Comparison of promoter-specific events during transcription initiation in mycobacteria

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DNA–protein interactions that occur during transcription initiation play an important role in regulating gene expression. To initiate transcription, RNA polymerase (RNAP) binds to promoters in a sequence-specific fashion. This is followed by a series of steps governed by the equilibrium binding and kinetic rate constants, which in turn determine the overall efficiency of the transcription process. We present here the first detailed kinetic analysis of promoter–RNAP interactions during transcription initiation in the σ^A -dependent promoters $P_{rrnAPCL1}$, P_{rrnB} and P_{gyr} of *Mycobacterium smegmatis*. The promoters show comparable equilibrium binding affinity but differ significantly in open complex formation, kinetics of isomerization and promoter clearance. Furthermore, the two *rrn* promoters exhibit varied kinetic properties during transcription initiation and appear to be subjected to different modes of regulation. In addition to distinct kinetic patterns, each one of the housekeeping promoters studied has its own rate-limiting step in the initiation pathway, indicating the differences in their regulation.

INTRODUCTION

Transcriptional regulation is one of the major mechanisms controlling gene expression in prokaryotes. RNA polymerase (RNAP), the central enzyme involved in bacterial transcription, consists of β , β' , ω and two α subunits along with one of several sigma factors. The transcription process is divided into three phases: initiation, elongation and termination (Helmann, 2009). The initiation event itself can be subdivided into multiple steps, including a series of sequence-specific DNA–protein interactions between RNAP and the promoter. Transcription initiation is the most frequent target for regulation by different transcription factors and small molecule regulators. The pathway has been most extensively studied for *Escherichia coli* RNAP (Browning & Busby, 2004; Haugen *et al.*, 2008).

Analysis of transcription and other essential molecular processes in mycobacteria has recently become important to better understand the biology of the organism due to the global re-emergence of tuberculosis and other mycobacterial infections. Genome sequencing and comparative sequence analysis have revealed the presence of 13 sigma factors in *Mycobacterium tuberculosis* and 26 in *Mycobacterium smegmatis* (Cole *et al.*, 1998; Manganelli

et al., 1999; Rodrigue *et al.*, 2006; Waagmeester *et al.*, 2005). The primary sigma factor, σ^A , is responsible for transcribing housekeeping genes and it is homologous to the *E. coli* σ^{70} class of sigma factors (Gomez *et al.*, 1998). Although the RNAP, sigma factors and several transcription factors from *M. smegmatis* and *M. tuberculosis* have been characterized (Gomez & Smith, 2000; Smith *et al.*, 2005), kinetic mechanisms of the promoter–RNAP interactions during transcription initiation and their effect on gene regulation are yet to be understood. While these processes have been well studied in several systems (Haugen *et al.*, 2008; Jia & Patel, 1997; Juang & Helmann, 1995), many aspects may differ significantly in mycobacteria due to the high GC content of the genome and the slow growth rate. Furthermore, the regulation of essential housekeeping functions may be different in mycobacteria. For example, factors such as Fis and DksA, which are well established regulators of rRNA transcription in *E. coli*, are absent in mycobacterial genomes (Brosch *et al.*, 2001; Cole *et al.*, 1998). To understand the different steps of promoter–polymerase interactions and to obtain the first glimpse of events during transcription initiation in *M. smegmatis*, three housekeeping promoters were chosen for the present study, viz. two *rrn* promoters (Gonzalez-y-Merchand *et al.*, 1998) and the *gyr* operon promoter (Unniraman & Nagaraja, 1999). *In vitro* promoter binding and transcription assays were carried out to analyse each step of transcription initiation, namely closed complex (RP_C)

Abbreviations: Ft, fraction of bound complex; PC_{90%}, 90 % promoter clearance; RNAP, RNA polymerase; RP_C, closed complex; RP_O, open complex; STB, standard transcription buffer.

A supplementary method and seven supplementary figures are available with the online version of this paper.

formation, isomerization to open complex (RP_O) and its stability, abortive transcription and promoter clearance. Our results show that the initiation and kinetics are characteristic of a given promoter in mycobacteria and that the strength of each of the promoters is governed at different steps of the initiation process.

METHODS

Bacterial strains, culture conditions and *in vivo* promoter activity. *M. smegmatis* mc²155 (used for *in vivo* β -galactosidase assays) and *M. smegmatis* SM07 (used for RNAP purification) were cultured in Middlebrook 7H9 medium (Difco) containing 0.05 % Tween-80 (Sigma) and 0.4 % glucose (Sigma) with shaking, at 37 °C. To measure the *in vivo* activity of the promoters, the fragments were cloned into the mycobacterial low-copy-number promoterless reporter vector pSD5b (Jain *et al.*, 1997). The fragments used for cloning were amplified by PCR using the primers listed in Table 1. The promoter sequences consist of nt -69 to +120 for P_{rrnAPCL1}, -125 to +180 for P_{rrnB} and -47 to +109 for P_{gyr}. Promoter strength was measured by using a β -galactosidase reporter assay and the activity is represented in Miller units {Miller units = 1000 $\times$ A₄₂₀/[time (min) $\times$ culture volume (ml) $\times$ OD₆₀₀] (Miller, 1992)}. *M. smegmatis* mc²155 transformed with pSD5b was used as the negative control.

RNAP purification, EMSA and preparation of transcription templates. *M. smegmatis* RNAP with a C-terminal hexa-histidine tag

in the β' subunit was purified after *in vivo* enrichment of σ^A , as described previously (China & Nagaraja, 2010). The σ^A content in the RNAP preparation used for the assays was >95 % stoichiometric to the $\beta \beta'$ subunits. The specific activity of the purified RNAP was determined by using two methods: (i) by transcription assays using the standard method of ³[H]-UTP incorporation and (ii) by EMSA using radiolabelled promoter DNA. The concentration of RNAP required for 50 % binding to promoter DNA was used for the binding and kinetic assays. Synthetic oligonucleotides (Sigma) containing promoter sequences of 95 (P_{rrnAPCL1}), 91 (P_{rrnB}) and 75 (P_{gyr}) nt were used in all the EMSAs. Oligonucleotides were 5'-end labelled with γ -³²P[ATP] (Perkin Elmer) using T4 polynucleotide kinase (NEB) and annealed with 2 mol excess of complementary strand and used for EMSAs (Table 1). The RNAP–promoter complexes were analysed by using 4 % native PAGE. The electrophoresis was carried out at 4 °C or at room temperature for RP_C and RP_O formation assays, respectively. The templates for *in vitro* transcription assays were prepared from the pUC18 promoter constructs by amplification using PCR, using a set of vector-specific primers followed by purification from the gel using a purification kit (Qiagen).

RP_C and RP_O formation and stability assays. For RP_C formation assays, 1 nM labelled DNA and increasing concentrations of RNAP (1.25–200 nM) were incubated on ice using standard transcription buffer (STB; 50 mM Tris/HCl, 5 mM magnesium acetate, 100 μ M DTT, 5 % glycerol, 50 μ g BSA ml⁻¹ and 50 mM KCl) for 15 min and loaded onto a native PAGE gel. To form the competitor-resistant open complexes, RNAP and promoters were incubated at 37 °C for 15 min followed by the addition of 50 μ g heparin ml⁻¹. The DNA–

Table 1. Oligonucleotides, strains and plasmids used in this study

Oligonucleotide/strain/ plasmid	Description*	Reference
<i>M. smegmatis</i> SM07	Hyg ^R , <i>rpoC</i> is replaced with <i>rpoC</i> with a hexa-histidine coding tag at the 3' end	Mukherjee & Chatterji (2008)
<i>M. smegmatis</i> mc ² 155	A high-efficiency transformation strain of <i>M. smegmatis</i>	Snapper <i>et al.</i> (1990)
P _{gyr} For	CGGAGCTCCAGAATCGGTGCTGTC	This work
P _{gyr} Rev	GAATGAGCTCGGATCCGGCGCCATACTCC	This work
P _{rrnAPCL1} For	GCGAGCTCGAGAAAACAACCCGGT	This work
P _{rrnAPCL1} Rev	CAAAAGAGCTCACCTCTAGACGGGAAAAAG	This work
P _{rrnB} For	CTTCTAGAGAGCTCGCTGGTGTGCGGCGTG	This work
P _{rrnB} Rev	CTGAGCTCGCATGCCGACTTTGCCGCGCAAG	This work
P _{gyr} Sense	AATTGAAACGCGGCTACAGAATCGGTGCTGTCGCTATCTCGCGGTAGAC-TGGACGACGGATCTCAGGCGGTGTCTG	This work
P _{gyr} Anti-sense	GATCCAGACACCGCCTGAGATCCGTCGTCCAGTCTACCGCGAGATAGCG-ACAGCACCGATTCTGTAGCCGCGTTTG	This work
P _{rrnAPCL1} Sense	AATTCGCGGAGCGGAGAAAACAACCCGGTCCAAGCGACTTGACAAGCC-AGACAAAGCAGTATTAAGCTGGCAGGGTTGCCCAAAACGGGGCG	This work
P _{rrnAPCL1} Anti-sense	GATCCGCCCCGTTTGGGGCAACCCTGCCAGCTTAATACTGCTTTGTCTG-GCTTGTCAGTTCGCTTGGACCGGGTGTCTTCTCCGCTCCGCGG	This work
P _{rrnB} Sense	GTCTGACCAGGGAAAATAGCCCTCTGACCTGGGGATTGACTCCCAGTTT-CCAAGGACGTAACCTATTCCAGGTACAGCGACACGGCCAG	This work
P _{rrnB} Anti-sense	CTGGGCCGTGTCGCTCTGACCTGGAATAAGTTACGTCCTTGGAAGTGGG-AGTCAAATCCCCAGGTACAGGGGCTATTTCCCTGGTCAGAC	This work
pJAM2 <i>mysA</i>	<i>M. smegmatis</i> <i>mysA</i> gene encoding σ^A cloned in pJAM2	Triccas <i>et al.</i> (1998); laboratory stock
pSD5b	<i>E. coli</i> –mycobacteria shuttle vector with promoterless <i>lacZ</i>	Jain <i>et al.</i> (1997)

*Oligonucleotide sequences are given in 5'–3' orientation.

protein complexes separated by PAGE from the free DNA were quantified using Multi Gauge software version 2.3 (Fujifilm). K_B values were determined by Prism software using the following equations, $D + P \rightleftharpoons DP$ and $K_B = [DP]/[D][P]$, where $[D]$ corresponds to the concentration of free DNA, $[P]$ corresponds to that of RNAP and $[DP]$ that of the DNA–protein complex. The $[DP]/[D]$ values were plotted as a function of RNAP concentration, where the slope of the linear plot was equal to K_B of RNAP binding (Chakraborty & Nagaraja, 2006). The K_B values were calculated from three independent sets of experiments and the mean was taken. The equilibrium dissociation constant for the heparin-resistant complex (K_d) was measured by the equation $Y = Y_{\max}[RNAP]/(K_d + [RNAP])$, where $Y_{\max}$ corresponds to binding maximum (Schroeder *et al.*, 2007). The salt sensitivity of RP_O was determined by carrying out the binding reactions in the presence of increasing concentrations of KCl. The temperature dependency of RP_O formation was determined by carrying out run-off transcription reactions using 25 nM template and 100 nM RNAP. The reactions were incubated at different temperatures (0, 7, 15, 22, 30, 37 and 44 °C) prior to the addition of NTPs. Transcription was initiated by the addition of NTPs, α - ^{32}P [UTP] and 50 μ g heparin ml^{-1} and the mixture was incubated further at 37 °C.

Association and dissociation kinetics.

RP_O formation. RNAP (100 nM) and labelled promoter DNA (1 nM) were mixed and incubated at 37 °C. Equal volumes were split into aliquots at different time points and challenged with heparin (50 μ g ml^{-1} for 1 min) before loading onto a 4% native PAGE gel which was run at room temperature (see Fig. 4a, upper path). The amount of RNAP–promoter complex obtained at various time points was normalized to that obtained after the longest incubation time to determine the fraction of bound complex (Ft). k' (pseudo first-order rate constant) was measured by using the equations described previously (Brunner & Bujard, 1987; Lutz *et al.*, 2001). The Ft values were fitted into a single exponential equation to determine the rate constants (Supplementary Methods, available with the online version of this paper).

RP_O decay. The rate of RP_O decay was determined by incubating 100 nM RNAP and 1 nM promoter DNA at 37 °C for 15 min followed by challenge with 50 μ g heparin ml^{-1} . Aliquots were taken out at different times and loaded onto a 4% native PAGE gel (see Fig. 4a, lower path). The level of radioactivity in the bound fraction was quantified and the fraction of bound complex was plotted as a function of time. k_{off} and $t_{1/2}$ of the promoters were calculated using the equation, $\ln(Ft) = -k_{\text{off}} \times t$. The half-life of the decay was calculated as $0.6932/k_{\text{off}}$ (Brunner & Bujard, 1987; Straney & Crothers, 1987; Tsujikawa *et al.*, 2002).

In vitro transcription.

RP_O half-life. Template (25 nM) was incubated with 100 nM RNAP in STB at 37 °C for 15 min to form RP_O in a reaction volume of 70 μ l. Heparin (50 μ g ml^{-1}) was added and incubation was continued for another 1 min. Aliquots were taken out at regular intervals and mixed with 100 μ M NTPs and 1 μ Ci α - ^{32}P [UTP] to initiate the RNA chain elongation. The reactions were stopped with $2 \times$ gel loading buffer [0.025% (w/v) bromophenol blue, 0.025% (w/v) xylene cyanol FF, 5 mM EDTA, 0.025% SDS and 8 M urea] and separated by using 8% urea PAGE.

Promoter clearance. After the open complexes were incubated with 50 μ g heparin ml^{-1} for 1 min, transcription was initiated by the addition of 100 μ M NTPs and 1 μ Ci α - ^{32}P [UTP]. Aliquots were withdrawn at different time intervals and the reaction mixtures were incubated for 1, 2, 5 or 10 min (Fig. 5a; Chakraborty & Nagaraja, 2006). The reactions were stopped and separated as above.

Abortive transcription. Single round transcription reactions were carried out as described above. Heparin was omitted from the reaction mix while the multiple round reactions were carried out. The transcripts were analysed by using 22% urea PAGE to resolve the abortive products (Hsu, 2009). Abortive transcripts resulting from different ratios of template (10 nM) and RNAP ranging from 2:1 to 1:20 in a single round transcription assay were also analysed by using 22% urea PAGE.

RP_O formation in the presence of initiating NTP and pppGpp.

Assays were carried out by adding increasing concentrations of iGTP (initiating nucleotide for all three promoters) and detecting the amount of RP_O formed by EMSA. To determine the fraction of closed complexes converted to the open complex, aliquots from the same assay mix incubated at 0 °C were moved to 37 °C and were incubated for 15 min. Two aliquots were taken out; one was treated with heparin and one was not. Both the samples were loaded onto a 4% native PAGE gel and run at 4 °C. The assay was carried out in both the absence and the presence of 200 μ M GTP. The ability to form initiation complexes at all three promoters was tested by adding only the initial three NTPs to the reaction, ensuring the formation of only a ternary initiation complex (Schneider *et al.*, 2003). The effect of pppGpp on RP_O was determined by incubating the RNAP and promoter DNA in the presence of 100 μ M pppGpp at 37 °C for 15 min. The complexes were analysed by EMSA after treating with 50 μ g heparin ml^{-1} for 1 min.

RESULTS

Promoter characteristics

The σ^A -dependent promoters from mycobacteria are architecturally similar to *E. coli* σ^{70} -dependent promoters (Gomez & Smith, 2000; Unniraman *et al.*, 2002). Three σ^A promoters involved in housekeeping functions were chosen for this study: $P_{rrnAPCL1}$, P_{rrnB} and P_{gyr} (Fig. 1a). Our earlier studies revealed that P_{gyr} is a strong promoter with comparable high activity to other strong promoters (Unniraman & Nagaraja, 1999). The two rRNA promoters chosen have been well characterized previously (Arnvig *et al.*, 2005; Gonzalez-y-Merchand *et al.*, 1998). Alignment of these mycobacterial promoter sequences with the σ^A consensus sequence shows their similarity, and two of the promoters (P_{rrnB} and P_{gyr}) exhibit strong *in vivo* activity (Fig. 1b) in accordance with previous observations (Arnvig *et al.*, 2005; Unniraman & Nagaraja, 1999). The *M. smegmatis* genome contains two *rrn* operons, *rrnA* and *rrnB*. The *rrnA* operon has two promoters, P_{rrnAP1} and $P_{rrnAPCL1}$, both of which are conserved across the genus (Stadthagen-Gomez *et al.*, 2008). Of the two, $P_{rrnAPCL1}$ is the major promoter in different species of mycobacteria and hence was chosen for the study. In contrast with the *rrnA* operon, a single promoter, P_{rrnB} , is known to transcribe the *rrnB* operon. P_{rrnB} is one of the strongest *rrn* promoters characterized in mycobacteria (Arnvig *et al.*, 2005; Ji *et al.*, 1994). *gyrB* and *gyrA* in *M. smegmatis* are organized as an operon driven by a single promoter P_{gyr} (Unniraman & Nagaraja, 1999). This is a strong promoter *in vivo* and responds to regulation by the topological status of DNA by the process termed as relaxation-stimulated transcription (Unniraman & Nagaraja, 1999). A compar-

ison of the -10 region of all three promoters shows that T at the first position, A at the second position and T at the sixth position are identical to the mycobacterial consensus sequence for the σ^A -dependent promoters (Unniraman *et al.*, 2002). These three bases are most conserved amongst the σ^A -dependent promoters of mycobacteria (Gomez & Smith, 2000) as well as σ^{70} promoters of *E. coli* (Lisser & Margalit, 1993) and are shown to play an important role during promoter DNA melting (McClure, 1985). The -35 site of $P_{rrnAPCL1}$ and P_{rrnB} shows similarity with the consensus sequence, while P_{gyr} has only two of six residues similar, although it shows strong *in vivo* activity. The spacer length between the -10 and -35 element of $P_{rrnAPCL1}$ and P_{rrnB} is 18 and 17 nt, respectively, compared with the 16 nt spacer present in all *E. coli* *rrn* promoters. Thus, the promoters selected have the following characteristics: (i) they transcribe housekeeping genes; (ii) the transcription start site is mapped, and -10 and -35 sequences are defined; and (iii) they show *in vivo* activity in exponentially growing cells. For a direct comparison, activities of the promoters were determined *in vivo* by transforming *M. smegmatis* with promoter-*lacZ* transcriptional fusion constructs (Fig. 1b). All the promoters were active at early exponential growth phase. In these assays, P_{rrnB} had the highest activity followed by P_{gyr} and $P_{rrnAPCL1}$. Although the promoter sequences closely match the consensus sequences, $P_{rrnAPCL1}$ and P_{rrnB} showed contrasting promoter strength *in vivo* in exponential growth phase. The expression patterns of the promoters were analysed at different growth phases (Supplementary Fig. S1, available with the online version of this paper). P_{rrnB} was down-regulated with no significant changes compared with $P_{rrnAPCL1}$ and P_{gyr} in stationary phase. The difference in the activity of the three promoters could be due to the variations in their interactions with RNAP. Hence, the

different steps of transcription initiation at these promoters were investigated.

Promoter binding and melting

The transcription initiation events begin with the sequence-specific binding of RNAP to the promoters (Brunner & Bujard, 1987; Buc & McClure, 1985; McClure, 1985), forming a closed complex (RP_C); Fig. 2 shows the sequential reaction pathway. To determine the equilibrium binding constants, promoter DNA and different concentrations of RNAP were maintained on ice for 15 min and the complexes were resolved by native PAGE (Fig. 3a). The K_B value of the RNAP is comparable for all three promoters (Fig. 3b and Table 2). Although P_{rrnB} was the strongest among the three promoters *in vivo* (Fig. 1b), its high strength was not evident at the RP_C formation step. Mycobacterial RNAP forms a promoter-specific complex at 0°C at all the promoters tested. The complex is stable and resistant to challenge by ~ 100 mM KCl (Supplementary Fig. S2). The RNAP also binds non-specifically to the double-stranded DNA. The promoter-non-specific complex is sensitive to treatment with 100 mM KCl (data not shown). In the next step of the transcription initiation pathway, RP_C is converted to RP_O . The equilibrium dissociation constant (K_d) was determined for the three promoters by measuring the extent of competitor-resistant complex formation with increasing RNAP concentrations (Fig. 3c). In contrast with RP_C formation, only a fraction of DNA was bound by the RNAP to form RP_O , even at saturating concentrations of the enzyme, indicating that only a subset of initially bound RNAP could form a competitor-resistant complex. RP_O formation was significantly different for each one of the promoters. $P_{rrnAPCL1}$ and P_{rrnB} had threefold differences between their K_d values and P_{gyr} was found to have a K_d

Fig. 1. Promoter structure and function. (a) Sequences of the three promoters aligned with the *E. coli* σ^{70} - and mycobacterial σ^A -dependent promoter consensus sequences. The -35 , -10 and $+1$ (transcription start site) sites of each promoter are represented in bold. (b) *In vivo* activity of the promoters. β -Galactosidase reporter activity of the promoters cloned in pSD5b vector was measured from early exponential phase cultures of *M. smegmatis*. Error bars, SD.

Fig. 2. Transcription initiation pathway. RNAP (R) binds to the promoter (P) through base-specific contacts mediated by the sigma factor to form a closed complex (RP_C). In the subsequent step, RP_C undergoes conformational changes to form the competitor-resistant open complex (RP_O), after melting of 12–14 bp of the duplex DNA around the +1 site. The first two NTPs complementary to the +1 and +2 positions on the template strand bind to RP_O , forming the pre-initiation complex ready for elongation. At this stage, RNAP synthesizes short abortive transcripts of 2–14 nt before proceeding into the elongation mode. Promoter clearance, the last step of transcription initiation, involves RNAP switching from abortive synthesis to the productive elongation complex (RP_E) (Haugen *et al.*, 2008; Helmann & deHaseth, 1999; McClure, 1985; Nudler, 2009). RP_{int} , transient intermediate complex; RP_I , ternary initiation complex.

value intermediate to these (Fig. 3d and Table 2). Surprisingly, a higher dissociation of RNAP from the P_{rrnB} promoter is in contrast with its high *in vivo* promoter strength (see Fig. 1b).

Thermal energy is required for the duplex unwinding during the isomerization process to form RP_O . Different promoters may need a different degree of thermal energy for DNA melting. For example, most *E. coli* promoters studied so far are inactive at temperatures below 20 °C (Burns *et al.*, 1996). *In vitro* transcription assays were carried out after incubating the promoter and RNAP at different temperatures ranging from 0–44 °C to determine the temperature at which the transition from closed to open complex occurs. Transcripts were not detected in reactions pre-incubated at temperatures less than 20 °C. This is an indication that the

complex formed below 20 °C is predominantly a closed complex (Supplementary Fig. S3).

Kinetics of RP_O formation and decay

The rate of formation of competitor-resistant RP_O is shown in Fig. 4(b). Interestingly, each of the promoters exhibited different kinetics for RP_O formation. The rate constant for RP_O formation was found to be $0.26 \pm 0.039 \text{ min}^{-1}$ at the P_{rrnB} promoter, $0.098 \pm 0.016 \text{ min}^{-1}$ at $P_{rrnAPCL1}$ and $0.11 \pm 0.024 \text{ min}^{-1}$ at P_{gyr} (Fig. 4c), showing that these two rRNA promoters have threefold differences between them in the rate of isomerization. Although the P_{rrnB} promoter showed the highest rate of open complex formation amongst the three promoters, it exhibited a higher dissociation of the enzyme (Table 2), indicating that

Fig. 3. K_B of RNAP binding and K_d of competitor-resistant complex. (a) Increasing concentrations of RNAP were incubated with the 5'-end labelled promoter fragments and resolved by using native PAGE. The smear in the gel resulted from the dissociation of complexes during electrophoresis. (b) The amount of free (D) and RNAP-bound (DP) DNA was quantified and analysed to determine the K_B of RNAP binding. $[DP]/[D]$ values (bound:free DNA) are shown; the slopes of the plots are a measure of K_B . (c) The K_d for the heparin-resistant complex was determined by incubating increasing concentrations of RNAP with the promoter fragments at 37 °C, followed by treatment with heparin and analysis using native PAGE run at room temperature. (d) The amount of RNAP-bound DNA (DP) was quantified and analysed to determine the K_d of the competitor-resistant complex.

Table 2. Summary of equilibrium binding and kinetic rate constants

Constant/property	P _{rrnAPCL1}	P _{rrnB}	P _{gyr}
Relative <i>in vivo</i> strength	1	12	5
K_B ($\times 10^6$ M ⁻¹)*	7.5	4.9	6.8
K_d ($\times 10^{-7}$ M ⁻¹)*	0.51	1.47	0.95
k' (min ⁻¹)*	0.098 ± 0.016	0.26 ± 0.039	0.11 ± 0.024
k_{off} (min ⁻¹)*	0.105 ± 0.015	0.177 ± 0.019	0.046 ± 0.009
$t_{1/2}$ (min)*	6.5	3.9	14.9
Abortive transcription†	++++	—	+
Promoter clearance, PC _{90%} (min)	10	2.3	3.7

*Kinetic constants are shown in the transcription initiation equation presented in Fig. 2.

†Qualitative description of the level of abortive transcripts formed at each promoter.

the complex is very unstable. The k_{off} of RP_O was measured from the exponential decay (Fig. 4d). The rate of decay of RP_O and its half-life ($t_{1/2}$) were analysed for all three promoters. In spite of its higher isomerization rate, the P_{rrnB} promoter showed the highest k_{off} (0.177 ± 0.019 compared with 0.105 ± 0.015 and 0.046 ± 0.009 for P_{rrnAPCL1} and P_{gyr} respectively). The $t_{1/2}$ of the P_{rrnB} open complex was approximately 3.9 min, followed by P_{rrnAPCL1} (6.5 min) and P_{gyr} (14.9 min) (Table 2).

To further examine the above findings, *in vitro* single round transcription assays were carried out. These assays also enabled the extent of RP_O stability to be determined. The run-off transcript length was 120 nt for P_{rrnAPCL1}, 180 nt for P_{rrnB} and 109 nt for P_{gyr}. The amount of run-off transcripts produced correlates with the fraction of transcriptionally active RP_O. The stability of RP_O at P_{rrnB} was found to be lowest, followed by P_{rrnAPCL1} and P_{gyr} (Fig. 4f). The values obtained in this set of assays are different from the earlier EMSA results, as the reaction conditions varied and included NTPs. However, the trend is similar irrespective of the assays used (Fig. 4e and f).

Promoter clearance

The last step in transcription initiation is promoter clearance. RP_O is converted to a pre-initiation complex in the presence of initiating NTPs before proceeding to the elongation step after the synthesis of abortive transcripts. Promoter clearance assays were carried out to analyse the kinetics of polymerase escape into elongation (Fig. 5b). In these assays, the fastest kinetics of promoter escape were observed from the P_{rrnB} promoter. Once RP_O was formed, transition into elongation was most rapid at this promoter [90% promoter clearance (PC_{90%})=2.3 min]. The promoter clearance at P_{rrnAPCL1}

(PC_{90%}=10 min) was more than fourfold slower than at P_{rrnB} while P_{gyr} (PC_{90%}=3.7 min) showed relatively fast promoter escape. The slower promoter clearance at P_{rrnAPCL1} appears to be its major rate-limiting step. To better understand these results and to further assess the late events during transcription initiation, abortive initiation assays were carried out. The results presented in Fig. 5(c) and Supplementary Fig. S4 reveal that the abortive transcription products were synthesized substantially at P_{rrnAPCL1} and were lower and not readily detectable at P_{rrnB} and P_{gyr}. The lower level of transcription seen at P_{rrnAPCL1} is thus due to the high level of abortive transcription. The multiple round transcription reactions (Fig. 5d) show that the overall transcriptions at P_{rrnB} and P_{gyr} promoters were higher than at the P_{rrnAPCL1} promoter, matching the *in vivo* promoter activity (see Fig. 1b).

Regulation by initiating NTP

Nucleotides serve as important effectors in positive regulation of the *rrn* promoters. The initiating nucleotide stabilizes the intrinsically short-lived RP_O by pairing with the template strand (Barker & Gourse, 2001). The RP_O formation assays were carried out in the presence of initiating nucleotide GTP at P_{rrnAPCL1}, P_{rrnB} and P_{gyr}. Initially, the effects of different combinations of NTPs (+1; +1 and +2; +1, +2 and +3; and all four NTPs) on RP_O formation were tested (Supplementary Fig. S5). The results indicate that the presence of +1 NTP is sufficient to activate RP_O formation at P_{rrnB} and to a lesser extent at P_{rrnAPCL1} (Fig. 6a). To assess the regulation of *rrn* promoters by other small molecule effectors, the role of pppGpp in RP_O stability was tested. The open complex at P_{rrnB} was destabilized in the presence of pppGpp, whereas pppGpp had no significant effect on P_{rrnAPCL1} and P_{gyr} (Fig. 6b). As expected, *in vitro* transcription assays revealed that the inhibition of P_{rrnB} promoter by pppGpp had no significant effect on P_{rrnAPCL1} and P_{gyr} (data not shown).

The effect of initiating NTP on open complex formation was tested further by determining the fraction of RP_C converted into RP_O in presence of iGTP. The extent of RP_O formation is lower at P_{rrnB} in the absence of iGTP (Fig. 6c). However, in the presence of the iGTP, RP_O formation at P_{rrnB} increased by nearly fivefold (Fig. 6d). The complex formation was also stimulated at P_{rrnAPCL1} by ~1.5-fold (Fig. 6c and d). The NTPs stimulate RP_O formation by increasing the stability of the complex, thus reducing the k_{off} and enhancing the half-life (Supplementary Fig. S6). Several-fold stimulation of RP_O formation by iGTP at the P_{rrnB} promoter also seems to contribute to its overall high promoter strength. As expected, iGTP did not stabilize RP_O at the P_{gyr} promoter (Fig. 6c and d and Supplementary Fig. S6). Once a ternary complex is formed with the synthesis of the first phosphodiester bond, RNAP could form a stable initiation complex at all three promoters (Supplementary Fig. S7).

Fig. 4. Kinetics of RP_O formation and decay. (a) Representation of the experimental procedure. (b) The formation of complexes between RNAP and the promoters was analysed under pseudo-first-order conditions by monitoring the formation of heparin-resistant RP_O . RNAP (100 nM) and promoter DNA (1 nM) were incubated at 37 °C and aliquots were removed at various time intervals (0.5–48 min). The samples were challenged with heparin and the extent of RP_O was measured after separating the complexes by using native PAGE. (c) The data were fitted into a pseudo-first-order kinetic equation. (d) The decay of specific complexes formed between RNAP and promoters in the presence of a competitor (heparin) was monitored by quantification of the heparin-resistant complex RP_O . Complex dissociation was monitored from 0 to 64 min by measuring the fraction of RP_O remaining after the addition of heparin. (e) The exponential fits of the data were plotted to measure dissociation rate constant, k_{off} , and the half-life of the decay. (f) Open complex decay by *in vitro* transcription. The stability of RP_O was analysed by an *in vitro* transcription assay using linear DNA templates, which generates run-off transcripts. RP_O was formed and then challenged with heparin. Aliquots were removed at different time intervals and transcription was initiated by the addition of NTP mix. The per cent transcription was plotted to determine the half-life of RP_O . Data shown are means $\pm$ SD based on three independent experiments.

DISCUSSION

After a promoter search, RNAP initiates a complex series of sequential interactions at the promoters culminating in polymerase escape and transcription elongation. The

complex functional pathway intrinsic to a given promoter is subjected to different rate-limiting substeps and the promoter strength is the end result of an optimization process involving many parameters. Thus, for each promoter, kinetic properties of the multi-step transcription

Fig. 5. Promoter clearance efficiency and abortive transcription. (a) Representation of the experimental procedure. (b) Promoter clearance assay. Open complexes were challenged with heparin, and then transcriptions were initiated by the addition of NTP. $PC_{90\%}$ is shown on the graph. (c) Abortive versus productive transcription. Promoter clearance assays were performed, with aliquots removed at different time intervals (1, 2, 5 and 10 min). The transcripts were separated by using 22 % urea-PAGE. Run-off transcripts are shown in separate boxes. The profiles of abortive transcripts at the three promoters are presented in the lower panel; prominent products are marked with asterisks. (d) Multiple round transcriptions from three promoters. Run-off transcripts are shown in the upper panels and abortive products are marked by asterisks.

initiation pathway are distinct and the rate-limiting steps may vary. In this first detailed analysis of mycobacterial promoter–polymerase interactions, we show that the three promoters studied possess different characteristics in spite of being housekeeping promoters with similar architecture. From the data presented, it is evident that each one of the promoters has its own characteristic interaction pattern with the RNAP.

Unlike *M. tuberculosis* and other slow-growing mycobacteria, which encode a single rRNA operon, *M. smegmatis* and other fast-growing mycobacteria have two *rrn* operons, *rrnA* and *rrnB* (Menendez *et al.*, 2002; Sander *et al.*, 1996; Stadthagen-Gomez *et al.*, 2008). In the *rrnA* operon, $P_{rrnAPCL1}$ is conserved across all mycobacteria and contributes about 5 % of rRNA transcripts in exponentially growing *M. smegmatis* cells. The expression levels of this promoter remain unaltered under nutrient starvation conditions (Gonzalez-y-Merchand *et al.*, 1998). The other promoter present in the *rrnA* operon (P_{rrnAP2}) contributes predominantly to rRNA synthesis during exponential growth phase. This promoter closely resembles P_{rrnB} used in this study in its architecture, expression pattern and the degree of rRNA synthesis (Gonzalez-y-Merchand *et al.*,

1997, 1998). *M. smegmatis* P_{rrnB} , which drives the transcription of the *rrnB* operon, contributes to more than 40 % of the total rRNA in exponential phase (Gonzalez-y-Merchand *et al.*, 1998).

Here, we provide mechanistic insights into the substeps of transcription initiation at two of the *rrn* promoters ($P_{rrnAPCL1}$ and P_{rrnB}), indicating their key regulatory features. In spite of having sequence similarity at the –35 and –10 regions, these two promoters contribute to the total rRNA synthesis to vastly different extents. In accordance with its known characteristics, RP_C formation (the first identifiable complex during the initiation pathway) was most prominent at $P_{rrnAPCL1}$. Furthermore, the isomerization of closed complex to open complex was very efficient at this promoter (Table 2). The extent of isomerization of RP_C to RP_O suggested that $P_{rrnAPCL1}$ is potentially a strong promoter. However, *in vivo* activity of this promoter at the exponential phase was relatively low. This study provides an explanation for this observation. Since the promoter escape was compromised due to the synthesis of abortive transcription products, the initial strength of RP_O formation was not reflected in the final productive transcription at this promoter. On the other

Fig. 6. Effect of iNTP binding on RP_O . (a) Open complex formation assays were performed as described in Methods. The fraction of productive RNAP–promoter complex formed in the absence and presence of initiating NTP was measured. (b) The inhibitory effect of pppGpp on RP_O . Open complexes were formed on the promoter following incubation with 100 μM pppGpp and the reactions were resolved by an EMSA. EMSAs were carried out in the presence and absence of 100 μM GTP. (c, d) The samples for the closed complex (open bars; 1), 37 °C complex (filled bars; 2) and heparin-resistant complex (shaded bars; 3) were taken from the same assay mix and separated on the gel as described in Methods. Assays were carried out in the absence (c) and presence (d) of iNTP. The values on the graph were normalized to the amount of closed complex formed in each assay.

hand, P_{rrnB} was found to be the strongest among all three promoters studied *in vivo* and is possibly one of the strongest promoters in the exponentially growing mycobacterial cells. The strength of this promoter was mediated at the later steps of transcription initiation, since the equilibrium binding affinity for closed and open complex formation was moderate in the absence of any other factors (Table 2). The faster promoter clearance facilitated the complex to proceed towards elongation rapidly. The RP_O formation at this promoter was stimulated by the binding of initiating nucleotide iGTP. The activation of the P_{rrnB} promoter in the presence of iGTP provides another explanation as to why this promoter is one of the strongest in the exponential growth phase, in spite of poor initial RNAP–promoter complex formation. P_{rrnB} is possibly regulated by growth-rate-dependent transcriptional control. The promoter activity is highest during exponential growth phase due to the presence of optimum concentrations of NTPs. In nutrient starvation conditions, NTP levels would go down, leading to a decrease in P_{rrnB} promoter activity (Gonzalez-y-Merchand *et al.*, 1998; Verma *et al.*, 1999). pppGpp is known to exert stringent control of transcription by decreasing the stability of unstable open complexes at *rrn* promoters (Haugen *et al.*, 2008). Accordingly, we observed inhibitory effects of the alarmone pppGpp at this promoter. In contrast, $P_{rrnAPCL1}$ is not inhibited to a significant extent by pppGpp and is also not stimulated by iGTP to the same extent as P_{rrnB} . The transcription at this promoter is further compromised at the promoter clearance step by high rates of abortive

transcription, possibly contributed by both its promoter recognition region and initial transcribed sequence. $P_{rrnAPCL1}$ may be responsible for carrying out the basal level of rRNA transcription activity required in stationary phase and in nutrient-starved conditions, as the total amount of RNA synthesized from this promoter remains unaltered. These conclusions were based on the primer extension analysis of RNA isolated from *M. smegmatis* cultures grown in complete medium or in carbon-limiting medium (Gonzalez-y-Merchand *et al.*, 1998). During balanced growth in the complete medium, P_{rrnB} was the major contributor towards pre-rRNA synthesis, whereas in stationary phase, activity of this promoter was reduced and $P_{rrnAPCL1}$ served as one of the major sources of rRNA transcripts. Detailed studies carried out with *rrn* promoter regulation in *E. coli* provide parallels to the results obtained with two mycobacterial promoters. In all the seven *E. coli* operons, the first of the two promoters (P_{rrnA1} , P_{rrnB1} etc.) contributes predominantly during exponential phase and is upregulated to a high level in response to growth-rate-dependent regulation by iNTP, whereas P_{rrnP2} appears to have a major role during stationary phase (Murray & Gourse, 2004). The open complex lifetime of P_{rrnP2} was found to be more than that for P_{rrnP1} , and it is not significantly regulated by iNTP (Murray & Gourse, 2004). *M. smegmatis* *rrnB* promoter is very much like the *E. coli* *rrnP1* promoter in overall properties, thus revealing common features shared by the bacteria for stable RNA transcription. Promoters driving transcription for the genes encoding DNA gyrase are, in general, regulated by

DNA topology to maintain the overall negative supercoiled nature of the genome. Earlier studies showed that P_{gyr} is a strong promoter in *M. smegmatis* during exponential growth (Unniraman & Nagaraja, 1999). From the present study, it is evident that P_{gyr} has moderately strong equilibrium and kinetic parameters (in between the two *rrn* promoters studied) and promoter clearance is fast, with minimal abortive transcripts (Table 2). P_{gyr} had the slowest rate of RP_O decay but also had fast promoter escape in the presence of NTPs. Thus, the rate-limiting step at this promoter appears to be the initial binding of RNAP to the promoter, which might depend on local DNA conformations separate from the sequence.

In conclusion, the analysis of the transcription initiation pathway of σ^A -dependent promoters of *M. smegmatis* provides the first insights into the general mechanism of promoter–RNAP interactions in mycobacteria. The steady-state kinetics are influenced by the nature of individual promoters and their interactions with RNAP. From studies in *E. coli* and other organisms, it is apparent that rate-limiting steps are targeted by regulatory proteins. Thus, it is conceivable that some of the cellular regulators in mycobacteria would target rate-limiting steps at these promoters. Finally, various assays to study the early steps of transcription, described here using mycobacterial RNAP and promoters, would be useful in elucidating the mechanism of action of various transcription activators and repressors of this important genus.

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