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HAL Id: inserm-02137260
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Submitted on 22 May 2019

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RbpA relaxes promoter selectivity of *M. tuberculosis* RNA polymerase

Ayyappasamy Sudalaiyadum Perumal\(^*\)¹, Rishi Kishore Vishwakarma\(^*\)¹, Yangbo Hu², Zakia Morichaud¹ and Konstantin Brodin\(\text{\textregistered}^1,^3\)

¹IRIM, CNRS, Univ Montpellier, 1919 route de Mende, 34293 Montpellier, France and ²Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan 430071, China

Received March 27, 2018; Revised July 21, 2018; Editorial Decision July 24, 2018; Accepted July 25, 2018

ABSTRACT

The transcriptional activator RbpA associates with *Mycobacterium tuberculosis* RNA polymerase (*Mt*RNAP) during transcription initiation, and stimulates formation of the *Mt*RNAP-promoter open complex (RPs). Here, we explored the influence of promoter motifs on RbpA-mediated activation of *Mt*RNAP containing the stress-response \(\sigma^B\) subunit. We show that both the ‘extended −10’ promoter motif (T\(_{17}\)G\(_{16}\)T\(_{15}\)G\(_{14}\)) and RbpA stabilized RPo and allowed promoter opening at suboptimal temperatures. Furthermore, in the presence of the T\(_{17}\)G\(_{16}\)T\(_{15}\)G\(_{14}\) motif, RbpA was dispensable for RNA synthesis initiation, while exerting a stabilization effect on RPs. On the other hand, RbpA compensated for the lack of sequence-specific interactions of domains 3 and 4 of \(\sigma^B\) with the extended −10 and the −35 motifs, respectively. Mutations of the positively charged residues K73, K74 and R79 in RbpA basic linker (BL) had little effect on RPo formation, but affected *Mt*RNAP capacity for *de novo* transcription initiation. We propose that RbpA stimulates transcription by strengthening the non-specific interaction of the \(\sigma\) subunit with promoter DNA upstream of the −10 element, and by indirectly optimizing *Mt*RNAP interaction with initiation substrates. Consequently, RbpA renders *Mt*RNAP promiscuous in promoter selection, thus compensating for the weak conservation of the −35 motif in mycobacteria.

INTRODUCTION

In bacteria, transcription is performed by the multi-subunit DNA-dependent RNA polymerase (RNAP) that is composed of the catalytic core (E, subunits 2\(\alpha\)β\(\beta\)\(\omega\)) and the \(\sigma\) subunit, required for promoter-specific initiation of RNA synthesis (reviewed in (1,2)). During exponential growth, expression of most genes is controlled by the housekeeping (principal) \(\sigma\) subunit (\(\sigma^70\) in *Escherichia coli*, and \(\sigma^A\) in *Mycobacterium tuberculosis*) that belongs to the Group 1 \(\sigma\) subunits. Alternative Group 2 \(\sigma\) subunits (\(\sigma^S\) in *E. coli*, and \(\sigma^B\) in *M. tuberculosis*) are the most similar to the principal \(\sigma\) subunit, and are responsible for the expression of specialized genes in response to stress, during the stationary growth phase and dormancy (3,4).

Most bacterial promoters recognized by Group 1 and 2 \(\sigma\) subunits belong to the −10/−35 class and contain two consensus elements: the −10 element (*E. coli* consensus motif: T\(_{12}\)A\(_{11}\)T\(_{10}\)A\(_{9}\)A\(_8\)T\(_7\)) and the −35 element (*E. coli* consensus motif: T\(_{35}\)T\(_{34}\)G\(_{33}\)A\(_{32}\)C\(_{31}\)A\(_{30}\)). These motifs are recognized by domain 2 (\(\sigma^2\)) and 4 (\(\sigma^4\)) of the \(\sigma\) subunit, respectively. The ‘extended −10’ motif of RPo consists of the extended −10 motif (T\(_{17}\)R\(_{16}\)T\(_{15}\)G\(_{14}\); R = purine) that is located one nucleotide upstream of the −10 element (5–7) and is recognized by domain 3 of the \(\sigma\) subunit (\(\sigma^3\)). It has been shown that the extended −10 motif bypasses the requirement of the \(\sigma^4/35\) element interaction (8,9). The percentage of promoters containing at least the downstream part of the extended −10 motif (T\(_{15}\)G\(_{14}\)) varies among bacteria, from ~18% in *E. coli* to ~45% in *Bacillus subtilis* (6,7,10).

During transcription initiation, RNAP binds to the promoter and forms an unstable ‘closed complex’ (RCp) that isomerizes spontaneously into a transcriptionally competent ‘open complex’ (RPo) through the formation of several intermediate complexes (RPI) (11–13). The concerted action of the RNAP core and \(\sigma\) subunit triggers the opening of ~13 bp of the promoter DNA around the transcription start site, and makes the single-stranded DNA template available for initiation of RNA synthesis (14–16).

*M. tuberculosis* RNAP (MtRNAP) differs from the extensively studied *E. coli* RNAP because it requires auxiliary factors (CarD and RbpA) to form stable RPo on house-
keeping gene promoters (17,18). RbpA is a global transcriptional activator essential for M. tuberculosis growth, and could be implicated in the control of its physiological state (19–22). RbpA selectively binds to the σ^A^ and σ^B^ subunits of MtbRNAP and stimulates RPo formation (19,23,24). It has been shown that the stress-response σ^B^-MtbRNAP displays stronger dependence on RbpA than σ^A^-MtbRNAP (24).

Structural studies demonstrated that RbpA C-terminal domain interacts with σ2 via its σ-interacting domain (SID), whereas RbpA basic linker (BL) interacts with promoter sequences upstream of the −10 element (25,26). RbpA seems not to recognize any DNA motif, although its requirement for transcription has been shown to be promoter sequence-dependent (18,24). Indeed, RbpA is required for the stable binding of σ^B^-MtbRNAP at promoters of the −10/-35 (rrnAP3, sigAP, lacUV5) and extended −10 class (galP1icons) (24). However, it is dispensable for RPo formation at the extended −10 class sinP3 promoter of B. subtilis (24). Recently, we demonstrated that RbpA stabilizes the 'open' conformation of the σ^B^ subunit in MtbRNAP. This is optimal for recognition of the −10/-35 promoters, but is dispensable for recognition of the extended −10 promoters (27). Here, to better understand the molecular basis of this promoter specificity, we explored the effect of mutations in σ^B^ and RbpA on MtbRNAP activity at promoter variants that harbor different combinations of the extended −10 and −35 motifs. We found that interaction between domain 3 of σ^B^ and the extended −10 motif strongly influences MtbRNAP activity, but has no effect on its ability to respond to RbpA activation. Furthermore, we found that RbpA modulates MtbRNAP selectivity for nucleotide substrates.

**MATERIALS AND METHODS**

**Proteins and DNA fragments**

*Mtb*RNAP, the σ^A^ subunit and RbpA were expressed and purified as described before (24). Mutations in σ^B^ and RbpA were introduced using the Agilent Quick Change Lightening Site-directed Mutagenesis Kit, following the manufacturer’s protocol. Variants of the sigAP promoter were prepared by annealing two oligonucleotides followed by primer extension and PCR amplification with Pfu using fluorescent primers (Table S1). The amplified promoter DNA fragments were resolved by 8% native PAGE and extracted using the Nucleospin® Gel and PCR Clean-up Kit (Macherey Nagel). The sigAP-TGTG promoter labeled with Cy3 at the +2 position was purified through 6% PAGE after primer extension.

**EMSA and KMnO₄ probing**

Core MtbRNAP (100 nM) was mixed with σ^B^ (300 nM) and RbpA (300 nM) in transcription buffer (TB, 40 mM HEPES pH 8.0, 50 mM NaCl, 5 mM MgCl₂, and 5% glycerol) and incubated at 37°C for 10 min. Then, fluorescein-labeled promoter DNA (50 nM) was added and samples were incubated at 37°C for 10 min. The competitor poly(dA-dT) was added to a final concentration of 20 ng/μl and incubated at 37°C for 5 min. Samples were resolved on 6% native PAGE in 1× TBE buffer. Gels were scanned with an Amersham Imager 600 (GE Healthcare) and quantified using the ImageQuant software. For KMnO₄ probing experiments, 5 mM KMnO₄ was added to the reaction mixtures formed at the indicated temperatures for 30 s, and quenched by addition of 1 M β-mercaptoethanol, 1.5 M Na(CH₃COO)₂ pH 7.0. Reactions were incubated with 0.5 M piperidine at 90°C for 15 min, and DNA fragments were precipitated by adding 1/10 volume of 5 M LiCl and 4 volumes of ice-cold ethanol. Precipitated DNA fragments were washed with 80% ethanol, vacuum-dried, dissolved in 90% formamide and analyzed on 8% sequencing gels. Gels were scanned with a Typhoon 9400 Imager (GE Healthcare) and quantified with the ImageQuant software. Graphs were plotted using the Graphpad7 and Grace-5.1.23 software (http://plasma-gate.weizmann.ac.il/Grace/) software. The apparent dissociation constants (K_d) were calculated from equation: RP = A0[RNPAP]/([RNPAP]+ K_d, where RP is the RNAP fraction bound to DNA.

**Transcription assays**

Multiple-round transcription assays were performed in 10 μl of TB with 50 μM each of ATP, GTP, CTP, 5 μM of UTP and 0.5 μM of [α^32P]-UTP at 37°C for 5 min. The GpC primer (Eurogentec) was added to 100 μM, when indicated.

Single-round transcription assays, to monitor RPo formation, were performed in 10 μl of TB. First, 180 nM MtbRNAP core, 590 nM σ^A^ and 590 nM RbpA were mixed and incubated at 37°C for 5 min. After addition of 50 nM of promoter DNA, samples were incubated at 37°C for 1, 2, 3, 5 and 10 min. Transcription was initiated by adding 50 μM each of ATP, GTP, CTP, 10 μM of UTP, 0.5 μM of [α^32P]-UTP and poly(dI-dC) (0.1 mg/ml final concentration) and performed at 37°C for 3 min. Single-round transcription assays, to monitor promoter escape, were performed using the same conditions as for RPo formation. MtbRNAP-promoter complexes were incubated at 37°C for 15 min (longer incubation at 37°C resulted in MtbRNAP inactivation). Then, after addition of the NTPs/poly(dI-dC) mixture, transcription was performed for 0.5, 1, 2, 5 and 10 min. Abortive transcription assays using the lacUV5 bubble template (28) were performed in 10 μl of TB. First, 180 nM MtbRNAP core was mixed with 590 nM σ^A^ or 1 μM σ^B^Δ4 and incubated at 37°C for 5 min. Then, 50 nM bubble DNA was added and incubated at room temperature (RT; 22°C) for 15 min. After addition of 0.5 mM ApA, 100 μM GTP, and 0.5 μM of [α^32P]-UTP, samples were incubated at RT for 10 min, and then reactions were stopped by addition of an equal volume of 7M urea/100 mM EDTA solution. RNA transcripts were analyzed on denaturing 18% PAGE/7M urea gels. Gels were scanned with a Molecular Dynamics STORM Imager. Bands were quantified using the ImageQuant software. For kinetics experiments, raw data were fitted in Grace-5.1.23 using the mono-exponential function A_t = A∞_t + A0·exp(-k·t), where A_t is the radioactive RNA signal at the time point t. The A∞ values determined from the fits were used for data normalization in each experimental set. Normalized data were used to calculate the mean and standard error (SE) values shown in
Fluorescent assay to determine the dissociation kinetics

Assays were performed in 60 μl of TB; 50 nM of RNAP was mixed with 5 nM of the sigA4P promoter fragment labeled with Cy3 at position +2 of the non-template DNA strand and incubated at 37°C for 10 min. To initiate dissociation of the MtbRNAP-promoter complexes, heparin was added to 10 ng/μl. Data were acquired using a PTQ Quantamaster spectrophotometer at room temperature. Data were fitted using the following bi-exponential equation: $A_t = A_0 + A_1 \cdot \exp(-k_{\text{fast}} \cdot t) + A_2 \cdot \exp(-k_{\text{slow}} \cdot t)$, where $A_t$ is the promoter DNA fraction bound to RNAP, calculated from the fluorescence fold change value ($A_t = (F - F_0)/F_0$), and $k_{\text{fast}}$ and $k_{\text{slow}}$ are the rate constants for the fast and slow phase, respectively; $F$ is the fluorescence signal of RNAP-bound DNA, and $F_0$ is the fluorescence signal of free DNA. The slow phase constant $k_{\text{slow}}$ was considered to be the rate constant $k_d$ for RNAP dissociation.

Native gel electrophoresis assay to study RbpA binding

RbpA was conjugated with the sulhydryl-reactive dye, Dylight 633 maleimide (Thermo Scientific), as described (24). Labeled RbpA (1.6 μM) was incubated with different concentrations of the B subunit (0.8, 1.6 and 3.2 μM) in 10 μl of TB at 37°C for 10 min. Samples were analyzed on 5–10% native PAGE in Tris–glycine buffer. Gels were scanned with a Typhoon 9400 Imager (GE Healthcare).

RESULTS

RbpA is dispensable for transcription from promoters containing the extended −10 motif

To explore the impact of the extended −10 motif on transcription initiation by the stress-response σB-MtbRNAP holoenzyme, we used four templates derived from the housekeeping, RbpA-dependent M. tuberculosis sigA promoter (sigA4P-WT) (Figure 1A). The four sigA4P variants carried the TG motif at different positions: −15 to −14 (T15G14) in sigA4P-TG1; −16, −17 (T16G17) in sigA4P-TG2; and −14 to −10 (T15G16,T14G15) in sigA4P-TGTG and in sigA4P-TGTGC. In addition, the sigA4P-TGTGC template carried a C nucleotide at position −13. We then tested whether σB-MtbRNAP could initiate transcription from these different sigA4P promoter variants using multiple round-run off-transcription assays (Figure 1B and C; Supplementary Figure S1A). In the absence of RbpA, σB-MtbRNAP was almost inactive at the sigA4P-WT promoter, in agreement with our previously published results (24). Introduction of any of the TG motifs stimulated transcriptional activity. Thus, the efficiency of transcription from the sigA4P-TGTG promoter in the absence of RbpA was similar to that observed at the sigA4P-WT promoter in the presence of RbpA. Introduction of a C nucleotide at position −13, which is known to stimulate promoter binding by the orthologous stress-response σS subunit from E. coli (29), had no effect on σB-MtbRNAP activity. We conclude that the T15G16,T15G14 motif can fully abolish RbpA requirement for transcription initiation. The fact that neither the TG1 nor the TG2 motif alone was sufficient to reach the level of transcription observed with the TGGT motif suggests that DNA bases at positions −17 to −14 interact cooperatively with σB. The RbpA-σB-MtbRNAP complex showed similar levels of transcription at all tested templates (Figure 1C), suggesting that RbpA makes MtbRNAP tolerant to sequence variations in the extended −10 motif.

RbpA activates transcription by stimulating MtbRNAP capacity to form RPo (24). To assess whether the TG motif makes RbpA dispensable for transcription initiation through stimulation of RPo formation, we performed KMnO4 probing of RNAP-promoter complexes formed at equilibrium. The template DNA strand thymines at positions −11, −9, −8 of the sigA4P promoter were KMnO4-reactive in RPo formed by the RbpA-σB-MtbRNAP complex (Figure 1D). In agreement with the result of the transcription assay, σB-MtbRNAP was able to open the sigA4P-TGTG promoter even in the absence of RbpA. However, the amount of RPo was ∼30% of that formed in the presence of RbpA, suggesting that RbpA stimulates RPo formation even at extended −10 promoters. In the absence of RbpA, sigA4P-TG1 promoter opening was barely detectable (Figure 1D), in striking contrast with the significant transcription activity of σB-MtbRNAP observed in the transcription assay (Figure 1B). This discrepancy likely indicates that RPo complexes were active but unstable (see below). We conclude that sequence-specific interaction of σB-MtbRNAP with the bases between −17 to −16 of the extended −10 motif is an essential determinant of promoter opening in the absence of RbpA.

Based on the observation that RbpA was not required for transcription from the perfect extended −10 consensus sinP3 promoter (24) and from the sigA4P promoter variants harboring TG motifs (24), we predict that any promoter harboring the extended −10 motif should be active in the absence of RbpA. To estimate the number of such presumably RbpA-independent or weakly RbpA-dependent gene promoters in M. tuberculosis, we performed a bioinformatic analysis using the set of 1658 promoters containing the σB-type −10 motifs (nAnnnT) (30). These promoters were reported to be active during exponential growth (30). The analysis demonstrated that 338 of 1668 promoters contained a partial or full-length extended −10 motif (Figure 1E). Specifically, 173 (10.4%) promoters contained the T15G14 and 135 (8.1%) the T17G16 motif and therefore, they expected to be loosely RbpA-dependent. Only 30 (1.8%) promoters contained the T17G16,T15G14 motif and therefore, they are expected to be constitutive. Accordingly, the majority of σB-dependent genes in M. tuberculosis are expected to be under the control of RbpA.

The extended −10 motif stabilizes MtbRNAP-promoter complexes in the absence of RbpA

Next, we used electrophoretic mobility shift assays (EMSA) to test whether σB-MtbRNAP can form stable RPo at sigA4P promoter variants in non-equilibrium conditions, in the presence of competitor poly(dI·dC) (Figure 2A and B). EMSA showed that in the absence of RbpA, the full-length extended −10 motif (T17G16,T15G14) and the downstream
Figure 1. RbpA is dispensable for transcription from promoters containing the extended −10 motif. (A) Schematic representation of the sigAP promoter and its derivatives. Mutated bases are underlined. (B) Representative gel of the run-off [32P]RNA products synthesized in the multiple-round transcription assay using sigAP-WT and the indicated derivatives in the absence and presence of RbpA. (C) Quantification of the run-off [32P]RNA products obtained in the transcription assay shown in panel B (mean values ± SE of three experiments except of TGTGC values which are from one experiment). All shown products were used for quantification. Values were normalized to the value obtained with the sigAP-WT promoter in the presence of RbpA. (D) KMnO4 probing of the open complexes formed at the sigAP-WT promoter and the indicated derivatives. Promoter DNA was fluorescein-labeled on the template strand. Traces of the gel lanes are shown at the bottom (E) Number and percentage of promoters harboring the indicated extended −10 motif variants in a subset of M. tuberculosis promoters active during the exponential phase (promoters from Cortez et al. (30)). The bioinformatic analysis was performed using the UniproUGENE software (49).
part (T13G14) stabilized the $\sigma^B$-MtRNAP-promoter complexes to a greater extent than the upstream T17G16 motif. As the A15 $\rightarrow$ T15 substitution was the only difference between sigAP-WT and sigAP-TG1, we conclude that the identity of the base at position 15 is critical for recognition of the extended $-10$ motif by the $\sigma^B$ subunit and for stabilization of the $\sigma^B$-MtRNAP-promoter complex. Titrations of the sigAP-TGTG template with increasing concentrations of $\sigma^B$-MtRNAP in the presence or absence of RbpA demonstrated that RbpA increased $\sigma^B$-MtRNAP affinity for the promoter by $\sim$3-fold (calculated apparent $K_d$ was 117 nM without RbpA and 42 nM with RbpA; Figure 2C, D, J). Thus, differently from run-off RNA synthesis, RPo formation at promoters that contain the extended $-10$ motif was still responsive to RbpA addition. This discrepancy is likely to arise from the different effects of RbpA and the TG motif on initiation and promoter escape in multiple-round transcription assays (see below). Similar uncoupling between RPo stability and transcriptional activity was previously reported for $\sigma^\infty$-MtRNAP at the sinP3 and sigAP promoters where the lack of stable promoter complexes contrasted with the relatively high transcription levels (18, 24).

To understand the nature of these discrepancies, we tested TGTG effect on the kinetics of RPo formation and on promoter escape in single-round run-off transcription assays (Figure 2E and F). To follow RPo formation, we incubated $\sigma^B$-MtRNAP and promoter DNA at 37°C for different lengths of time, before addition of nucleotides and poly(dI-dC). Transcription was performed for 3 min for each time point (Figure 2E). To follow promoter escape, we supplemented pre-formed RPo complexes with nucleotides and poly(dI-dC) before transcription for various lengths of time (Figure 2F). To quantitatively characterize the process, we calculated the half-time values ($t_{1/2}$) required to reach half of the maximum run-off RNA amount (Figure 2G; Supplementary Table S2). The kinetics of RPo formation on sigAP and sigAP-TGTG promoters were similar and unexpectedly slow ($t_{1/2} \sim 4$ min) (Figure 2G and H). The kinetic of promoter escape was $\sim$2-fold faster for the sigAP-TGTG promoter ($t_{1/2} \sim 1$ min) compared with the sigAP-WT promoter ($t_{1/2} \sim 2$ min). We conclude that, in our experimental conditions, the TGTG motif does not affect the rate of RPo formation, but stimulates promoter escape. Thus, the effect of the extended $-10$ motif on transcription differs from that of RbpA, which accelerates RPo formation (26, 32 and see below). Based on our results, we propose that RbpA and the extended $-10$ motif synergistically stimulate RPo formation, probably by stabilizing the transcription bubble within the RPo.
RbpA stabilizes open promoter complexes formed at the sigAP-TGTG promoter

To quantitatively characterize the impact of RbpA on RPo formation at extended −10 promoters, we studied the dissociation kinetics of MtbRNAP-sigAP-TGTG promoter complexes using a fluorescence-based assay (31,32). We incubated the sigAP-TGTG promoter, labeled with Cy3 at position +2 of the non-template DNA strand, with MtbRNAP (E; control), σ^B^-MtbRNAP (Eσ^B) or the RbpA-σ^B^-MtbRNAP complex (Eσ^B + RbpA). Then, we monitored the fluorescence intensity change before and after addition of the competitor heparin that neutralizes free RNAP (Figure 2I). Binding of σ^B^-MtbRNAP or RbpA-σ^B^-MtbRNAP to the promoter DNA induced a 2-fold and 2.5-fold change in fluorescence intensity, respectively. The MtbRNAP core enzyme also induced a ~1.8-fold increase in fluorescence, possibly due to non-specific binding and the high sensitivity of Cy3 fluorescence to the environment (33). Indeed, after heparin addition (Figure 2I and J). As the MtbRNAP core enzyme, which cannot bind specifically to the promoter, also produced a significant change in fluorescence, we conclude that this assay can detect not only RPo, but also RPc and RPi. This result also suggests that the fluorescence signal increase observed in the presence of the holoenzyme could arise in part from non-specific binding. Indeed, after heparin addition to the complexes formed by the σ^B^-MtbRNAP holoenzyme or RbpA-σ^B^-MtbRNAP, we observed a first rapid fluorescence decrease (few seconds), followed by a slow decay (Figure 2I and J). Based on the results obtained with the MtbRNAP core enzyme, we attributed the first signal decrease to the dissociation of non-specific complexes (e.g., formed upon MtbRNAP binding to the DNA fragment ends), and the subsequent slow decay phase (fitted by a single exponential) to dissociation of the specific promoter-RNAP complexes. Considering the three-step model of open complex formation (Figure 2I), RNAP dissociation rate from the promoter (RPo → k^−1 RPi → k^−2 RPc → undetectable species) is determined mainly by the slow isomerization of RPo to RPc, and is characterized by the dissociation rate constant k_d (k_d = k_1/(1+K_2)) which is determined from the exponential fit of the slow decay phase (see Methods section) (34,35). Because the RPc complex formed without RbpA dissociated ~50-fold faster than the complex formed in its presence (Figure 2J), we conclude that RbpA acts on the isomerization step and may stabilize the final ‘open’ state of DNA in RPo (24,25).

The synergy between RbpA and the extended −10 motif allows promoter opening at 0°C

Promoter melting by E. coli RNAP containing the σ^70 subunit is strictly temperature-dependent (11,36). This feature reflects RNAP capacity to undergo temperature-dependent isomerization, leading to the formation of a stable transcription bubble. To assess the effect of RbpA and the extended −10 motif on bubble formation, we performed KMnO_4 probing of MtbRNAP complexes at the sigAP-WT and sigAP-TGTG promoters at increasing temperatures, from 0 to 37°C (Figure 3). RPo formation by RbpA-MtbRNAP at the sigAP-WT promoter displayed weak temperature dependence. Furthermore, RbpA-MtbRNAP could open the sigAP-WT promoter even at 0°C (Figure 3C). At the sigAP-TGTG template, DNA melting was detected at temperatures as low as 16°C even without RbpA. RbpA boosted MtbRNAP capacity to open the promoter, thus rendering it temperature-independent (Figure 3D). Indeed, the amounts of RPo formed at 0 and at 37°C were quite similar. This result suggests that MtbRNAP interaction with either RbpA or TGTG decreases the thermal energy requirement for RPo formation, while interaction with both leads to a strong cooperative effect.

The σ^H166A mutant abolishes MtbRNAP-promoter interaction at the extended −10 motif

The H455 residue in the σ^70 subunit domain 3 (σ3) interacts with the −17GC base pair (C on the template strand) of the extended −10 motif (8,37). On the basis of the structure of Mycobacterium smegmatis RNAP, the homologous residue H166 in σ^B should interact with the upstream (T-17G-16; TG2) and downstream (T-13G-14; TG1) parts of the TGTG motif in the major DNA groove (see model Figure 4A). RbpA interacts with the TGTG motif on the opposite DNA face, and could affect its interaction with σ3. To determine whether RbpA affected the σ3-TGTG motif interaction, we generated the σ^B subunit containing the His → Ala substitution in position 166. In the absence of RbpA, the H166A substitution abolished run-off transcription from the sigAP promoter variants that contain the extended −10 motif (Figure 4B and D; Supplementary Figure S1B). Addition of RbpA restored the activity of σ^H166A-MtbRNAP to a level even higher than that of the wild type holoenzyme, possibly due to increased RNAP recycling. In agreement with the result of the transcription assay, EMSA showed that the H166A substitution abolished RPo formation at the sigAP-TGTG promoter (Figure 4C). Compared with the wild type holoenzyme, σ^H166A-RNAP formed less RPo at the sigAP-TGTG promoter also in the presence of RbpA (Figure 4C and D). Thus, we conclude that the sequence-specific interaction between the H166 residue of the σ^B subunit and the TGTG motif is pivotal for efficient transcription initiation by σ^B-MtbRNAP at promoters of the extended −10 class. This interaction enhances RbpA capacity to stabilize RPo, but is not essential for RbpA-mediated transcription activation.

Domain σ^4 is essential for transcription initiation from extended −10 promoters

Interaction of domain 4 of σ^70 (σ4) with the −35 element in the promoter is dispensable for transcription from promoters of the extended −10 class (9,38). To test whether the σ^4/-35 element interaction contributes to transcription initiation in the presence of RbpA, we introduced mutations in the −35 motif of the sigAP-WT and sigAP-TGTG promoters (Figure 5A). EMSA and multiple-round run-off transcription assays demonstrated that substitutions in the −35 element did not significantly affect promoter binding and transcription (Figure 5B-E; Supplementary Figure S2A),
Figure 3. *Mtb* RNAP forms open promoter complexes at 0°C. (A–C) Temperature-dependence of promoter melting by *Mtb* RNAP probed with KMnO₄. DNA was labeled with fluorescein on the template strand. *Mtb* RNAP-promoter complexes were formed at the indicated temperatures in the absence (−) or presence (+) of RbpA. (D) Quantification of the results shown in panels A and B. The open DNA fractions were calculated as the cleaved DNA to total DNA ratio.

suggesting that sequence-specific recognition of the −35 element by σ⁴ is dispensable for transcription initiation in the presence of RbpA or of the TGTG-motif. However, we cannot exclude that, in the absence of the perfect −35-motif, a non-specific interaction of σ⁴ with promoter through contacts with the DNA phosphate backbone (39) contributes to transcription initiation.

If the σ⁴/-35 element interaction is dispensable for transcription initiation in the presence of RbpA, σ⁴ deletion should not affect the activity of the *Mtb*RNAP-RbpA complex. To test this hypothesis, we generated a σ⁴ subunit mutant in which the C-terminal residues 252–323 were deleted (σ⁴Δ4) (Figure 4A). Using a native PAGE-based protein-protein interaction assay (24), we demonstrated that this deletion did not affect the σ⁴ subunit capacity to form a stable complex with RbpA (Figure 5F). To test whether mutant σ⁴Δ4-*Mtb*RNAP was catalytically active, we performed abortive transcription assay using the synthetic lacUV5 promoter harboring a heteroduplex region between position −11 and −5 (28) (Figure 5G). Initiation of transcription on lacUV5 promoter by addition of dinucleotide RNA primer, ApA, and two nucleotides (GTP and [α²⁵P]-UTP) resulted in formation of short RNA products, up to 7nt in length (14). In these experimental conditions, wild type σ²⁵-*Mtb*RNAP initiated transcription (with similar efficiency) both in the presence and absence of RbpA, which is in line with the fact that RbpA acts at the promoter melting step. The mutant σ²⁵Δ4-*Mtb*RNAP displayed reduced (~18%), but detectable catalytic activity, compared with the wild type enzyme. This could be caused by defects in promoter binding, holoenzyme σ²⁵Δ4-*Mtb*RNAP assembly, and structural disturbance in the σ²⁵ region 3.2, which is implicated in transcription initiation and promoter escape (40,41). As expected, the mutant σ²⁵Δ4-*Mtb*RNAP holoenzyme was inactive in run-off transcription assays performed with the sigAP-WT promoter in the absence and presence of RbpA (Figure 5H). Surprisingly, the mutant σ²⁵Δ4-*Mtb*RNAP holoenzyme was also inactive at the sigAP-TGTG promoter and at the sinP3 promoter (24) that harbors the perfect extended −10 consensus motif (5′-T-17GTGcTATAAT-7–3′) and lacks the −35 element. Addition of RbpA partially restored σ²⁵Δ4-*Mtb*RNAP activity at the sinP3, but not at the sigAP-TGTG promoter, possibly due to differences in promoter architectures. Because mutant *Mtb*RNAP showed low but detectable activity on the lacUV5 bubble template, irrespective of RbpA, the lack of transcription at the sigAP promoter in the presence of RbpA and at the sigAP-TGTG in the absence of RbpA cannot be explained only by defects in initiation of RNA synthesis. Altogether, these results suggest that RbpA cannot compensate for the lack of σ⁴ interaction with the core enzyme or/and the promoter, and that σ⁴ per se is an essential component of the RbpA-mediated activation mechanism at the −10/−35 class promoters. Based on these results we hypothesize that even for promoters of the extended −10 class, interaction of the σ⁴ domain with the *Mtb*RNAP core enzyme, and/or non-specific interaction with DNA contribute to transcription initiation.
Figure 4. The substitution H166A in region 3 of σβ abolishes MtbRNAP interaction at the extended −10 motif. (A) Structural model of Mycobacterium smegmatis RNAP in complex with RbpA and promoter DNA (PDB code: 5TW1). Red ribbon, RbpA; green ribbon, σβ subunit; gray semitransparent molecular surface, RNAP core; blue, DNA template strand; red, DNA non-template strand; orange, TG1-motif (T−15G−14); yellow, TG2-motif (T−17G−16). Residues in σβ (H166) and RbpA (K73, K74, R79) that were mutated are shown in CPK rendering. Schematic representations of the RbpA and σβ domains are shown at the bottom. The positions of the mutated residues are indicated. (B) Run-off [32P]-RNA products synthesized in run-off transcription assays using sigA onset promoter derivatives in the presence or not of RbpA. (C) EMSA analysis of promoter complex formation by RbpA-MtbRNAP and fluorescein-labeled sigA promoter variants. Complexes were resolved in native 5% PAGE. (D) Quantification of the experiment shown in panel C (mean values ± SE of two experiments).

RbpA-BL modulates σβ-MtbRNAP selectivity for initiating transcription substrates

Based on the structure of RPo and RbpA-SID fragment, it was proposed that the residues R79 (in contact with nucleotides −13 and −14 of the non-template DNA strand), K73 and K74 in RbpA-BL interact with DNA at the TGTG element in the DNA minor groove (25,26). We assessed whether mutations in these residues (R79A and the double substitution K73A, K74A (KKAA)) affected RPo formation at the sigA-WT and sigA-TGTG promoters. The EMSA results showed that in agreement with previous findings (25), the R79A substitution in RbpA decreased RPo stability by ∼2-fold at the sigA-WT and by ∼1.5-fold at the sigA-TGTG promoter (Figure 6A and C). Conversely, the KKAA substitutions had no effect on RPo stability (Figure 6B and D). Furthermore, KMnO4 probing demonstrated that neither the R79A nor the KKAA substitution hinders sigA-WT promoter opening (Figure 6E). Opening of the sigA-TGTG promoter was even enhanced in the presence of the RbpA mutants. This discrepancy between KMnO4 probing and EMSA results can be explained by the formation of an unstable RPo that dissociates in the non-equilibrium conditions of EMSA, but can be detected in the equilibrium conditions of KMnO4 probing. Thus, we conclude that R79 contributes to RPo stabilization, while K73 and K74 are dispensable.

Next, we tested whether the RbpA mutants could stimulate de novo transcription. In multiple-round transcrip-
Figure 5. Impact of σ4 and the −35 element on Mtb RNAP activity. (A) Scheme showing the −35 motif of the sigAP promoter with the introduced mutations underlined. (B) Run-off [32P]-RNA products synthesized by wild type σ4-MtbRNAP from sigAP-WT and sigAP-TGTG and the respective variants lacking the −35 element (Δ-35). (C) Quantification of the results shown in panel B (mean values ± SE of two experiments). (D) EMSA analysis of promoter complex formation by σ4-MtbRNAP using the sigAP-WT and sigAP-TGTG promoters and the respective variants lacking the −35 element (Δ-35). (E) Quantification of the results shown in panel D (mean values ± SE of two experiments). (F) Analysis of the RbpA-Δ4 subunit interaction by native gel electrophoresis. RbpA, labeled with DyLight 633, was incubated with increasing concentrations (0.8, 1.6, 3.2 μM) of wild type σ4-MtbRNAP (WT) or the mutant in which domain 4 residues 252–323 were deleted (Δ4). (G) Abortive transcription activity of wild type σ4-MtbRNAP (WT) and mutant σ4Δ4-MtbRNAP (Δ4) on the lacUV5-bubble template harboring a heteroduplex region. (H) Run-off [32P]-RNA products synthesized in the presence of wild type σ4-MtbRNAP (WT) or mutant σ4Δ4-MtbRNAP (Δ4) and the sigAP-WT, sigAP-TGTG or B. subtilis sinP3 promoter that lacks the −35 element.

To better understand the effect of substitutions in RbpA on RPo formation and promoter escape, we performed single-round transcription assays as described above (Figure 7). The half-time of RPo formation in the presence of RbpA was ~4-fold higher than without RbpA (compare Figure 2G, H and Figure 7C, D). The half-time of promoter escape was also 2-fold greater in the presence of RbpA (Figure 7, Table S2), suggesting that RbpA regulates not only
promoter melting but also the initial transcription. We detected no effect of the R79A substitution on RPo activation and escape rates, at least in the time resolution range of our assay. Therefore, we cannot presently explain why this substitution affects the yield of run-off RNA in multiple-round transcription assays. In agreement with the result of the multiple-round transcription assays, the KKAA substitutions led to a ∼3-fold decrease in escape rate ($t_{1/2} = 2.8$ min), while addition of GpC stimulated promoter escape. Based on our results and on the recent structure of the $\sigma$-RNAP-RbpA complex (42), we propose that RbpA, can indirectly modulate $\sigma$-RNAP selectivity for the priming substrates (NTPs and short RNAs) through contact with the $\sigma$3.2 finger (42) which controls transition from initial transcription to productive elongation (28,40,41).

**DISCUSSION**

Transcription initiation from most of the bacterial promoters requires simultaneous binding of the RNAP $\sigma$ subunit to the $-10$ and $-35$ elements. The weak conservation of the $-35$ element in *M. tuberculosis* promoters (30) predicts the existence of compensatory mechanisms. Here, we found that RbpA abolishes the requirement of sequence-specific interactions between the domains 3 and 4 of the $\sigma$ subunit and promoter DNA upstream of the $-10$ element. Therefore it converts $\sigma$-RNAP into a hyperactive enzyme with promiscuous promoter selection. Moreover, the presence of the T-17G-16T-15G-14 motif fully abolishes RbpA requirement for transcription initiation, suggesting that mycobacterial promoters bearing the extended $-10$ motif are constitutive. Our estimation is that at least 2% of the known *M. tuberculosis* promoters belong to this group. Moreover, ∼14% of *M. tuberculosis* promoters contain the T-15G-14 motif (43), and thus should be loosely dependent on RbpA, or hyperactive in the presence of RbpA. Based on the similarities between the effects of RbpA and of the TGTG motif on transcription, we propose that RbpA could strengthen the interaction of the $\sigma$ subunit domains 3 and 4 with promoter DNA upstream of the $-10$ element. This hypothesis...
is supported by our smFRET study showing that σB in the MtbRNAP holoenzyme adopts a conformation incompatible with binding to −10/-35 promoters and that RbpA stabilizes σB in a conformation compatible with binding (27). The finding that σ4 was still required for transcription initiation in the context of the extended −10 sinP and sigAP-TGTG promoters even in the presence of RbpA suggests that σ4 may have additional roles in initiation, probably in organizing RNAP clamp or β subunit flap domains for RPo formation, as proposed for E. coli σ70 (44). The σ4/β-flap contact may be essential for correct positioning of σ3 (e.g., region 3.2) in RPo, which in turn affects RPo stability and RNA synthesis initiation (28,40).

We observed that RbpA increases MtbRNAP affinity for promoters bearing the extended −10 motif (~3 fold decrease in apparent Kd) and stabilizes RPo (~50-fold decrease in k4). Thus, we propose that RbpA acts on two initiation steps: promoter binding (RPc formation), and RPc isomerization to RPo. Our result differs from a previous study reporting no effect of RbpA on the k4 of Mycobacterium bovis RNAP (MboRNAP) binding at the rnap3 and vapB10 promoters (26). We found that these promoters harbor the T_{17}G_{16} motif and therefore, belong to the extended −10 class. Consequently, the properties of these promoter templates should be close to those of the sigAP promoter templates used in our study. However, Hubin et al. used MboRNAP assembled with the σA and not the σB subunit. The difference in the structures of these two σ subunits could explain the discrepancy in the observed RbpA effect on k4. As the σA and σB residues that interact with the extended −10 and −35 elements are almost identical (supplementary Figure S3 in Hu et al. (24)), they are unlikely to explain their different behavior. The major difference in structure between σA and σB is the long N-terminus (202 amino acids) of σA that could be involved in RPo formation, as reported for the N-terminus of E. coli σ70 (45). In addition, the N-terminus of RbpA (RbpA^NTT) interacts with the variable region σ3.2 (42) which controls bubble stability in RPo and transition from initial transcription to productive elongation (28,40,41).

**Role of the extended −10 motif in promoter melting at suboptimal temperatures**

Studies on the E. coli σ70-RNAP and B. subtilis σA-RNAP holoenzymes demonstrated that the extended −10 motif stabilizes RPo and allows promoter melting triggered by RNAP at low (6 and 10°C), suboptimal temperatures (46,47). Also it has been proposed that the T_{15}G_{14} motif can decrease the thermal energy requirement for RPo formation by MtbRNAP (48). Our results demonstrate that the identity of the nucleotides at positions −17 to −16 is critical for melting of promoters of the extended −10 class. The combination of TGTG motif and RbpA strongly decreased the thermal energy requirement for promoter melting and allowed MtbRNAP to form open complexes even at 0°C. The effect of RbpA on Tm supports its action on isomerization of the closed-to-open complex. We speculate that interaction at the TGTG motif could promote DNA bending around RNAP. This will direct promoter DNA to the downstream channel in RNAP, thus facilitating distor-
tion of the −10 element DNA by the region 2 of σ and then formation of the transcription bubble.

Role of RbpA-BL in transcription initiation

Previous studies on σA-MtbRNAP suggested that interaction of the residue R79 in RbpA-BL with promoter DNA at positions −13, −14 is critical for RbpA function in vitro and in vivo (25,26). We observed a moderate effect of this mutation on RPo stability and no effect on promoter opening and on transcriptional activity. Furthermore, the RbpAΔ79/79 mutant was even more active than wild type RbpA in multiple-round transcription assays without a 2-mer RNA primer. This discrepancy indicates that the σA-MtbRNAP and σB-MtbRNAP holoenzymes respond differently to RbpA. Indeed, differently from σB-MtbRNAP, the σA-MtbRNAP holoenzyme cannot form stable RPo at the extended −10 σnP3 promoter without RbpA and at the sigAσ promoter in the presence of RbpA (18,24). The molecular basis of these differences, lying in the above mentioned structural properties of σA and σB, is an intriguing subject for future studies.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

We thank L. Makrini for technical assistance in protein purification.

FUNDING

French National Research Agency [Mycobacterium ANR-16-CE11-0025-01]; CNRS [PRC Russie CNRS/RFBR to K.B.]; Fondation pour la recherche médicale (FRM) (to Y.H.) during his stay in the laboratory of K.B.; ERASMUS MUNDUS Svagata fellowship (to A.S.P.); Infectopole Sud (to R.K.V.). Funding for open access charge: MycoMaster ANR-16-CE11-0025-01.

Conflict of interest statement. None declared.

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