1992; Kim and Patel, 1999). There exists three strong and three weak binding sites for ATP and RNA (Geiselmann and von Hippel, 1992; Seifried *et al.*, 1992) with the tightly bound ATP less catalytically active than the weakly bound (Kim *et al.*, 1999). ATP hydrolysis requires nucleophilic water. Bicyclomycin, a naturally occurring antibiotic and a non-competitive inhibitor of Rho, prevents ATP turnover by blocking the binding of the nucleophilic water required for ATP hydrolysis. The water molecule that attacks ATP is coordinated by Glu near Walker A and B motifs of ATP-binding domain. Bicyclomycin binds to Glu112 and physically occlude the attacking water molecule (Skordalakes *et al.*, 2005), thus hindering ATP hydrolysis. Fig. 1 summarizes the different functional domains of Rho protomer demarcated so far.

Rho-dependent termination signals: The Rho utilization (rut) site defines a rho-dependent terminator

Rho terminates transcription in response to specific DNA signals transcribed into RNA called the rho-dependent terminators. There are two fundamental features of a rho-dependent terminator; a proximal Rho binding site called the '*rut* site' (Rho utilization) and a distal sequence comprising the termination zone. Rho binding site is a highly degenerated region of ~ 70-80 nucleotides (nt) that is rich in cytosine and devoid of secondary structures (Morgan *et al.*, 1985; Bear *et al.*, 1988; Alifano *et al.*, 1991). The highly degenerated nature of *rut* site poses problems in scanning genomes for rho-dependent terminators. Schneider *et al.* carried out a 'SELEX' approach to scan for Rho recognition sites in *E. coli* genome and finally found that two categories of potential Rho recognition sites, one with 35%-65% C residues in 30 nt stretch and other, surprisingly, had six base pairs hairpin structure (Schneider *et al.*, 1993). The observations that not all C-rich sequences or longer RNAs can activate ATP hydrolysis equally, the actual role of 'cytosine' in the loading step of termination by Rho still remains unclear.

From deletion analyses of rho-dependent terminators like λ *tR1* and *trp t'* it could be interpreted that the *rut* site is an essential part that dictates the termination zone downstream

in a rho-dependent terminator (Morgan *et al.*, 1983; Chen and Richardson, 1987; Zalatan *et al.*, 1993; Richardson and Richardson, 1996). That Rho specifically binds to the *rut* site of a terminator was demonstrated by H_2O_2 - Fe EDTA cleavage protection assays. Rho was protected from cleavage when λ *cro* RNA with the *rut* site was used, but no protection was found with a partial transcript lacking *rut* site (Wei and Richardson, 2001).

The termination zone begins about 60-90 nt downstream the *rut* site within which the termination occurs at different sites. When RNA polymerase scans these sequences, it temporarily halts at specific sites, called pause sites. Most frequently Rho termination sites are the points where RNA polymerase pauses. However, not all pause sites are termination points, and these include some of the strongest pause along a terminator (Kassavetis and Chamberlin, 1981).

Sequential events in Rho-dependent termination

Transcription termination mechanism of Rho can be expressed grossly in the following steps (Fig. 2); (a) Rho binds to naked, non translating RNA at a C-rich site called Rho utilization site (rut site), (b) The rut site binding of Rho is achieved by the N-terminal or primary binding domain, (c) The bound RNA, passing through primary binding site reaches the secondary binding site that resides on C-terminal, (d) RNA bound secondary sites sensitize Rho for ATP hydrolysis, (e) The energy derived from ATP hydrolysis powers the translocase / helicase activity of Rho to unwind RNA/DNA duplex.

All these function in concert to finally release nascent transcript and disengage the transcribing RNA polymerase. The process, however, is more complex than the simplistic steps mentioned above. To understand Rho, it is important to consider that apart from the Rho as an ATPase, helicase and RNA binding protein involved in transcription termination, it is largely dictated by the terminators and the elongation complexes that are the mechanistic components of the Rho action.

Events of Rho loading and formation of ATPase competent intermediates

Rho recognizes the *rut* site on a terminator template. The loading of Rho, however, does not require a free 5' end on the template (Burgess and Richardson, 2001b). The binding kinetics of RNA to Rho in a real time window using the stopped-flow experiments (Kim and Patel, 2001) indicated a three step mechanism involving at least three intermediate RNA bound Rho conformations before forming a stable active association. RNA randomly collides with the Rho molecule with a diffusion limited rate of 7.5 ± 10^{-8} $M^{-1}S^{-1}$ forming a dynamic Rho-RNA complex with a dissociation rate of 12 S^{-1}. RNA then wraps around the primary binding site of each of the five protomer of the Rho open complex forming an isomeric, comparatively stable moiety with association rate of 26.2 S^{-1} and dissociation rate of 2.8 S^{-1}. The RNA subsequently passes towards the center of the open Rho ring making contact with the secondary binding site of the Rho molecule in a slow yet steady rate of 5 S^{-1}. This complex is a steady complex with almost no detectable dissociation rates. These intermediates lack ATPase activity. Once the RNA comes in contact with the secondary binding site, Rho gets activated for ATP hydrolysis. Thus, there exists a time lag between the initial contact of Rho with RNA and the beginning of ATP hydrolysis and translocation. It is during this time window these various intermediates are formed. This became evident by presteady state kinetics of ATP hydrolysis that showed an initial burst of ATP hydrolysis coinciding with the contact of RNA with Rho followed by a lag of three seconds during which no ATP hydrolysis takes place. Kinetic simulations further cleared that after the RNA enters the 'ring' of Rho, the open conformation gets closed by a sixth subunit, leading to formation of a highly stable ATPase competent moiety capable of translocation (Kim and Patel, 2001). Fig. 3, adapted from Kim and Patel 2001, sums up these steps.

Rho couples ATP hydrolysis with the translocase/helicase activity leading to the unwinding of a RNA-DNA duplex

The concluding step in the transcription termination is the unwinding of the RNA-DNA duplex to release the nascent transcript. Rho has the inherent property to translocate along a RNA and unwind a RNA-DNA duplex (Richardson, 2002; Richardson, 2003). The ATPase competent Rho-RNA complex formed after RNA binds to the secondary binding site of the Rho, is capable of helicase activity and therefore unwinding of RNA-DNA duplex from a 5' → 3' direction (Brennan *et al.*, 1987).

The translocation rate of Rho along either a single stranded RNA template or a RNA-DNA duplex, remain unchanged to ~20 nt/sec at 37°C. At 50 mM salt concentration, Rho can translocate upto 300 nt. The ATP hydrolysis rate of Rho whether it is translocating along a single stranded RNA template or a RNA-DNA duplex, remain unchanged (Walstrom *et al.*, 1997a; Walstrom *et al.*, 1997b).

The helicase property of Rho outside the context of termination has been studied extensively. The helicase reaction of Rho consists of two phases. First is the burst phase that results in rapid release of RNA with a single round of helicase activity per Rho molecule. The subsequent phase is a slow phase that is largely limited by the separation of Rho from the RNA after the completion of the helicase activity. The slow phase thus depends on the processivity of the Rho. Increasing the salt concentration in the helicase reaction increases dissociation of Rho from the RNA, but the overall translocase rate also becomes slow as Rho dissociates from the RNA even before it could reach RNA-DNA duplex. Thus the rate-limiting step in the helicase activity of Rho may be the dissociation of Rho from the RNA and rebinding to a new helicase substrate (Walstrom *et al.*, 1997a). ATP hydrolysis continues even after the first round of helicase activity indicating that Rho remains productively bound to the RNA (Steinmetz *et al.*, 1990).

The *in vitro* studies and characterization of ATPase defective *rho* mutants couple ATP hydrolysis to the helicase activity of Rho (Pereira and Platt, 1995b). That is, the Rho defective for ATPase, eventually is defective for helicase. However, mutants exemplified by *rho nitA18* (Shigesada and Imai, 1978) and Rho201 (Pereira and Platt, 1995a) suggest the existence of one more step, i.e; coupling of energy production and energy utilization by Rho for helicase and eventually termination. Rho201 is severely defective for RNA-DNA unwinding despite an 'ultra' ATPase activity compared to wild type Rho. A ten times lower affinity to RNA binding is sufficient for this mutant to elicit an ATPase activity higher than wild type. This uncoupling of ATPase with helicase may also suggest that 'energy coupling' is yet another inherent property of Rho by which it harnesses the energy produced by ATP hydrolysis for helicase/translocase activity. The defect in this 'energy coupling' can also attribute to the termination defects of Rho.

Involvement of NusG in Rho-dependent termination

Rho can work efficiently in a purified *in vitro* system; however, supplementary factors like NusG immensely influence its termination efficiency and pattern across a terminator.

NusG was originally identified as a component of λ mediated antitermination (Li *et al.*, 1992). A close physiological relationship between NusG and Rho became evident when NusG over-expression could overcome some of the *nusD* class of *rho* mutants (Sullivan and Gottesman, 1992; Sullivan *et al.*, 1992) and its depletion decreased Rho-dependent termination at specific terminators. Retardation of Rho by a NusG column (Li *et al.*, 1993) suggests a physical contact between the two.

NusG causes early termination at several terminators including *trp t'* and can overcome the defects of certain *rho* mutants (Burns *et al.*, 1999). When each property of Rho is taken uncoupled with termination, NusG does not improve either rate of unwinding or ATP hydrolysis, or RNA binding (Nehrke *et al.*, 1993). Ironically, NusG at the same time is also responsible for increasing the rate of RNA polymerase elongation by ~25% (Burns *et al.*, 1998), therefore it is difficult to conceive that it assists Rho in overcoming kinetic limitations. The line of evidence that inability of Rho to terminate transcription with fast moving RNA polymerase can be overcome by NusG came from Rho deletion variant of *Micrococcus luteus* (Burns *et al.*, 1999). Unlike *E. coli* Rho, *M. luteus* Rho has higher efficiency to terminate at tR1, terminating at more proximal points than *E. coli* Rho. A deletion variant of it was examined to behave like *E. coli* Rho, with almost similar pattern of termination across tR1. This deletion variant, however, was shown to lack the kinetic limitation at the first termination site of tR1 that the *E. coli* Rho could only overcome either with higher concentrations of NTPs or in presence of NusG.

So far, the role of NusG in Rho dependent termination is not conclusive, but the evidences hint to these possibilities: NusG increases the retention of Rho on nascent transcript (Nehrke and Platt, 1994), thereby might increase the local concentration of Rho. Also, NusG increases the rate of RNA release at termination sites (Burns *et al.*, 1999).

Models explaining the mechanism of the Rho-dependent termination

In a nut-shell, Rho-dependent termination has three basic steps, a) Rho loading b) Rho translocation and c) Rho unwinding of DNA-RNA duplex to release RNA. These basic steps are often influenced by several known (like NusG) and unknown cofactors. Fig. 2 gives an overview of the steps involved in the termination and points out some of the queries that still remain unanswered. Over the years, several speculations and hypotheses have been put forward to explain the mechanism of Rho action. Some of them are supported by direct or indirect experimental evidences, while others are still questioned and unexplored. In the section below, we discuss some of these models and also propose new theories that can be tested to completely understand the Rho mechanism.

The kinetic coupling between Rho and RNA polymerase

Every step in Rho-dependent termination has a kinetic component. From loading of Rho, to activation of Rho for ATPase activity, to tracking along the nascent transcript, all work in a time window, before it actually makes contact with the transcribing RNA polymerase. This probably explains why, most often, the RNA polymerase pause sites along a rho-dependent terminator coincide with the termination sites of Rho. Therefore, it is advocated that a 'kinetic coupling' between a transcribing RNA polymerase and the translocating Rho exists and whenever Rho 'catches up' with the elongation complex, it can disengage and release RNA. This model of 'kinetic coupling' is supported both by *in vivo* and *in vitro* data using slow and fast RNA polymerase variants. The termination defects of several *rho* mutants could be overcome by a slow moving RNA polymerase RpoB8 *in vivo* and the effect could be mimicked by decreasing the elongation rate of the wild type Rho by reducing NTP concentration (Jin *et al.*, 1992).

But there are accumulating evidences against the 'kinetic coupling' model. Such as, termination defects of several mutants could not be suppressed by RpoB8 (Jin *et al.*, 1988) or strong pauses are not necessarily the termination points (Kassavetis and Chamberlin, 1981; Richardson and Richardson, 1996) and antagonistic affects of NusA and NusG on Rho and RNA polymerase (Burns *et al.*, 1998). This is indicative that Rho-dependent termination is probably more complex than just 'catching' up with RNA polymerase.

Translocation models of Rho along the template

Amongst the helicases, what makes Rho different is its high selectivity for a loading site on a template. Once loaded, there have been several proposals as to how Rho tracks along a nascent RNA to reach the elongation complex. Since the binding efficiency to the Rho utilization site (*rut* site) is maximum than the rest of the RNA, it is often questioned if Rho remains bound to the *rut* site while translocating. There have been following proposals explaining Rho movement (Fig. 4): a. Tracking model: Rho leaves the *rut* site and translocates to reach elongation complex. b. Looping model: Rho remains attached to the *rut* site and loops over to the elongation complex at the site of termination, a proposal that has been overruled by the observation that a block of RNA-RNA could act as a conditional block to unwinding of a RNA-DNA duplex downstream to it (Steinmetz *et al.*, 1990). c. Tethered tracking model: Rho remains attached to the *rut* site and moves along with the RNA in a zipper like manner. This is called the tethered tracking of Rho. The model was tested by Steinmetz and Platt. They monitored the helicase mediated release of an RNA oligo annealed between *trp t'* terminator and a neutral spacer sequence. The released RNA oligo was replaced by a DNA oligo used in excess in the helicase reaction. This was followed by a RNAse H cleavage that generated a 5' *trp t'* and 3' spacer sequence. The distribution of *rho* on the *trp t'* and spacer sequence was assessed by filter retention assays. They observed that even after helicase activity majority of the Rho remains bound to *trp t'* probably to the *rut* site (Steinmetz and Platt, 1994). Interaction with Rho secondary site being more transient and unstable, it could not be detected by the filter binding. The present crystal structure also showed that N-terminal domain opens out from the center of the ring creating a depression that can allow a single stranded RNA to remain attached while a second single stranded RNA is spooled by Rho (Skordalakes and Berger, 2003). This model could explain why rho retains ATPase activity after helicase activity is over. The model also explains some of the previous observations where mutations in the primary binding site decrease the efficiency of termination, while mutations in the secondary binding site influence the location of termination (Tsurushita *et al.*, 1989). This may be because DNA remain bound to the primary binding site while the helicase / translocase depends more on tracking along secondary binding site.

Proposals to explain Rho's access to RNA-DNA hybrid to release RNA

Spooling of RNA by Rho: One of the methods by which Rho may release RNA from elongation complex is by acting like a spool with the centre of axis passing through its hole to wind RNA from the binding pocket of RNA polymerase (Fig. 5A).

Other mechanisms that can be further explored may be:

- The translocating Rho pushes the RNA polymerase forward to get an access to RNA-DNA hybrid and release RNA (Fig. 5B).
- Rho interaction with RNA polymerase changes the conformation of the RNA exit channel, thus allowing Rho to get access to the hybrid (Fig. 5C).
- Rho disengages RNA polymerase to get access to the RNA-DNA hybrid.

Properties of the elongation complex affecting Rho-dependent termination

While all the models talk about how Rho behaves, less emphasis has been given to the state of elongation complex that Rho uses as a substrate. Several lines of evidence actually point to a highly specific conformation of elongation complex preferred by Rho, for instance:

- RNA polymerase pausing or arresting outside the terminator zone is not acted upon by Rho,

- Strong pause or an arrested elongation complex may not be a termination point of Rho (Kassavetis and Chamberlin, 1981),
- While NusA causes decrease in RNA polymerase elongation rate and NusG increases it, these render opposite effects on Rho (Burns *et al.*, 1998). Does it suggest that these factors influence the conformation of elongation complex making it suitable or unsuitable for Rho action?
- Heterogenous system using *E. coli* Rho factor to induce RNA release from yeast RNA polymerase shows that RNA polymerase II, but not I and III is affected by Rho (Lang *et al.*, 1998) again points to a preferential state of conformation of RNA polymerase by Rho,
- Mutation in RNA polymerase can suppress termination defects of *rho* mutants (Guarente and Beckwith 1978; Jin *et al.*, 1988; Jin *et al.*, 1992; Sparkowski and Das, 1992).

Understanding biology of Rho through phylogeny: does it have any other function in vivo?

Phylogenetically, Rho belongs to the RecA like NTPase super family. This family is characterized by NTP binding domain of F1 and V1H+ ATPases. This family includes DnaB and related helicases, bacterial RecA and related eukaryotic and archeal recombinases. It also bears significant similarity with eukaryotic RNPs. Fig. 6 summarizes schematically the conserved domains of *E. coli* Rho through NCBI Blast. Genes with sequences related to Rho are wide spread even amongst the most diverged organisms suggesting a common ancestor for the bacterial, archeal and eukaryotic Rho homologues.

N-terminus, the domain for RNA binding, amongst Rho and Rho homologs is more variable than its ATP binding domain. The region with eukaryotic RNP1 like motif at N-terminus is highly conserved. RNP1 like sequence play a critical role in Rho loading to RNA, with the two Phe residues invariant amongst Rho from a wide range of bacteria (Opperman and Richardson, 1994). The RNP2-like motif, characteristic of eukaryotes, lacks in *E. coli* Rho. With regard to the RNP2-like domain, *E. coli* Rho shares sequence homology with single stranded DNA binding proteins of other bacteria.

The RNA binding domain of the GC-rich Gram-positive bacteria as compared to *E. coli* has long insertions of up to 260 residues. These insertions are highly diverse in nature (Nowatzke and Richardson, 1996). The observation that *M. lutues* Rho could terminate transcription along λ *cro* DNA with *E. coli* RNA polymerase at sites that are not accessible to *E. coli* Rho, points to the possibility that these inclusions in RNA binding domain help Rho to bind to structured GC-rich sequences in these organisms (Nowatzke and Richardson, 1996).

The ATP binding domain is the most conserved segment. The ATPaseA and ATPaseB motifs have blocks of conserved residues and are very similar to parts conserved in F1 ATPase β subunits. However, within the ATP binding domain there exist blocks specific to Rho protein and not to any other members of RecA superfamily, suggesting that these residues are specific to Rho function and therefore have been conserved exclusively among Rho molecules by strong selection during evolutionary process (Opperman and Richardson, 1994). Surprisingly, as a RNA-DNA helicase, Rho has insignificant similarity with other RNA helicases. DEAD box the signature of RNA helicases (Linder *et al.*, 1989) has no homology in Rho protein making it more diverse from RNA helicases than F1 ATPases (Opperman and Richardson, 1994).

With the fact that it has closer resemblance to proton pumps one can think of additional roles of Rho/Rho-like proteins in building a protein gradient across membrane or act like a proton driven rotatory molecule device like those of flagellated prokaryotes. Even though Rho homologues are present throughout bacterial world most of them have not been characterized for Rho functions as terminators (for instance, RV1297 in *Mycobacterium tuberculosis* is still annotated as a probable Rho even though it is an essential gene based on HIMAR 1 mutagenesis). The observation that Gram-positive bacteria are resistant to bicyclomycin and that Rho is not essential in certain bacteria like *Bacillus subtilis* and *Staphylococcus aureus* (Quirk *et al.*, 1993; Washburn *et al.*, 2001), points to its extra-terminator role in these organisms.

Despite of Rho being so conserved across the prokaryotic world, the Rho utilization (*rut*) sites are highly degenerated and unconserved. Probably, the only common feature of the *rut* sites amongst different rho-dependent terminators is it being rich in cytosine residues. Random nucleotide substitutions in the *rut*A site of λ *tR1* showed that Rho is able to recognize a wide variety of similar *rut* site sequences (Graham, 2004). An artificial terminator sequence of ~ 30bp encoding $(rUrC)_n$ sequence on the transcript is sufficient to the formation of rho-dependent terminator at a downstream non terminator site (Guerin *et al.*, 1998).

Physiological importance of Rho and rho-dependent terminators

As mentioned earlier, Rho protein is a transcription termination factor in prokaryotes (Roberts, 1969; Adhya and Gottesman, 1978; Platt, 1981). Rho originated early in the evolutionary time in prokaryotes, probably giving some adaptive advantages to the organisms. By far, the intragenic terminators (the terminator sequences within the coding region) are predominantly rho-dependent terminators. Under environmental stress, when translation machinery does not get sufficient amino acid substrates, translation gets uncoupled with transcription leading to accumulation of untranscribed naked RNAs. These untranslated RNAs may form duplex with single strand of DNA, displacing the other DNA strand as a loop. This condition, called ‘R’ loop formation, is unfavourable for the cell viability. It has been argued that factor-dependent termination have evolved to rescue cells from such transcription - translation uncoupling. Rho factor is thought to constitute one of such ‘rescue systems’ where, whenever ribosomes fall off a translating RNA, they bind to the exposed RNA and terminate the transcription, thus preventing a condition of the ‘R’ loop formation (Harinarayanan and Gowrishankar, 2003).

Rho recognition sites or the *rut* sites along a DNA sequence are also highly unstructured and degenerated. Physiologically, this might have an advantage over the intrinsic terminators. Owing to their non-structured nature, rho-dependent terminators can occur at sites where sequence of an intrinsic terminator would be incompatible. Further, the unconserved nature of *rut* sites helps Rho to bind more indiscriminately to these untranslated RNAs and halt the synthesis of such transcripts (Richardson, 1991).

Questions to be answered

The anatomy of Rho as a molecule has been studied fairly well in the last few years. However, how the individual properties of Rho are linked to release RNA in a transcription termination setup is not yet clear. Rho, in addition to having the present characteristic properties, may have more intrinsic features like capacity to harness energy production and utilization for translocation. There is no genetic or biochemical evidence specifying domains for the helicase activity in the structure of Rho. Also, the mutations outside the specified domains of Rho affecting its primary properties like RNA binding and ATPase activity need to be further explored.

The preference of ‘cytosine’ residues in the Rho recognition site is still ambiguous. The function of cytosines in the loading step of termination by Rho should be further studied.

Some of the other mechanistic areas in Rho-dependent termination that has not been explored sufficiently include queries like how RNA binding elicits an ATPase activity or how Rho interacts with RNA polymerase to get access to the RNA inside a transcribing RNA polymerase to unwind and release it. Further, we need to test some of the proposed models to confirm if RNA release is due to spooling of RNA by Rho from the binding pocket of RNA polymerase or the tranlocating Rho pushes the RNA polymerase to get access and release RNA. We strongly feel that not all state of conformation of elongation complex can be accessed by Rho for termination. Therefore the precise needs of Rho from an elongation complex should be systematically studied.

The most challenging area would be to find other physiological functions of Rho, for instance, if it works like a molecular pump. Also, in some prokaryotes Rho is dispensable, or absent altogether. These suggest that Rho may have other ‘dispensable’ function(s) than being an essential partner in the termination machinery.

Rho is a mysterious and interesting molecule, catering an essential function to the cell. Even after thirty five years of its discovery we stand with more questions than answers. Understanding the mechanism of termination of Rho in detail could further highlight the steps that could serve as the probable site of action of Rho inhibitors. Since Rho is essential for the viability of most of the bacteria, including some pathogenic ones, the whole spectrum of information on Rho-dependent termination and anti-termination can be harnessed to design inhibitors systematically.

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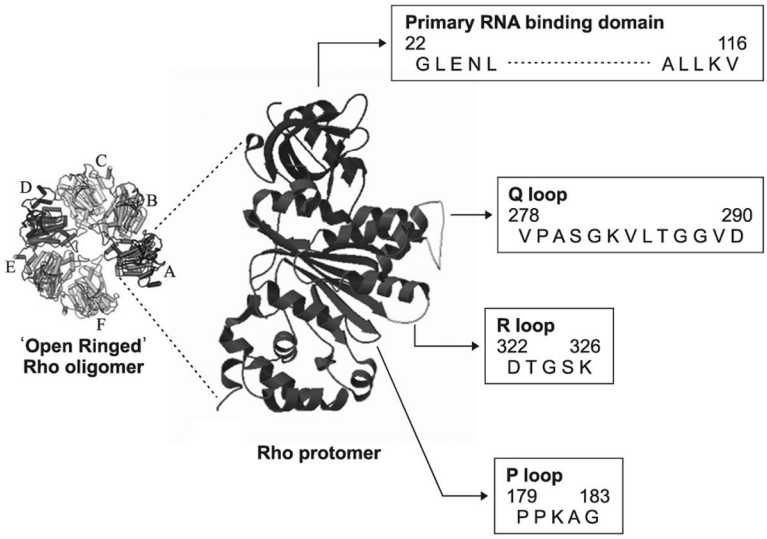

Fig. 1.

Functional domains of a Rho protomer of *E. coli* Rho. The figure shows an open 'key washer' like pentameric form of Rho. Each protomer has four defined functional domains: Primary RNA binding domain that can bind to a single stranded DNA molecule as well as single stranded RNA molecule extending from residues 22 to 116; P-loop, for ATP binding and ATPase activity, includes residues from 179-183; Q-loop, from 278-290 and R-loop from 322-326 comprising the secondary RNA binding site of Rho.

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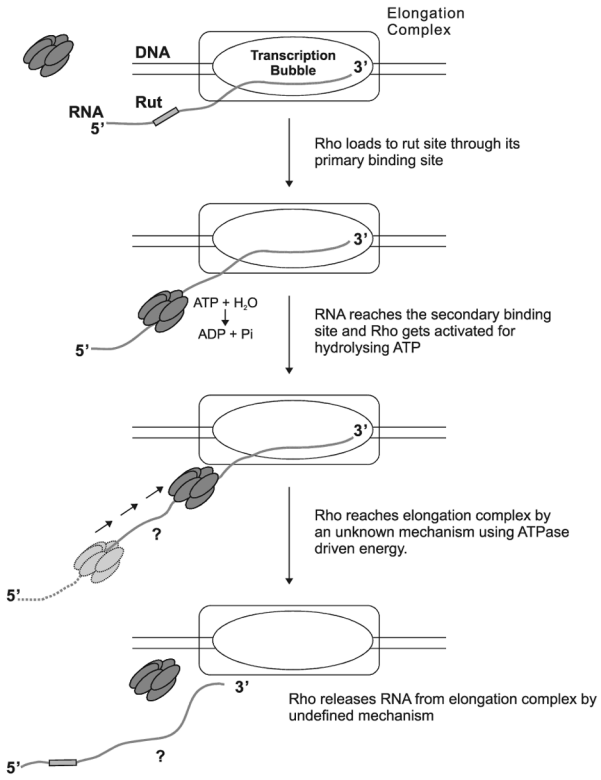

Fig. 2.

An overview of the steps involved in the termination by Rho. The exact phenomenon of Rho translocation, interaction with elongation complex and finally unwinding and releasing RNA are still ambiguous.

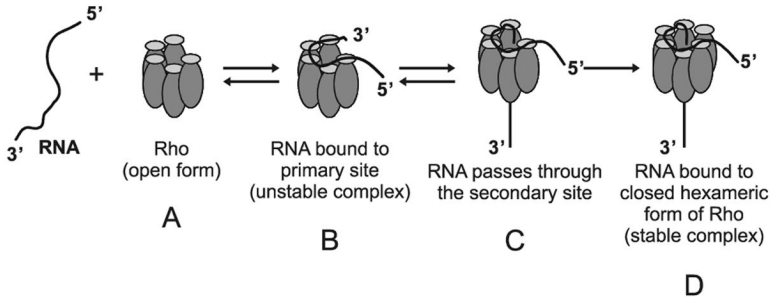

Fig. 3.

Events of Rho loading and formation of ATPase competent intermediates (based on Kim and Patel 2001). RNA randomly collides with the Rho molecule forming a dynamic Rho-RNA complex. RNA then wraps around the primary binding site of each of the five protomer of the Rho open complex forming a stable moiety. The RNA subsequently passes towards the center of the open Rho ring making contact with the secondary binding site of the Rho molecule forming a steady complex. Once RNA enters the 'ring' of Rho, the open conformation gets closed by a sixth subunit, leading to the formation of a highly stable ATPase competent moiety.

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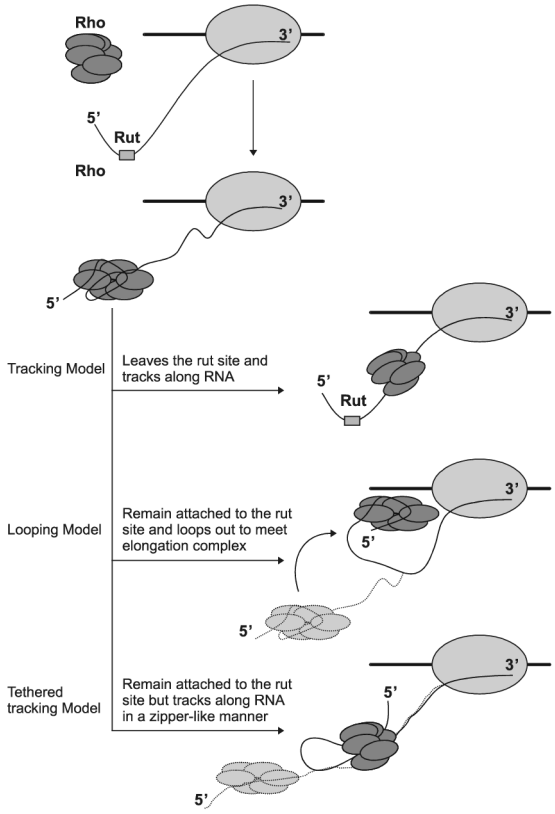

Fig. 4.
Translocation models of Rho along the template. Tracking, looping, and tethered tracking models.

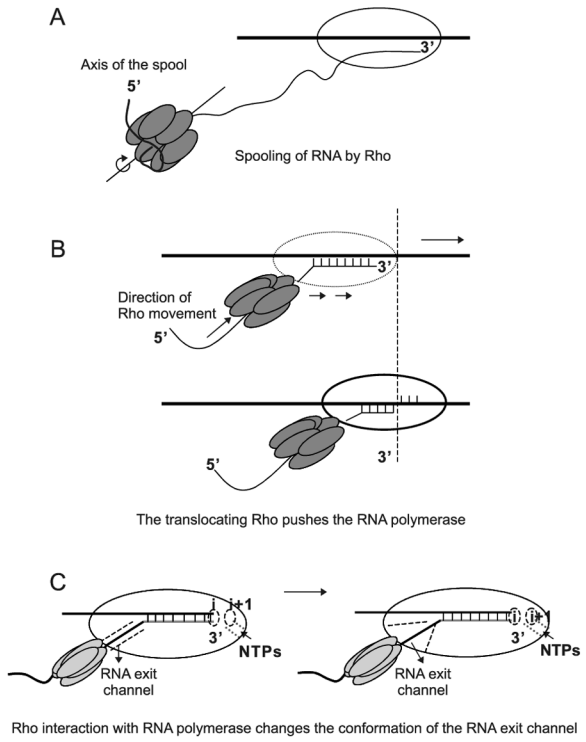

Fig. 5.

Models to explain Rho's access to RNA-DNA hybrid and release RNA. A, it spools RNA from a distance without making contact with elongation complex. B, Rho forward translocates the elongation complex to get access to the RNA. C, Rho contact with elongation complex changes the conformation of RNA exit channel.

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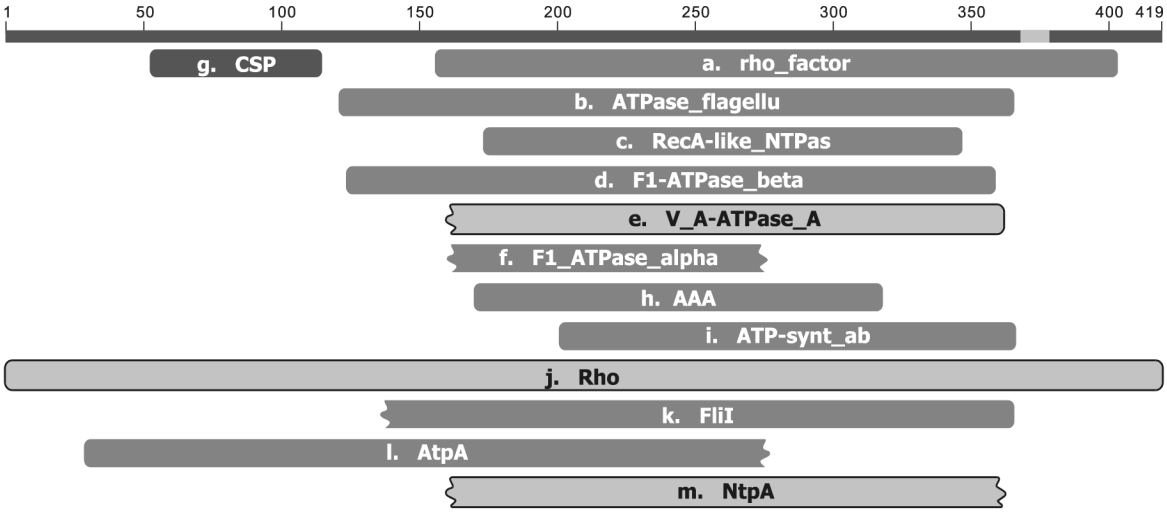

Fig. 6.

Schematic representation of the conserved domains of *E. coli* Rho through NCBI Blast. **a.** Rho-factor: Transcription termination factor Rho is a bacterial ATP-dependent RNA/DNA helicase. **b.** ATPase-fllagellum or Flagellum-specific ATPase/type III secretory pathway virulence-related protein: This group of ATPases are responsible for the export of flagellum and virulence-related proteins. The bacterial flagellar motor is similar to the F_0F_1-ATPase, in that they both are proton driven rotary molecular devices. **c.** RecA-like NTPases: This family includes the NTP binding domain of F1 and V1 H^+ATPases, DnaB and related helicases as well as bacterial RecA and related eukaryotic and archaeal recombinases. **d.** F_1 ATP synthase beta subunit, nucleotide-binding domain: The F-ATPase is found in bacterial plasma membranes, mitochondrial inner membranes and in chloroplast thylakoid membranes. It uses a proton gradient to drive ATP synthesis and hydrolyzes ATP to build the proton gradient. **e.** V/A-type ATP synthase catalytic subunit A. These ATPases couple ATP hydrolysis to the build up of a H^+ gradient, but V-type ATPases do not catalyze the reverse reaction. The Vacuolar (V-type) ATPase is found in the membranes of vacuoles, the golgi apparatus and in other coated vesicles in eukaryotes. **f.** F_1 ATP synthase alpha, central domain. The F-ATPase is found in bacterial plasma membranes, mitochondrial inner membranes and in chloroplast thylakoid membranes **g.** CSP, Cold shock protein domain; RNA-binding domain that functions as a RNA-chaperone in bacteria and is involved in regulating translation in eukaryotes. Contains sub-family of RNA-binding domains in the Rho transcription termination factor. **h.** AAA-ATPases associated with a variety of cellular activities. **i.** ATP synthase alpha/beta family, nucleotide-binding domain. This family includes the ATP synthase alpha and beta subunits the ATP synthase associated with flagella. **j.** Rho: Transcription termination factor. **k.** Flagellar biosynthesis/type III secretory pathway ATPase: involved in cell motility and secretion/Intracellular trafficking and secretion. **l.** AtpA: F_0F_1-type ATP synthase, alpha subunit involved in energy production and conversion. **m.** NtpA: Archaeal/vacuolar-type H^+-ATPase subunit A involved in energy production and conversion.