CHAPTER VII

Conclusions and Future perspectives
7.1 Conclusions

Discovery of the Rho-dependent transcription termination helped to envision the antitermination function of the N protein (Gottesman et al., 2004) that was subsequently established by showing the N-mediated suppression of the polarity in gene expression caused by Rho (Adhya et al., 1974). More recently it has been proposed that the requirement of the well conserved Rho function in bacteria is to prevent the unwanted gene expression from the invading foreign DNAs, like bacteriophage DNA (Cardinale et al., 2008) and as a corollary to this hypothesis, it can be stated that the bacteriophages evolved the antitermination machinery to overcome the ubiquitous Rho-dependent termination of the host.

The exact molecular mechanism by which N protein brings about transcription antitermination at Rho dependent terminators remains unknown, despite efforts to this end for the last four decades or so. The objective of this study was to get a better understanding of the mode of antitermination of N protein at the Rho dependent terminators and to understand the mechanistic details of this process. Antiterminators like N in λ and in related lambdoid phages convert RNA polymerase into a termination resistant form, during early phases of transcription elongation. N protein binds to its RNA ligand nut boxB and to RNA polymerase and together with host factors called Nus factors, transforms it into a highly processive, termination resistant transcription apparatus. We used the transcription antitermination system from a lambdoid bacteriophage H-19B, due to its reduced requirement of Nus factors for processive antitermination, which made it more suitable for in vivo and in vitro studies. Genetic as well as Biochemical studies were carried out to underline the various mechanistic aspects of this process.

7.1.1 A multi-pronged strategy of N to overcome Rho

The small antiterminator protein N has three interacting regions. They interact 1) with the nut site on mRNA (Lazinski et al., 1989), 2) with the nut-bound NusA (Mah et al., 1999) and 3) with the RNAP (Mah et al., 1999). Here we show that N uses all these three interaction modules to develop a multi-pronged strategy to overcome the Rho action.

Strategy I: In Chapter III and IV, we show that N-NusA-Rho co-occupies the nut/rut site and this configuration slows down the initiation of ATP hydrolysis process of Rho.
(discussed in Chapter IV). This in turn slows down the translocase as well as the RNA release kinetics of Rho. Most likely the presence of N and NusA at the nut site affects the proper placement of the downstream RNA sequence into the central hole of the Rho hexamer. This strategy of N functions independent of the N-CTD-RNAP interaction (discussed in chapter III).

**Strategy II:** N-CTD interacts with RNAP near the RNA exit channel and may use this channel to penetrate a part of its "thread-like" CTD into the interior of the EC (Cheeran et al., 2005; 2007). This interaction becomes important to prevent Rho action when the EC moves away from the nut site which allows Rho to freely translocate along the nascent RNA present between the nut site and the EC (discussed in Chapter III). Although it has not been proved, the RNA exit channel could be the likely area of EC through which Rho gains access to the RNAP. Presence of N-CTD in the vicinity of the exit channel may function as a lid to the Rho-access point and prevent/delay the putative Rho-RNAP interaction.

**Strategy III:** In Chapter V, we report the unusual dependence of the E134K Rho on NusA for efficient termination and its suppression activity of N function. This led us to propose that Rho and N compete for the same NusA molecule. N-NusA interaction removes NusA from the Rho-dependent termination pathway and makes the latter process less efficient.

"**NusA remodelling**, a major antitermination mechanism of the antiterminators**

NusA interacts with RNA polymerase and also with the nascent RNA emerging out of the EC (Sen et al., 2008; Roberts et al., 2008). It is an important component for both the termination and the antitermination processes. NusA improves the efficiency of hairpin-dependent termination likely by stabilizing the RNA hairpins of the terminators (Roberts et al., 2008; Schmidt et al., 1987; Artsimovitch et al., 2000). It is also involved in Rho-dependent termination (Burns et al., 1998; Cardinale et al., 2008; Saxsena et al., 2011). On the other hand, antiterminators like N- and Q- functions are highly NusA-dependent (Santangelo et al., 2011; Roberts et al., 2008). N makes NusA more specific to nut site (Prasch et al., 2009) and also changes its mode of interaction with RNAP (Gusarov et al., 2001), whereas in the presence of Q protein, NusA forms a shield at the RNA exit channel (Shankar et al., 2007). This specific interaction of the antiterminators with NusA
leads to a "NusA-remodelling" and its subsequent removal from the termination pathway by following means.

1) As Rho and NusA binding sites ("spacer" region; discussed in chapter IV) at the nutR/tR1 overlap, the high affinity N-NusA interaction at the nut site may make NusA unavailable to Rho during its loading to and activation by the nut RNA.

2) The proposed N- (Gusarov et al., 2001) or Q-induced (Shankar et al., 2007) changes in the interaction surface of NusA on the RNAP is likely to affect the putative Rho-RNAP interaction or the terminator hairpin folding at the RNA exit channel.

We propose that "NusA-remodelling" is a major mechanism to overcome both the Rho-dependent and –independent terminations by the antiterminators in addition to stabilizing the transcription elongation complexes.

What is the role of NusA in the Rho-dependent termination?

Involvement of NusA in Rho-dependent termination has been implicated in different reports (Zheng et al., 1994; Burns et al., 1998; Cardinale et al., 2008; Saxsena et al., 2011). The role of NusA in this process is still unknown. Here for the first time, we report a Rho mutant, E134K, whose function is highly dependent on NusA both in vivo and in vitro (Chapter V). NusA improves the termination efficiency of E134K by increasing the rate of RNA release and stimulating the NusG function. We suggest that the secondary RNA binding defect of E134K (discussed in Chapter V) is rectified by NusA-mediated chaperoning of the RNA into the secondary channel and this stabilization of RNA in the central hole may also stimulate the NusG function. Based on these results we propose that the role of NusA is important for a subset of Rho-dependent terminators, where Rho-loading onto the RNA and subsequent activation step(s) are rate-limiting. In these terminators, due to the structural constraints, the nascent RNA cannot be placed properly into the central hole of the hexameric Rho, thereby affecting its "open" to "close" isomerization step(s) as well as the rate of initiation of ATP hydrolysis. Analogous to the chaperoning role of NusA for the Rho-independent terminators with imperfect RNA hairpins, we envision that it also functions as a RNA-chaperone to guide the nascent RNA into the central hole of the hexameric Rho.
7.1.2 Understanding the role of NusG in N mediated antitermination at factor dependent terminators

The two Nus factors, NusA and NusG, which are integral part of N mediated antitermination, are also engaged by Rho to form a termination competent configuration. While isolation of E134K mutant of Rho brought an understanding of the importance of NusA in this regard, the importance of NusG in this respect was accessed by first establishing the surface of contact between Rho and NusG. Once this was known, the effect of NusG mutants that were not capable of interacting with Rho was assessed on N mediated antitermination, with the logic that N may remove NusG from Rho-dependent termination pathway by competing for interaction with NusG-CTD at the same site where Rho interacts with it. Sequestration of NusG by N has been speculated earlier (Li et al., 1993; Washburn et al., 1996).

We observed that in vivo NusG-CTD mutants defective for Rho binding did not have any effect on N function. Hence we concluded that N functions independent of Rho-NusG CTD interaction. However, it is not clear to us whether the requirement of NusG in the N-antitermination machinery perturbs the Rho-NusG complex formation.

7.2 Future perspectives

Though the study presented here has given an indication of the mechanistic details of the mode of antitermination by N at Rho dependent terminators, it also brings up some interesting pursuable ideas for future.

In Chapter IV we report how N slows down the kinetics of RNA release of Rho from a stalled Elongation Complex. This effect of N can be re-probed by Fast Kinetic Approaches, and it possibly will help in understanding the mechanism of antitermination by N better. Similarly, single molecule experiments to probe the behavior of individual Rho and N molecules during this process can be carried out.

In chapter V, we report about a termination defective Rho E134K, which becomes extremely termination proficient in presence of NusA and NusG, and can also suppress N function in presence of these host factors at Rho dependent terminators. Further studies can be carried out to understand its termination defect. There is a possibility that the interaction of this Rho mutant with RNA polymerase may be different than WT Rho. This interaction can be probed by biochemical means as well as by biophysical studies like
fluorescence spectroscopy. Genetic studies for the same may involve looking for RNAP mutants that can suppress the termination defect of E134K Rho. The influence of NusA on the termination properties of E134K Rho mutant is strikingly different than its effect on WT Rho, and moreover we propose that remodeling of NusA contacts with E134K Rho and N to be a possible mode of the antitermination suppression observed in presence of E134K. Therefore, interaction and functional influence of NusA on this Rho mutant can be taken up in more detail. Mutants in NusA that will restore the properties of E134K Rho back to the WT Rho phenotype can be looked for. We also report a slow RNA release kinetics of E134K from stalled Elongation Complex which is improved in the presence of NusA. RNA release kinetics of E134K Rho in presence and absence of NusA and/or NusG must be studied by ‘Fast Kinetic Approaches’ to underline the subtle changes caused by these host accessory factors.