**In vivo regulation of bacterial Rho-dependent transcription termination by the nascent RNA**

Bacterial Rho is a RNA-dependent ATPase that functions in the termination of transcription. The *in vivo* nature of the bacterial Rho-dependent terminators, as well as the mechanism of the Rho-dependent termination process, are not fully understood. Here, we measured the *in vivo* termination efficiencies of 72 Rho-dependent terminators in *Escherichia coli* by systematically performing qRT-PCR analyses of cDNA prepared from mid-log phase bacterial cultures. We found that these terminators exhibited a wide range of efficiencies, and many behaved differently *in vivo* compared to the predicted or experimentally determined efficiencies *in vitro*. Rho-utilization sites (rut sites) present in the RNA terminator sequences are characterized by the presence of C-rich/G-poor sequences or C > G bubbles. We found that weaker terminators exhibited a robust correlation with the properties (size, length, density, etc.) of these C > G bubbles of their respective rut sites, while stronger terminators lack this correlation, suggesting a limited role of rut sequences in controlling *in vivo* termination efficiencies. We also found that *in vivo* termination efficiencies are dependent on the rates of ATP hydrolysis as well as Rho-translocation on the nascent RNA. We demonstrate that weaker terminators, in addition to having rut sites with diminished C > G bubble sizes, are dependent on the Rho- auxiliary factor, NusG, *in vivo*. From these results, we concluded that *in vivo* Rho-dependent termination follows a nascent RNA-dependent pathway, where Rho-translocation along the RNA is essential and rut sequences may recruit Rho *in vivo*, but Rho-rut binding strengths do not regulate termination efficiencies.

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quantitative measurements of in vivo Rho-dependent termination efficiencies in different genes and in vivo regulation of these terminators by the nature of their cognate rut sites are not known. The in vivo Rho-dependent terminator strength, as well as the efficiency of the termination process, should be different from those obtained by in vitro measurements because the in vivo process could be affected by the mRNA-binding of ribosomes and other RNA-binding proteins, availability of NusG-CTD (NusG-CTD also binds to the ribosome) to interact with Rho, presence of different cis-acting elements like RNA-hairpins and riboswitches, Rho-antagonists, etc. It is also important to know how the nature and pattern of the C > G bubbles (the “descriptors”, (9)) are correlated with the in vivo termination efficiencies of the various Rho-dependent terminators, which in turn would decipher whether the rut sites regulate the vivo-termination process as well as the existence of the RNA-dependent pathway in vivo.

In this study, we have employed RT-qPCR analyses of 72 genes to identify the presence of Rho-dependent terminators and in vivo termination efficiencies of these terminators using E. coli strains expressing termination-defective Rho mutants and Rho-binding defective NusG mutants. These terminators exhibited a wide range of efficiencies and many of them behaved differently in vivo compared to that predicted or experimentally shown in vitro (9, 17). The weak or moderately strong terminators, under in vivo conditions, showed a positive and robust correlation with the nature (size, length, density, etc) of the C > G bubble stretches (described as “descriptors”) of the rut sites. This correlation disappeared as the terminator strength increased suggesting a limited role of the nature of rut sequences in controlling the termination efficiencies in vivo, even though the binding affinities of Rho to the rut sites were dependent on the nature of the C > G bubbles. The termination efficiencies of the majority of the terminators showed good positive correlations with their respective rates of ATPase activities of Rho on the RNA templates having the terminator-zone sequences. This indicates the Rho-translocase activity-dependence of the majority of the terminators in vivo. Consistent with the earlier proposals (18, 19), the in vivo termination efficiencies for the weak and moderately strong terminators exhibited dependence on NusG. The highly NusG-dependent terminators have significantly reduced the area, length, and density of the C > G bubbles. These data indicate the existence of nascent-RNA–dependent pathways in vivo, where Rho translocation along the RNA is essential for the termination.

Results

Measurements of in vivo termination efficiencies of the Rho-dependent terminators

Several genomics studies revealed the existence of genes as well as operons, expressions of which are under the control of Rho-dependent termination in vivo (10, 29–31). More recently, Nadiras et.al., 2018 has predicted genomic regions, which function as Rho-dependent terminators based on the dataset obtained from the in vitro transcription termination assays. We chose genes that are shown by the above-mentioned studies to be under the regulation of Rho-dependent termination (Fig. S1). We primarily chose the genes that are upregulated in the microarray profiles under the conditions when Rho function is compromised (detailed in Fig. S1). Later, more genes upstream or downstream of these chosen genes as well as some genes whose functional characterizations were ongoing in our laboratory were added to the primary list. We compared the level of transcribed RNA from these genes in the WT MG1655 strain relative to those obtained in the strains expressing the Rho mutant N340S and the NusG mutants, G146D, L158Q, and V160N (Fig. S2). This Rho mutant is defective in secondary RNA binding as well as severely defective in ATPase activity (17), whereas, the NusG mutants have strong binding defects for Rho (14). And hence, both the Rho and NusG mutants have defective Rho-dependent termination functions resulting in read-through transcription into the genes, which otherwise is terminated in the WT variants. This is manifested as upregulation of the expressions of the downstream genes in an RT-qPCR assay (Fig. 1A). Multiple primers probes were used to experimentally validate the presence of Rho-dependent termination zones upstream of these probes sites (Fig. 1, A–C). The length of the RNA transcripts of different genes that were analyzed in these assays varied from 91 to 5914 nt (Figs. 1, B and C, and S3). The enhancements of the RT-qPCR signals of each gene in the Rho or the NusG mutants relative to the WT strain give a measure of in vivo gene expression in those strains. The relative enhancements of gene expressions are expressed in fold-change as indicated in Figure 1, D and E. As the termination zones are present upstream of these genes (Fig. 1, A–C), the fold-change values of each gene give a semiquantitative measure of the in vivo termination efficiencies of the terminators present in these terminator zones. The higher fold change values indicate the higher termination efficiency and the presence of a stronger terminator.

The rut sites within a terminator zone are characterized by C > G sequence bubbles in the RNA sequences (sequence of non-template DNA) (2). We scanned and analyzed the sequences upstream of 72 genes that were used in the RT-qPCR assays for the presence of C > G bubbles (Fig. 1, F and G; see Experimental procedures section). We followed the methodology described by Nadiras et al. 2018 to define and calculate the descriptor values based on the length, size, width, etc of these bubbles (Fig. S4; definitions of each descriptor are stated in the Experimental procedures section under “sequence selection and analyses of rut sites” and also in the Fig. S4).

In vitro termination efficiencies of many Rho-dependent terminators have been measured, and an algorithm developed from these experimental data was used to predict their strength (9). However, in vivo termination efficiencies of these genes should be quite different as Rho-dependent termination faces many competing processes inside the cell (see Introductory paragraphs). We measured the upregulation (in terms of fold-change) of 72 genes in a strain expressing the Rho N340S mutant relative to that expressing the WT variant by RT-qPCR (Fig. 2A). Among them, some regions were
previously identified to have Rho-dependent terminators in an in vivo 3’-end mapping study (31). The fold change values as well as the in vivo termination efficiencies varied over 2 log scales. Based on these fold-change values, we classified the terminators into the following groups: weak or nonterminator (<2 fold), moderate (2–10 fold), strong (10–30 fold), and very strong (>30) (Fig. 2A). Out of the 54 terminators that belong to the very strong to the moderately strong category, 31 were predicted to be strong from the in vitro data (9), whereas out of 17 terminators in the weak or nonterminator category, only 8 of them were predicted to be weak. Interesting to note that out of five terminators that could not be predicted properly (white bars), four were good terminators in vivo. This mismatch between the in vitro and in vivo might indicate that
rut sequences *per se* do not control Rho-dependent termination *in vivo*. Since we expressed the *in vivo* termination efficiencies as fold change of the RNA expression levels relative to the WT strain, possible interferences from the presence of the multiple transcription start-sites or Rho-independent terminator signals should be nullified. However, these measurements assumed that both the mutants and the WT strains behaved in a similar manner other than the Rho-dependent termination.

We observed that terminators present upstream of the prophage genes (CP 4-6: *ykfG*, *yafZ*, *ykfl*, *yagM*, and *yagL*; Qin prophage: *ydfl*; CP 4-57: *yflfl*) are the strongest Rho-dependent terminators, which are essential to suppress the toxic prophage genes (31–34). Two terminators located upstream of the tRNA
genes (thrW and selC) also belonged to the very strong/strong category. They were not categorized under any group in Nadiras et al. 2018. An RNA folding analysis of these two genes revealed the presence of extensive secondary structures, which could have affected theirs in vivo characters. It has been reported that tRNA genes are highly regulated by Rho-dependent termination (29, 35). We also observed the presence of a very strong terminator in the riboswitch region of thiC and thiM (36). The functional data of these genes correlate well with our measurements of their termination efficiencies (Fig. 2A).

We tested how the three Rho-dependent terminators, trpt', λT_R, and f_rac, that are widely used in in vitro studies behave under in vivo conditions. We observed that in vivo efficiencies of these terminators fall under the moderate to strong categories. Interestingly, the prophage terminators are much stronger than these standard terminators.

The fold change values obtained in these RT-qPCR assays could be influenced by the in vivo stability and the length of the RNA transcripts used for preparing the complementary DNAs (cDNAs). We calculated the length of the RNA from the transcription start site (TSS; considering only the σ70 promoters that are predominantly experimentally verified as mentioned in the EcoCyc database) to the sites of the reverse primer (RP) probe of the gene (Fig. S3). Since we have harvested the mid-log phase cultures for our measurements, the majority of transcription is expected to be from σ70 promoters. And hence, we chose only the σ70 promoter-initiated transcripts in our analyses. The lengths of the theoretically calculated RNA and the free energy (ΔG) for their duplex formation did not correlate with the measured fold changes obtained in Figure 2A (Fig. 2, B and C). This indicated that the high RT-qPCR values did not arise from longer and more stable RNA. Next, we experimentally determined the in vivo stabilities of the 12 RNA transcripts transcribed from the selected genes that showed a wide range of fold change values in Figure 2A. We isolated RNA at different time points from the mid-log phase culture of an MG1655 strain expressing Rho N340S mutant and performed RT-PCR to quantitate the time-dependent decay of the RNA (Fig. S5, A and B) and calculated their half-lives (Fig. 2D). A plot of half-lives against their corresponding fold change values (obtained from Fig. 2A) did not show any correlation. These data strongly indicated that the fold change values described above were not influenced by the in vivo RNA stability and they truly represent the in vivo termination efficiencies of the 72 terminators. It should also be noted that the general stability of RNA transcripts in the Rho termination efficiency (72 terminators). It should also be noted that the general stability of RNA transcripts in the Rho-dependent regulation of Rho-dependent termination

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Next, we experimentally verified whether the rut sites with higher C > G descriptor values have higher binding constants (K_R) for the Rho protein (Fig. S9, A–D). We synthesized radiolabeled RNAs having the terminator zone sequences of selected genes, termination efficiencies (fold change values) of which were measured in Figure 2A (Fig. S9, A and B). We have synthesized >200 nt RNA sequences so that all the putative rut sites of a terminator zone are accommodated. These
Figure 3. Correlation between descriptors and the in vivo termination efficiencies. The fold change values obtained from Figure 2A are plotted against the indicated descriptor values. In (A), scattered values of the fold changes (x-axis in log10 scale) are plotted against different descriptors, in (B), fold change values up to 10-fold (x-axis in linear scale) are plotted, whereas in (C), those with >10-fold values (x-axis in linear scale) are plotted against the indicated descriptors. In all the plots, the scattered plots were fitted to straight lines to establish the linear relationship and the correlation coefficients. The correlation coefficient is indicated. Calculations of values of descriptors are described in the Experimental procedures section.
terminators have a wide range of fold change values (0.7–78 fold). We performed gel-shift assays using WT Rho to calculate the $K_d$ values of each of the Rho–RNA transcript interactions (Figs. S9, A, B, F and G). The sigmoidal nature of the binding isotherms indicated the presence of multiple Rho-binding sites in these RNA transcripts (38). We used a Rho-PBS mutant, Y80C, that has reduced affinity for the rut sites (17), as a control. On some of the tight-binding rut sites, this mutant exhibited significantly reduced affinity compared to the WT (Fig. S9G), which indicated that the Rho-interactions with the high-affinity rut sites are specific. However, it should be noted that the difference between specific and nonspecific binding at the weaker rut sites would be much less. Upon plotting the $K_d$ values against the descriptor values of the rut sites present in these synthesized RNA transcripts, we observed a moderate correlation ($r^2 = 0.44$) with the length of the C > G bubbles (Fig. S9, C and D). This indicates that the higher descriptor value of a rut site could reflect higher binding affinity (lower $K_d$) toward Rho. An increase in the length of the C > G bubble increases the length of C-rich sequences, and hence the binding constant increases. Interestingly, these measured $K_d$ values did not show any correlation with the fold change values of the respective terminators that these rut sites belong to (Fig. S9E). This indicates that the binding strength of the rut sites for Rho does not influence the termination efficiency, even though these sites are required to recruit Rho to the elongation complex at some stage. Post-RNA recruitment steps such as Rho-isomerization (18) and translocation along the RNA could be more important parameters to determine the termination efficiencies. It should be noted that the in vitro synthesized RNA could have a different folded structure compared to the in vivo RNA-folding pattern, which could have contributed to the apparent mismatch between the in vitro binding constants and the in vivo termination efficiencies.

**Dependence of in vivo termination efficiencies on the rate of ATP hydrolysis of WT Rho**

Rho is an efficient molecular motor that can translocate along the RNA driven by its RNA-dependent ATP hydrolysis activity, and this translocation activity has been implicated in its transcription termination function (3, 4, 6). We tested the extent of dependence of in vivo termination efficiency of WT Rho on its rate of translocase activities. As ATP hydrolysis is the driving force behind translocation, the rate of ATPase activity of Rho would be proportional to the rate of its translocase activity. We synthesized RNA templates having the rut sites of the terminator zones of several genes, in vivo termination efficiencies of which were measured in Figure 2, and measured the rates of ATPase activities of WT Rho on these templates (Fig. 4A). These templates were able to induce ATPase activity of WT Rho with a wide range of rates in the in vitro assays (172–0.12 pmol/min; Fig. 4B). The template with the sequence of terminator zones of gfcA yielded the highest rate that was still 3-times lower than that we achieved with the best synthetic substrate of Rho, polyC RNA. This data indicates that the Rho most likely translocates with a wide range of rates on the different nascent RNAs from different operons in vivo, which could have variable effects on its termination efficiencies.

We next plotted these rates of ATPase activities against the fold change values of the corresponding genes (see Fig. 2A) from which these RNA transcripts were prepared. We observed a positive correlation ($r^2 = 0.62$) between these two parameters for most of the terminators, except those having very high in vivo termination efficiencies (fold change values >40-fold; yagM, sugE, ydeT, and thiC; Fig. 4, C and D). Among them, the thiC terminator is part of the thymine pyrophosphate–activated riboswitch (36), which might have affected its termination efficiency in addition to the translocase rate. These data strongly indicate that in general, the in vivo termination efficiency is dependent on the rate of translocase activity of Rho, and the RNA sequences of the terminator zone induce variable translocation speed. However, terminators with very high termination efficiency could also be under the control of in vivo factors in addition to the rate of translocase activity.

**Dependence of rate of ATPase activities on the descriptor values**

Next, we explored the dependence of the rate of ATPase activity on the C > G bubble sequence patterns of the rut sites. This also enabled us to understand how the descriptor values of the rut sequences influence the Rho-termination efficiency via controlling the translocase activity. Upon plotting the rates of ATPase activities (shown in Fig. 4) against the descriptor values of the corresponding terminators, we observed a moderately good positive correlation ($r^2 > 0.4$) with the descriptor values that describe the area and length of the C > G bubbles (Fig. 5, A and B). This correlation was observed for the ATPase rates < 50 pmol/min. The highest correlation was observed with the descriptor that defines the length of the longest C > G bubble. The descriptors defining other parameters of the C > G bubbles did not show good correlations (Figs. S10 and S11). These data indicate that the C > G bubble patterns of the rut sites can influence the ATPase rates to a certain degree (up to 50 pmole/min). As the rate of ATP hydrolysis is dependent on the Rho-binding and isomerization on the rut sites, this correlation is expected. These two steps are rate-limiting and are dependent on the C > G bubble sequences. Hence, the bubble sequences primarily influence the rate of ATPase via these initial steps. However, a very high rate of ATPase activity on certain terminators like gfcA, setC, etc, could be dependent on the postrecruitment steps such as the presence of duplex structures or the presence of long unhindered stretches of single-stranded regions on the RNA transcripts encountered by Rho during the translocation.

**Effects of intrinsic pause sequences on the Rho-dependent termination efficiencies**

So far, we have described the dependence of the in vivo termination efficiencies on the Rho–rut interaction, Rho translocation, and the sequence patterns of the C > G bubbles.
However, the very high termination efficiencies of certain terminators did not correlate with these parameters, indicating their regulation by other factors. The transcription EC faces numerous pause sites that slow down its rate of elongation, which could facilitate the Rho-dependent termination efficiency. We reasoned that the EC could encounter pause sequences in the genes that allowed Rho to function with very high termination efficiencies (>30 fold, Fig. 2). Using the database that describes the elemental pause sequences, GGcataatTG(C/T)GGCcg (39), we found that these pause sites are present in thiC, sbp, and yegQ (Fig. S12). We inferred that these pause sequences might have contributed to their respective termination efficiencies in addition to the above-mentioned nascent RNA-dependent parameters.

**NusG dependency of the in vivo termination efficiencies of various Rho-dependent terminators**

A subset of Rho-dependent terminators is dependent on NusG (10). We explored the in vivo NusG-dependence of the Rho-dependent terminators associated with 72 genes that we described in Figure 2A and the existence of NusG-specific
Figure 5. Relationship between rates of ATPase activity and the descriptor values. In (A), scattered plots of rates of ATPase (x-axis in log_{10} scale) activities against the indicated descriptor values, and in (B), the rates of ATPase activities (x-axis in linear scale) up to 50 pmol/min were plotted against the indicated descriptor values and were fitted to a linear equation to obtain the correlation coefficients. The correlation coefficients are indicated in each plot.
signature(s) in the C > G bubble sequences of these terminators. We chose MG1655 strains that express the Rho-binding defective NusG mutants, G146D, V160N, and L158Q (14) that cause reduced in vivo termination efficiency of Rho. We measured the gene expressions of these genes in the NusG mutants using RT-qPCR and calculated the fold changes in gene expression relative to the WT strain in the same way as described in Figure 2 (Fig. S13, A and B). We calculated the average fold change values from the fold change data of all the three NusG-mutants (Fig. 6A).

A wide range of average fold change values of the 72 genes was obtained in the presence of the NusG mutants (Figs. 6A, S13, A and B). However, the values were less compared to that obtained in the presence of the Rho mutants, N340S (compare with Fig. 2A), and also the NusG- and Rho-mutant data sets did not follow a linear correlation. In general, for the strong terminators, the fold change values obtained in the Rho mutant are much higher than that observed in the NusG mutants (e.g., yagL showed 15.6 fold in the NusG mutants and 66.6 fold in the Rho mutant). But in cases of weak or moderately strong terminators, these differences in the upregulation (fold change) were observed to be much less. As NusG-dependent terminators are a subset of Rho-dependent terminators and the NusG dependency of all the Rho-dependent terminators varies considerably (Figs. 6A and S13), we reasoned that a quantitative measure of the in vivo NusG-dependency would be obtained properly if the ratios of average fold change obtained in the NusG mutants to that in N340S Rho are plotted against the fold changes obtained in the N340S Rho mutants (Fig. 6B). The plot showed a biphasic nature, where the ratio of fold-change values > 0.5 was considered to be highly NusG-dependent (21 genes). Values within 0.2 to 0.5 were classified as moderately NusG-dependent (31 genes) and the values < 0.2 were classified as NusG-independent (20 genes). Most of the terminators that are categorized as none or weak terminators in Figure 2A were also observed to be highly NusG dependent in vivo. Hence, the nature of in vivo NusG dependency of these 72 terminators was consistent with the mechanism of NusG action proposed from the in vitro studies (18, 19).

**Signatures of NusG-dependent terminators**

It is not clear which rut site characteristics are important for a Rho-dependent terminator to become NusG-dependent. So, we explored the descriptor values of different C > G bubble stretches of the NusG-dependent terminators obtained from Figure 6B to identify their rut-site signatures. We analyzed the distribution of different descriptor values for all the NusG-dependent and NusG-independent terminators and expressed them in the form of box plots (Figs. 7, A–D and S14). We observed a significant difference in the mean and median of some of the descriptor values between the NusG-dependent and NusG-independent categories. The most prominent difference was observed in four descriptors: cumulated area of all C > G bubbles, area of the longest C > G bubble, cumulated length of all C > G bubbles, and density of C > G bubbles (in the area) (Fig. 7). We expressed the difference in terms of percentage reduction in the median between the two categories. In all the cases, the median values reduced significantly in the NusG-dependent terminators. The maximum difference between the two categories was seen in the cumulated area of all C > G bubbles (58%). We concluded that the NusG dependent terminators, in general, have rut sites with the reduced area, length, and density of the C > G bubbles. A Rho-dependent terminator with lower termination efficiencies and marked by rut sites with diminished C > G bubble patterns should be a likely candidate to be a NusG-dependent terminator. Our conclusion is consistent with what was proposed earlier (10).

**Discussion**

Even though detailed analyses of the in vitro mechanism of bacterial Rho-dependent transcription termination have been reported (1, 3, 4, 6), understanding of this termination process in vivo is limited. Here, we measured the in vivo termination efficiencies of 72 Rho-dependent terminators in the mid-log phase cultures of E. coli strains that express either a Rho mutant (N340S) or NusG mutants (G146D, L158Q, and V160N) and established their correlation or lack of it with the...
C > G bubble patterns of their respective rut sites, binding constants of Rho–rut site interactions, the Rho translocase activity on the nascent RNA, and in vivo NusG-dependence of these terminators. We made the following observations. (1) These terminators exhibited a wide range of terminator strengths in vivo where the prophage terminators exhibited to be the strongest ones. Many of the terminators behaved differently in vivo compared to that predicted or experimentally shown in vitro (Fig. 2). (2) Only the weaker terminators under in vivo conditions showed a positive and robust correlation with the nature (size, length, density, etc) of the C > G bubble stretches of the rut sites. This correlation disappeared as the terminator strength increased suggesting a limited role of the nature of rut sequences in controlling the termination efficiencies in vivo (Figs. 3, S7, and S8). This observation was despite the fact that the intrinsic binding affinities of Rho for the rut sites were dependent on the nature of the C > G bubbles (Fig. S9). (3) The in vivo termination efficiencies of the majority of the terminators showed good correlations with the rates of ATPase activities of Rho on the RNA templates made of the sequence of the respective terminator-zones (Figs. 4, S10 and S11) indicating the Rho-translocase activity-dependence of the terminators in vivo. (4) Consistent with the earlier propositions (18, 19), the weaker Rho-dependent terminators in vivo showed significant dependence on NusG (Figs. 6 and S13). (5) The signatures of highly NusG-dependent terminators are marked by reduced area, length, and density of the C > G bubbles, the pattern which could be used to predict unknown NusG-dependent terminators from the genome sequence (Figs. 7 and S14). We concluded that Rho-dependent termination follows a nascent-RNA dependent pathway in vivo, where Rho translocation along the RNA is essential and rut sequences may be required to recruit Rho but the binding strengths of the latter for the rut sites have limited influence on the outcome of the termination events.

Based on the in vitro Rho-dependent transcription termination assays, two types of termination pathways have been proposed: RNA-dependent and RNAP-dependent pathways (1). In the RNA-dependent pathway, Rho loads onto the rut sites of the nascent RNA and following translocation along with the RNA catches up the EC before dislodging the latter via a direct interaction. In the more recently reported RNAP-dependent pathway, Rho is directly recruited onto the RNAP of the EC, inactivates the EC, and terminates the transcription (25, 27). In this model, Rho may be transferred to the exiting RNA after loading onto the EC. In the recently solved EC–Rho complex structures, Rho-induced catalytically inactivated state(s) of RNAP was observed, which was coined as a preterminated state(s) of the EC (26, 28). As these inactivated ECs were observed in the absence of ATP hydrolysis function of Rho, the requirement of Rho-translocation for its

Figure 7. Signatures of NusG dependent terminators. A–D, boxed plots showing the mean differences in indicated descriptor values between highly NusG dependent and independent groups (Fig. 6B). A, cumulated area of all C > G bubbles shows a reduction of 58%, (B) area of the longest C > G bubble shows a reduction of 43%, (C) cumulated length of all C > G bubbles shows a reduction of 42%, and (D) density of C > G bubbles (in the area) shows a reduction of 32% in the highly NusG-dependent group. The highly NusG-dependent group includes the terminators that have ratios of average fold change values obtained from the NusG mutants and that obtained from the N340S Rho > 0.5 (obtained from Fig. 6B).
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In vivo termination function was questioned. Here, we show that the in vivo termination efficiencies of the majority of the 72 terminators are correlated and dependent on the Rho ATPase as well as its translance function. However, the in vivo termination efficiencies, especially at the genes where the efficiency values are high, are not dependent on the binding strength of the *rut* sites for Rho. Our results support the model that Rho follows the RNA-dependent pathway to execute the in vivo transcription termination. To this end, we cannot rule out the possibility that the EC might play an anchor to gravitate the Rho protein onto the nascent RNA and thereby facilitate the Rho-recruitment process in vivo.

In vitro analyses suggested that NusG-CTD-Rho facilitates the conversion of open to close complex formation of Rho at the *rut* sites of a subset of terminators (18, 19). However, in the recently solved Rho-EC structures (26, 28), the NusG-CTD was not visible indicating its lack of stable interaction with Rho when they are part of an EC. Our in vivo termination data show that NusG-dependence is highly pronounced for the terminators that have weak termination efficiencies and the size, length, area, etc. of the C > G bubble of their *rut* sites are significantly diminished. A reduced C > G bubble stretch could pose a higher activation energy barrier for the isomerization steps at the *rut* sites, and NusG reduces this energy barrier. Hence, our in vivo functional data is inconsistent with the Rho-EC structure, rather it supports the proposed mechanism of NusG action in the Rho-dependent termination (18, 19) and further supports the existence of an RNA-dependent pathway of termination in vivo.

### Experimental procedures

#### Materials

\( [\gamma^{32}\text{P}] \) ATP (3000 Ci/mmol) and \( [\alpha^{32}\text{P}] \) UTP (3000 Ci/mmol) were from Jonaki, BRIT. Antibiotics, lysozyme, DTT, and bovine serum albumin were from USB. Primers for PCR and dC34 were obtained from Eurofins. Restriction endonuclease, T4 polynucleotide kinase, and T4 DNA ligase were from New England Biolabs. Taq DNA polymerase was from Roche Applied Science. RNAeasy kit, following which the residual genomic DNA in the RNA preparations was removed by the DNase I treatment. One microgram RNA was used for the synthesis of cDNA using Superscript III Reverse Transcriptase following the standard procedures. The amount of cDNA produced during the PCR cycles was monitored in real-time using SYBR green dye in the Bio-Rad CFX96 RT-PCR system. The threshold cycle ‘Ct’ was calculated from the midpoint of the sigmoidal curve obtained by plotting the fluorescence intensity against the number of PCR cycles. \( 2^{-\Delta\Delta Ct} \) was used to calculate the fold change in the mRNA level for the mutants relative to the WT, where Ct = the number of threshold cycles; \( \Delta Ct = \) Ct of target gene \( - \) Ct of internal control; and \( \Delta\Delta Ct = \) Ct of mutant \( - \) Ct of WT. The level of *rpoC* mRNA was used as an internal control. Primer pairs were designed to probe various regions of an operon so that the PCR products were within 200 nt in size. For the terminators, \( \alpha_{trc}, trp' \), and \( \lambda_{R1} \), the primer probes were in lacZ gene fused downstream to them.

#### RNA purification and qPCR reaction

The RS862 was transformed with pC1920 expressing WT and the mutant derivatives of Rho and NusG following by deletion of chromosomal copies of these two genes. RNA was isolated from mid-log phase (\( A_{600} \sim 0.3 \sim 0.4 \)) cultures of these strains using Qiagen’s RNAeasy kit, following which the residual genomic DNA in the RNA preparations was removed by the DNase I treatment. One microgram RNA was used for the synthesis of cDNA using Superscript III Reverse Transcriptase following the standard procedures. The amount of cDNA produced during the PCR cycles was monitored in real-time using SYBR green dye in the Bio-Rad CFX96 RT-PCR system. The threshold cycle ‘Ct’ was calculated from the midpoint of the sigmoidal curve obtained by plotting the fluorescence intensity against the number of PCR cycles. \( 2^{-\Delta\Delta Ct} \) was used to calculate the fold change in the mRNA level for the mutants relative to the WT, where Ct = the number of threshold cycles; \( \Delta Ct = \) Ct of target gene \( - \) Ct of internal control; and \( \Delta\Delta Ct = \) Ct of mutant \( - \) Ct of WT. The level of *rpoC* mRNA was used as an internal control. Primer pairs were designed to probe various regions of an operon so that the PCR products were within 200 nt in size. For the terminators, \( \alpha_{trc}, trp' \), and \( \lambda_{R1} \), the primer probes were in lacZ gene fused downstream to them.

#### Sequence selection and analyses of rut sites

Reference genomes used for bioinformatics analysis is the E. coli genome U00096.3. The selection of sequences was done
based on qPCR analysis. Multiple primer probes were designed for each operon that was upregulated in our microarray data profiles (GEO ID: 200077176 and 200077177; see Fig. S2); list of primers is given in Table S1) and those described in a study using global RNA profiling techniques (10) in the presence of Rho and NusG mutants and upon treatment with bicyclomycin. Few operons that are under the control of Rho-dependent termination were also chosen from the in vitro study of Rho-dependent terminators (9); see Fig. S2). The sequences in the genes for primer binding were selected based on the following: if the upregulation was observed in the first gene of an operon, the sequences from the TSS (from the EcoCyc database, we considered the experimentally determined promoters) till the end of the RP probes inside the first gene were taken for analysis, whereas if upregulation was seen in the genes downstream of the first gene, the sequences from the RP probe of the downstream gene to the RP of the upstream gene was taken for analysis (see Fig. S3). Sequences were checked for the presence of %C > %G bubbles using a python script and calculating the %C and %G using a sliding window of 70 nt with 10 nt increment along with the selected sequences. RNA folding free energies were determined with the Vienna RNA folding software (40) using default settings with a window size of 70 nt.

The descriptor values were calculated using the python script “MakeDescriptors” as described in Nadiras et al., 2018. The script analyses certain sequence characteristics named “descriptors” and gives a value. It uses a sliding window of 78 nt on the sequence for the calculation of descriptor values such as %C and %G (Figs. S4 and S6). Among the various descriptors (111 descriptors) described in Nadiras et al, 2018, we chose descriptors that are related to C > G bubbles and were among the ‘most differentiating’ ones. These chosen descriptors are length of the longest C > G bubble, cumulative length of all C > G bubbles, density of C > G bubbles (along the sequence), area of the longest C > G bubble, cumulative area of all C > G bubbles, area density of C > G bubbles, maximum %C in a longest C > G bubble, maximal %C - %G in a longest C > G bubble, average %C - %G in a longest C > G bubble, number of [(YC)N9→13]1 motif (YC dimers) in longest C > G bubble, number of [(YC)N9→13]2 motifs in longest C > G bubble, number of [(YC)N9→13]3 motifs in longest C > G bubble, and the density of [(YC)N9→13]1 motif (%YC) (see Fig. S6 for the details). The meaning of each descriptor is as follows: The length of the longest C > G bubble calculates the length of the longest bubble (L1). The cumulated length of all C > G bubbles calculates the sum of all C > G bubbles’ length (L1+L2) within the sequence of 78 nt window. The density of C > G bubbles (along the length) describes the fraction of cumulated length of all C > G bubbles relative to the total length of the sequence (Lt) ((L1+L2)/Lt). The area of a bubble (Si in Fig. S6) was determined by summing up %C - %G values over the length of the bubble (area are thus expressed in % x bp). The area of the longest C > G bubble describes the area that comes under the longest C > G bubble (S1). The cumulated area of all C > G bubbles describes the sum of the area under all C > G bubbles (S1+S2). The area density of C > G bubbles describes the fraction of the cumulated area of all C > G bubbles along the length of the sequence under study (S1+S2)/Lt. Maximal %C in the longest C > G bubble describes the maximum %C value among all the 78 nt sequence windows within the longest C > G bubble.
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Maximum %C - %G in the longest C > G bubble describes the point of largest difference between the %C and %G ((C-G)max % Bub). Average %C - %G in the longest C > G bubble describes the average of the difference between %C and %G at each point within a C > G bubble (C-G)av % Bub. Structural data suggested that 5’-YC (Y = pyrimidine) dimer sequences in the G-C stretches are important for Rho-PBS-RNA binding. The number of [(YC)N9→13] 1/2/3 motifs in the longest C > G bubble counts the number ofYC dimer motifs present inside the longest C > G bubble. When the dimer motifs are repeated, the distances between the two consecutive motifs will be 9 to 13 bases. Number of [(YC)N9→13] 1 counts all YC dimers. [(YC)N9→13] 2 counts the occurrence of two consecutive YC dimers. [(YC)N9→13] 3 counts the occurrence of three consecutive YC dimers. The density of [(YC)N9→13]1 motif (%YC) calculates the fraction of the number of YC dimers within the length of the sequence under study (Number of [(YC)N9→13]1)/Lt).

Calculation of correlation coefficients

In all the correlation graphs, scattered points were plotted using SigmaPlot 13 and they were fitted to straight lines by linear regression analyses using the formula (y = y0 + ax). The goodness of fit for the linear regression (r²) values was also calculated using Sigmaplot 13 and the probability of a hypothesis (p-value) was calculated using GraphPad Prism. The plots with higher correlation coefficients (r² > 0.3) had p-values in the range of > 0.0001 to 0.041. Linear regression plots with lower r² values were found to have higher p-values (>0.05) and hence considered to be not significant.

Box plot analyses of the distribution of descriptors

Box plots were used to represent the difference in the range of descriptor values of different terminator classes. The Box plot was plotted in Sigmaplot 13 using linear interpolation to determine the percentile value and computing the median of the data that is represented by a straight line in the box. Data was divided into four quartiles, each having 25% of the data. Outliers (10th percentile and 25th percentile) are represented as dotted symbols.

Templates for in vitro binding assay and in vitro ATPase assay

Different linear DNA templates for RNA synthesis containing the predicted rut site sequences were synthesized by PCR in such a way that in all the cases, transcription starts from T7 Φ10 promoter. This promoter sequence was incorporated in the forward primers. The reverse primer was designed in such a way that all the PCR products were ~300 bp in length. The PCR amplifications were performed using high fidelity Deep Vent polymerase from NEB and the chromosomal DNA was used as a template for the PCR reactions. These templates were transcribed using the AmpliScribe T7 High Yield Transcription Kit following the manufacturer’s protocol. For the Rho-RNA binding assays, the RNAs were labeled with [α-32P]UTP during the synthesis. For the ATPase assays, the RNAs were synthesized in the same way, except that radioactive NTP was not used. The poly (dC)34 was labeled with [γ-32P] ATP using T4 polynucleotide kinase.

Binding assay

WT Rho-RNA binding constants were measured by gel-shift assays using radio-labeled RNA. In each assay, 5 nM of RNA was used in the transcription buffer (25 mM Tris–HCl (pH 8.0), 5 mM MgCl2, 50 mM KCl, 1 mM DTT and 0.1 mg/ml of bovine serum albumin) with the increasing concentrations of the WT Rho (0–50 nM). The binding reactions were performed at 37 °C for 5 min before loading onto a running 4% (w/v) native acrylamide gel under cold (4 °C). Gels were scanned in phosphor-imager Typhoon 9200 and fractions of unbound species were quantified by ImageQuant software. Gel-shift assays with radio-labeled poly(dC)34 were also performed in the same way. The dissociation constant (Kd) values were calculated by fitting the binding curves to either sigmoidal or hyperbolic equations using the SigmaPlot 13.

ATPase assay

The colorimetric determination of ATPase activity was performed by an MG assay using the procedure previously described in an in vitro ATPase study (41) with slight modification. The MG reagent was freshly prepared every day and consisted of a mixture of MG solution (0.00812%, w/v in water), polyvinyl alcohol aqueous solution (2.32%, w/v), ammonium molybdate (5.72%, w/v) in 6M HCl, and water in 2:1:1:2 ratios. The MG reagent was left standing for 3 h to get a stable green/golden solution, which was filtered through a 0.45 μm filter before use. All the ATPase assays were performed in the transcription buffer. The release of inorganic phosphate was measured at various time points colorimetrically by the MG method. ATPase reaction was stopped by the addition of 50 μl MG solution at each time point, and volume was made up to 196.875 μl by adding 136.875 μl of assay buffer and incubated for 1 min and then 3.125 μl of sodium citrate aqueous solution (34% w/v) to limit ATP hydrolysis (42). The solution mixture was incubated for 30 min, following which absorbance was measured at A630.

In vivo RNA stability assays

E. coli WT strain, MG1655ΔracΔrho having pCL1920 plasmid expressing N340S Rho was grown in LB broth at 37 °C until the early-log phase (A600~0.3–0.4). Two milliliters of culture was harvested (time point 0) and immediately RNAprotect Bacteria (Qiagen) were added to stabilize the RNA in vivo. At that point, transcription was stopped by treatment with 50 μg/ml rifampicin and the cells were incubated for an additional 2, 4, 6, 8, and 10 min, respectively, following which cells were harvested and were resuspended in RNAprotect Bacteria, and RNA was isolated using the Qiagen RNEasy Kits. Residual genomic DNA in the RNA preparations was eliminated by DNase I treatment. One microgram RNA was used for the synthesis of cDNA using Superscript III Reverse Transcriptase following the standard procedures. The
cDNA was used for RT-PCR amplification for estimating the amount of RNA left at different time points. PCR products were run on 1.5% agarose gels that were visualized on Alpha Imager Gel Imaging System. The decrease of DNA band intensities relative to the control (time point 0) was estimated by ImageQuant software. Band intensities were plotted and the curves were fitted to an exponential decay equation to calculate the half-life and degradation constants of the RNAs in vivo.

Data availability
All the raw datasets are available upon request. Computer codes (in python script) are written based on that described in Nadiras et al., 2018 and are available upon request.

Supporting information—This article contains supporting information (9, 10, 17, 30, 39).

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Abbreviations—The abbreviations used are: EC, elongation complex; MG, malachite green; NTP, nucleotide triphosphate; NusG-CTD, NusG interacting with C-terminal domain; PBS, primary binding site; RNAS, RNA polymerase; RP, reverse primer; TSS, transcription start site.

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