**Mycobacterium** RbpA cooperates with the stress-response $\sigma^B$ subunit of RNA polymerase in promoter DNA unwinding

Yangbo Hu, Zakia Morichaud, Ayyappasamy Sudalaiyadum Perumal, Françoise Roquet-Baneres and Konstantin Brodolin*

CNRS UMR 5236 – UMI 1 - UM2, Centre d’études d’agents Pathogènes et Biothechnologies pour la Santé (CPBS), 1919 route de Mende, 34293 Montpellier, France

Received April 15, 2014; Revised August 01, 2014; Accepted August 3, 2014

ABSTRACT

RbpA, a transcriptional activator that is essential for *Mycobacterium tuberculosis* replication and survival during antibiotic treatment, binds to RNA polymerase (RNAP) in the absence of promoter DNA. It has been hypothesized that RbpA stimulates housekeeping gene expression by promoting assembly of the $\sigma^A$ subunit with core RNAP. Here, using a purified in vitro transcription system of *M. tuberculosis*, we show that RbpA functions in a promoter-dependent manner as a companion of RNAP essential for promoter DNA unwinding and formation of the catalytically active open promoter complex (RPo). Screening for RbpA activity using a full panel of the *M. tuberculosis* $\sigma$ subunits demonstrated that RbpA targets $\sigma^A$ and stress-response $\sigma^B$, but not the alternative $\sigma$ subunits from the groups 3 and 4. In contrast to $\sigma^A$, the $\sigma^B$ subunit activity displayed stringent dependency upon RbpA. These results suggest that RbpA-dependent control of RPo formation provides a mechanism for tuning gene expression during the switch between different physiological states, and in the stress response.

INTRODUCTION

*Mycobacterium tuberculosis* is one of the most successful human pathogens that can persist in human tissues for years in a dormant state which is not sensitive to a majority of antibiotics (1). Adaptation of the pathogen to the hostile environments that it meets inside host cells and its tolerance to drugs are regulated at the level of gene transcription (2,3). Transcription in bacteria is performed by the multisubunit DNA-dependent RNA polymerase (RNAP) holoenzyme composed of the catalytic core (E, subunits $\alpha_2\beta\beta'\omega$) and one of the $\sigma$ subunits which are required for promoter-specific initiation of RNA synthesis (4,5). Recognition of the double-stranded DNA of the -10 and -35 consensus promoter elements by $\sigma$ subunit domains 2 ($\sigma_2$) and 4 ($\sigma_4$) leads to the formation of the unstable ‘closed complex’ (RPc) between RNAP and promoter. RPc isomerizes into a transcriptionally competent ‘open complex’ (RPo) through several intermediate complexes (RPi) (6). During isomerization, a concerted action of the $\sigma$ subunit and core RNAP triggers unwinding (melting) of $\sim$13 bp of the promoter DNA surrounding the transcription start site and makes the single-stranded DNA template available for initiation of RNA synthesis (7–10).

Each bacterial species has a characteristic library of $\sigma$ subunits. The housekeeping (principal) $\sigma$ subunit ($\sigma^{70}$ in *Escherichia coli* or $\sigma^A$ in *M. tuberculosis*) controls transcription of genes during exponential growth. Alternative $\sigma$ subunits activate the transcription of specialized genes that are implicated in the stress response, virulence and the switch from exponential to stationary growth phase or to the persistent state (11,12). The *M. tuberculosis* genome encodes 12 alternative $\sigma$ subunits, of which $\sigma^B$ is a putative stationary phase subunit that is structurally similar to $\sigma^A$ and orthogonal to *E. coli* $\sigma^B$ (13–15). Competition between $\sigma$ subunits for binding to the core RNAP ($\sigma$-swapping) provides the basal regulatory mechanism for tuning bacterial gene expression in response to environmental signals (12). In addition, RNAP is regulated by a number of the non-DNA binding factors which interact with $\sigma$ subunit and repress or activate transcription (reviewed by (16,17)). The molecular mechanisms that regulate activity of different $\sigma$ subunits in *E. coli* are extensively studied, while the mechanisms employed by *M. tuberculosis* remain largely unknown.

*M. tuberculosis* RNA polymerase binding protein A (RbpA), which is present only in *Actinomycetes* species, has been assigned to the group of non-DNA binding factors (18,19). RbpA binds to the $\sigma$ and $\beta$ subunits of...
RNAP (20–23) and stimulates transcription dependent on the housekeeping σ subunit (19,23). RbpA is essential for *M. tuberculosis* growth and may play a critical role in control of pathogen physiological states because the *rpbA* gene was shown to be upregulated 8-fold during stationary phase, starvation, the stress response and rifampicin or vancomycin treatment (24,25). Yet, the role of RbpA in tolerance to rifampicin is not understood (22,23,26).

Three models describing the mechanism of action of RbpA are compatible with the available experimental data and include RbpA stimulating (1) holoenzyme assembly, (2) promoter complex formation or (3) promoter escape and include RbpA stimulating (1) holoenzyme assembly, (2) promoter complex formation or (3) promoter escape (23). To discriminate between these models, we developed a highly efficient *in vitro* transcription system for *M. tuberculosis* that allowed us to determine the step in transcription initiation regulated by the activator. Furthermore, we demonstrated that RbpA acts as promoter-specific and σ-selective activator controlling the activity of the σ^A^ and σ^B^ subunits of *Mycobacterium*.

**MATERIALS AND METHODS**

**Proteins and DNA templates**

Recombinant *M. tuberculosis* RNAP core enzyme containing 6×His-tag at the C-terminus of the β subunit was expressed in *E. coli* BL21(DE3) cells from the pMR4 plasmid and purified as described in the supplementary file (Supplementary Figure S1). The plasmids used for expression of σ subunits are listed in Supplementary Table S1. The pSR01 plasmid coding for σ^A^ and pSR5 plasmid coding for σ^B^ were a generous gift from Dr H. O’Hare and Dr A. Bortoluzzi. The DNA fragments bearing the *M. tuberculosis* promoters were amplified from genomic DNA (23) using corresponding primers (Supplementary Table S2). The forward primers were labeled with fluorescein at the 5′-end. The H37Ra genomic DNA was obtained from BEI Resources. The H37Ra genomic DNA was purchased from ATCC. *lacUV5* and *sinP3* promoter DNA fragments were prepared as described (10,23). Synthetic galP1AA promoter with the substitutions A, T, T, A, G, A to T, G, C, T, T, C, T, G, A, T, A, G, A was prepared by annealing two oligonucleotides (Supplementary Table S2). *sinP3* promoter with the substitution G, T, T, A, T, A, G, A to A, A, A, A was prepared by annealing two oligonucleotides (Supplementary Table S2) followed by PCR amplification with the primers used for amplification of the corresponding wild type promoter DNA fragments (ref. (23) and Supplementary Table S2).

**In vitro transcription and electrophoretic mobility shift assays**

Transcription was performed in 5 μl of transcription buffer (TB, 20 mM Tris–HCl pH 7.9, 50 mM NaCl, 5 mM MgSO_4_, 1 mM dithiothreitol (DTT), 0.1 mM ethylene-diaminetetraacetic acid (EDTA) and 5% glycerol). The RNAP holoenzyme was assembled by mixing 600 nM σ subunit with 200 nM core RNAP and incubation for 5 min at 37°C. RbpA at 600 nM or at the concentrations indicated on the figures was added to the mixtures and incubated for 5 min. Promoter DNA fragment (15 nM) was added and incubated at 37°C for 10 min. Transcription was initiated by the addition of 50 μM adenosine triphosphate (ATP), guanosine triphosphate (GTP), cytidine triphosphate (CTP) and 3 μCi [α–32P] uridine triphosphate (UTP) and carried out for 5 min at 37°C. The reactions were stopped by adding 8 M urea and the synthesized RNA products were analyzed on denaturing (7 M urea) 18% polyacrylamide gels electrophoresis (PAGE). For the electrophoretic mobility shift assays (EMSA) experiments, the fluorescein-labeled promoter fragments were mixed with RNAP or RNAP-RbpA complex, prepared as described above, in 10 μl TB and incubated for 10 min at 37°C. Then, 20 μg/ml of poly(dA–dT) was added and incubated for 5 min at 37°C. Afterwards, samples were resolved on 5% native 0.5×TBE-PAGE. Gels were scanned by Typhoon 9200 Imager (GE Healthcare) and quantified using ImageQuant software (Molecular Dynamics).

**DNase I footprinting and KMnO₄ probing**

For DNase I footprinting experiments, fluorescein-labeled promoter DNA fragment (40 nM) was mixed with 400 nM RNAP in 50 μl TB. RbpA was added before promoter complex formation as described for *in vitro* transcription. The samples were treated with 2 U/ml DNase I (Promega) for 1 min at 37°C. The reactions were stopped by addition of 10 mM EDTA (pH 8.0) and 400 ng poly(dA–dT). For KMnO₄ probing, RNAP-promoter complexes were formed as described for DNase I footprinting. The samples were treated with 5 mM KMnO₄ for 30 s at 37°C. Reactions were quenched by addition of 1/10 volume of 1 M 2-mercaptoethanol, 1.5 M Na(CH₃COO) pH 7.0. Samples were treated with 0.5 M piperidine and DNA fragments were analyzed on 8% sequencing gel.

**RbpA labeling and native gel analysis**

Purified RbpA protein was conjugated with the sulphydryl-reactive dye, DyLight633 maleimide (Thermo Scientific), at the single cysteine site (C56) according to the manufacturer protocol. Briefly, 1 μg of RbpA protein was incubated with the 20 μl maleimide-activated dye in the conjugation buffer (0.1 M phosphate, 0.15 M NaCl, 2 mM EDTA, pH 7.4) at 24°C for 2 h. The excess dye reagent was then removed from the sample by dialysis. Protein was concentrated to 4 mg/ml by Ultracel-10 membrane filter unit (Millipore) and stored at −20°C in 50% glycerol. For the native gel analysis, the labeled RbpA (1.6 μM) was incubated with 2.4 μM of indicated σ subunit in 10 μl TB at 37°C for 15 min. The complexes were analyzed on 5–10% native PAGE in Tris–Glycine buffer. Gels were scanned by Typhoon 9200 Imager (GE Healthcare) and quantified using ImageQuant software (Molecular Dynamics).
RESULTS

RbpA stimulates transcription by RNAP containing either the $\sigma^A$ or $\sigma^B$ subunit

To explore the role of RbpA in regulation of M. tuberculosis transcription, we designed a plasmid co-overexpressing the M. tuberculosis RNAP core subunits $\alpha$, $\beta$, $\beta'$ and $\alpha$ (Supplementary Figure S1). The plasmid allowed us to achieve high expression levels of core RNAP in E. coli cells, and in vitro-assembled enzyme was more stable and displayed higher specific activity than RNAP that was assembled in vitro from individually expressed subunits (23,27). To define the full range of $\sigma$ subunits that are regulated by RbpA, all 13 $\sigma$ subunits from M. tuberculosis were expressed, purified and assembled with the core RNAP (Supplementary Figure S1). The ability of the $\sigma$ subunits to drive promoter-specific transcription initiation was tested in multiple-round transcription assays using DNA fragments ($\approx$100 bp in length) bearing M. tuberculosis promoters that were reported to be recognized by their corresponding $\sigma$ subunits (Figure 1A, Supplementary Figure S2, Supplementary Table S3). Transcription was performed either in the presence or in the absence of RbpA, and core RNAP alone was used to control the specificity of the initiation reaction. All $\sigma$ subunits except the $\sigma^G$ and $\sigma^K$, supported transcription initiation by the RNAP holoenzyme, while no or little RNA synthesis was detected when using core RNAP alone. The lack of detectable activity for $\sigma^G$ and $\sigma^K$ may be caused by misfolding of the recombinant proteins, or the inability of these $\sigma$ subunits to recognize the tested promoters without auxiliary transcription activators. The transcription patterns varied significantly between $\sigma$s and displayed a large amount of RNA products that were shorter than the expected 40–50 nucleotides (nt) run-off products that could form due to transcription pausing or arrest. Noticeably, the $\sigma^C$- and $\sigma^H$-containing RNAPs displayed defects in promoter escape and produced mainly short RNA products. The $\sigma^B$ subunit and, to a lesser extent, $\sigma^A$ were only weakly active in initiation of RNA synthesis (sigA and rrnAP promoters without RbpA (Figure 1A, Supplementary Figure S2A). The addition of RbpA strongly stimulated transcription initiated by the $\sigma^B$-containing RNAP ($\geq$10-fold), while the activity of none of the other alternative $\sigma$ subunits was increased in the presence of the activator. The activation level for $\sigma^A$-RNAP was weaker ($\approx$3-fold) and corresponded to the one previously reported for the in vitro assembled holoenzyme (23).

RbpA stimulates stable promoter complex formation by the $\sigma^B$-RNAP

Previously, we showed that RbpA stimulates the formation of stable promoter complexes by the $\sigma^A$-RNAP (23). To explore if RbpA can affect promoter binding of RNAPs containing alternative $\sigma$ subunits, we performed EMSA using the above-mentioned promoter DNA fragments that were end-labeled with fluorescein (Figure 1B, Supplementary Figure S2 showing complete images of the gels). To suppress non-specific DNA binding, the promoter complexes were challenged by adding poly(dA–dT) as a competitor. No formation of competitor-resistant complexes between the $\sigma^B$-RNAP and sigA promoter was detected without RbpA, which was in agreement with the results of the transcription assay. Meanwhile, all RNAPs containing alternative $\sigma$s, that were active in transcription, formed competitor-resistant complexes at their corresponding promoters (Figure 1B). The addition of RbpA induced the formation of competitor-resistant promoter complexes of $\sigma^B$-RNAP but did not affect complexes formed by RNAPs bearing other alternative $\sigma$ subunits. The competitor-resistant complex of $\sigma^A$-RNAP at the $rrnAP$ promoter was formed even without RbpA, but its formation was stimulated if RbpA was present in the reaction (23). Surprisingly, we observed no stable complex formation between $\sigma^A$-RNAP and the sigA40 promoter fragment, even in the presence of RbpA (Supplementary Figure S2B), which was in striking contrast to the stimulation effect that was observed using the transcription assay (Supplementary Figure S2A). Therefore, $\sigma^A$-RNAP and $\sigma^B$-RNAP recognized the same promoter sequence but formed structurally different open complexes. Together, the results of the transcription and EMSA assays suggest that RbpA only stimulates the activity of the structurally similar $\sigma^70$-like group 1 and group 2 $\sigma$ subunits (Figure 1C) and has a strong bias for the stress-response $\sigma^B$ subunit.

RbpA binds $\sigma^B$ but none of the other alternative $\sigma$ subunits

RbpA has been shown to bind to free $\sigma^A$ and $\sigma^B$ subunits (20,21). To test if the ability of RbpA to activate transcription correlates with its ability to bind $\sigma$, we tested the interactions between RbpA and free $\sigma$ subunits using native gel electrophoresis (Figure 1D). To monitor RbpA-$\sigma$ complex formation RbpA was labeled with the Dylight633 dye at the single Cys66 residue whose modification does not influence RbpA activity (23,26). Each of the 12 M. tuberculosis $\sigma$s was mixed with RbpA in the presence of BSA to reduce non-specific binding, and the resulting complexes were resolved on a native gel. In agreement with the results of the transcription assay, we observed that RbpA formed stable complexes with $\sigma^A$ and $\sigma^B$, but not with any of the other alternative $\sigma$ subunits. Because the concentrations of $\sigma$s were equal (2.4 $\mu$M) in all samples but only 20% of RbpA (at 1.6 $\mu$M) was bound to $\sigma^A$ compared with 100% that was bound to $\sigma^B$, we concluded that RbpA has higher affinity for the latter subunit. Therefore, the ability of RbpA to activate transcription correlated with its ability to bind free $\sigma$ subunits and indicated that the interaction with $\sigma$ may be a part of the activation mechanism. In support of this conclusion, RbpA lacking its C-terminal domain, which is required to interact with $\sigma$ subunits (20), was unable to stimulate promoter complex formation (Supplementary Figure S2N).

Stabilization of the $\sigma^B$-RNAP holoenzyme by RbpA is not the basis of transcription activation

Previously, we proposed that the basis of RbpA-mediated transcription activation was an increased affinity of $\sigma$ for the core, which leads to stabilization of the RNAP holoenzyme (23). Because RbpA binds $\sigma$ and the core, it may affect the stability of the holoenzyme by bridging the partners. Therefore, the lack of a stable promoter complex without
RbpA can be explained by low stability of the σB-RNAP holoenzyme. To explore this idea, we compared the stability of a σB-RNAP holoenzyme that was assembled with or without RbpA using chromatography with a Superose-6 gel-filtration column (Figure 2A). The fractions containing RNAP holoenzyme (Figure 2A, peak ‘P1’) were pooled and analyzed using SDS-PAGE (Figure 2B). Quantification of the gel showed that RbpA increased the retention of σB in the holoenzyme (Figure 2B, gel profiles on the right), which was in agreement with the previous result for the σA subunit (23). Additionally, RbpA co-eluted with RNAP suggesting that it was stably bound to the holoenzyme. However, even without RbpA, the RNAP holoenzyme exhibited 60% binding to σB compared with the one assembled in the presence of RbpA suggesting that RbpA is not obligatory for holoenzyme assembly. To test if the assembled σB-RNAP holoenzyme that was collected using gel-filtration was responsive to RbpA, EMSA assay and a run-off transcription assay using the sigAP promoter were performed (Figure 2C and D). The experiment showed that purified σB-RNAP holoenzyme could not form a stable promoter complex and initiate transcription using the sigAP promoter, while addition of RbpA stimulated both events. Because RbpA activates transcription more than it stimulates formation of the holoenzyme (>10-fold versus 1.7-fold correspondingly) we proposed that stabilization of the holoenzyme is not a major cause of transcription activation. If the function of RbpA is to compensate for the low affinity of σ for the core, then it could be bypassed by increasing the concentration of σ.
Figure 2. RbpA stabilizes the σ^B-RNAP holoenzyme. (A) Superose-6 elution profile of the σ^B-RNAP holoenzyme assembled in the presence or absence of RbpA. P1 indicates the peak corresponding to the RNAP core and holoenzyme. (B) SDS-PAGE of the RNAP holoenzyme that was assembled in the presence (lanes 4 and 5) or absence of RbpA (lanes 2 and 3) and fractionated on a Superose-6 column as shown in panel A. Samples of the input (IP) before fractionation and of the pooled peak fractions (P1) are shown. Profiles on the right show the scan of lanes 2 and 3 (black) and 4 and 5 (gray). (C) Effects of RbpA on promoter complex formation by the pre-assembled σ^B-RNAP (from the panel B, lane 3). (D) Run-off RNA products that were synthesized by the pre-assembled σ^B-RNAP in a single round of transcription from the sigA^P promoter DNA. (E) EMSA of the σ^B-RNAP complexes with the sigA^P promoter formed in the presence of increasing concentrations of σ^B (0.4, 0.8, 1.6, 3.2 μM). RbpA (800 nM) was added where indicated.

To test this assumption, we performed EMSA using the sigA^P promoter in the presence of different amounts of σ^B (Figure 2E). No stimulation of promoter complex formation was observed in the absence of RbpA, even when 16-fold excess of σ^B (3.2 μM) over the RNAP core (200 nM) was used. Thus, we concluded that activation of σ^B-dependent transcription is not caused by increased affinity of σ^B for the core RNAP, but is due to a RbpA-mediated conformational change in the holoenzyme, σ subunit, or both that stimulates promoter complex formation.

RbpA is required for promoter binding and RP_o formation

To determine which of the steps on the pathway to the open promoter complex (RP_o) formation is targeted by RbpA, DNA–protein interactions in the σ^B-RNAP promoter complexes were probed with KMnO_4 and DNaseI at equilibrium conditions in the absence of competitor. As a control we followed binding of σ^{1'-RNAP to the sigl promoter, which is non-responsive to RbpA. First, we tested whether σ^B-RNAP could form RP_o at the -10/-35 consensus sigA^P promoter. Promoter DNA melting was monitored by probing the accessibility of the thymine at position -4 (T_4) of the non-template DNA strand to KMnO_4 (Figure 3A). The experiment showed that T_4 was accessible to KMnO_4 only when RbpA was present in the reaction. Therefore, even at equilibrium conditions (without competitor) at 37°C, the σ^B-RNAP was unable to melt promoter DNA without RbpA. RNAP assembled with the σ^J subunit formed RP_o at the siglP promoter in the absence of RbpA. Furthermore, the siglP promoter melting, marked by the presence of unpaired thymines at positions -4, -7 and -8 of the non-template DNA strand, was not affected by addition of the activator, which was in agreement with the results of the transcription and EMSA assays (Figure 3B).

The lack of detectable promoter melting does not exclude that RNAP can bind to a promoter and form RP_c or RP_i complexes. Thus, the closed promoter complexes, which do not have a melted DNA region, were detected on several E. coli promoters using DNase I footprinting (29–32). To test if M. tuberculosis σ^B-RNAP could also bind to the sigA^P promoter in the absence of RbpA, we performed DNase I footprinting (Figure 3C) under the same conditions as those used for KMnO_4 probing. No protection of the promoter DNA from DNase I was detected without RbpA, while addition of the activator resulted in protection of positions -39 to +18. The RNAP holoenzyme containing the σ^J subunit protected positions -39 to +18 of the promoter DNA equally well with or without RbpA (Figure 3D). The lack of a detectable footprint at the sigA^P promoter without RbpA indicates that closed promoter complex is highly unstable and cannot be detected by DNase I due to its short life time. Thus, we concluded that σ^B-RNAP is ineffective in
The inability of \( \sigma^B \)-RNAP to form \( R_P^\circ \) without RbpA may arise from the low affinity of \( \sigma^B \) to the -10 and -35 elements of promoter. Indeed, the sequences of the -10 element of the \( \sigma^A \) promoter (5'-TACAAT-3') and the \( \text{sigP} \) promoter (5'-TTGACA-3') differ from the -10 consensus sequence 5'-TATAAT-3'. Furthermore, the -35 element of the \( \sigma^A \) promoter (5'-TGTACT-3') displays only weak similarity to the -35 consensus 5'-TTGACA-3'. To examine whether the variations in promoter sequence can modulate the efficiency of \( R_P^\circ \) formation, we performed EMSA using 'strong' \( E. coli \) promoters: lacUV5 and the 'extended -10' \( galP1\alpha \) (derivative of \( galP1 \)) containing the perfect -10 consensus sequence. We reasoned that using of the 'extended -10' promoter, which does not contain the -35 element, allows to neglect the impact of upstream interaction between the \( \sigma \) domain 4 and the -35 element on \( R_P^\circ \) formation. However, stable complex formation with both promoters showed RbpA-dependence (Supplementary Figure S2P). These results suggest that neither a perfect match to the -10 consensus nor the presence of the extended -10 motif ‘TG’ can stimulate \( R_P^\circ \) formation or suppress the requirement in RbpA. Interestingly, the hybrid enzyme comprising the core RNAP of \( E. coli \) and \( \sigma^B \) formed stable promoter complex with the \( \text{lacUV5} \) promoter in the absence of RbpA, suggesting that requirement in RbpA for \( R_P^\circ \) formation depends on the interplay between \( \sigma^B \) and core RNAP (Supplementary Figure S3A).

We showed previously that \( \sigma^A \)-RNAP, without RbpA, was unable to form stable promoter complexes with the 'extended -10' consensus \( \text{sinP} \) promoter from \( B. subtilis \) (23). Strikingly, in contrast to \( \sigma^A \)-RNAP, the \( \sigma^B \)-RNAP formed a competitor-resistant complex with the \( \text{sinP} \) promoter and successfully initiated transcription without RbpA, although RbpA stimulated both reactions approximately 2-fold (Figure 4A). The KMnO4 probing of the \( \text{sinP} \) promoter complex, formed by \( \sigma^B \)-RNAP without RbpA, showed that thymine at position -5 of the non-template strand was accessible to KMnO4 and a weak reactivity was observed at the position -10 (Figure 4B). In addition, a clear protection of the \( \text{sinP} \) promoter from DNaseI was observed (Figure 4C) suggesting that RbpA is not essential for \( R_P^\circ \) formation at the \( \text{sinP} \) promoter. Noticeably, addition of RbpA stimulated unwinding of the upstream part of transcription bubble (thymines at positions -7, -10, -12) and enhanced DNaseI protection, indicating that activator still contributes to the promoter melting and stabilization of the complex. These data suggest that \( \sigma^B \)-RNAP activity is restricted to a limited set of promoters while RbpA broadens the range of promoters recognized by RNAP.

Cytosine at position -13 (C-13) was shown to be required for efficient recognition of promoters by the \( E. coli \) stress-response \( \sigma^S \) (33) which is orthologous to \( \sigma^B \). \( \text{SinP} \) pro-
motecarriescatposition-13whileTisfoundatthecorres-
pondingpositionofthesigAPpromoter.ToexploreifC-13
is responsible for weak dependence of the sinP3 promoter
on RbpA, we constructed two mutant templates: sinP3 with
the substitution C-13 → T (sinP3mut) and sigAP with the
substitution A-13 → C (sigAPmut) (Supplementary Figure
S4A). The activity of the mutant promoters was tested in
EMSA and transcription assays with the βAB-RNAP (Sup-
plementary Figure S4B, C and D). The assays showed that
the mutant sinP3 promoter was still active in transcription
in the absence of RbpA, yet an overall activity was reduced
to 70% of that observed with the wild type template. The
mutant sigAP promoter was unable to support transcription
in the absence of RbpA. No effect of the substitutions
on the promoter activity was observed in the presence of
RbpA. These results suggest that C-13, while contributes
to recognition of sinP3 promoter by σ8-RNAP, does not ob-
viate the requirement for RbpA in transcription initiation.

The fact that σ8-RNAP recognized the same promoters
as σA-RNAP and σ70-RNAP suggests that these σs have
similar promoter consensus sequence specificity. In support
to this conclusion, alignment of the σ subunits regions 2,
3 and 4 revealed that residues important for recognition of
the DNA bases of the -10 and -35 elements are identical
between σA, σ8 and σ70 (Supplementary Figure S3B).

**RbpA remains bound to RNAP in the promoter complex**

Two scenarios could describe the fate of RbpA during ini-

tiation: (1) RbpA binds to RNAP transiently and disso-

ciates as soon as the active promoter complex is assem-

bled (chaperone-like function), or (2) RbpA remains bound
to RNAP in the promoter complex as a true transcrip-
tion initiation factor. To determine which model is cor-

rect, we performed EMSA using a fluorescein-labeled sigAP
promoter and DyLight633-labeled RbpA (RbpADL) (Fig-

ure 5A). This approach allowed for simultaneous detection
of the binding of promoter DNA (fluorescein channel) and
RbpA (DyLight633 channel) to RNAP. RbpADL induced
open complex formation by the βAB-RNAP with the same
efficiency as the unlabeled RbpA (Figure 5A, fluorescein
channel). Scanning of the gel using the DyLight633 channel
revealed that nearly all RbpA was shifted and co-localized
with the RNAP-sigAP promoter complex. This finding sup-
ported the idea that RbpA is an integral component of the
initiation complex and does not dissociate after open com-
plex formation. In support of this view, ChIP analysis per-
formed on Streptomyces showed that RbpA co-localized
with RNAP at promoter regions in vivo (21).

**DISCUSSION**

In the previous work we proposed that RbpA helps the
σA subunit to compete with the alternative σs for bind-
ing to core RNAP by stimulating assembly of the σA-
RNAP holoenzyme (23). Our present study demonstrated
that RbpA induces formation of the catalytically competent
RPo complex by the RNAP containing the stress-response
σB subunit. In addition, the assembly of the σB with core
RNAP was also stimulated by RbpA but did not contribute
significantly to the activation of transcription. Based on
these results we suggest that control of the σ factors activity
by RbpA occurs through the stimulation of RPo formation but not through the stimulation of the assembly of a particular σ with core RNAP.

RbpA comprises two structural domains connected by a flexible linker (20,21). The structured core domain interacts with the β subunit (22,23) while the C-terminal domain binds to the σ subunit domain 2 (20,21). We propose a model where RbpA interaction with σ and β subunits remodels RNAP holoenzyme structure, induces an optimal fit between the σ subunit and promoter consensus elements and thus facilitates the isomerization from the RPc to RPo (Figure 5B). In support to this model, the requirement in RbpA for RPo formation can be bypassed by changing promoter DNA sequence or by assembly of σB heterologous core RNAP and σ subunit domains 4 (brown), 3 (green) and 2 (blue) are represented as ellipsoids. RbpA is shown by two red ellipsoids representing two structural domains of the protein (20). The σ subunit domains 4 are represented by black lines with the sequences of the -10 and -35 elements in boxes. Transcription start site (+1) is indicated by arrow.

Regulation of σB activity by RbpA and the stress response

The σB subunit is responsible for the expression of genes during stationary phase, starvation and the stress response (14,24–25,37–39). The expression of the rbpA gene is also upregulated at these physiological states and induced by antibiotics (19,25). The strong dependence of σB-RNAP activity on RbpA leads to a proposal that RbpA is a principal regulator of the σB-dependent stress response in Mycobacterium. Also, this finding supports the view that role of RbpA in tolerance to rifampicin is indirect (23) and may be linked to the stress response leading to inactivation or elimination of the drug (2). Because, according to our results, σB displayed the same promoter sequence specificity as σA, we predict that the genes that are controlled by σA could be efficiently transcribed by σB-RNAP in the presence of RbpA. The σB–RbpA pair might serve as an alternative system to support the expression of housekeeping genes during conditions when σA gene expression is repressed or σA is sequestered. Noticeably, none of the DNA templates that were used in our work and recognized by σB-RNAP displayed any similarity to the sequence, 5′-NNGNNG-3′, which was suggested as a -10 consensus for σB-dependent promoters (14). That discrepancy may reflect a high ‘flexibility’ in the usage of the -10 consensus by σB or its erroneous attribution.
RbpA differs from other global regulators of transcription

Previously we proposed that RbpA functions similar to the Crl protein (23), which was found in γ-Proteobacteria and belongs to a small group of transcription factors that do not bind DNA (16). Indeed, similar to RbpA, Crl stimulates the activity of the E. coli stationary phase σ^5 by binding the region 2 and increasing the affinity of σ^5 for core RNAP (40,41). However, our current study revealed that RbpA acts though a different mechanism because stabilization of the holoenzyme by RbpA is not a basis for the RbpA-driven stimulation of transcription. The mode of RbpA action also differs from the recently described transcription factor CarD, which was suggested to be a global regulator of transcription in M. tuberculosis (42). In contrast to RbpA, CarD was shown to bind to double-stranded DNA and proposed to act through β1-lobe interactions by stimulating RP_formation (43).

Lastly, our results delineate RbpA as an essential co-factor of M. tuberculosis RNAP and a global regulator of the expression of housekeeping genes in Mycobacteria and likely in other Actinomycetes. A challenge for future studies will be to define the full set of genomic targets of RbpA and to explore its role in pathogenesis, tolerance to antibiotics and bacterial fitness.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGMENTS

We thank Dr M. Buck for critical reading of the manuscript and Dr H. O’Hare and Dr A. Bertoluzzi for providing a sample of the RbpA mutant.

FUNDING

CNRS and INSERM: Fondation pour la Recherche Médicale (FRM) [to Y.H.]; ERASMUS MUNDUS Svagata fellowship [to A.S.P.].

Conflict of interest statement. None declared.

REFERENCES


