Distinct and Contrasting Transcription Initiation Patterns at *Mycobacterium tuberculosis* Promoters

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Abstract

Although sequencing of *Mycobacterium tuberculosis* genome lead to better understanding of transcription units and gene functions, interactions occurring during transcription initiation between RNA polymerase and promoters is yet to be elucidated. Different stages of transcription initiation include promoter specific binding of RNAP, isomerization, abortive initiation and promoter clearance. We have now analyzed these events with four promoters of *M. tuberculosis* viz. $P_{gyrB1}$, $P_{gyrB}$, $P_{metU}$ and $P_{metD}$. The promoters differed from each other in their rates of open complex formation, decay, promoter clearance and abortive transcription. The equilibrium binding and kinetic studies of various steps revealed distinct rate limiting events for each of the promoter, which also differed markedly in their characteristics from the respective promoters of *Mycobacterium smegmatis*. Surprisingly, the transcription at $gyr$ promoter was enhanced in the presence of initiating nucleotides and decreased in the presence of alarmone, pppGpp, a pattern typically seen with rRNA promoters studied so far. The $gyr$ promoter of *M. smegmatis*, on the other hand, was not subjected to pppGpp mediated regulation. The marked differences in the transcription initiation pathway seen with $rrn$ and $gyr$ promoters of *M. smegmatis* and *M. tuberculosis* suggest that such species specific differences in the regulation of expression of the crucial housekeeping genes could be one of the key determinants contributing to the differences in growth rate and lifestyle of the two organisms. Moreover, the distinct rate limiting steps during transcription initiation of each one of the promoters studied point at variations in their intracellular regulation.

Introduction

*M. tuberculosis* is one of the most formidable pathogens known to mankind. The resurgence of the pathogen, its alliance with HIV infection and emergence of the drug resistant strains has resulted in a global challenge to combat tuberculosis [1]. The distinctive features of the bacterium such as slow growth rate, dormancy, unique cell wall composition, resistance towards phagocytosis by macrophages etc demand a thorough investigation of its biology at the molecular level. A number of studies carried out so far reveal significant differences in the transcription process in mycobacteria when compared to *E. coli* and other bacteria [2,3]. Presence of as many as 13 sigma factors for transcription from promoters with diverse architecture [2,4,5] and inability of the mycobacterial promoters to function in *E. coli* [5–7] are some of the key features warranting a detailed study of the transcription process in the pathogen.

Transcription constitutes the first stage in gene expression and comprises of multiple steps viz initiation, elongation and termination (Figure 1A). During the initiation, RNA polymerase (RNAP) binds to the promoter, leading to the formation of several intermediates which differ from each other in their kinetic properties [8–11]. After initial binding of RNAP to the promoter to form the closed complex, the DNA strands unwind to form a catalytically competent open complex, associated with a series of conformational changes in the enzyme as well as DNA [8–11]. Binding of the initial ribonucleotides (iNTPs) to the RNAP results in the formation of ternary complex, poised to enter into the elongation mode [8–11]. After the synthesis of abortive transcripts of 2–15 nucleotides in most of the promoters studied, the RNAP leaves the promoter to enter into the elongation phase of transcription [12]. Because of these elaborate orchestrated steps, the initiation pathway is also fine-tuned by a number of regulatory mechanisms to meet the requirements posed by various physiological conditions of the cell [11,13]. Typically, a few promoters ($rrn$, initiator tRNA) achieve higher promoter strength in exponential phase due to the stabilization of open complex by initiating nucleotides (iNTPs). In contrast, during the stationary phase, increase in the concentration of guanosine tetra/penta phosphate ([p]pppGpp), leads to inhibition of transcription from these promoters [14,15]. Thus the strength of these promoters varies with the growth phase as they are subjected to growth phase dependent regulation.

The present work is the first detailed kinetic analysis of the events during transcription initiation in *M. tuberculosis*. We have carried out promoter-polymerase interaction studies using a few of the house-keeping promoters to characterize the mechanisms of transcription initiation. The kinetics of RNAP-DNA interactions
Figure 1. Transcription with *M. tuberculosis* promoters. **A** Scheme of transcription initiation. **B** Sequences of promoters used in this study. The -35, -10 elements and the transcription start sites are underlined. Sequences are aligned with *E. coli* σ70 and mycobacterial σA dependent promoter consensus. **C** *In vivo* reporter assays. Transcriptional activities of the promoters were determined by β-galactosidase reporter assays.
was measured in different promoter sequence contexts to determine their key rate-limiting steps. Further, the role of iNTPs and pppGpp in regulating transcription initiation was studied. While the *M. tuberculosis* ribosomal RNA promoter exhibited the characteristics seen with *E. coli* and other bacteria, the promoters for the gyr operon showed unusual and hitherto unknown pattern of transcription initiation. Most significantly, the promoters for the same genes from *M. smegmatis* and *M. tuberculosis* differed markedly in their kinetic properties and response to the effectors.

**Results**

**Promoter Characteristics and Activities**

For comparison of the promoter–RNAP interactions, two stable RNA promoters viz., ribosomal RNA, initiator tRNA (P*rrnPCL1*, P*metU*) and another house-keeping promoter (P*gyrB1*) were chosen in addition to a weak promoter (P*gyrB*) (**Figure 1B**). *M. tuberculosis* has only a single operon for rRNA transcription driven by two promoters [16–18]. Amongst the two promoters, P*rrnPCL1* is the major house-keeping promoter and is stronger than P*metU* [16]. Moreover, P*rrnPCL1* is found in the genome of every sequenced species of mycobacteria and appears to be conserved across the genus [18]. P*metU* is the only promoter driving the transcription of the single initiator tRNA gene in *M. tuberculosis* [19]. The -10 and -35 elements of these two stable RNA promoters resemble the mycobacterial σ^70_\text{consensus sequence} [6]. Two promoters from the *gyr* operon of *M. tuberculosis* included in the study are illustrated in **Figure 1B** [6]. P*gyrB1* is the major promoter of the *gyr* operon that directs the high levels of transcription from the *gyrB1–gyrA* dicistron [6]. The -10 and -35 elements of these two stable RNA promoters resemble the mycobacterial σ^A_\text{consensus sequence} [6]. Two promoters from the *gyr* operon of *M. tuberculosis* included in the study are illustrated in **Figure 1B** [6]. P*gyrB1* is the major promoter of the *gyr* operon that directs the high levels of transcription from the *gyrB1–gyrA* dicistron [6]. The -10 and -35 elements of these two stable RNA promoters resemble the mycobacterial σ^70_\text{consensus sequence} [6]. Two promoters from the *gyr* operon of *M. tuberculosis* included in the study are illustrated in **Figure 1B** [6]. P*gyrB1* is the major promoter of the *gyr* operon that directs the high levels of transcription from the *gyrB1–gyrA* dicistron [6]. 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shown), which could be attributed to the intrinsic stability of the complexes at these promoters in the absence of initiating nucleotides. In vitro assays carried out in the presence of pppGpp showed that transcription at P_{rrnPCL1} and P_{gyrB1} was inhibited (Figure 5C, D). There was no significant effect of pppGpp on transcription at P_{metU} (Figure 5C, D), revealing that the promoter is not subjected to a similar kind of regulation.

**Inefficient Promoter Clearance at Gyr Promoters**

In addition to the efficiency in DNA binding and melting, overall promoter strength also depends on the rate of promoter clearance by RNAP. Thus the extent of abortive initiation during the transition from the initiation to elongation also has an important bearing on transcription initiation [20]. To determine the contribution of the post DNA-melting steps in overall transcription efficiency, the rate of promoter clearance and formation of abortive as well as run-off transcripts were measured (Figure 6A, B, Table 1). The rate of promoter clearance was faster at two stable RNA promoters in contrast to the gyr operon promoters. The promoter which drives the dicistron transcription (P_{gyrB1}) had 2.5 and 10 times slower clearance rate compared to P_{metU} and P_{rrnPCL1} respectively. The lower clearance rate seen with the gyr promoters seems to be resulting out of higher abortive transcription delaying the escape of RNAP from these promoters (Figure 6). When the amount of run-off transcripts synthesized at these promoters were compared in the single and multiple round conditions, fewer run-off transcripts were synthesized at P_{metU} in the single round transcription compared to the P_{gyrB1} and P_{rrnPCL1} (data not shown). However, after multiple rounds of transcription, the accumulation of run-off transcripts at P_{metU} was comparable to that of P_{gyrB1} and P_{rrnPCL1}.

**Discussion**

Kinetics and equilibrium binding studies provide an insight into the strength and the mechanism of transcription initiation at the promoters. In addition to the sequence of promoter elements and overall promoter architecture, the strength of a given promoter is governed by events occurring at various stages of the transcription initiation process and the in vivo strength is the net result of cumulative effect of all the steps. In the present study with the four promoters of *M. tuberculosis*, we have dissected the individual steps in the transcription initiation to understand their characteristic rate limiting steps.

Generally, in every organism as if by a rule, the n operons are transcribed by the strongest house-keeping promoters. Very high frequency of initiation at m operons is a characteristic feature that contributes to the abundance in rRNA transcritps [21] and the P_{rrnPCL1} of *M. tuberculosis* is no exception to this paradigm. The high strength of the promoter can be attributed to its −10 and −35 elements, which closely resemble to the σ^54 consensus sequence [6,16]. The instability of the open complex and increase

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**Figure 2. Determination of K_B.**

A Scheme of assay, R represents RNAP, P represents promoter fragment and RP_c represents closed complex. B Promoter fragments were incubated with different RNAP concentrations for 20 min and the complexes formed were resolved using 4% native-PAGE. C The amount of radioactivity in bound and free fragments was measured by densitometry and indicated as RP_c and P respectively. RP_c/RP_c+P ratios were plotted as function of RNAP concentrations. K_B was calculated from the slope of the graph. The values obtained are mean of three independent experiments (Table 1).

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in half-life in the presence of iNTPs seen with the promoter is a characteristic property of any typical rRNA promoter analyzed so far including promoters from \textit{M. smegmatis} [15,22–24]. However, the comparison of kinetics at P\textsubscript{rrnPCL1} of \textit{M. tuberculosis} with that of \textit{M. smegmatis} also revealed interesting differences (Figure 7). P\textsubscript{rrnPCL1} of \textit{M. smegmatis} showed slower promoter clearance and greater amount of abortive transcription in addition to an intrinsically unstable open complex [24]. The cumulative effect of these kinetic events results in (10 fold) lower transcriptional activity of \textit{M. smegmatis} P\textsubscript{rrnPCL1} in comparison to \textit{M. tuberculosis} (24, Figure 7, Figure S2). The two promoters also differed significantly in their response to iNTPs and pppGpp (24 and this work); the stimulation and inhibition by the two effectors was much more pronounced at P\textsubscript{rrnPCL1} of \textit{M. smegmatis} showed slower promoter clearance and greater amount of abortive transcription in addition to an intrinsically unstable open complex [24]. The cumulative effect of these kinetic events results in (10 fold) lower transcriptional activity of \textit{M. smegmatis} P\textsubscript{rrnPCL1} in comparison to \textit{M. tuberculosis} (24, Figure 7, Figure S2). The two promoters also differed significantly in their response to iNTPs and pppGpp (24 and this work); the stimulation and inhibition by the two effectors was much more pronounced at P\textsubscript{rrnPCL1} of \textit{M. tuberculosis}. However, inadequacy of the P\textsubscript{rrnPCL1} of \textit{M. smegmatis} appears to be compensated by the very strong P\textsubscript{rrnB}, which appears to be one of the strongest promoter in the organism. Moreover, the presence of a second functional rRNA operon also ensures adequate rRNA transcription. All these observations indicate the importance of species specific variations in promoters to meet the cellular requirements. The constitutive high level transcripts synthesized from the single rRNA operon of \textit{M. tuberculosis} seem to fulfill the need of the metabolic machinery of the cell possibly due to the slow growth characteristics of the organism. As a consequence, the present day \textit{M. tuberculosis} strains and other closely related

\textbf{Table 1.} Summary of equilibrium binding constants and kinetic parameters.

<table>
<thead>
<tr>
<th>Constant/Property</th>
<th>P\textsubscript{gyrB1}</th>
<th>P\textsubscript{rrnPCL1}</th>
<th>P\textsubscript{gyrR}</th>
<th>P\textsubscript{metU}</th>
</tr>
</thead>
<tbody>
<tr>
<td>K\textsubscript{B} (x 10\textsuperscript{7} M\textsuperscript{-1} M\textsuperscript{2})</td>
<td>2.1±0.03</td>
<td>3.1±0.16</td>
<td>0.27±0.001</td>
<td>0.7±0.08</td>
</tr>
<tr>
<td>K\textsubscript{B} (x 10\textsuperscript{7} M)</td>
<td>68.05±12.16</td>
<td>40.14±6.09</td>
<td>146.2±44.58</td>
<td>127.6±27.19</td>
</tr>
<tr>
<td>k’ (fast) (min\textsuperscript{-1})</td>
<td>1.4±0.7</td>
<td>1.2±0.08</td>
<td>0.54±0.20</td>
<td>0.24±0.15</td>
</tr>
<tr>
<td>k’ (slow) (x 10\textsuperscript{-7} min\textsuperscript{-1})</td>
<td>0.24±0.4</td>
<td>0.21±0.74</td>
<td>0.61±0.24</td>
<td>0.04±0.04</td>
</tr>
<tr>
<td>k\textsubscript{off} (fast) (min\textsuperscript{-1})</td>
<td>2.1±0.17</td>
<td>2.7±0.17</td>
<td>1.3±0.17</td>
<td>1.35±0.17</td>
</tr>
<tr>
<td>k\textsubscript{off} (slow) (x10\textsuperscript{-1} min\textsuperscript{-1})</td>
<td>0.74±0.27</td>
<td>2.63±1.41</td>
<td>1.35±0.92</td>
<td>0.48±0.16</td>
</tr>
<tr>
<td>t\textsubscript{1/2} (fast) (min)</td>
<td>5.17</td>
<td>1.51</td>
<td>7.75</td>
<td>4.4</td>
</tr>
<tr>
<td>t\textsubscript{1/2} (slow) (min)</td>
<td>17.78</td>
<td>6.12</td>
<td>13.32</td>
<td>34.84</td>
</tr>
<tr>
<td>Abortive transcription</td>
<td>++++</td>
<td>–</td>
<td>+++</td>
<td>–</td>
</tr>
<tr>
<td>Promoter clearance (min)</td>
<td>23</td>
<td>2.3</td>
<td>18</td>
<td>8.93</td>
</tr>
</tbody>
</table>

Figure 3. Determination of K\textsubscript{B}. \textbf{A} Scheme of assay, R represents RNAP, P represents promoter fragment and RP\textsubscript{o} represents open complex. \textbf{B} Promoter fragments were titrated using a range of RNAP concentrations, incubated at 37°C for 10 min, challenged with heparin and analyzed using 4% native-PAGE. \textbf{C} The amount of radioactivity in bound and free fragments was measured by densitometry and indicated as RP\textsubscript{o} and P respectively. RP\textsubscript{o}/RP\textsubscript{o}+P ratios were plotted as a function of RNAP concentrations to obtain the hyperbolic graph. The values (Table 1) are mean of three independent experiments.

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Figure 4. Kinetics of association and dissociation. A Scheme of assay, R represents RNAP, P represents promoter fragment and RPₜ represents open complex. B The association of RNAP and promoter DNA to form open complex was monitored over time intervals ranging from 0 to 48 min, as indicated. C The amount of radioactivity in bound and free fragments was measured by densitometry and indicated as RPₜ and P respectively. RPₜ/P ratios were plotted against time and k’ was measured by double exponential association analysis. The values of k’ are mean of three independent experiments and are shown in Table 1. D Open complex at each promoter fragment was formed by incubating RNAP and promoter fragments for 15 min at 37˚C. Dissociation of RNAP was monitored by challenging the pre-formed open complex with heparin for time intervals ranging from 0 to 48 min, as indicated. E The data was fit into the double exponential decay equation to measure k_{off}. The biphasic nature of the double exponential decay curve is suggestive of the existence of two complexes decaying at different rates. The steeper and the trailing parts of the curve represent the faster and slower decaying phases respectively. The values of k_{off} are mean of three independent experiments (Table 1).
Figure 5. Effect of iNTPs on open complex. **A** Open complex was formed in the presence of 100 μM of iNTPs (+1 and +1, +2) and the transcription was initiated by adding heparin and all the four NTPs. The run-off transcripts were resolved on 8% urea–PAGE. The initial transcribed sequence (ITS) for all the promoters is shown. **B** The graph shows the quantification of transcripts formed in the absence and presence of +1 iNTP and +1, +2 iNTPs. The amount of run-off transcripts formed was measured by densitometry and indicated as AU on Y axis. **C** Effect of pppGpp on open complex. Open complex was formed in the presence of increasing concentrations of pppGpp as indicated and the transcription was initiated as described before. The run-off transcripts were resolved on 8% urea–PAGE. **D** The graph shows the quantification of transcripts formed in the presence of increasing concentrations of pppGpp. The amount of run-off transcripts formed was measured by densitometry and indicated as AU on Y axis.
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Figure 6. In vitro transcription assays. A Promoter clearance assay was carried out as described in Materials and Methods. (α-32P)-2 nucleotide of ITS was used to label the transcripts. A 10 µl aliquot from the same assay mix was loaded onto 8% urea-PAGE (19:1) to resolve the run-off transcripts.
pathogens seem to have lost the second rRNA operon (rrnB) found in the fast growing species of the genus [16, 17]. The down-regulation of the operon by the action of the pppGpp during transcription initiation would ensure the fine tuning of rRNA expression to lower levels sufficient in the stationary phase. One would expect a very low level of rRNA expression in the dormant state of the organism during its intracellular survival. The positive regulation of the PmetZL by iNTPs and inhibition of its transcription initiation by pppGpp point out at the remarkable conservation of rRNA transcription regulation across the diverse bacterial species and this sensing mechanism appears to provide a unified theme for the growth phase dependent regulation of rRNA transcription. However, as summarized in Table S1, the rRNA operons are subjected to diverse controls, in addition to conserved features, adding another level of complexity.

Surprisingly, in contrast to the rrn promoter, the other stable RNA promoter, PmetU, which drives the transcription from a single initiator tRNA gene in M. tuberculosis, does not appear to be influenced by iNTPs and pppGpp. In this context, studies with E. coli tRNA promoters provide interesting parallels. E. coli has two initiator tRNA promoters, Pinit and Pinit2, transcribing the single initiator tRNA and an operon containing three tandemly repeated tRNA

promoters provide interesting parallels. While the transcription from Pinit is unaffected by pppGpp, the Pinit2 is subjected to inhibition [26]. Thus, it is apparent that unlike the rrn operon, regulation of promoters of initiator tRNA is not universally conserved. Also in contrast to the PmetZL, the isomerization step was found to be the rate limiting step at PmetU in M. tuberculosis. The lower amounts of run-off transcripts from the promoter in single round transcription assays, compared to the gyr and rrn promoters could be due to the slower rate of formation of the open complex. However, the high promoter strength of the PmetU under in vivo conditions could be accounted for by its higher open complex stability, faster promoter clearance and low levels of abortive transcription. The accumulation of large amounts of transcripts after multiple rounds of transcription at PmetU (Figure 4D) could be explained not only because of its promoter strength but also possibly due to frequent recycling of RNAP likely to occur at the tRNA gene. The process of re-initiation of transcription could be facilitated because of the presence of an intrinsic terminator at the end of the short gene to allow RNAP to fall off at a distance not far away from the promoter [19, 27]. Notably, high levels of transcripts seen with class III transcripts in eukaryotes is attributed to efficient recycling of the Pol III on tRNA genes [28].

The extent of closed complex formation and rate of isomerization are the major determinants of promoter strength of PgyrR1, the major promoter transcribing the gyr operon of the organism. The rate limiting step at PgyrR1 seems to be the promoter clearance by RNAP. The promoters efficient at early steps of promoter-polymerase interaction tend to be inefficient in promoter clearance [29]. The present data with PgyrR1 supports this hypothesis. The stimulation of open complex formation upon addition of the iNTPs and the opposing effect of inhibition by pppGpp is an unusual property of the promoter, a feature distinct from Pgyr, of M. smegmatis, which do not appear to respond in a similar fashion [24]. To our knowledge, this is the first ever description of nucleotide mediated activation and pppGpp mediated inhibition of transcription initiation of gyr promoter in any organism or for that matter promoter of any topoisomerase gene.

Surprisingly, the present studies reveal that the process of transcription initiation at M. tuberculosis PgyrR1 is markedly distinct from the Pgyr, a single promoter transcribing gyrase operon of M. smegmatis, which is a non-pathogenic member of the same genus often used as surrogate host for a variety of studies (Table S2). Slower isomerization rates, faster promoter clearance, lower abortive initiation are the characteristic features of the transcription initiation at Pgyr, from M. smegmatis [24]. In contrast, the principle gyr promoter of M. tuberculosis, the subject of the present analysis, exhibited entirely opposite effects viz. faster rate of open complex formation, slower promoter clearance and higher abortive transcription. Notably, the two promoters exhibit markedly distinct mode of ‘Relaxation Stimulated Transcription’ (RST), a homeostatic control employed by cells to regulate gyrase activity and topological status of the genome [6, 30, 31]. In addition, distinct influence exerted by iNTPs and pppGpp on the promoter strength of PgyrR1 could ensure control of the promoter linked to the growth phase. These differences in the regulation of the gyr operons between the two different species may indeed reflect their growth rates, physiology and contrasting life-style.

To conclude, during initiation of transcription, each of the M. tuberculosis promoters studied is subjected to different rate-limiting steps and regulation. While unstable open complexes appear to serve as the sensors of initiating nucleotide concentration in rRNA promoter, distinctly tRNA promoter is rate-limited at open complex formation and not subjected to growth phase dependent control. The opposing effects of the regulatory effectors, on the principle promoter of the gyr operon of the organism indicate the fine control connecting growth phase to supercoiling homeostasis of the genome, a mechanism probably required for metabolic shut down.

Materials and Methods

Promoter DNA, Transcription Templates and RNAP

The strains, plasmids and the sequences of the promoter fragments used for this study are listed in the Table S3. Since promoters of gyr operon are divergent and overlap, the sequences of these promoters were altered such that only one of the two promoters was functional. The sequence of -10 element of PgyrR1 was rearranged from TACAGT to ACTTAG in the fragment containing PgyrR1 and the sequence corresponding to -10 element of PgyrL was changed from TCTTCT to GTGCGT in fragment containing PgyrL (Figure 1B). pARN104, a derivative of pUC18 was used as a vector to clone the promoter fragments amplified from M. tuberculosis H37Ra genomic DNA with specific primers. For in vitro transcription assays, templates were prepared by PCR amplification from the constructs using a set of vector specific primers followed by gel purification. The primers used in this study are listed in Table S4. RNAP was isolated from M. smegmatis SM07 [32] by a modified procedure involving in vivo reconstitution of the enzyme with σ70 [33]. The σ70 content in the RNAP preparation was 95%
stoichiometric to the β, β’ subunits. The specific activity of the purified RNAP was determined both by the standard method of \(^{3}H\)-UTP incorporation and by titrating the promoter fragment with a range of RNAP concentrations as described [34,35]. ppGpp was synthesized as described [36].

**β-galactosidase Reporter Assays**

The cells were grown in MB7H9 (Difco) medium supplemented with 2% glucose (Sigma) and 0.05% Tween80 (Sigma). Promoter strength was measured by β-galactosidase reporter assay and the activity represented in Miller units (Miller units = \(1,000 \times A_{420}/(\text{time (min)} \times \text{volume of culture (ml)} \times \text{optical density at 600 nm})\) [37]. *M. smegmatis mc \(^{2}155\) transformed with the vector pSD5B [38] was used as the negative control. To determine the *in vivo* promoter strength in different growth phases, the cultures of *M. smegmatis mc \(^{2}155\) transformed with the promoter fusion constructs were grown for 12, 18, 24, 30, 48 hours and the β-galactosidase reporter assay was carried out as described before.

**Electrophoretic Mobility Shift Assay (EMSA)**

For EMSA, oligonucleotides having the individual promoter sequences were used. The 5’ promoter fragments were end labeled at their 5’ ends of one of the strands with (γ -\(^{32}\)P) ATP and T4 polynucleotide kinase (New England Biolabs) at 37°C for 30 min. The labeled strand was annealed with two molar excess of complementary strand. The binding reactions were carried out in transcription buffer containing 50 mM Tris HCl, pH-8.0 at 25°C), 5 mM magnesium acetate, 100 μM EDTA, 100 μM DTT,
were subjected to heparin challenge (50 μg ml−1 BSA, 5% glycerol [27]. The buffer used for pppGpp assays also included 35 mM of potassium glutamate. The electrophoresis was carried out either at 4 °C or room temperature on a 4% native-PAGE. The amount of radioactivity in bound and free promoter fragments was measured by phosphorimager (Fujifilm) and densitometry analysis by Image Guage ver. 2.54.

Determination of Equilibrium Constants (Kθ and Kθ)  
To study the RNAP-DNA closed complexes, 1 nM of promoter fragments were titrated with varied amounts of RNAP. The incubation was carried out at ice for 20 min and the fractions were resolved on 4% native-PAGE at 4 °C. The Kθ was determined by Prism software from three independent sets of experiments as described [39]. For determination of the Kθ of the open complex, different concentrations of RNAP and 1 nM of promoters were incubated at 37 °C for 10 min followed by heparin (50 μg ml−1) challenge for 1 min. The fractions were resolved on 4% native-PAGE at 37 °C. The equilibrium dissociation constant for the heparin resistant complexes (Kθ) was measured by the equation Y = Ymax[RNAP]/Kθ[RNAP], where Ymax corresponds to binding maximum [40,41].

Determination of Association and Dissociation Rate Constants  
For determination of association rate constants, closed complexes were pre-formed as described. The aliquots (9 μl) from the assay mixture were withdrawn at different time points (0 to 48 min) and challenged with heparin (50 μg ml−1) followed by immediate loading onto 4% native-PAGE electrophoresed at room temperature to analyze the bound fractions. For dissociation assays, open complexes were formed by incubating promoter fragments and RNAP for 15 min at 37°C and the assay mixtures were subjected to heparin challenge (50 μg ml−1). Aliquots (10 μl) were withdrawn at time intervals ranging from 0 to 48 min followed by loading onto 4% running native-PAGE electrophoresed at room temperature. The first order and dissociation rate constants were calculated by fitting the values as described earlier [34].

Assays to Determine the Effect of Ribonucleotides on Isomerization and Stability  
Initially promoter DNA was incubated with RNAP (50 nM; 100 nM in case of Pgal) in the presence of ribonucleotides. The ribonucleotides were added to a final concentration of 100 μM in different combinations (+1, +1+2). The reactions were incubated to form competitor resistant complex as described above and supplemented with NTP mix (100 μM), 1 μCi (α-32P) UTP and incubated at 37°C for 15 min. The reactions were terminated with 2x stop dye (95% formamide, 0.025% (w/v) bromophenol blue, 0.025% (w/v) xylene cyanol, 5 mM EDTA and 0.025% SDS and 8 M urea). The samples were heated at 95°C for 1 min and snap chilled before loading on 8% urea-PAGE. The amount of RNAP-promoter complex formed in the equilibrium dissociation constant for the heparin resistant complexes (Kθ) was measured by the equation Y = Ymax[RNAP]/Kθ[RNAP], where Ymax corresponds to binding maximum [40,41].

In vitro Transcription Reactions  
After RNAP [100 nM] and promoter DNA [50 nM] were incubated at 37°C for 10 min for open complex formation, RNA synthesis was initiated by the addition of NTP mix (100 μM), 1 μCi (α-32P) UTP and incubated at 37°C for 15 min and terminated with 2x stop dye (95% formamide, 0.025% (w/v) bromophenol blue, 0.025% (w/v) xylene cyanol, 5 mM EDTA and 0.025% SDS and 8 M urea). The samples were heated at 95°C for 1 min and snap chilled before loading on 8% urea-PAGE. For single round transcription, 30 μg ml−1 heparin was added along with NTP mix (100 μM) and 1 μCi (α-32P) UTP. For promoter clearance analysis, promoter DNA (50 nM) and RNAP (100 nM) were incubated in transcription buffer and the reactions were carried out as described (13, 32). +2 NTP in the Initial Transcribed Sequence (ITS) of each promoter was used as the labeled nucleotide. (α-32P)ATP was used to label the transcripts in case of PaoB, PmetL: (α-32P) UTP for PmetPCL1 and PpapB. The samples were analyzed in 23% urea-PAGE (10:1) to resolve abortive transcripts. A 10 μl aliquot from the same assay mix was loaded onto 8% urea – PAGE (19:1) to resolve the run-off transcripts. The run-off transcripts were quantified as arbitrary units (AU) and plotted against time. The time corresponding to 90% of the maximum transcript formed, at each promoter, was calculated as the promoter clearance rate (PC90%).

Assays with pppGpp  
For the assays with pppGpp, RNAP (100 nM) was incubated with pppGpp (1 mM) in transcription buffer (with 35 mM potassium glutamate) for 15 min. In vitro transcription assays to study the effect of pppGpp were carried out as described above.

Supporting Information  
Figure S1 Effect of iNTPs on formation and dissociation of open complex. A iNTPs were incubated with promoter fragments and RNAP as described in Materials and Methods to determine their effect on isomerization. The initial transcribed sequence of each template is shown on the left side of the picture. B The amount of RNAP-promoter complex formed in the presence and absence of iNTPs was quantified (AU) and plotted. Slower moving complex was quantified in case of PmetPCL1. (TIF)

Figure S2 In vivo promoter activity of PmetPCL1 from M.smegmatis and M.tuberculosis. In vivo activities of PmetPCL1 from M. smegmatis (M.smeg) and M. tuberculosis (M.tb) was measured by β galactosidase assay and plotted on Y axis as Miller units. (TIF)

Table S1 Comparison of transcription at rRNA promoters of E. coli, M. smegmatis and M. tuberculosis. (PDF)

Table S2 Comparison of transcription at gyr promoters of E. coli, M. smegmatis and M. tuberculosis. (PDF)

Table S3 Strains, plasmids and oligonucleotides used in this study. (PDF)

Table S4 Sequence of primers used in this study. (PDF)

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Author Contributions
Conceived and designed the experiments: PT. Analyzed the data: VN PT AC. Contributed reagents/materials/analysis tools: VN AC. Wrote the paper: PT VN.

References


