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- Promoter escape with bacterial two-component sigma factor suggests retention of sigma region two in the elongation complex.
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- Bacillus subtilis δ functions as a transcriptional regulator by facilitating the open complex formation
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- “Optimization of recombinant Mycobacterium tuberculosis RNA polymerase expression and purification”
  Rajdeep Banerjee, Paulami Rudra, Ranjit Kumar Prajapati, Shreya Sengupta, Jayanta Mukhopadhyay.
  tuberculosisjournal.com/article/S1472-9792 (2014)

- “Novel mechanism of gene regulation: the protein Rv1222 of Mycobacterium tuberculosis inhibits transcription by anchoring the RNA polymerase onto DNA”
Promoter Escape with Bacterial Two-component σ Factor Suggests Retention of σ Region Two in the Elongation Complex*

Received for publication, May 20, 2015, and in revised form, September 23, 2015. Published, JBC Papers in Press, September 23, 2015, DOI 10.1074/jbc.M115.666008

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Background: The proposed model for promoter escape predicts the destabilization of interactions of σ region 4 with RNA polymerase and DNA.

Results: Using a two-component σ factor, we show that YvrI, mimicking the σ region 4, is released, whereas YvrHa, mimicking σ region 2, is retained after promoter escape.

Conclusion: This study validates the proposed mechanism for promoter escape.

Significance: This study suggests the possibility of certain σ-factors to be retained in elongation complex.

The transition from the formation of the RNA polymerase (RNAP)-promoter open complex step to the productive elongation complex step involves “promoter escape” of RNAP. From the structure of RNAP, a promoter escape model has been proposed that suggests that the interactions between σR4 and RNAP and σR4 and DNA are destabilized upon transition to elongation. This accounts for the reduced affinity of σ to RNAP and stochastic release of σ. However, as the loss of interaction of σR4 with RNAP results in the release of intact σ, assessing this interaction remains challenging to be experimentally verified. Here we study the promoter escape model using a two-component σ factor YvrI and YvrHa from Bacillus subtilis that independently contributes to the functions of σR4 and σR2 in a RNAP-promoter complex. Our results show that YvrI, which mimics σR4, is released gradually as transcription elongation proceeds, whereas YvrHa, which mimics σR2 is retained throughout the elongation complexes. Thus our result validates the proposed model for promoter escape and also suggests that promoter escape involves little or no change in the interaction of σR2 with RNAP.

Transcription is the first step in gene regulation that involves initiation, elongation, and termination. For transcription initiation in bacteria, a σ factor must bind to RNA polymerase (RNAP) rendering the enzyme capable of promoter recognition and nucleation of DNA prior to the open complex formation (RPo). Upon addition of NTP, RNAP in the open complex begins synthesis of RNA as an initial transcribing complex and releases short RNA transcripts between 2 and 8 nucleotides. Once the length of the nascent RNA becomes greater than 9–11 nucleotides, RNAP is able to escape from the promoter and start RNA synthesis productively as a transcription elongation complex (EC). The step between the open complex formation and elongation complex formation is known as “promoter escape.” Initially, it was proposed that promoter escape is possible only upon the release of σ from RNAP (1–5). The conclusion was based on biochemical assays with Escherichia coli σ70, in which σ70 was observed to be present in the open complex, but absent in the elongation complex. This observation lead to the generalized proposal that promoter escape in bacteria involves “σ-cycle” in which σ binds to RNAP to initiate transcription and is released from RNAP upon transition from transcription initiation to elongation (1–5). This obligatory release of σ70 for promoter escape was subsequently challenged by several observations that suggested that the release of σ70 does not occur immediately upon transition from transcription initiation to elongation, but rather occurs slowly during the course of transcription elongation (6–12). In contrary to the “σ-release model,” it was further reported that a fraction of RNAP of E. coli in stationary phase does not release σ70 throughout the elongation step on some genes (13, 14). When RNAP covalently tethered with σ70 was expressed in E. coli, there was no adverse effect on the cell growth. Because there was no release of σ70 in vivo in these cells, this observation argues that the σ-cycle may not be essential for bacteria.

The principal σ factors of bacterial species contain four conserved regions: (σR1.1, σR2, σR3, and σR4), all of which interact extensively with β and β′ subunits of RNAP (15–17). In addition, σR4 and σR2 are responsible for recognition and interaction with promoter elements −35 and −10, respectively. σR1.1 is located inside the active center cleft in the RNAP holo but is displaced from the cleft once the open complex is formed (15). The structures of RNAP holoenzyme of different bacterial species show that the σR3/σR4 linker occupies the “RNA exit channel” that mediates the egress of nascent RNA. This linker encounters a steric clash with the nascent RNA once it reaches a threshold length of ~9–11 nucleotides and tries to enter the RNA exit channel (15–17). Thus, the transition from transcription initiation to elongation involves the displacement of the
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σR3/σR4 linker from the RNA exit channel. From the structure of RNAP, Murakami and Darst (18) proposed a model for the promoter escape, which predicts that displacement of the σR3/σR4 linker could destabilize the interactions between σR4 and β, which in turn, further destabilizes the interactions of σR4 and the ~35 element, allowing RNAP to escape from the promoter and start productive elongation. Although the interaction of σR2 with RNAP possibly remains intact at this stage, this transition into elongation is responsible for the reduced affinity of σ for RNAP (19) and results in a stochastic release of σ.

In Bacillus subtilis σ-like factors YvrI and coregulator YvrHa activate transcription from a small set of conserved promoters (PoxdC, PyvrI, and PyvrJ) (20). YvrI includes a σ region 4 domain that interacts with the β subunit of RNAP and is responsible for recognizing the ~35 element of specific promoters. On the other hand, YvrHa functions as an R2 and is responsible for recognizing the ~10 element and DNA melting of the promoter (20–22). Thus, these two proteins together function as a σ factor in an RNAP-promoter complex and is referred to as a two-component σ factor. This two-component σ factor could posit an ideal system to study the proposed promoter escape model that predicts that the interaction between RNAP and YvrI (which mimics σR4) may break once nascent RNA reaches a length of 9–11 nucleotides, whereas interaction between YvrHa (which mimics σR2) and RNAP may remain intact. Here, we observed that YvrI was released as RNAP proceeds through elongation, whereas YvrHa was fully retained in the elongation complex. Thus, our study validates the predictions of the proposed model for promoter escape and establishes that σR2 retains its interaction with RNAP in the elongation phase.

Materials and Methods

Cloning Strategies—The genes encoding YvrI and YvrHa were cloned from plasmids pSM11 and pSM17, respectively (a kind gift from Dr. Shawn Maclellan), into pET32a with NcoI and BamHI, for the incorporation of the C-terminal histidine tag. DNA fragments containing oxdc, pyvrI, and pyvrj (20) (20). YvrI includes a σ region 4 domain that interacts with the β subunit of RNAP and is responsible for recognizing the ~35 element of specific promoters. On the other hand, YvrHa functions as an R2 and is responsible for recognizing the ~10 element and DNA melting of the promoter (20–22). Thus, these two proteins together function as a σ factor in an RNAP-promoter complex and is referred to as a two-component σ factor. This two-component σ factor could posit an ideal system to study the proposed promoter escape model that predicts that the interaction between RNAP and YvrI (which mimics σR4) may break once nascent RNA reaches a length of 9–11 nucleotides, whereas interaction between YvrHa (which mimics σR2) and RNAP may remain intact. Here, we observed that YvrI was released as RNAP proceeds through elongation, whereas YvrHa was fully retained in the elongation complex. Thus, our study validates the predictions of the proposed model for promoter escape and establishes that σR2 retains its interaction with RNAP in the elongation phase.

Purification of YvrI and YvrHa Proteins of B. subtilis—pET32a-yvrI and pET32a-yvrHa were transformed into E. coli BL21(DE3) cells and grown in 2 liters of 2 × YT (16 g of tryptone, 10 g of yeast extract, and 5 g of NaCl per liter) with 0.1% dextrose and 100 μg/ml of ampicillin at 37 °C until the OD reached 0.4. Protein expressions were induced with addition of 0.5 mM isopropyl-1-thio-β-D-galactopyranoside and the cells were grown for another 3 h. The cells were lysed with TGB (50 mM Tris–HCl, 5% glycerol, 2 mM β-mercaptoethanol) and 0.2 M NaCl, 0.25% deoxycholate, 1 mM PMSF, followed by sonication and centrifugation at 14000 rpm for 20 min at 4 °C. The pellets were washed with TGB, 0.2 M NaCl, and 0.5% Triton X-100 followed by centrifugation at 8000 rpm for 20 min at 4 °C. The pellets were solubilized in buffer A (TGB + 8 M urea) and incubated for 1 h, followed by centrifugation at 14000 rpm at room temperature. The supernatant containing the solubilized protein was passed through 5-mL nickel-nitrilotriacetic acid-agarose, pre-equilibrated with buffer A, washed with 25 mL of buffer A, and eluted with 5 mL each of buffer A containing 10, 20, 40, 80, and 160 mM imidazole. Both proteins were eluted at 40 mM or higher concentration of imidazole. The purified YvrI sample was then dialyzed in 4 liters of TGB buffer for 24 h with two changes at 8-h intervals and the renatured protein was stored with 50% glycerol at −80 °C.

The 5 mL of YvrHa protein sample was renatured by serial dialution with an equal volume of TGB buffer to make a final volume of 80 mL. The sample was applied to a MonoQ HR 10/10 column in Akta Purifier (GE Healthcare), preequilibrated with TGB buffer. The column was washed with 16 mL of TGB buffer.
and eluted in 1-ml fractions with a linear gradient 0 to 1 M NaCl in 160 ml of TGB buffer. The protein was eluted at 0.4 M NaCl. The purified protein sample was added with an equal volume of 100% glycerol and stored at −80 °C.

Preparation of E. coli RNAP Core—E. coli RNAP core was purified as in Mukhopadhyay et al. (7).

Purification of E. coli σ70—E. coli σ70 (a derivative with a single-Cys residue at position 578) was purified as in Mukhopadhyay et al. (7).

Purification of E. coli σ70-R2 and σ70-R4—Plasmids pET32α-rpoD-R2 and pET32α-rpoD-R4 containing Cys at positions 396 and 578, respectively, were transformed into E. coli C43 cells and grown in 1 liter of 2YT containing 100 µg/ml of ampicillin. After growing the cells to OD of 0.4 at 37 °C, the protein induction was initiated at 16 °C with the addition of 0.5 mM isopropyl-1-thio-galactopyranoside, and the cells were further grown for 12 h. The cells were lysed and the proteins that appeared in soluble forms were purified essentially following the same method as for YvrI except that protein samples were passed through a nickel-nitriilotriacetic acid column using TGB + 0.2 M NaCl buffer, and no denaturation/renaturation steps were involved.

Labeling of YvrI and YvrHa mutants of B. subtilis and σ70-R2, and σ70-R4 of E. coli with TMR-6-maleimide—For labeling of YvrI and YvrHa, we generated single Cys derivatives of each protein. Because YvrI does not contain any cysteine, this residue was incorporated at amino acid position 25 (originally Ser) using a site-directed mutagenesis. YvrHa contains two cysteines at amino acid positions 24 and 61. First these cysteine residues were mutated to serine to generate a no-Cys protein derivative. Then Cys was incorporated at amino acid position 25 (originally Thr) of YvrHa using a site-directed mutagenesis derivative. Then Cys was incorporated at amino acid position 15 (originally Thr) of YvrI using a site-directed mutagenesis. YvrHa contains two cysteines at amino acid position 25 (originally Ser) using a site-directed mutagenesis. YvrHa contains two cysteines at amino acid position 25 (originally Ser) using a site-directed mutagenesis. YvrHa contains two cysteines at amino acid position 25 (originally Ser) using a site-directed mutagenesis.

YvrHa T15C mutants of B. subtilis and σ70-578C, σ70-R2–396C, and σ70-R4–578C of E. coli (containing single Cys residues) were labeled with TMR-6-maleimide as described by Rudra et al. (24, 25). The labeled protein fractions were confirmed by running on SDS-PAGE and viewed in a Fluorescence Imager. The efficiency of labeling was detected by the ratio of the dye concentration (estimated by absorbance at 555 nm) and the protein concentration (estimated by Bradford assay). The labeling efficiencies of YvrI S25C, YvrHa T15C, σ70-578C, σ70-R2–396C, and σ70-R4–578C were found to be 90, 95, 98, 72, and 60% respectively.

Preparation of 5’-Biotinylated Promoter DNA Fragments—The promoter DNA templates (Table 2) were generated by PCR amplification of either genomic DNA (Bs168) or from synthetic oligonucleotides with their respective 5’-biotinylated forward and reverse primers (Table 1).

Formation of Open Complexes on Streptavidin Beads—E. coli RNAP holoenzyme was prepared by incubation of 200 nM unlabeled RNAP core with 500 nM labeled σ70 (or σ70-R2 and σ70-R4) for 20 min at 25 °C in transcription buffer (TB: 40 mM Tris, 10 mM MgCl2, 0.1% Triton-X-100, 2 mM DTT). Open complexes were formed by incubating the E. coli RNAP holoenzymes sample with 100 nM 5’-biotinylated DNA fragments containing either T7A1 or lacUV5 promoter at 37 °C for 20 min.

B. subtilis RNAP holoenzyme was prepared by incubation of 200 nM unlabeled RNAP core and 500 nM each of TMR-labeled YvrI and YvrHa in transcription buffer (18 mM Tris (pH 8.0), 10 mM NaCl, 8 mM β-mercaptoethanol, 10 mM MgCl2, 0.01% Triton-X-100, 2 mM DTT). Open complexes were formed by incubating the RNAP holoenzyme sample with 100 nM 5’-biotinylated DNA probes containing either PoxyC or PpyrI promoter at 37 °C for 20 min. 10 µl RP samples were immobilized on streptavidin beads pre-equilibrated with the respective transcription buffer, by incubating the samples at 25 °C for 1 h with regular tapping. The beads were washed 4 times with the respective transcription buffer; each time the samples were centrifuged at 4000 × g for 1 min.

Formation of Stalled Elongation Complexes—For formation of stalled elongation complex EC+n, we used the DNA derivative that contained the first non-template strand CTP or UTP at position n + 1, (Table 2). After formation of the open complex as above, transcription reactions were initiated with subset of NTPs and 0.25 µg/µl of heparin as following: EC + 11 on PoxyC, 500 µM ApG, GTP, UTP, ATP, EC + 20 and EC + 39 on PoxyD,

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<th>Bs PoxyC EC +39</th>
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| Table 2 
Promoter DNA templates used in the assays

The bold letter A denotes the transcription start site. Other bold letters denote the first non-template strand T or C nucleotide.
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These complexes were washed thoroughly several times with TB to remove the excess unbound proteins, followed by centrifugation at 4000 × g for 1 min each time. A set of reactions containing RPα and EC are run on 10% SDS-PAGE and scanned in the TMR channel (excitation 530 nm, emission 580 nm) of a fluorescence scanner, (Typhoon Trio+ , GE Healthcare). The amounts of YvrI, YvrHa, and σ70 in each of the complexes were quantitated from the fluorescence intensities of the corresponding protein bands on the fluorescence scanned gel, and the amount of RNAP was quantitated from the intensity of the βB' band on the same gel stained with Coomassie Blue. Previously, a standard curve with intensity versus concentration was generated for each protein sample by running various amounts of each protein on a 10% SDS-PAGE and measuring the intensity of each band on the gel either scanned by fluorescence on the TMR channel or stained by Coomassie Blue. The fractional occupancies of YvrI, YvrHa, and σ70 in each of the complexes were determined from the ratio of the amount of protein to the amount of RNAP.

Determination of the Transcription Efficiency of RPα—To quantitate the amount of transcripts in stalled EC, an in vitro transcription assay was performed with each RPα, as above but with 32p-labeled ATP. The samples were run on 12% urea PAGE and scanned by phosphorimaging (Typhoon Trio+ , GE Healthcare). The amounts of transcripts were determined from the intensity of band for each stalled EC as described by Mukhopadhyay et al. (7). The amounts of open complexes formed were determined from the quantity of DNA bound to the beads by a fluorescence based assay with SyBr Gold as per the manufacturer’s protocol (Life Technologies). The subpopulation of RPα competent to form EC (f) was determined from the ratio of the amount of transcripts to RPα.

The fractional occupancies of YvrI, YvrHa, σ70, σ70-R2, and σ70-R4 were corrected for the subpopulation of the RPα competent to form EC using the formula: \( F_{EC} = \frac{(E_{RPα}+NTPs)-(1-f)E_{RPα}}{f} \), where f = fraction of molecules competent to undergo the transition to elongation, E = fractional occupancies of YvrI, YvrHa, σ70, σ70-R2, and σ70-R4.

Fluorescence Anisotropy Assay—20 nm TMR-labeled YvrI and unlabeled YvrHa in 60 μl of TB was titrated with an increasing concentration of RNAP at 37 °C and the anisotropy values were monitored with excitation at 540 nm and emission at 580 nm using a PTI Fluorescence master QM400 system fitted with automatic polarizer (25, 26). The anisotropy values remained unchanged on the addition of unlabeled YvrHa to labeled YvrI, in the absence of RNAP (considered as anisotropy value \((A_e)\) of free YvrI. When saturation was reached with the addition of around 50 nm RNAP, PoxDC promoter DNA was added at a concentration equal to the saturating level of RNAP core, to form the open complex. The addition of DNA did not change the anisotropy value (considered as \(A_s\) of fully bound YvrI).

Stalled elongation complexes (EC + 11, EC + 20, and EC + 39) were formed by adding appropriate NTP and heparin (as indicated above) and incubating with RPα and the anisotropy values of the samples were monitored. The fractions of bound YvrI in RPα and ECs with anisotropy value A (with both free and bound molecules) were determined from the equation: \( f A_e + \frac{(1-f) A_s}{A} = A \), where f is the fraction of free molecules of YvrI.

The subpopulation of RPα competent to form EC was estimated using in vitro transcription assay as above but in solution. The fractional occupancies of YvrI and YvrHa in the stalled elongation complexes were corrected for the subpopulation of RPα competent to form EC using the formula mentioned earlier. A similar set of experiments were repeated with labeled YvrHa (and unlabeled YvrI) to determine the fractional occupancies of this protein in RPα and ECs.

Results

Previously the occupancy of σ relative to RNAP in the context of the open complex and elongation complex had been determined by separating the complexes from the free components either by gel electrophoresis or chromatography and subsequently analyzing their contents by SDS-PAGE and Coomassie staining. In this report, we use a similar technique that involves immobilization of open complex (RPα) on streptavidin beads using biotin-labeled promoter DNA fragments. First RNAP core and TMR-labeled σ or two-component σ factor were incubated to form RNAP holo before further incubation with promoter DNA fragments to form RPα. The beads were washed several times to remove excess RNAP and σ. A part of the open complexes were used to form the stalled elongation complexes (EC + n, where n is the length of RNA) and further washed. To form EC, the promoter DNA fragment derivatives first having UTP or CTP residues at the position n + 1 base on the non-template strand of the transcribed region (sequences listed in Table 2) were used to form the RPα and added with ATP, GTP, and CTP or UTP, so that RNAP synthesizes RNA of length n nucleotide and halts. Both RPα and EC + n were resolved on SDS-PAGE gel, scanned with a fluorescence imager at the TMR channel, stained with Coomassie Blue, and scanned further. The amounts of RNAP in the complexes were quantitated by comparing the intensity of βB’ band on the Coomassie-stained gel with the intensity of the same band of RNAP of known quantity run in parallel on the same gel. The amount of labeled YvrI and YvrHa (or σ) in the complexes were quantitated in the same way by estimating the fluorescence intensity of the band on the fluorescently scanned gel. Because the size of YvrI and YvrHa is small (much less as compared with βB’), the protein bands from the above complexes were not visible on the Coomassie-stained gel. Therefore these small proteins were labeled with fluorescent dye for better quantification. The labeled YvrI and YvrHa were active in transcription (results not shown). The fractional occupancy of these factors in RPα and EC were determined from the ratio of the amount of protein to RNAP in the complexes. The fractional occupancy was further corrected for the subpopulation of RPα that was unable to form EC. The amount of RPα was determined from the quantity of DNA bound to the beads as the DNA was the limiting factor in the formation of RPα. The amount of EC was determined from the quantity of radiolabeled transcripts formed with RPα using
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the in vitro transcription assay identical to the formation of EC as above, but with $^{32}$P-labeled NTP.

Study with E. coli $\sigma^{70}$—Because it was previously shown that $\sigma^{70}$ in E. coli is released upon transition from transcription initiation to elongation, we first tested whether the result was reproducible using the above assay. Two different promoter DNA fragments, lacUV5 and T7A1, were used to form RPo. Subsequently EC + 23 and EC + 15 were formed with T7A1 and lacUV5 promoter DNA fragments, respectively (Fig. 1A). Six replicates of a set of RPo and EC were formed for each promoter. Fractional occupancy of $\sigma^{70}$ in these complexes were estimated for each set. The mean value of fractional occupancy of $\sigma^{70}$ was found to be 0.61 for the T7A1 DNA fragment and 0.57 for the lacUV5 DNA fragment (Fig. 1B). Fractions of RPo that were competent to form elongation complexes were estimated for each set and the typical values were 0.55 of RPo in T7A1 and 0.60 in lacUV5 (Fig. 1C). After correcting the subpopulation that were competent to undergo the transition from transcription initiation to elongation, the fractional occupancy of $\sigma^{70}$ was estimated to be 0.19 and 0.13 in T7A1 and lacUV5 promoters, respectively (Fig. 1D). Thus, the majority of $\sigma^{70}$ was released from the elongation complexes on both promoters in this assay, consistent with previous observation that $\sigma^{70}$ was released during transition from transcription initiation to elongation using a similar assay.

Study with B. subtilis YvrI and YvrHa—We then tested the fate of two-component $\alpha$ factors YvrI and YvrHa of B. subtilis during the transition from transcription initiation to elongation. RPo were formed on the PoxDC promoter DNA fragments with RNAP core, YvrI and YvrHa. Three PoxDC promoter DNA derivatives were used in the assay that permitted formation of EC + 11, EC + 20, and EC + 39, respectively, using a subset of NTPs (Fig. 2A). The assay was repeated six times and the mean value of the fractional occupancies (Fig. 2B) of YvrI with respect to RNAP were estimated to be 0.85, 0.85, and 0.69 in EC + 11, EC + 20, and EC + 39, respectively. The subpopulation of RPo, competent to undergo transition to elongation was estimated to be 0.45 for EC + 11, 0.36 for EC + 20, and 0.30 for EC + 39 (Fig. 2C). After correction, the fractional occupancies of YvrI were estimated to be 0.48 in EC + 11, 0.16 in EC + 20, and 0.0 in EC + 39 (Fig. 2D). On the other hand, the fractional occupancy of YvrHa for all three ECs was estimated to be around 0.99 and 0.97, respectively, before and after the correction. Please note that as the fraction of RPo, that was competent to undergo transition to elongation was estimated only from the intensity of stalled EC, not taking into account the intermediate paused EC, and therefore there could be a 10–20% error in the estimation, and these errors could be incorporated in the estimation of fractional occupancies of YvrI and YvrHa. The data indicate a gradual release of YvrI from RNAP as transcription elongation proceeds, whereas there is no release of YvrHa from RNAP for all the ECs.

FIGURE 1. Fractional occupancy of $\sigma^{70}$ of E. coli in EC on T7A1 and lacUV5 promoters. A, representative data assessing the components of RPo and EC. RPo were formed by incubating 200 nM E. coli RNAP core, 500 nM each of TMR $\sigma^{70}$, and 100 nM biotinylated promoter DNA fragment at 37 °C for 20 min, and immobilized on streptavidin beads. ECs were formed by adding NTP (indicated under “Materials and Methods”). The beads containing RPo and EC were washed before resolving on 10% SDS-PAGE, followed by fluorescence scanning and Coomassie staining. Left panel, data for T7A1 promoter: EC + 23 were formed. Right panel, data for lacUV5 promoter: EC + 15 were formed. B, fractional occupancies of $\sigma^{70}$ with respect to RNAP core: determined by quantifying the amount of each protein from the gel. Data were average of six replicates. Left panel, data for T7A1 promoter: EC + 23 were formed. Right panel, data for lacUV5 promoter: EC + 15 were formed. C, representative data for in vitro transcription assay with RPo immobilized on streptavidin beads. Stalled elongation complexes were generated using $^{32}$P-labeled NTP; chase, all four NTP were added to EC to produce runoff products. The samples were run on 12% urea PAGE and scanned on a phosphorimager. Left panel, data for T7A1 promoter: EC + 23 were formed. Right panel, data for the lacUV5 promoter: EC + 15 were formed. D, fractional occupancies of $\sigma^{70}$ after correction for the subpopulation that are competent to form EC. Data were average of six replicates. Left panel, data for T7A1 promoter: EC + 23 were formed. Right panel, data for lacUV5 promoter: EC + 15 were formed.
Identical results were obtained with these factors using another promoter DNA PyvrI. Two elongation complexes, EC10 and EC19, were formed using the PyvrI promoter derivatives (Fig. 3A). The fractional occupancy of YvrI at EC10 and EC19 was estimated to be 0.79 and 0.72 before correction (Fig. 3C); and 0.45 and 0.11 after correcting for the subpopulation not competent to undergo transition to elongation (fraction of RPo efficient in transcription were 0.39 for EC10 and 0.30 for EC19, Fig. 3D). The fractional occupancy of YvrHa for the above ECs was estimated to be around 1 and 1.1, respectively, before and after the correction.

Study with B. subtilis YvrI and YvrHa Using Anisotropy—In addition to the above method that involved a separation step, we used another complimentary approach involving fluorescence anisotropy that does not involve any separation step for RPo or EC from the free components. As the fluorescence anisotropy value of a free molecule ($A_0$) is different from the anisotropy value of the molecule bound to another protein ($A_1$), the fraction of free molecules ($f$) in a mixture of samples with anisotropy value $A$ (both free and bound molecules) can be monitored from the equation: $A = fA_0 + (1 - f)A_1$. The anisotropy values of TMR-labeled YvrI were monitored in the presence of an equal amount of unlabeled YvrHa (taken as an anisotropy value of free YvrI, Fig. 4A) before and after titration with RNAP core. Once saturation was reached, the PoxdC promoter DNA fragment was added (at a concentration equal to the saturating level of RNAP core) to form the open complex. After addition of DNA, the anisotropy value of the complex did
Mechanism of Promoter Escape

Our results show that the amount of YvrI relative to RNAP is gradually decreased in the elongation complexes as elongation proceeds, whereas the amount of YvrHa relative to RNAP remains constant in the elongation complexes (Fig. 6). Because a certain amount of YvrI is present in the early elongation complexes (fractional occupancy is 0.48 at EC+11), our result is consistent with previous observations that shows a significant fraction of 70 remains associated with RNAP in the early elongation complex (6, 12). The result (fractional occupancy of YvrI are 0.48, 0.16, and 0, respectively for EC+11, EC+20, and EC+39) is also consistent with the observation that demonstrated the stochastic release of 70 in transcription elongation (11). The RNAP structure-based proposed model for promoter escape predicts that, upon transition from transcription initiation to elongation, the interaction of 70 with RNAP is destabilized upon transition to elongation reducing the overall affinity of 70 to RNAP and this accounts for stochastic release of 70. However, as the destabilization of interactions of 70 with RNAP in EC results in the release of intact 70-R4, the release of intact 70-R4 is not possible using a simple biochemical assay. In principle, assessing the above interaction could be possible using a FRET assay involving fluorescently labeled RNAP and promoter derivatives of 70-R4 and 70-R4 with respect to RNAP was estimated to be 0.67, 0.52, and 0.45 for EC+15, EC+25, and EC+40, respectively (Fig. 5B). After correction, the fractional occupancies of 70-R4 were estimated to be 0.45 in EC+15, 0.15 in EC+25, and 0.0 in EC+40 (Fig. 2D). On the other hand, the fractional occupancies of 70-R2 for all three ECs were estimated to be around 0.99 and 0.98, respectively, before and after the correction.

Discussion

Study with E. coli 70-R2 and 70-R4—To test whether the promoter escape study with YvrI and YvrHa is valid for any other two-component 70 factor, we prepared two truncated derivatives of 70, comprising amino acids 130–500 of the 70 region 2/3.1 (70-R2) and amino acids 501–613 of the 70 region 3/2 (70-R4), respectively. Interestingly, these two truncated derivatives of 70 are able to initiate transcription from the 70-dependent promoter. Each protein derivative contained a single Cys residue and was labeled with TMR. The promoter escape study with these two 70 derivatives was performed essentially as with YvrI and YvrHa of B. subtilis except that the lacIIVS promoter derivatives and E. coli RNAP core were used to form RPo and ECs (Fig. 5A). The mean value of the fractional occupancies of 70-R4 with respect to RNAP was estimated to be 0.67, 0.56, and 0.49 in EC+15, EC+25, and EC+40, respectively (Fig. 5B). The subpopulations of RPo, competent to undergo the transition to elongation were estimated to be 0.60 for EC+15, 0.52 for EC+25, and 0.45 for EC+40 (Fig. 5C). After correction, the fractional occupancies of 70-R4 were estimated to be 0.45 in EC+15, 0.15 in EC+25, and 0.0 in EC+40 (Fig. 2D). On the other hand, the fractional occupancies of 70-R2 for all three ECs were estimated to be around 0.99 and 0.98, respectively, before and after the correction.

not change and was taken as the anisotropy value of bound YvrI (Fig. 4A). Three sets of RPo and ECs (EC+11, EC+20, and EC+39) were formed, and the anisotropy values were monitored. Each set was repeated six times and the mean values of fractional occupancies of YvrI in ECs were estimated to be 0.75 for EC+11, 0.65 for EC+20, and 0.52 for EC+39 (Fig. 4C). The competent subpopulation of RPo, efficient in forming ECs in solution was determined to be 0.42 for EC+11, 0.36 for EC+20, and 0.30 for EC+39 (Fig. 4D), which in turn, determined the corrected fractional occupancy of EC+11, EC+20, and EC+39, respectively, as 0.52, 0.22, and 0 (Fig. 4E). Similar assays were performed with TMR-labeled YvrHa and unlabeled YvrI (Fig. 4B), in which the fractional occupancy of YvrHa remained around 0.98 for all three ECs. Observation of the fluorescence anisotropy assay was consistent with the previous assay.

FIGURE 4. Fractional occupancy of YvrI and YvrHa of B. subtilis in EC:fluorescence anisotropy assay. A, fluorescence anisotropy values of 20 nM TMR-labeled YvrI (and 20 nM unlabeled YvrHa) upon titration with B. subtilis RNAP core. A1 (0.151) and A0 (0.03) were the values of free and fully bound YvrI, respectively. B, fluorescence anisotropy values of 20 nM TMR-labeled YvrHa (and 20 nM unlabeled YvrI) upon titration with B. subtilis RNAP core. A1 (0.051) and A0 (0.104) were the values of free and fully bound YvrHa, respectively. C, open complexes were formed by incubating 50 nM B. subtilis RNAP core, 20 nM each of labeled Yvr and YvrHa with 50 nM PonDc promoter in solution at 37 °C. Stalled elongation complexes were generated by adding 500 μM each of ApG, heparin, and other NTPs at +11 (without CTP), +20 (without UTP), and +39 (without UTP), The mean anisotropy values of RPo, EC+11, EC+20, and EC+39 were 0.297, 0.26, 0.24, and 0.22, respectively, for YvrI: 0.104, 0.102, 0.1, and 0.1, respectively, for YvrHa. The fractional occupancies of YvrI and YvrHa were determined by estimating the amount of bound proteins in RPo, and EC from the anisotropy values of the protein in the respective complexes. Gray bar, YvrI; black bar, YvrHa. D, representative data for in vitro transcription assay in solution. Stalled elongation complexes EC+11, EC+20, and EC+39 were generated using [32P]-labeled NTP; chase, all four NTP were added to EC to produce runoff products. The samples were run on 12% urea PAGE and scanned on a phosphorimager. E, fractional occupancies of YvrI and YvrHa after correction for the subpopulations that are competent to form EC. Data were average of six replicates. Gray bar, YvrI; black bar, YvrHa.
whereas YvrHa (that mimics σR2) is retained in the EC. Similar results were obtained with the truncated σ70-R2 and σ70-R4 with respect to RNAP core: determined by quantifying the amount of each protein from the gel. The data were the average of six replicates. Gray bar, σ70-R2; black bar, σ70-R4. C, representative data for in vitro transcription assay with RNAP immobilized on streptavidin beads. Stalled elongation complexes EC + 15, EC + 25, and EC + 40 were generated using [32P]-labeled NTP; chase, all four NTP were added to EC to produce runoff products. The samples were run on 12% urea-PAGE and scanned on phosphorimager. D, fractional occupancies of σ70-R2 and σ70-R4 after correction for the subpopulation that are competent to form EC. Data were average of six replicates. Gray bar, σ70-R2; black bar, σ70-R4.

FIGURE 6. Proposed model for promoter escape: YvrI (σR4) is released from RNAP, whereas YvrHa (σR2) is retained by RNAP upon transition from transcription initiation to elongation.

whereas YvrHa (that mimics σR2) is retained in the EC. Similar results were obtained with the truncated σ70 derivatives: σ70-R4 is released, whereas σ70-R2 is retained in the EC. Therefore, our results validate the proposed model for promoter escape in bacteria. As YvrHa is not covalently attached to YvrI, the release of YvrI does not alter the interaction of YvrHa with RNAP in EC. This further suggests that there is no or little change in the interaction between σR2 with RNAP upon transition to elongation. But this interaction is not strong enough to retain the whole σ70 in the elongation complex as the rest of σ regions, σR1.1, σR2, and σR3/4 linker, lose their interaction with RNAP. However, the observation raises the possibility that the σR2-RNAP interaction of a certain σ factor from other bacterial species could be strong enough to retain the σ factor in the EC throughout the elongation phase.

References


Bacillus subtilis δ factor functions as a transcriptional regulator by facilitating the open complex formation

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Keywords: δ factor, Bacillus subtilis, transcription, RNA polymerase, transcriptional regulators

Running title: δ functions as a transcriptional regulator.

Abstract:
Most bacterial RNA polymerases (RNAP) contain five conserved subunits viz. 2α, β, β’ and ω. However, in many gram positive bacteria, especially in fermicutes, RNAP is associated with an additional factor, called δ. Over three decades since its identification, it had been thought that δ functioned as a subunit of RNAP to enhance the level of transcripts by recycling RNAP. In support of the previous observations, we also find that δ is involved in recycling of RNAP by releasing the RNA from the ternary complex. We further show that δ binds to RNA and is able to recycle RNAP when the length of the nascent RNA reaches a critical length. However in this work we decipher a new function of δ. Performing biochemical and mutational analysis we show that Bacillus subtilis δ binds to DNA immediately upstream of the promoter element at A-rich sequences on the abrB and rrnB1 promoters and facilitates open complex formation. As a result, δ facilitates RNAP to initiate transcription in the second scale, compared to minute scale in the absence of δ. Using transcription assay we show that δ mediated recycling of RNAP cannot be the sole reason for the enhancement of transcription yield. Our observations that δ does not bind to RNAP holo enzyme but is required to bind to DNA upstream of the -35 promoter element for transcription activation, suggest that δ functions as a transcriptional regulator.

Introduction:
Transcription is the first step in gene regulation in bacteria in which RNA polymerase (RNAP) together with different sigma factors and transcriptional regulators control the gene expression. Bacterial RNAP core enzyme contains five conserved subunits: 2 α, β, β’ and ω. A specificity factor sigma associates with RNAP core enzyme to form RNAP holo enzyme that is able to recognize and initiate transcription at promoters.

In certain gram positive bacteria, including Bacillus subtilis and Staphylococcus aureus, an additional factor, called δ, is associated with RNAP. The δ factor was first identified in 1975 during the purification of RNAP from phage (SP01) infected B. subtilis (1). The protein copurified with RNAP, and therefore it was thought that δ functions as a subunit of RNAP. Attempts were made to characterize the functional role of the protein in transcription. Several reports suggested that δ was involved in promoter selection (2-5) and functioned together with σA as an initiation subunit of RNAP (6,7), or as an allosteric modulator of RNAP conformation in both initiation and the RNAP core recycling phase (5). Other reports showed that δ and σA bind to RNAP core with negative cooperativity (8,9) and δ has no effect on transcription initiation, the rate of elongation or termination (5). Using in vitro transcription assays, several groups showed that δ enhances the production of transcripts from certain promoters. This increase in transcript yield in the presence of δ is attributed to the recycling of RNAP possibly by δ mediated release of RNAP from the elongation complex following transcription termination or by inhibiting the formation of stable RNAP core-DNA/RNA complex (5,10).
In order to characterize the role of δ in vivo, strains lacking δ were studied. It was found that these mutants did not exhibit any distinctive phenotype, except an altered cell morphology and a delayed exit from stationary phase (9) or a block in sporulation (11). Recently, Rabatinova et al. (12) demonstrated that mutant strains lacking δ displayed a decreased ability to survive under a condition where iNTP concentration changes rapidly. This was explained by a condition where iNTP concentration changes rapidly. This was explained by a condition where iNTP concentration changes rapidly. This was explained by a condition where iNTP concentration changes rapidly. ITP concentration changes rapidly. This was explained by a condition where iNTP concentration changes rapidly. This was explained by a condition where iNTP concentration changes rapidly. This was explained by a condition where iNTP concentration changes rapidly. This was explained by a condition where iNTP concentration changes rapidly. This was explained by a condition where iNTP concentration changes rapidly. This was explained by a condition where iNTP concentration changes rapidly. This was explained by a condition where iNTP concentration changes rapidly. This was explained by a condition where iNTP concentration changes rapidly. This was explained by a condition where iNTP concentration changes rapidly. This was explained by a condition where iNTP concentration changes rapidly. This was explained by a condition where iNTP concentration changes rapidly. This was explained by a condition where iNTP concentration changes rapidly. This was explained by a condition where iNTP concentration changes rapidly. This was explained by a condition where iNTP concentration changes rapidly. This was explained by a condition where iNTP concentration changes rapidly. This was explained by a condition where iNTP concentration changes rapidly. This was explained by a condition where iNTP concentration changes rapidly. This was explained by a condition where iNTP concentration changes rapidly. This was explained by a condition where iNTP concentration changes rapidly. This was explained by a condition where iNTP concentration changes rapidly. This was explained by a condition where iNTP concentration changes rapidly. This was explained by a condition where iNTP concentration changes rapidly. This was explained by a condition where iNTP concentration changes rapidly. This was explained by a condition where iNTP concentration changes rapidly. This was explained by a condition where iNTP concentration changes rapidly. This was explained by a condition where iNTP concentration changes rapidly. This was explained by a condition where iNTP concentration changes rapidly. This was explained by a condition where iNTP concentration changes rapidly. This was explained by a condition where iNTP concentration changes rapidly.
Purification of Bs RNAP core:

E. coli B834 (DE3) cells were transformed with plasmids pNG545 (encoding β and α) and pNG540 (encoding ω and β') (kind gift from Peter J. Lewis, University of Newcastle, Australia (14)) and were grown in 3L LB at 37°C up to 0.4 OD. Temperature of the growth media was lowered to 16°C before addition of 0.5 mM IPTG. The cells were further grown at 16°C for 12 h. Cells were harvested and RNAP was purified as in Mukhopadhyay et al. (15).

For purification of Bs RNAP from δ-knockout strain of B. subtilis HB6010 (CU1065 ∆rpoE::cm; a kind gift from Dr. Helmann (9)), cells were grown in 2L LB at 37°C up to 1.2 OD. The cells were harvested and the protein was purified as recombinant Bs RNAP excluding Ni-affinity chromatography.

Purification of σ8:

E. coli C43 (DE3) cells containing pET28-sigA were grown in 1 L LB at 37°C till OD reached 0.5. Protein production was induced by adding 0.5 mM IPTG, followed by growth at 25°C for 5 h. Cells were harvested by centrifugation (6,000 g, 10 min, 4°C), were resuspended in 20 ml TG buffer (50 mM Tris-Cl, 5% Glycerol, 4°C), were resuspended in 20 ml TG buffer (50 mM Tris-Cl, 5% Glycerol) containing 200 mM NaCl, 5 mM βME, 1 mM PMSF and were disrupted by sonication. The lysates were spun at 18000g for 30 min at 4°C. The supernatant was diluted to 100 ml with TG buffer and was loaded onto Q-sepharose column (GE Healthcare) pre-equilibrated with TG buffer. Protein was eluted using a step gradient of NaCl in TG buffer. The eluted fractions enriched with σ8 were further purified on a MonoQ HR10/10 column in an AKTA purifier (GE Healthcare) using a 0.1-1.0 M NaCl gradient in TG buffer. The purified σ8 sample was concentrated and kept at -80°C after addition of glycerol to a final concentration of 30%.

Preparation of DNA and RNA Fragments:

abrB promoter sequence -95 to +136 and rrnBP1P2 promoter sequence -92 (with respect to +1 of P1) and +112 (with respect to +1 of P2) (sequence listed in Table 1) were amplified by PCR from genomic DNA (isolated from Bs168) using oligo primers (Table 1) and were cloned in pUC19 vector using EcoRI-BamHI.

Promoter DNA fragments having different lengths of upstream regions were also amplified by PCR using oligo primers (Table 1) and purified by PAGE.

abrB promoter DNA fragment containing mutation at positions -40,-41; -44,-45; -40,-41,-44,-45 or GC-rich upstream sequence was prepared by PCR using oligo primers (Table 1). 144 nt RNA was prepared by in vitro transcription assay as below and was purified after treating the reaction mixture with DNaseI.

In vitro transcription assays:

Multi-round transcription: 100 nM RNAP core was mixed with 400 nM σ8 in 10 µl transcription buffer [18 mM Tris-Cl (pH 8.0), 10 mM NaCl, 8 mM βME, 10 mM MgCl2] and was incubated on ice for 30 min followed by 10 min at 25°C to form the holoenzyme. 50 nM promoter DNA fragments (unless stated otherwise) was added to RNAP holo and incubated at 37°C for 20 min to form the open complex. Transcription was initiated with NTP (final concentration: 250 µM of ATP, GTP and UTP, and 25 µM of α-P32-CTP (0.2 µCi)) at 37°C for 30 min. The reactions were terminated by the addition of 2.5 µl of FLB dye (80% formamide, 10 mM EDTA, 0.01% Bromophenol Blue, 0.01% Xylene Cyanol), resolved in 8% or 12% Urea-PAGE. When transcription assays were performed in the presence of δ, the protein was added to the open complex mixtures, following incubation at 37°C for 5 min before the addition of NTP (unless stated otherwise).

Single round transcription: carried out as the multiround assays described above except that heparin (0.25 µg/µl) was added to the reaction mixtures along with NTP.

In a separate assay, the open complexes were allowed to form for 1, 5 and 30 min by incubating RNAP and promoter DNA as above followed by the addition of heparin (0.25 µg/µl) and NTP.

Stalled elongation complex assays: performed as above except that transcription initiation reactions were carried out with the abrB promoter derivatives that contains first C residues at +14, +24 and +45 respectively and with addition of 250 µM GTP, UTP and 25 µM α-32P-ATP.

In vitro transcription assay using Kool NC-45™ template: 1.75 pmol of Kool NC-45™ (Epicentre) template was incubated at 37°C with 200 nM RNAP core and increasing concentrations
of δ in 10 µl transcription buffer, along with 0.5 mM NTP at 37°C for 30 min and fluorescence were monitored as per manufacturer’s protocol (Epicentre Biotechnologies).

**Fluorescence anisotropy assays:**

**Labelling of δ with TMR**

δ protein does not contain any cysteine residue.

Single cysteine derivative of δ was prepared by introducing a cysteine residue at the amino acid residue 51 of rpoE in pET28a by point mutation, using a site directed mutagenesis kit (Stratagene Inc).

The single-cysteine derivative of δ was purified following the protocol used for δ. 50 µl of 11 µM protein sample was reduced with 10 mM DTT as in Kim et. al. (16) before the labelling reaction. The sample was dissolved in 100 µl of Buffer A (100 mM Na-Phosphate pH 7.3, 1 mM EDTA) and reacted with tetramethyl rhodamine (TMR)-6-maleimide for 1 hr at 4°C. The protein sample was centrifuged for 10 min at 18,000g and loaded onto a 10 ml BioGel P6 column (Bio-Rad) pre-equilibrated with buffer A to remove the free dye. The labelled δ was eluted in the void volume, mixed with an equal volume of 100% glycerol and was stored at -80°C. The labelling efficiency is 98%, and activity of the labelled protein was confirmed by in vitro transcription assay.

**Fluorescence anisotropy measurements:** 20 nM of TMR-labelled δ in 60 µl transcription buffer was titrated with increasing concentrations of RNAP or DNA or RNA at 37°C and fluorescence intensity and anisotropy values were measured (λex = 540 nm, λem = 580 nm) using a PTI Fluorescence Master QM400 system fitted with automatic polarizers. Normalized fluorescence anisotropy increments (ΔA/Ao, where A and A₀ are the anisotropy value of δ at a particular concentration of RNAP [or DNA] or zero RNAP [or DNA] respectively, and ΔA = A-A₀) were plotted against titre concentration using the Sigmaplot software (Systat software Inc.). The dissociation constants (Kₐ) of the bindings for RNAP or DNA were determined by fitting the data to single parameter hyperbolic or sigmoidal functions.

**EMSA assay:**

The primer for abrB DNA fragment was labelled using γ-P³² ATP and T4 polynucleotide kinase (NEB) following the manufacturer’s protocol. The promoter DNA fragment was amplified by PCR and was purified using agarose gel elution. In the first set, 200 nM RNAP holo samples were incubated with 25 nM P³²-labelled DNA in 10 µl transcription buffer at 37°C for given time intervals, challenged by 400 nM unlabelled DNA before resolving at 5% PAGE in 0.5X TBE buffer. In the second set, 200 nM of δ was mixed with the RNAP samples before addition of DNA. The gels were scanned by phosphorimaging (Typhoon trio+, GE Healthcare).

EMSA assays with promoter DNA fragments of different upstream length were performed as above after labelling the DNA with γ-P³² ATP.

**DNasel footprinting assay:**

0.2 µM abrB promoter DNA fragment (-95/+30) labelled with P³² at the 5’ end of the template strand was mixed with 0, 0.5, 1.0, and 2.0 µM δ in 50 µl transcription buffer and incubated at 37°C for 30 min. 1 µl of 100 mM CaCl₂ and 0.01 U of DNase I was added to the reaction mixtures at room temperature. The reactions were stopped after 90 sec by the addition of 10 µl of 0.5 M EDTA. The DNA samples were extracted following Sambrook and Russel (2001). Samples containing equal counts were resolved on an 8% urea PAGE gel. The bands were visualized by phosphorimaging.

**FeBABE-mediated protein–DNA footprinting assay:**

The single-cysteine (at 51 residue) derivative of δ was reacted with FeBABE (Dojindo Molecular Technologies Inc., Japan) in 1:5 molar ratio as in Rudra et. al. (17). The unused probes were removed by gel filtration P-6 column (Bio-Rad), pre-equilibrated with buffer (20 mM Tris-Cl, pH 8.0, 0.2M NaCl). Labelled proteins were distributed in 20 µl aliquots and stored at -80°C. 0.2 µM of abrB promoter DNA fragment (-95/+30) labelled with P³² at the 5’ end of the template strand was mixed with 0, 0.1, 0.2 µM FeBABE labelled δ in 100 µl transcription buffer and incubated at 37°C for 15 min. Note that, 0.1-0.2 µM labelled δ was used in FeBABE footprinting assay, 10 fold less than the amount used in DNasel footprinting to reduce the nonspecific binding of δ to DNA. The cleavage reaction and purification of the products were performed as in Rudra et. al. (17). The products were run on 6% Urea- PAGE gel.
Recombinant in vivo reporter assay:
A recombinant in vivo reporter assay using three-plasmid expression system in *E. coli* was employed essentially as in Banerjee et al. (18). Plasmid pNG 219 (a kind gift from Dr Lewis (14)) containing the genes *rpoA*, *rpoB* and *rpoC* respectively of *B. subtilis* was used for Bs RNAP core expression. The plasmids *pYcACDuet-rpoD* and *pYcACDuet-rpoD-rpoE* were used for expression of Α and both Α and δ respectively. *abrB* promoter fragment (-95/15) and its four mutant derivatives were inserted at the upstream of the *mCherry* gene in the *pFPVmCherry* vector. Cells (*E. coli* B834 (DE3)) were transformed with the following plasmids (i) *pFPVmCherry-abrB* alone (for background); (ii) *pFPVmCherry-abrB + pAcYCuet-rpoE* (for control; δ only, no Bs RNAP); (iii) *pFPVmCherry-abrB + pNG219* for Bs RNAP core; (iv) *pFPVmCherry-abrB + pNG219 + pAcYCuet-rpoD*; (v) *pFPVmCherry-abrB + pNG219 + pAcYCuet-rpoD-rpoE*. Similar set of assays were performed with *pFPVmCherry-abrB* derivatives carrying mutations at (i) -40,-41; (ii) -44,-45; (iii) -40,-41,-44,-45; and with (iv) GC-rich upstream sequence. The cells (in 50 ml LB medium supplemented with 100 μg/ml ampicillin, 35 μg/ml chloramphenicol) were grown at 37°C up to 0.5 OD, added with 0.5 mM IPTG and were grown further for 16 hr at 16°C. Cells from each set were diluted to obtain equal OD and their fluorescence intensities were measured at 610 nm with excitation at 592 nm.

Results:
δ binds to RNAP core but not to RNAP holo:
Using electrophoretic mobility shift assay, it was previously shown that the binding of δ and σ to RNAP are mutually exclusive, either due to overlapping binding sites of these factors on RNAP or due to negative cooperativity between these factors for their binding to RNAP (8,9). Using EMSA assay, Lopez de Saro et. al.(9) determined the binding affinity of δ to RNAP (Kδ = 400 nM). Since EMSA involves a separation step, the observed affinity may not reflect the actual affinity in the cases of low-affinity binding or nonspecific binding. Here, we employed a fluorescence anisotropy assay with TMR labelled δ to determine the binding constants of the protein to RNAP core and to RNAP holo (Fig 1A, B). As δ does not have any cysteine residue, we first introduced a cysteine residue in δ by site-directed mutagenesis at amino acid residue 51 and subsequently labelled the protein derivative by cysteine specific reaction with TMR maleimide. The labelled δ retained its activity as judged by in vitro transcription assay (data not shown). The dissociation constant (Kδ) for binding of δ to RNAP core by the anisotropy assay was estimated to be 96 ± 18 nM which is 4-fold lower than the previously reported value (9). However, we did not observe any affinity of δ to RNAP holo, consistent with the previous observation (8,9).

δ has no effect on RNAP core mediated transcription, but enhances the yield of RNAP holo mediated transcription:
Since δ binds to RNAP core, we tested whether the protein has any effect on RNAP core mediated transcription. Since Bs RNAP core does not produce any transcripts from double-stranded linear DNA fragments or tailed template DNA fragments, we monitored the yield of transcripts in a fluorescence based in vitro transcription assay in which RNAP core generates transcripts from a Kool-template. It is reported that RNAP core enzyme produces transcripts from the Kool template by an unknown mechanism (19). We observed that δ has little or no effect on the level of transcripts produced (Fig 1C). On the other hand, when a radioactive based in vitro transcription assay with RNAP holo and *abrB* (20) or *rrnBP1* (21) promoter DNA fragments were performed, we observed a significant increase in the level of transcripts in the presence of δ (Fig 1D and E). In these transcription assays, 100 nM RNAP and 50 nM promoter DNA were used in the presence of increasing concentrations of δ from 10 nM to 200 nM. The result showed that sub-stoichiometric amount of δ (50 nM Vs 100 nM) relative to RNAP had the same effect on the yield of transcription as compared to the yield in the presence of equimolar or higher level of δ (Fig 1D and E). To test whether the results were not due to any artifact arising from the use of recombinant Bs RNAP purified from *E. coli*, we isolated Bs RNAP from a δ-knockout strain of *B. subtilis* (HB6010 (CU1065 ∆rpoE :: cm, (9)) and performed an identical assay. The result showed that δ activated transcription from the *rrnBP1* promoter by the native Bs RNAP with similar efficiency as the recombinant version (Fig 1F). We
note that this result contradicts the previous observation by Rabatinova et al. (12).

The above observations that (i) δ binds to RNAP core, but fails to induce core mediated transcription; (ii) δ does not show any affinity towards RNAP holo, but requires RNAP holo for its function, suggested that δ may function as a transcriptional regulator.

**δ binds to DNA upstream of the promoter element:**

Since most transcriptional regulators bind to DNA at specific sites, we, therefore, examined the ability of δ to bind DNA. We used a fluorescence anisotropy assay with TMR-labelled δ to monitor its ability to bind to any promoter DNA. The data showed that δ exhibits strong affinity for the *abrB* promoter DNA fragment (apparent Kd = 13 ± 0.4 nM, Fig 2A) that spans from an upstream position of -95 to a downstream position of +144. Since we used 20 nM labelled δ in this assay and the Kd value was estimated using the sigmoidal function, the actual dissociation constant of binding of δ to DNA should be less than the estimated value. The sigmoidal nature of binding data also suggests cooperative binding of δ to the promoter DNA, possibly due to binding of the protein at multiple sites of DNA. To map the binding site of δ on DNA, DNase I footprinting was performed with radio-labelled promoter DNA fragment (*abrB* promoter, -95/+30). In the presence of δ, a broad region upstream of the -35 region is protected (Fig 2B). As the exact location of the binding site of δ on DNA could not be mapped from this assay, we carried out protein–DNA footprinting assay using FeBABE labelled δ. The results showed a δ induced cleavage at -41 of *abrB* promoter (Fig 2C) DNA suggesting that the possible binding site of δ on the promoter DNA lies around -41 bp of the promoter. To verify whether this upstream element has any effect on the function of δ, we used several derivatives of the same promoter DNA fragment in which the upstream region was deleted stepwise from the 5’ end and monitored the yield of transcripts from these DNA fragments in the presence and absence of δ. Our results showed that removal of DNA beyond -41 completely abolished the δ mediated increase in the transcript yield (Fig 2D). To test whether removal of the upstream DNA region impaired the ability of RNAP to form the open complex on these DNA fragments, we performed EMSA assay. It was found that the removal of upstream DNA did not affect the open complex formation by RNAP (Fig 2E). The effect of the upstream region on δ mediated transcription was also tested with *rrnBP1* promoter DNA fragments with a similar result: a complete loss of transcriptional activity by δ was observed when the upstream region beyond -41 was removed from the promoter DNA fragment (Fig 2F). As the stretch of DNA immediately upstream of the -35 element of both the promoters contain AT-rich regions, we tested whether these AT-rich sequences are required by δ for transcriptional activation. We prepared four derivatives of the *abrB* promoter DNA fragment where (i) AA sequence at positions -40 and -41 were replaced by CC (*abrB* mut(i)), (ii) AA at positions -44 and -45 were replaced by CC (*abrB* mut(ii)), (iii) AA at positions -41 and -42 and AA at positions -44 and -45 both were replaced by CC (*abrB* mut(iii)), and (iv) a 17 bp GC-rich sequence was inserted immediately upstream of -38 bp (*abrB* mut(iv)) (Fig 3). We observed that the replacement of AA by CC around -40 abrogated δ mediated increase in transcript yield, but, did not have any effect on the initial transcript level without δ (Fig 3.). On the other hand, the replacement of AA by CC around -44 had no adverse effect on δ function (Fig 3.). Similarly for the same promoter, when both the AA bases around both -40 and -44 were replaced by CC, or the 17 bp AT-rich sequence upstream of the -35 element was replaced by GC-rich sequence, the effect of δ on transcription at this promoter derivative was completely lost (Fig 3). Therefore, we infer that the A-rich sequence around -40 is required for δ mediated increase in transcript yield on the *abrB* promoter. This also explains why δ was unable to enhance the transcription yield at the *rrnBP1* promoter DNA derivative in which the 6 A bases at upstream of the -35 element (starting from -40) were removed (Fig 2E). The data clearly indicates that binding of δ at the upstream DNA site is essential for its function.

To test the effect of these mutations at *abrB* promoter on the activity of δ in vivo, we employed a recombinant reporter assay in *E. coli* using three-plasmid expression system as in Banerjee et al.
The abrB promoter fragment (-95/+15) and its four mutant derivatives were inserted upstream of the mCherry gene in pFPVmCherry vector. The plasmid pNG219 was used for expression of Bs RNAP core along with either pYcACDuet-rpoD or pYcACDuet-rpoE or pYcACDuet-rpoD-rpoE for expression of $\sigma^{A}$ or $\delta$, or both. All three plasmids were transformed in E. coli B834 (DE3) and the cells were grown at 16°C for 16 hr after IPTG induction. To rule out the possible interference by E. coli (Ec) RNAP on the mCherry expression, we performed control assays by omitting Bs RNAP expressing plasmid, pNG219. The assays were carried out with the wild type abrB promoter and its all four derivatives (Fig 3, middle panels). The levels of mCherry expression from all the promoter derivatives by Bs RNAP holo were comparable and normalized to 1. Our results showed that mCherry fluorescence from the control assays (without Bs RNAP) were comparable to the background fluorescence obtained with E. coli harbouring pFPVmCherry-abrB only, thus establishing the fact that $\delta$ does not function with Ec RNAP as observed in our in vitro transcription assay (data not shown). However, expression of of Bs RNAP core increased the level of mCherry expression compared to the background. This increase probably occurred due to leaky expression. In contrast, expression of Bs RNAP holo resulted in at least 5 times increase in the levels of mCherry expression from the abrB promoter and its derivatives. The presence of $\delta$ increased the mCherry expression from the wild type abrB promoter by 3 fold compared to Bs RNAP holo. However, the presence of $\delta$ did not change the level of mCherry fluorescence for all three abrB mutant derivatives: abrB mut(i), abrB mut(iii), and abrB mut(iv). On the other hand, expression level on abrB mut(ii) showed a wild type (2.5 fold) like increase upon co-expression of $\delta$. We note that the levels of mCherry expression from the promoters in this assay could be different if being performed in B. subtilis. However, we previously (18) showed that the recombinant reporter assay in E. coli is sufficient to test the in vitro interactions of promoters with RNAP and transcriptional regulators if there is no interference from E. coli RNAP. These experiments, therefore, provide further evidence that the mutation on abrB promoter around -40 abrogates the $\delta$ mediated transcriptional activation in vivo as well, corroborating with our in vitro findings that A-rich sequence around -40, immediately upstream of -35 element is required for $\delta$ function.

To test whether $\delta$ is able to bind the small DNA fragment containing the proposed DNA binding site, we prepared double-stranded (ds) DNA fragment spanning from -57 to -30 of the abrB promoter and its mutant derivatives (see Fig 3, right panels for sequence). The binding of $\delta$ to these DNA fragments were monitored using fluorescence anisotropy assay. For GC-rich abrB promoter derivative, we used the DNA fragment spanning from -52 to -30. (see Fig 3 right panel). The data (Fig 3) showed that $\delta$ was unable to bind to abrB mut(iii) containing the double mutation (around -40 and -44) and abrB mut(iv) that contains GC-rich sequence , but was able to bind to the wild type abrB promoter (~ Kd value 0.52 $\mu$M), abrB mut(i) (~ Kd value 0.69 $\mu$M), and abrB mut(ii) (~ Kd value 0.34 $\mu$M). The Kd value for $\delta$ to the small DNA fragments are, much less than the apparent Kd (13 $\mu$M) observed with longer abrB DNA fragment spanning from -95 to +144.

We presume that this is possibly due to the binding of $\delta$ at multiple sites within the DNA. Interestingly, $\delta$ binds to the abrB mut(i) that contains mutation around -40, but is unable to activate transcription from this mutant promoter. The result further confirms that binding of $\delta$ to A-rich sequence at -40, not at -44 is critical for its function.

The requirement of $\delta$ to bind DNA for transcriptional activity was further demonstrated by the in vitro transcription assay carried out at higher salt concentration (100 mM as opposed to 10 mM of NaCl, Fig 4A and B). At 100 mM salt, $\delta$ retained its affinity towards RNAP core as observed by EMSA assay (Fig 4C), but lost its ability to bind DNA as observed by fluorescence anisotropy assay (Fig 4E). At high salt concentration, RNAP was able to bind promoter DNA as confirmed by EMSA assay (Fig 4D) and was able to produce transcripts from both the promoters used in the assay (Fig 4A and B), although there was lower yield of transcripts compared to that at 10 mM NaCl concentration. In the presence of $\delta$, there was no change in the yield of transcripts from both the promoter DNA
fragments (Fig 4A and B). Therefore, at high salt concentration, δ loses its ability to enhance the yield of transcripts. This result further demonstrates that the binding of δ to DNA is essential for the δ-mediated transcription regulation.

δ affects transcription initiation at the open complex formation, not at the promoter escape state:

From the above results, it is apparent that δ functions as a transcriptional regulator. We, therefore, tested the possibility whether the protein has any role in transcription initiation. As transcription initiation involves two rate limiting steps, namely, open complex formation and promoter escape, we wished to monitor the effect of δ on both of these steps and therefore, performed two types of in vitro transcription assays. In the first assay, we incubated all the components of the transcription reaction, e.g., RNAP, promoter DNA, NTP and δ (or no δ), except σ^7 and initiated transcription reactions were with the addition of σ^7 (Fig 5A). This reaction involved open complex formation as well as promoter escape steps. In the second assay, we first formed the open complex by incubating RNAP holo and promoter DNA for 20 min at 37°C, and subsequently initiated the reactions with NTP. δ (or no δ) was added to the reactions along with NTP (Fig 5B). Previously it had been shown that the multi-round transcription with Ec RNAP does not occur successfully on linear DNA template containing no transcriptional terminator (22,23). It is plausible that the level of transcripts in the presence of δ is higher than that in the absence of δ at each time point, due to the occurrence of multi-round transcription in the presence of δ (Fig 5B). However, a remarkable observation of this assay is the appearance of the first bands of transcripts within 1 min, both in the presence or absence of δ. Since the open complex was formed prior to transcription initiation in both the cases, these results indicate that δ does not have any role in the promoter escape. On the other hand, in the first assay, the first band of transcripts appeared at 1 min in the presence of δ and at 10 min in the absence of δ (Fig 5A). In this assay, the time taken by RNAP to synthesize run-off transcripts reflects the time taken by RNAP to form open complex as well as for promoter escape. Since δ has no role in promoter escape, the difference in the time taken by RNAP to synthesize transcripts may be attributed to the difference in the time taken by RNAP to form open complex in the presence or absence of δ. This result allows us to conclude that δ may facilitate the open complex formation, possibly by reducing the DNA melting time. This was further confirmed by EMSA assay as well as single round transcription assay. In the EMSA assay, we used 20 nM radio-labelled abrB promoter DNA fragment (-66/+30). After forming the open complex, the complexes were challenged with the same unlabelled DNA fragment at 400 nM concentration to remove any non-specific complexes. The EMSA data showed that the open complex was formed within 1 min in the presence of δ but required approximately 15 min to reach the same level in the absence of δ (Fig 5C). Identical results were observed with the rrnBP1 promoter (data not shown). With the present experimental setup, we were unable to study the open complex formation in less than 1 min. To monitor the minimum time required by RNAP to synthesize transcripts from a promoter, a single round transcription assay was performed. Heparin was added to the reaction at the time of transcription initiation by NTP to prevent recycling of RNAP. We also used 1.5-fold higher concentrations of RNAP and DNA in this assay to increase the amount of transcripts for easy visualization. The data showed that RNAP is able to synthesize transcripts as early as 10 sec in the presence of δ (Fig 5D). Thus the presence of δ reduces the time of transcription initiation by RNAP to the second scale, compared to the minute scale as observed in the absence of δ. When single-round transcription assays were further carried out by forming the open complex for 1 min, 5 min, and 30 min in the presence and absence of δ, transcription activation by δ were observed with the open complexes of 1 min and 5 min, not significant with the open complex of 30 min (Fig 5E). At 30 min, the amount of the open complex in the presence and absence of δ were the same and at the saturating level, as a result, there were no change in the amount of transcript.
δ involves in recycling of RNAP from stalled elongation complex:

The previous study (10), as well as our observation, shows that δ is involved in recycling of RNAP in transcription. To test whether the protein is able to recycle RNAP in the stalled elongation complex, we used three sets of complexes stalled at positions +13, +23, and +44 on the abrB promoter derivatives. To our surprise, we observed an increase in the transcript yield in multi-round transcription assay with a stalled elongation complex at +44 (Fig 6A). Ideally, since RNAP does not dissociate from the stalled elongation complex, recycling of RNAP does not occur under normal condition. Therefore, the increase in the transcription yield can only be explained by the recycling of RNAP. However, when we formed stalled elongation complexes with short nucleotides (13 nt, 23 nt), we did not find any increase in the transcript yield (Fig 6 B and C) in the presence of δ. Next we, tested whether δ binds to RNA, using fluorescence anisotropy assay with 20 nM labelled δ and 144 nt RNA (Fig 6D). The apparent $K_d$ value of δ binding of RNA is~ 8 nM. Since we used the hyperbolic function to fit the data, the actual $K_d$ value should be less than estimated value. Helmann group (10) suggested that the recycling of RNAP occurs through δ mediated release of RNA and a concomitant release of RNAP from the elongation complex. Our observation additionally suggests that δ mediated release of RNA is only possible if the length of the RNA reaches a critical length at least greater than 23 nt.

Discussion:

The δ protein is present in most gram positive bacteria (especially in firmicutes). Since δ co-purifies with RNAP, it has been thought to be a subunit of RNAP (1,5-10). Using several biochemical assays, we elucidate that δ binds to RNAP core, but not to RNAP holo. We further show that δ functions as a transcriptional regulator and binds to DNA upstream of the promoter region. Structural studies on the N-terminal domain of δ revealed that it contains an HARE-HTH motif (10,24,25), known to interact with DNA(26). The upstream DNA to the -35 element of the two promoters reported in this study contains AT-rich sequences, as found in most σ70-dependent promoters in B. subtilis (27). Replacement of A bases around -40 by C-rich ones or removal of the AT sequences from the promoter DNA fragments impaired transcription initiation by δ, indicating that A-rich sequences immediately upstream of the -35 element are required for the binding of δ to DNA, as well as its function. Since δ does not bind RNAP holo and since δ mediated transcription assay was performed with RNAP holo, the possibility that δ binds to DNA as a subunit of RNAP is unlikely.

The binding of δ to DNA region upstream of the promoter allows RNAP to form the open complex much faster. As a result, the synthesis of transcripts is found to occur in the second scale in contrast to an order of magnitude slower (minutes scale) rate observed in the absence of δ. The location of binding of δ is immediately upstream of the -35 element, where RNAP binds to the promoter DNA. Thus, the protein may facilitate the open complex formation possibly by interacting with RNAP.

Our observations that δ facilitates open complex formation and activates transcription from certain promoters including rrnBP1 do not support the proposition by Rabatinova et. al. (12) that δ destabilizes the open complex at the rrnBP1 promoter and mediates changes in the requirement of iNTP by RNAP to stabilize the formation of open complexes. We further observed that not only transcription initiating nucleotide (iNTP, GTP for rrnBP1) but increasing concentration of ATP also resulted in a higher amount of transcript from the same promoter (data not shown). Thus, the observed rise in the level of transcript in the presence of higher GTP amount is an effect of nucleotide concentration on the transcript yield, not due to stabilization of the open complex by iNTP in presence of δ. On the other hand, their conclusion that δ is required for competitive fitness of the cell could be explained by the involvement of δ in the transcriptional regulation of essential genes under stress condition.

The fact that δ is involved in both up-regulation and down-regulation of genes can be explained by its role as a transcriptional regulator (13,28-30). In this study, δ is found to act as an activator of transcription on the rrnBP1 and abrB promoters. However, it is also possible that δ binds to other promoters and interacts with RNAP in a way that inhibits transcription initiation, as has been
observed in the case of certain repressors (31,32). We have observed that δ acts as a repressor on the spo0B promoter (33), although the mechanism of repression by the protein needs to be ascertained (unpublished results).

Since δ was able to release RNA from the RNAP-RNA binary complex, it was suggested that δ is also involved in the release of RNAP from the RNAP-DNA-RNA ternary complex, by releasing RNA from the complex (10). This lead to the suggestion that δ was involved in recycling of RNAP in multi-round transcription yielding higher amounts of transcripts. However, this recycling of RNAP was only observed when a linear DNA fragment was used in the assay without having any transcription terminator. δ was unable to enhance the transcript yield when a promoter DNA fragment contained a terminator. This was observed in a previous study (5) as well as in our study (data not shown). Since recycling of RNAP occurs upon transcription termination, the effect of δ was not apparent on the DNA template that contains a terminator sequence. However in the linear template without a terminator sequence, Ec RNAP forms a dead-end complex upon synthesis of full-length RNA, and no recycling of RNAP occurs (22,23). It is likely that Bs RNAP also forms dead end-complex like Ec RNAP. In the event, δ releases the RNAP from the dead-end complex and, therefore, allows it to rebind to the promoter for multi-round transcription. This explains the higher level of transcript in the presence of δ in Fig 5A and 5B. When the interaction of δ with DNA was abrogated by mutating or removing the upstream region of the promoter, the protein was unable to increase the level of transcripts. Although, RNAP is available to rebind to the promoter DNA due to recycling of RNAP by δ in this assay, the polymerase is unable to initiate rapid synthesis of transcript without the binding of δ at the promoter DNA and therefore, is unable to increase the yield of transcript. Thus, this observation further indicates that δ mediated recycling of RNAP could not be the sole reason for δ function.

The results of the single-round transcription assay show that δ has no effect on the overall yield of transcripts (Fig 5E), when sufficient time (30 min) was available for the formation of the open complex. However, δ increases the yield of transcripts by several fold in multi-round transcription assays even if the open complex formation was allowed for 30 min (Fig 1D). This observation could be explained by the effect of δ on the rate of open complex formation. As δ facilitates the open complex formation, the protein drastically reduces the time to initiate RNA synthesis: from the minute scale to the second scale. Thus after each round of transcription, an RNAP molecule could rebind to the promoter, quickly form an open complex and synthesize a transcript. Therefore, recycling of RNAP occurs rapidly in multi-round transcription and increases the yield of transcripts.

Interestingly, we also observed δ mediated recycling of RNAP in the stalled elongation complex. Since no recycling of RNAP is expected from the stalled elongation complex, our observation of δ mediated increase in the level of transcripts from the complex provides direct evidence for the recycling of RNAP from the ternary complex. The increase of transcript yield was observed only when the stalled complex contained large transcripts (44 nt), and not when the transcripts were shorter (13 nt or 23 nt). Since the nascent transcripts (> 15 nt) emerges from the RNA exit channel in RNAP (34-36), it is likely that the elongation complex containing 13 nt RNA remains within the RNA exit channel and thus remains inaccessible to δ. For the complex containing the 23 nt RNA, approximately 9 nt long RNA would lie outside the exit channel, and accessible to the solution. However, it may not be fully accessible to δ. Therefore the above results suggest that δ mediated recycling of RNAP in the stalled elongation complex is possible only when the nascent RNA is fully accessible to δ, and the interaction of the transcript with δ is essential for the release of RNA (and the concomitant release of RNAP) from the ternary complex. This is consistent with our observation that δ binds to RNA with high affinity. However, whether the interaction of δ with RNAP is required during δ mediated release of RNA from stalled ternary complex, further study is required. Previously, a single strand nucleic acid binding protein from E. coli, SSB, has been shown to recycle N4 virion RNAP by releasing the nascent transcript from the elongation complex when its size is greater than 32 nt (37). Since δ has no effect on DNA template
with a terminator sequence as recycling of RNAP occurs even without δ, the utility of δ mediated recycling is not clearly understood at this point. The only plausible explanation could be the involvement of δ in releasing RNAP from the unwanted paused complex for efficient transcription.

Overall, we propose a model of transcription activation by δ in which δ binds to DNA at A-rich sequence immediately upstream of the -35 element of promoter DNA and facilitate the open complex formation that leads to rapid synthesis of transcripts.
Acknowledgements: We thank K Murakami (Penn State University), R Sur (University of Calcutta), AB Datta, and P Parrack (Bose Institute) for critically reading the manuscript and comments. This work is supported by research grants from Department of Biotechnology, India: BT/PR 5345/MED/29/648/2012 and BT/PR 5270/BRB/10/1066/2012. RKP, and PR are the recipients of fellowships from CSIR India.

Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions: Conceived and designed the experiments: JM, Performed the experiments: RKP (purification of RNAP, in vitro transcription assays, EMSA assays, fluorescence anisotropy assays, site-directed mutagenesis), SS (cloning and purification of δ), PR (fluorescence based in vitro transcription assays), Analysed the data: RKP, JM, Wrote the paper: JM.

References:


**Figure Legends:**

**Fig 1. δ binds to RNAP core, but has no effect on core mediated transcription.**

A) Binding of δ to RNAP: Fluorescence anisotropy assay: 20 nM TMR labelled δ was added with core RNAP. Fluorescence anisotropy of the labelled δ was monitored at Ex 540 nm and Em 580 nm. Each data set represents mean of three replicates. The dissociation constant (K_d) of δ to RNAP core was estimated by fitting the data using a single parameter hyperbolic function.

B) Same as A, but for RNAP holo.

C) Effect of δ on RNAP core mediated transcription: Fluorescence based transcription assays were performed using 100 nM RNAP core and 1.75 pmol Kool NC-45™ template in presence of δ. Each data set represents mean of three replicates.

D) Effect of δ on RNAP holo mediated transcription: radioactive based transcription assay with 100 nM RNAP holo and 50 nM abrB promoter DNA fragment in presence of δ. Run-off transcripts were 144 nt. Each experiment was repeated thrice and the mean of fold increase in the amount of transcript at each concentration of δ with respect to the amount in absence of δ were plotted as a bar graph (shown in lower panel).

E) Same as D, but 50 nM rnbP1 promoter DNA fragment was used. Run-off transcripts were 47 nt in length.

F) Same as E, but using Bs RNAP purified from δ− Knock-Out strain of *B. subtilis*. Run-off transcripts were 47 nt in length.

**Fig 2. δ binds to DNA upstream of promoter element.**

A) Binding of δ to promoter DNA fragment: Fluorescence anisotropy assay: 20 nM TMR labelled δ was added to the abrB promoter DNA (-95/+144) (Table 1). Each data set represents mean of three replicates. The K_d values of δ to abrB promoter was estimated by fitting the data using the sigmoidal function.

B) Protection of upstream promoter DNA sequence in presence of δ: DNase I foot-printing assay: 0.2 μM P^{32} labelled (at the 5’ end of the template strand) abrB promoter DNA (-95/+30) were incubated with δ in transcription buffer as indicated. The products were separated on 8% Urea- PAGE gel. Line indicates the protected region on DNA by δ.

C) Location of binding region of δ on abrB promoter DNA: FeBABE induced protein-DNA foot-printing assay: 0.2 μM of abrB promoter DNA fragment (as above) was incubated with FeBABE labelled δ in transcription buffer. The products were separated on 6% Urea- PAGE gel. G+A DNA ladder and samples were run on same gel but visualized with different contrast. Arrow indicates the cleavage product.
D) Effect of upstream DNA on δ mediated transcription: *in vitro* transcription assay: 200 nM RNAP holo, 100 nM *abrB* promoter DNA fragments having different length of upstream region were used, in absence and presence of δ. Run-off transcripts sizes were 144 nt.

E) Removal of upstream DNA has no effect on open complex formation: EMSA assay: 200 nM of RNAP holo samples were incubated at 37°C for 20 min with 20 nM P32 labelled promoter DNA fragments having different length of upstream region. The products were challenged with 400 nM of the same unlabelled DNA and run on 5% PAGE. The bands were visualized by phosphor imager scanning.

F) Same as D, but for *rrnBP1* promoter DNA. Run-off transcripts were 47 nt in length.

**Fig 3. Effect of upstream A- rich sequences on δ function:**

A) *in vitro* transcription assay: 200 nM of RNAP holo, 50 nM promoter DNA both (wt and mutants) and in presence and absence of δ. Sequences of the promoter DNA templates were shown at each panel; the mutated bases are highlighted. Run-off transcripts were 144 nt. Each experiment was repeated thrice and the mean of fold increase in the amount of transcript at each concentration of δ with respect to the amount in absence of δ were plotted as a bar graph.

B) *in vivo* recombinant reporter assay; three-plasmid expression system in *E. coli*:
The bars represent relative mCherry fluorescence of *E. coli* cells containing the pFPvmCherry-*abrB/abrB* mutants and plasmids encoding (i) δ (pAcYcDuet-rpoE), (ii) Bs RNAP core (pNG219), (iii) Bs RNAP holo (pNG219 + pAcYcDuet-rpoD), and (iv) Bs RNAP holo + δ (pNG219 + pAcYcDuet-rpoD-rpoE). DNA fragments (-95/+15) of *abrB* or *abrB* mut(i)-(iii) and fragment (-52/+15) of *abrB* mut(iv) were inserted upstream of mCherry gene. Each set of assay was repeated thrice and the mean values of relative mCherry fluorescence of the cells were plotted. Fluorescence of the cells containing Bs RNAP holo were normalized to 1.

C) Binding of δ to A-rich DNA fragments: Fluorescence anisotropy assay: 20 nM TMR labelled δ was added with dsDNA (sequence shown above the graph) containing putative δ binding sites and its mutants. Each data set represents mean of three replicates. The Kd values of δ to A-rich DNA template was estimated using the sigmoidal function.

**Fig 4. Effect of salt on the activity of δ.**

A) *in vitro* transcription assay: 200 nM of RNAP holo, 50 nM of *abrB* promoter DNA in absence and presence of δ at 10 mM and 100 mM NaCl concentration. Run-off transcript of 144 nt in length.

B) Same as in A, but for the *rrnBP1* promoter DNA. Run-off transcript of 47 nt in length.

C) Binding of δ to RNAP core at different NaCl concentration: EMSA assay: 40 nM of TMR labelled δ protein was incubated with 400 nM of RNAP core in transcription buffer at different NaCl concentration as indicated at 37°C for 30 min and the product were separated of 5% TBE PAGE. Bands were visualized by scanning the gel using fluorescence scanning.

D) Binding of RNAP holo to promoter at different salt concentration: EMSA assay: 100 nM or 200 nM RNAP holo were incubated with 20 nM of P32 labelled *abrB* promoter DNA in transcription buffer containing either 10 mM or 100 mM NaCl at 37°C for 30 min. The products were challenged with 400 nM of unlabelled DNA and separated on 5% TBE PAGE. Bands were visualized by phosphorimaging.

E) Binding of δ to promoter DNA fragment at 100 mM NaCl: Fluorescence anisotropy assay: 20 nM TMR labelled δ was added to the *abrB* promoter DNA fragment (length -95/+144) in transcription buffer containing 100 mM NaCl. Each data set represents mean of three replicates.

**Fig 5. Effect of δ on the open complex formation and promoter escape:**

A) Effect of δ on open complex formation: *in vitro* transcription assay: 200 nM RNAP core, 50 nM *abrB* promoter DNA and NTP were incubated in absence or presence of 200 nM δ. Transcription reactions were initiated by addition of 800 nM of σ7. Reactions were stopped by FLB at given time points as indicated. Products were run on a same gel and visualized by phosphorimaging with same contrast.
B) Effect of δ on promoter escape: in vitro transcription assay: First open complex was formed by incubating 200 nM RNAP holo with 50 nM abrB promoter. Transcription reaction was initiated with addition of NTP. δ (200nM) was added to the reaction at the time of NTP addition. 10µl of the reaction samples were aliquoted at given time points, mixed with FLB and denatured by heating at 95°C for 5 min. obtained product were separated by running in 8% urea PAGE gel. Run-off transcripts sizes were 144 nt. Products were run on a same gel and visualized by phosphorimaging with same contrast.

C) Effect of δ on open complex formation: EMSA assay: 200 nM of RNAP holo samples were incubated with 25 nM P\textsuperscript{32} labelled abrB promoter DNA fragments (length -66/+30) in absence and presence of 200 nM δ for the time points indicated in the figure to form complexes, the products were challenged with 400 nM unlabelled DNA fragments. The samples were separated on 5% native PAGE.

D) Single-round in vitro transcription assay: 300 nM of RNAP holo, 75 nM of abrB promoter DNA were incubated at 37°C for 30 min in presence of 300 nM δ. Reactions were initiated by addition of NTP and 0.25 µg/µl heparin and stopped by FLB at time indicated. Products were separated on 12% urea PAGE. Run-off transcripts sizes were 30 nt.

E) Single-round in vitro transcription assay: 100 nM RNAP holo samples were incubated with 25 nM abrB promoter DNA fragments in absence or presence of 100 nM δ at 37°C for the time as indicated in the figure. Single-round transcription reactions were initiated by addition of NTP and 0.25 µg/µl heparin at the time indicated points. The reactions were stopped by addition of FLB after 30 min and resolved on 8% urea-PAGE. Run-off transcripts sizes were 144 nt.

**Fig 6. Effect of δ on recycling of RNAP in stalled elongation complex.**

A) Effect of δ on stalled elongation complex: in vitro transcription assay: 100 nM RNAP holo were incubated with 25 nM abrB promoter DNA fragment in which the first C base appear at +45 of the template strand, in the presence of δ. Transcription reactions were initiated by addition of ATP, UTP and GTP and kept it for 15 min before the reactions were stopped by addition of FLB and resolved on 12% urea-PAGE. Transcripts sizes were 44 nt.

B) Same as A except using abrB promoter DNA fragment in which first C base appear at +14. Transcripts sizes were 13 nt.

C) Same as A except using abrB promoter DNA fragment in which first C base appear at +24. Transcripts sizes were 23 nt.

D) Binding of δ to RNA: Fluorescence anisotropy assay: 20 nM TMR labelled δ was titrated with RNA. Each data set represents mean of three replicates. The K\textsubscript{d} values of δ to RNA is 7.6 ± 1.4 nM.
Table 1.

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</tr>
<tr>
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<td>rrnBP1 (-92/47)</td>
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Bold letters denote transcription start site. -10 and -35 elements of the promoters are underlined. The dotted line represents the DNA region protected by δ in DNaseI footprinting assay.
Fig 1
Fig 2

A

\[ K_d = 32.2 \pm 0.7 \text{ nM} \]

B

C

D

E

length of \( \alpha \beta \)B promoter DNA

RNAP

- - + +

- + - +

- + - +

- - + +

144 nt

F

length of \( \alpha \beta \)B promoter DNA

- - + +

- + - +

- + - +

- - + +

47 nt
Fig 3

\[ \text{in vitro transcription assay} \quad \text{in vivo reporter assay} \quad \text{DNA binding assay} \]
<table>
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<tr>
<th></th>
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<th>rrrBP1</th>
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<tr>
<td></td>
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<td>10</td>
<td>+</td>
<td>10</td>
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</table>

![Figure 4](image)

**Fig 4**

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<td>2</td>
</tr>
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<td>3</td>
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- RPo
- free DNA

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</table>

Free DNA
Fig 6

A  \( \delta \) (nM)  
0  25  50  100  200

B  \( \delta \) (nM)  
0  10  25  50  100  200

C  \( \delta \) (nM)  
0  25  50  100  200

D  
\[ K_d = 7.6 \pm 1.4 \text{ nM} \]
Novel mechanism of gene regulation: the protein Rv1222 of *Mycobacterium tuberculosis* inhibits transcription by anchoring the RNA polymerase onto DNA

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Received February 18, 2015; Revised May 01, 2015; Accepted May 06, 2015

**ABSTRACT**

We propose a novel mechanism of gene regulation in *Mycobacterium tuberculosis* where the protein Rv1222 inhibits transcription by anchoring RNA polymerase (RNAP) onto DNA. In contrast to our existing knowledge that transcriptional repressors function either by binding to DNA at specific sequences or by binding to RNAP, we show that Rv1222-mediated transcription inhibition requires simultaneous binding of the protein to both RNAP and DNA. We demonstrate that the positively charged C-terminus tail of Rv1222 is responsible for anchoring RNAP on DNA, hence the protein slows down the movement of RNAP along the DNA during transcription elongation. The interaction between Rv1222 and DNA is electrostatic, thus the protein could inhibit transcription from any gene. As Rv1222 slows down the RNA synthesis, upon expression of the protein in *Mycobacterium smegmatis* or *Escherichia coli*, the growth rate of the bacteria is severely impaired. The protein does not possess any significant affinity for DNA polymerase, thus, is unable to inhibit DNA synthesis. The proposed mechanism by which Rv1222 inhibits transcription reveals a new repertoire of prokaryotic gene regulation.

**INTRODUCTION**

Gene regulation is one of the most important requirements of microorganisms for their survival under a wide variety of fluctuating environmental niche (1). The majority of the genes are regulated during transcription and RNA polymerase (RNAP), the key enzyme for mRNA synthesis, together with different sigma factors and transcriptional regulators orchestrates gene expression in bacteria. The level of expression of a given gene generally depends on the cellular demand: the basal level gene expression could be sometimes activated or inhibited. The wide variety of mechanisms by which transcriptional regulators function have been unravelled over the years. While multiple mechanisms exist for the activation of gene expression, inhibition of transcription in prokaryotes could be classified mainly into four categories: (i) a ‘repressor’ occupies the promoter element, either partially or fully, preventing the binding of RNAP to the promoter (2,3), or binds to DNA beside RNAP blocking the promoter escape of RNAP during transcription initiation (4,5), (ii) a transcription factor binds to downstream DNA, thereby blocking the translocation of RNAP (6,7), (iii) an anti-sigma factor binds to a specific sigma factor, inducing a conformational change to the cognate sigma factor and making it deficient in recognizing the promoter elements (8,9) and (iv) a factor that alters the DNA architecture rendering it inaccessible for RNAP (10–12) or unfavourable for RNAP translocation (13). In majority of the above cases, repressors or anti-sigma factors function at specific promoters, and hence gene-specific transcriptional regulation occurs. In addition to the above four, there are transcription factors or small-molecule effectors that do not require any interaction with DNA for transcription inhibition. Hence, the inhibition of transcription by these factors occurs at any gene. For example, DkSA (14,15), Gfh1 (16,17) and ppGpp (18,19) inhibit transcription by binding at the secondary channel of RNAP and modulating the function of RNAP. Several bacteriophage proteins have been reported to inhibit transcription by different mechanisms. gp2 from the bacteriophage T7 binds to RNAP and induces a conformational change in the polymerase making it deficient for RNA synthesis (20). Bacteriophage HK022 Nun protein binds to the transcription elongation complex (EC) through a nut site and prevents the translocation of RNAP (21).

Rv1222, a *Mycobacterium tuberculosis* transcriptional factor, was reported to function as an anti-sigma factor for...
σE. Based on the facts that Rv1222 gene is located immediately downstream of sigE gene, Rv1222 binds to σE of the same bacteria, and exclusively inhibits transcription by σE-RNAP holoenzyme, it has been inferred that Rv1222 is a regulator of sigma E factor (RseA) (22,23). However, our study reveals that Rv1222 is not an anti-sigma factor, but inhibits transcription by a completely different mechanism.

Rv1222 is a small protein (16.25 kD) whose function is not known. Microarray mapping of transposon insertions shows that the protein is nonessential (24). Transcriptome analysis of M. tuberculosis, in dormant state or under conditions leading to dormant state, reveals that the mRNA of Rv1222 is upregulated (25–28).

Here, we show that Rv1222 anchors the RNAP onto DNA and thereby slows down the translocation of RNAP along the DNA and RNA synthesis. The inhibition of transcription requires the simultaneous binding of the protein to both RNAP core and DNA. The interaction of the protein with DNA is not sequence specific, hence the Rv1222-mediated inhibition of transcription can occur at any DNA template. When the interactions between Rv1222 and DNA are abrogated by removing 10 residues from the C-terminus, the protein loses its ability to inhibit transcription. On the other hand, as the protein does not bind to DNA polymerase (DNAP), Rv1222 is unable to anchor DNAP onto DNA. Interestingly, when Rv1222 is expressed in Mycobacterium smegmatis or Escherichia coli, the growth rate of the bacteria is significantly reduced due to reduction in the level of RNA synthesis. The way, Rv1222 inhibits transcription, represents a novel mechanism and reveals a new repertoire of prokaryotic gene regulation.

MATERIALS AND METHODS

Cloning strategies

The M. tuberculosis rv1222 gene was amplified by polymerase chain reaction (PCR) from H37Rv genomic DNA (a kind gift from ATCC, USA) using primers (Supplementary Table S1) and subsequently cloned in pBluescript SK(+) using primers and was cloned in pBluescript SK(+). Rv1222 is upregulated (25–28).

Plasmid using primers and was cloned in pBluescript SK(+) and subsequently cloned in pAcYC-rpoB-rpoC in pAcYC-Duet vectors using NdeI-HindIII and NcoI-HindIII (NEB), respectively. Rv1222ΔC was created by inserting a stop codon by site-directed mutagenesis, at 10 residues prior to the original stop codon of the protein. For Rv1222 expression in M. smegmatis, rv1222 gene was cloned in pLAM12 vector using restriction enzymes NdeI-EcoRI.

Previously, we purified M. tuberculosis (Mt) RNAP-σA holoenzyme, by co-expressing all MtRNP subunits using two-plasmid expression system (pETDuet-rpoB-rpoC and pAcYC-rpoA-sigA) in E. coli (29). For production of recombinant Mt RNP-σE holo, we followed the same strategy as above except sigA gene was replaced by sigE in pAcYC-Duet-rpoA-sigA. First M. tuberculosis rpoA gene was cleaved with NcoI-BamHI from pET16b-rpoA (30) and cloned in pAcYC Duet. The M. tuberculosis sigE gene was amplified from Mt genomic DNA H37Rv using primers (Supplementary Table S1) and subsequently cloned in pAcYC-Duet-rpoA using EcoRV-XhoI restriction enzymes.

σE-dependent promoter Bpr (31) was amplified from H37Rv using primers and was cloned in pBluescript SK(+) plasmid using EcoRV restriction site. E. coli lacCONS promoter DNA (32) was amplified from 79 bases oligonucleotide template and cloned in pUC19 using KpnI-BamHI restriction enzymes. The lacCONS promoter was amplified from this construct (pUC19-lacCONS) using primers and subsequently cloned in pFPVmcherry with KpnI-XbaI enzymes. sinP3, rnaA (29) and abrB (33) promoters were prepared by PCR with synthetic primers and template and purified by PAGE elution.

Rv1222 protein purification

Using denaturation/renaturation method. E. coli BL21 (DE3) cells were transformed with pET28-rv1222 and grown in Luria Broth (LB) media overnight at 37°C. 2L LB media was inoculated with 1% of overnight culture and was supplemented with 0.5 mM IPTG after cells reached OD600 0.5 and was further grown for 3 h at 37°C. Harvested cells were suspended in buffer A (100 mM sodium phosphate (pH 7.0), 100 mM NaCl and 2 mM β-mercaptoethanol) containing 0.25% deoxycholic acid, protease cocktail inhibitor (Roche), lysed by sonication and centrifuged. The pellet was washed with buffer A + 0.25% triton-X100 + 1 mg/ml lysozyme and further centrifuged. The pellet was dissolved in buffer B (buffer A+ 8M urea) and loaded on Ni-NTA column (Rv1222 gene fused with 6X histidine at the N-terminus) pre-equilibrated with buffer B, washed with five column volumes of buffer B and eluted with buffer B + 100 mM imidazole. The Rv1222 was purified to near-homogeneity by nickel affinity chromatography under denaturing conditions as judged by 15% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and Coomassie blue staining. The eluted protein was dialysed against buffer A containing 10 μM ZnCl2 with three changes at an interval of 15 h at 4°C. The dialysed protein was concentrated using concentrator (Amicon Ultra 10K), mixed with equal volume 100% glycerol and stored in −80°C. All assays were performed with this refolded Rv1222 protein.

By expressing the protein in soluble form. The E. coli SoluBL21 (Ambso) cells were transformed with pET28-rv1222 and were grown in M9 minimal media (HiMedia) overnight. One litre fresh M9 media was inoculated with 1% of overnight cultures and was supplemented with 0.5 mM IPTG after cells reached OD600 0.5 and was further grown overnight at 37°C. Cells were harvested, lysed by sonication and purified by Ni-NTA chromatography using buffer A as above. In vitro transcription assay shows that the activity of this Rv1222 is similar to the activity of Rv1222 purified by denaturation/renaturation method (Supplementary Figure S1).

Purification of Mt RNAP core, Mt RNAP-σA holo, Mt RNAP-σE holo and Mt σA. Mt RNAP core, Mt RNAP-σA holo, Mt RNAP-σE holo and Mt σA were purified following the protocol as in (29).

Purification Bs RNAP core and Bs σA. The proteins were purified essentially as in (34).
**Purification of $\sigma^E$.** *E. coli* BL21 (DE3) cells containing pET30-sigE (gift from Dr Rodrigue) were grown in 1L LB (containing 50 $\mu$g/ml Kanamycin) at 37°C till OD$_{600}$ reached 0.5. Protein production was induced by adding 0.5 mM IPTG, followed by growing them for 3 h at 37°C. Cells were harvested by centrifugation (6000 rpm, 10 min, 4°C), resuspended in 20 ml buffer (50 mM Tris-Cl, 200 mM KCl, 10 $\mu$M ZnCl$_2$, 5 mM BME, 1 mM PMSF, 20% Glycerol) and disrupted by sonication. The lysates were spun at 25,000 rpm for 30 min at 4°C. The supernatant was loaded onto Ni-NTA column pre-equilibrated with the above buffer and protein was eluted at 400 mM imidazole. The protein was further purified on MonoQ HR10/10 in Akta purifier (GE Healthcare) using a 0.2–1.0 M NaCl gradient in buffer (20 mM Tris-Cl and 5% Glycerol). The purified protein was concentrated and kept at −80°C after adding equal volume of glycerol.

**Purification of Ec holo RNAP.** *E. coli* RNAP holo was purified as in (35).

**EMSA assay.** The forward primer for Bpr DNA fragment was labelled using $^{32}$P γ ATP and T4 polynucleotide kinase (NEB) following manufacturers’ protocol. The promoter DNA fragment was then amplified by PCR and was precipitated with equal volume of isopropanol and 0.1 volume DNA fragment was then amplified by PCR and was pre-

**In vitro transcription assay**

Two sets of *in vitro* transcription assays were performed with RNAP-$\sigma^E$ holo. In the first set, 100 nM RNAP-$\sigma^E$ holo was incubated respectively with 0, 100, 200 and 400 nM Rv1222 in Tx buffer [45 mM Tris-Cl (pH 8), 5 mM MgCl$_2$, 70 mM KCl, 1 mM DTT, 10% Glycerol, 1.5 mM MnCl$_2$] at 37°C for 5 min and the samples were added with 100 nM RNAP and 20 nM $^{32}$P-labelled DNA and further incubated at 37°C for 15 min to form open complex. In the second set, 100 nM RNAP-$\sigma^E$ holo was incubated respectively with 0, 100, 200 and 400 nM Rv1222 in Tx buffer at 37°C for 5 min and then added with 20 nM $^{32}$P-labelled DNA, following incubation at 37°C for 15 min. In both cases, heparin was added to the samples at 0.5 $\mu$g/$\mu$L before resolving on 5% PAGE in 1X TBE buffer (89 mM Tris base, 89 mM Boric acid, 2 mM EDTA) for 1 h at 4°C and then examined by phosphor imaging (Typhoon Trio + GE Healthcare).

*In vitro* transcription assay using 100 nM Mtb RNAP-$\sigma^A$ holo and 50 nM rrnA promoter DNA fragment was performed essentially as above except Rv1222 was incubated with open complex for 5 min at 37°C before heparin was added. The transcription reactions were terminated after 5 min following the addition of NTP unless stated otherwise.

*In vitro* transcription assay using 100 nM Ec RNAP-$\sigma^70$ holo and 50 nM lacCONS DNA was performed essentially as in Mukhopadhyay *et al.* (35), except Rv1222 was incubated with open complex for 5 min at 37°C before heparin was added. The transcription reactions were terminated after 5 min following the addition of NTP unless stated otherwise.

For nuclease activity assay, $^{32}$P-labelled transcripts (81 nt) were formed using *in vitro* transcription assay with 100 nM Ec RNAP-$\sigma^70$ holo and 50 nM T7A1 promoter DNA fragment. Rv1222 was added after the formation of transcripts and incubated for 5 min before resolving on 12% Urea-PAGE.

Single-round transcription assays to monitor the rate of RNA synthesis in the absence and presence of Rv1222 by Mtb RNAP or Ec RNAP were performed as above except that the reaction was stopped at different time points before resolving on 12% Urea-PAGE.

*In vitro* transcription assay using Bs RNAP-$\sigma^A$ holo was performed as following: 100 nM Bs RNAP core was incubated 200 nM Bs $\sigma^A$ in buffer [18 mM Tris (pH 8.0), 10 mM NaCl, 8 mM $\beta$ME, 10 mM MgCl$_2$] and incubated on ice for 30 min to form holo enzyme. Fifty nanomolar *abrB* promoter DNA was added to the holoenzyme to form open complex at 37°C for 20 min and further added with Rv1222 (0, 100, 200 and 400 nM) and incubated at 37°C for 5 min. Transcription was initiated with NTP (final concentration: 250 mM of ATP, GTP, UTP and 25 mM of $\alpha^{32}$P-CTP (0.4 $\mu$Ci) at 37°C for 5 min.

Approximate IC$_{50}$ of transcription inhibition for each of the three RNAPs were estimated as follows: the intensity on radioactive band of the run-off transcript at each Rv1222 concentration was quantified from the phosphor imaging of the gels and the mean values of the intensities from two or three replicates were plotted against the concentration of Rv1222.

*In vitro* transcription assay using 100 nM Mtb RNAP core and 50 nM tailed-template DNA was performed essentially as in Gnatt *et al.* except that 100 nM Mtb RNAP core was incubated with varying concentrations of Rv1222 and then was added with 50 nM tailed-template DNA fragment. Transcription was initiated with NTP (final concentration: 250 $\mu$M of ATP, GTP, UTP and 25 $\mu$M of $\alpha^{32}$P-CTP (0.2 $\mu$Ci)) at 37°C for 5 min.

*In vitro* transcription assay with 0.2U of T7 RNA Polymerase (Bio-Bharati India Ltd) and 100 nM T7 promoter DNA fragment was performed as in (36).

*In vitro* transcription assay using Ec RNAP core and Kool NC-45TM Template was performed as per manufacturers’ protocol (epicentre).
 Fluorescence anisotropy assays

Labelling of Rv1222 with TMR. Cysteine labelling was performed with Rv1222 protein having three cysteine residues at positions 70, 73 and 109. One hundred micromolar purified protein in 200 µl was reduced as in (37). The sample was reacted with 5-fold molar excess of tetramethylrhodamine (TMR)-6-maleimide in buffer [100 mM sodium phosphate (pH 7.3), 200 mM NaCl, 1 mM EDTA]. The protein sample was centrifuged for 10 min at 13 000 rpm and then loaded onto 10 ml BioGel P6 column (Bio-Rad) pre-equilibrated with buffer C to remove the free dye. The labelled Rv1222 was eluted in void volume, mixed with equal volume of 100% glycerol and kept at −80°C. The activity of the TMR labelled Rv1222 derivative was confirmed by its inhibition of in vitro transcription by RNAP. The labelling efficiency of the protein was 0.85.

Fluorescence anisotropy measurements. Twenty nanomolar of TMR labelled Rv1222 in 60 µl Tx buffer was titrated with increased concentrations of RNAP and/or DNA at 37°C and fluorescence intensities and anisotropy measurements were monitored with Ex at 540 nm and Em at 580 nm on a PTI Fluorescence Master QM400 System fitted with automatic polarizers. Normalized fluorescence anisotropy increments ∆A/∆n, where A and n, are the anisotropy value of Rv1222 at x nM RNAP [or DNA] or zero RNAP [or DNA], respectively, and ∆A = A–A0, were plotted against Rv1222 concentration using Sigma Plot software. The dissociation constants (Kd) of the bindings for RNAP or DNA were determined by fitting the curves with single parameters hyperbolic function (f = aX/(Kd+X), where f is the concentration of complex and X is the concentration of Rv1222). To monitor the binding affinity of Rv1222 to RNAP and DNA simultaneously, first equal concentration of Mtb RNAP core and Bpr DNA fragment were incubated together for 10 min at 25°C before titrating with Rv1222. The dissociation constant (X0) for binding of Rv1222 to both RNAP and DNA simultaneously was determined by fitting the curve with sigmoidal function (f = a/(1+exp(-X–X0)/b)), where f is the concentration of complex, X is the concentration of Rv1222 and X0 is the half saturation constant).

In vivo recombinant reporter assays. E. coli BL21 (DE3) cells were transformed with three sets of two plasmids: (i) pFPVmCherry-lacCONS + pAcYc (for control), (ii) pFPVmCherry-lacCONS + pAcYC Rv1222 and (iii) pFPVmCherry-lacCONS + pAcYC Rv1222ΔC. The cotransformed cells were grown in 50 ml LB media at 37°C with antibiotics (35 µg/ml Chloramphenicol and 100 µg/ml Ampicillin) until OD600 reached 0.4 and induced by the addition of 0.5 mM IPTG and were grown further for 14 h at 37°C. Cells were diluted to make up equal number of cells in each sample and fluorescence properties of the cells were analysed by FACS Aria (Becton Dickinson) (38).

Effect of Rv1222 on bacterial growth

M. smegmatis. M. smegmatis MC2-155 cells containing pLAM12 or pLAM12-Rv1222 were grown at 37°C in Middlebrook 7H9 broth (HiMedia) with 0.2% glycerol and 0.05% tween 80 and 20 µg/ml kanamycin for 18 h. Fifty milliliter fresh 7H9 media supplemented with 0.2% acetyamide was inoculated with 0.2% of the saturated culture and growth of the cells were monitored for 18 h by measuring the OD600 at 1 h interval.

E. coli. E. coli BL21 cells harbouring plasmid pACYC-Rv1222 was grown overnight at 37°C in LB containing 35 µg/ml chloramphenicol. Fifty millilitre fresh LB media was inoculated with 1% of the saturated culture and treated with different IPTG concentrations (25 µM, 50 µM, 100 µM). OD600 of the cells was monitored at ½ h interval. For control, identical assays were performed with E. coli harbouring the plasmid pACYC Rv1222ΔC at identical IPTG concentration.

In vivo 3²P-labelling of RNA. Each of two separate 120 ml 7H9 media was inoculated with M. smegmatis (one with pLAM12 and other with pLAM12-Rv1222) as above. When OD600 of the cells reached 0.2, 25 µl of 3²P-orthophosphoric acid (5Ci/ml) was added to the culture and cells were grown for another hour before 5 ml of cell culture was aliquoted and pelleted at 30 min intervals. OD600 of the cells was measured at each of the time points. RNA was isolated from the sample of each aliquot using RNA kit (Agilent technologies). Amount of 3²P-labelled RNA in each sample was measured by liquid scintillation counter (Perkin Elmer TriCarb 2800TR). Radioactive count of each sample RNA was divided by the OD600 of cells at each time point to get the relative amount of 3²P-labelled RNA per cell (in arbitrary units).

Fe-BABE foot-printing

lacCONS promoter DNA fragment was labelled with ³²P γATP at 5’ of upstream end as described above for Bpr promoter DNA.

Preparation of Rv1222 derivatives. The three cysteine residues of Rv1222 at positions 70, 73 and 109 were mutated to alanine or glycine residue to create a no-cysteine Rv1222 derivative. Previously, Cys 70 and Cys 73 residues were shown to be essential for the function of Rv1222 by Barik et al. Contrary to this observation, we found that the no-Cys derivative of Rv1222 remains active in inhibition of transcription. Using this protein derivative, we generated two single-cysteine derivatives of Rv1222 by incorporating a cysteine residue at position A23C (near N-terminus) and G133C (near C-terminus) respectively using site-directed mutagenesis (Stratagene Inc). Each of the single-Cys derivatives of Rv1222 was purified by denaturation/renaturation following the protocol as described above. Activities of the Rv1222 derivatives were confirmed by assessing the ability of each protein to inhibit transcription by in vitro transcription assay.

Labelling of Rv1222 with Fe-BABE. The single-Cys Rv1222 derivatives were treated and reacted with Fe-BABE (ThermoFischer) in 1:5 molar ratio as per manufacturers’ protocol. The unreacted Fe-BABE was removed by passing the reaction samples through P6 column that was pre-
equilibrated with buffer [20 mM Tris (pH 8.0), 5% glycerol and 0.2 M NaCl]. The protein was distributed in small aliquots and stored at −80°C.

Fe-BABE-mediated protein–DNA foot-printing assay. First, we tested the ability of each of Fe-BABE labelled Rv1222 derivatives to induce cleavage on the DNA. Each of Fe-BABE labelled Rv1222 derivatives (800 nM) was incubated with 1 μM DNA in buffer [50 mM Tris-Cl (pH 8), 100 mM KCl, 10 mM MgCl₂, 1 mM DTT, 5% Glycerol, 5 μg/ml BSA] for 15 min at 37°C. The cleavage reactions were initiated as described below. As only Rv1222 derivative labelled at G133C (C-terminus end) is able to produce cleavage on DNA, we performed subsequent Fe-BABE-mediated protein–DNA foot-printing assay (39,40) using this protein derivative.

Ec RNAP holo (2 μM) was incubated with 1 μM 32P-labelled DNA in the above buffer at 37°C for 10 min to form open complex (RPo). Four hundred nanomolar of Rv1222 was added to RPo and incubated further for 15 min at 37°C. Twenty five micromolar each of ATP, GTP, UTP was added to sample and kept for 15 min to form stalled EC with 15 nt RNA (EC₁5). The cleavage reactions were initiated by adding 0.22 mg/ml ascorbate and 0.1 mM H₂O₂ to each sample. After incubation for 30 s at 37°C, reactions were quenched with 0.3 mg/ml thiourea. Then 70 μl sterile H₂O, 10 μl 3M sodium acetate (pH 5.2) and 200 μl chilled ethanol were added to each sample and kept at −20°C for 1 h. The samples were centrifuged at 13,000 rpm for 15 min, washed with 200 μl chilled 70% ethanol and dried in vacuum. The samples were dissolved in 10 μl of FLB [95% formamide, 50 mM EDTA, 0.01% xylene cyanol], denatured by heating at 95°C. Samples (equal counts) were resolved by electrophoresis on 8% Urea-PAGE in TBE and scanned by storage phosphor imager in Typhoon Trio+ (GE Healthcare). As control reaction, unlabelled Rv1222 was incubated with RPo as above and the cleavage reaction was performed. In another control, Fe-BABE labelled Rv1222 was incubated only with the DNA fragment (no RPo formed) prior to cleavage reaction.

In vitro replication assay

The single-stranded DNA template and the Cy5-labelled primer was annealed in buffer [50 mM Tris (pH 7.5) and 100 mM NaCl] by heating to 95°C followed by cooling to 25°C. Fifty nanomolar annealed DNA template was incubated increasing concentrations of Rv1222 (0, 100 nM, 200 nM, 400 nM) and for 5 min at 37°C. 0.5 U of Klenow (Thermo Scientific) and 0.25 mM dNTP mix were added to the sample and incubation was carried out for another 1 min (41). The reactions were stopped by adding 2 μl FLB (80% formamide, 10 mM EDTA). The reaction samples were then run in 12% Urea-PAGE and the gel was scanned in Typhoon Trio+ (GE Healthcare) scanner at Cy5 channel. To assess the effect of Rv1222 on the kinetics of DNA synthesis, the assay was performed as above except the reactions were stopped at different time points.

RESULTS

Rv1222 does not function as an anti-sigma E

Previous reports suggest that M. tuberculosis (MtB) Rv1222 protein functions as an anti-sigma factor for σE. In principle, binding of an anti-sigma factor with sigma inhibits transcription either (i) by preventing the association of the sigma factor with RNAP (42) or (ii) by preventing the association of RNAP to the promoter DNA by inducing a conformational change in sigma. In both cases, as RNAP cannot bind to DNA, the polymerase does not initiate open complex formation. However, once the RNAP promoter open complex is formed, anti-sigma is not able to act on sigma and thus is unable to inhibit transcription. To test how Rv1222 inhibits transcription, we performed EMSA and in vitro transcription assays. In both the assays, we performed two sets of experiments. For EMSA, in the first set, we incubated Rv1222 and σE to form a complex before adding to RNAP and subsequently formed the open complex (Figure 1A). In the second set, we incubated RNAP and σE to from the holo enzyme, and then added Rv1222 to the RNAP holo before forming the open complex (Figure 1B). Heparin was added to the samples to remove any non-specific RNAP–DNA complexes other than open com-
plex (RPo). In both the cases, Rv1222 did not inhibit open-complex formation. For the in vitro transcription assay, in the first set, we incubated RNAP-σE holo with Rv1222 and added promoter DNA fragment to form an open complex, followed by transcription initiation (Figure 1C). In the second set, we first formed the open complex and then added Rv1222 before transcription initiation (Figure 1D). In both the cases, Rv1222 inhibited transcription with similar efficiency (full gels of Figure 1C and D were shown in Supplementary Figure S2A). To eliminate the possibility that the inhibition of transcription could be due to the presence of RNase in the preparation of Rv1222, first P32-labelled RNA was formed by in vitro transcription assay and subsequently was incubated with Rv1222 at identical conditions used in Figure 1C or D. As no degradation of the transcript was observed in the presence of Rv1222, it was confirmed that the inhibition of transcription by Rv1222 was not due to the presence of any RNase in the preparation (Supplementary Figure S2B).

The fact that Rv1222 does not inhibit open-complex formation but inhibits transcription when added after open-complex formation clearly indicates that the protein does not function as an anti-sigma factor as suggested previously.

Rv1222 inhibits transcription by both RNAP holo and RNAP core from M. tuberculosis, E. coli and B. subtilis

As Rv1222 does not function as an anti-sigma factor, but still inhibits RNAP-σE holo, we investigated whether the protein would inhibit transcription by an RNAP holo enzyme that contains a sigma factor other than σE. Using in vitro transcription assay, we tested the effect of Rv1222 on Mtb RNAP-σA holo in which the σA, the principal sigma factor of M. tuberculosis was associated with RNAP core (Figure 2A). The data showed that Rv1222 inhibited transcription by Mtb RNAP-σA holo. Subsequently, we performed the same assay using the E. coli (Ec) RNAP-σ70 holo (Figure 2B) and Bacillus subtilis (Bs) RNAP-σA holo (Figure 2C). The data further showed that Rv1222, despite being a transcription factor of M. tuberculosis, inhibited transcription by both E. coli and B. subtilis RNAP and inhibited transcription by all three RNAP with similar efficiencies. Approximate values of IC50 of transcription inhibition by Mtb, Bs and Ec RNAP were ~77 nM, ~62 nM, ~70 nM respectively as estimated from two to three replicates of the assays. The results further confirm that the inhibition of transcription by Rv1222 is not σE specific. Since the structure and sequence of RNAP core is conserved among the different bacterial species (43), it is likely that Rv1222 targets RNAP core enzyme for transcriptional inhibition and not any specific sigma factor.

To test whether Rv1222 targets RNAP core enzyme for transcription inhibition, we performed in vitro transcription assay with the Mtb RNAP core and a tailed-template DNA fragment (a double-stranded DNA fragment that contains a single-strand overhang of ~10 bases) (44,45). RNAP core is able to bind the overhang junction and to perform transcription from this DNA. The assay data showed that Rv1222 inhibited transcription by RNAP core (Figure 2D) with efficiency similar to RNAP holo (IC50 ~75 nM). To further validate this observation, we performed another complimentary fluorescence based in vitro transcription assay using Ec RNAP core and Kool-template DNA (46). The data also confirmed that Rv1222 was able to inhibit transcription by Ec RNAP core (Supplementary Figure S3) and did not require any sigma factor for its function.

Rv1222 binds to RNAP core and DNA

Since Rv1222 targets RNAP core, and not any sigma factor for transcriptional inhibition, we argued that the protein should bind to RNAP core with high affinity and should exhibit little or no affinity towards the sigma factors, namely σE and σA. To test this hypothesis, we monitored the binding affinities of Rv1222 to Mtb RNAP core and different sigma factors by fluorescence anisotropy assay (47). We used TMR labelled Rv1222 protein for this assay (Figure 3). The binding affinities of Rv1222 to RNAP core (Figure 3A), σE and σA were estimated to be ~60 ± 10 nM, ~270 ± 45 nM and ~690 ± 270 nM respectively (Figure 3A and also Supplementary Figure S4). Data showed that the protein binds to RNAP core with at least 5-fold higher affinity compared to both the sigma factors. Interestingly Rv1222 displayed ~2-fold higher affinity for the σE over σA. This comparatively higher affinity for σE is consistent with the western blot data obtained by previous groups (22,23) demonstrating σE-Rv1222 interaction. The fact that Rv1222 showed binding affinities to sigma factors could be explained by the possible ionic interaction among the proteins. Rv1222 is a highly positively charged protein (pI = 8.68) whereas the sigma factors are negatively charged in neutral buffer condition. Therefore, it is possible that the sigma factors contain negatively charged residues at the surface of the proteins whereas Rv1222 contains positively charged residues at its surface.

As Rv1222 is a positively charged protein, we speculated that the protein might bind to the DNA fragment due to its negative charge. To test this idea, we investigated whether the protein possessed any affinity for DNA (Figure 3B). We used two DNA fragments: one containing a promoter element and another containing no promoter element. Our anisotropy assay with the labelled Rv1222 showed that the protein bound to these DNA fragments with similar affinities (for promoter DNA fragment, KD = 60 ± 20 nM, Figure 3B; for promoterless DNA fragment, KD = 52 ± 8 nM, Supplementary Figure S5). When the binding assays were performed at high salt (200 mM NaCl, instead of 100 mM NaCl), Rv1222 completely lost its ability to bind to DNA (Supplementary Figure S6), whereas the protein retained its affinity to RNAP. Therefore, we conclude that interaction of Rv1222 to DNA is non-specific, possibly ionic, whereas its interaction to RNAP is specific. As Rv1222 exhibits affinity for RNAP and DNA, on the other hand RNAP is known to bind DNA fragment, we expected that the presence of RNAP would alter the binding affinity of Rv1222 to the DNA fragment. The anisotropy assay showed that Rv1222 binds to RNAP core and DNA simultaneously (nature of the binding curve is sigmoidal indicating simultaneous binding of ligands (Figure 3C), whereas for individual ligand binding, the nature of the curve is hyperbolic (Figure 3A and B) and the presence of RNAP increased the binding affinity of Rv1222 to DNA (KD = 119 ± 5 nM).
Figure 2. Rv1222 inhibits transcription by RNAP from *M. tuberculosis*, *E. coli*, *B. subtilis*. (A) 100 nM Mtb RNAP-$\sigma^A$ holo and 50 nM *rnrA* promoter. Concentrations of Rv1222 were indicated. Run-off transcripts 316 nt. (B) 100 nM Ec RNAP-$\sigma^70$ holo, and 50 nM *lacCONS* promoter. Run-off transcript sizes were 30 nt. (C) 100 nM Bs RNAP holo and 50 nM *abrB* promoter. Run-off transcript sizes were 70 nt. (D) 100 nM Mtb core RNAP and 50 nM tailed-template DNA. Transcript sizes were 60 nt.

Figure 3. Rv1222 simultaneously binds to RNAP and DNA: fluorescence anisotropy assay. Twenty nanomolar TMR labelled Rv1222 was added with (A) Mtb RNAP core, (B) DNA, (C) Mtb RNAP core + DNA. Fluorescence anisotropy of the labelled protein was monitored at Ex 530 and Em 580 nm. Each data set represents mean of three replicates.

C-terminal tail of Rv1222 is critical for its interaction with DNA

To identify which part of the protein interacts with DNA, we performed DNA–protein foot-printing assay with Fe-BABE labelled Rv1222. We generated two single Cys derivatives of the protein: the position of cysteine is either at residue 23 (close to N-termini) or at residue 133 (close to C-termini) (Figure 4A). The protein derivatives were subsequently labelled with Fe-BABE and were subjected to DNA–protein foot-printing assay. Both the labelled Rv1222 derivatives were active in inhibiting transcription (Supplementary Figure S7). We observed only the C-terminus-labelled protein derivative, not the N-terminus-labelled protein derivative, produced the Fe-BABE induced cleavage on the DNA (Supplementary Figure S8). The reason could be due to the proximity of the Fe-BABE labelling site of the protein to its DNA binding site. Thus, the result indicates that the protein may contain DNA binding determinant near or at the C-terminus. Sequence analysis of the protein by BindN+ (freeware) predicted that 12 residues at the C-terminal tail of Rv1222 could contain determinants for DNA binding. The protein contains five positively charged residues at the C-terminal tail. Indeed, when the 10 C-terminal residues were deleted from Rv1222, the resultant protein (Rv1222$\Delta$C) lost its ability to bind DNA (Figure 4B). As a result, Rv1222$\Delta$C lost its ability to inhibit transcription, although retained its affinity for RNAP ($K_d = 72 \pm 26$ nM) (Figure 4C and D). Thus, we conclude that the positively charged C-terminal tail of Rv1222 is critical, or possibly responsible for its interaction with DNA. The result showing the loss of ability of the protein to bind DNA at high salt (Supplementary Figure S6) indicates that the interactions among Rv1222 and DNA are weak and possibly ionic. At this salt concentration, although Rv1222 retains its affinity to RNAP, the protein loses its ability to inhibit transcription (Supplementary Figure S6). However, the possibility that deletion of the C-terminus tail alters putative DNA binding site of the protein, if any, could not be excluded. Nevertheless, our data clearly show that the interaction of Rv1222 to DNA is essential for the inhibition of transcription.

Rv1222 binds DNA adjacent to RNAP in the open complex (RPo) and stalled EC

To test the location of Rv1222 on DNA in the context of open complex (RPo) and stalled EC (containing 15 nt RNA), we performed protein–DNA foot-printing assay with Fe-BABE labelled Rv1222 (40). We used sub-stoichiometric level of labelled Rv1222 (400 nM) than DNA (1 $\mu$M) and RNAP (2 $\mu$M), so that majority of the labelled protein remained bound at the DNA adjacent to RNAP while avoiding sites of DNA where there is no RNAP (Figure 5A). This is evident from comparison of the results of Supplementary Figures S5A and S8. In Supplementary Figure S8, Fe-BABE labelled Rv1222 produces nicks on free DNA at multiple sites (lane 3) as the concentration of the protein (800 nM) was 2-fold higher than DNA concentra-
Figure 4. C-terminal tail of Rv1222 is critical for DNA binding and inhibition of transcription. (A) Schematic representation of Rv1222 WT and Rv1222ΔC. A23C and G133C were the positions in two single-cysteine protein derivatives at which Fe-BABE was conjugated. (B) 20 nM TMR labelled Rv1222ΔC was added with DNA. Fluorescence anisotropy of the labelled protein was monitored at Ex 530 nm and Em 580 nm. Each data set represents mean of three replicates. (C) Rv1222ΔC does not inhibit transcription: in vitro transcription assay. Ec RNAP holo and lacCONS promoter DNA fragments were used; Rv1222ΔC was incubated with the open complex before initiation of transcription. Run-off transcript sizes were 30 nt. (D) Binding of Rv1222ΔC to RNAP core: Same as B except that labelled Rv1222ΔC was added with Mtb RNAP core instead of DNA.

Figure 5. Location of Rv1222 in open complex (RP0) and stalled-EC (EC15). (A) Fe-BABE induced protein–DNA foot-printing assay. Four hundred nanomolar of Rv1222 labelled with Fe-BABE at G133C (near C-termini) was used in this assay. Two micromolar Ec RNAP and 1 μM lacCONS15 promoter were used to form open complex. In both cases (lanes 3 and 4), Rv1222 was added to the mixture after open-complex formation. For the formation of stalled EC at +15 (EC15, lane 4), transcription was initiated with ATP, GTP and UTP (no CTP), so that RNAP could synthesize 15 nt RNA and stalled (EC15). We observed a Fe-BABE induced nick at +3 position (with respect to transcription start site) on the DNA template in the open complex, in which the RNAP active centre is located at +1, whereas in the EC, the position of the nick was shifted to +17 position, in which the RNAP active centre is located at +15 (Figure 5B). This 14 nt shift in the nick site closely corresponds to the translocation of RNAP on DNA template by 15 nt. Since Rv1222 was added before transcription initiation and sub-stoichiometric amount of labelled Rv1222 was used for this assay, it is likely that Rv1222 binds to the open complex and translocates with the elongating RNAP that stalls at +15 and subsequently produces a nick on DNA at +17. However, the possibility of re-binding of Rv1222 to the stalled EC cannot be completely ruled out from this assay. In this context, it is important to note that Rv1222 inhibits transcription elongation when added after the formation of stalled EC (Supplementary Figure S9). In the stalled EC, in addition to the nick at +17, Rv1222 also produced a nick at +3 on DNA that corresponds to the nick for open complex (lane 4, Figure 5). The result indicates that, as expected, only a part of the open complexes formed were able to initiate transcription and form ECs. The result also suggests that Rv1222 binds to RNAP at a site that faces downstream DNA.

Rv1222 slows down RNA synthesis by RNAP in vitro and in vivo

As Rv1222 was found to bind to RNAP and DNA simultaneously, it could be possible that the protein anchors RNAP onto DNA and thus could exert frictional force as RNAP translocates along the DNA during transcription. If this is the case, we might expect Rv1222 not to arrest or prohibit transcription, rather influence RNAP to slow
down RNA synthesis. To test this hypothesis, we performed single-round in vitro transcription assay in the presence and absence of Rv1222 and monitored the amount of transcripts produced at each fixed time point. As Rv1222 neither affects the open-complex formation (Figure 1A and B), we first formed open complex prior to incubation with Rv1222 and followed by transcription initiation. Heparin was added to the sample to ensure single-round transcription. We performed the assay with Mtb RNAP as well as Ec RNAP. For Mtb RNAP, in the absence of Rv1222, the amount of transcripts was saturated at 5 min, whereas in the presence of 200 nM Rv1222, the time required for saturation increased to 20 min (Figure 6A and B). The total amount of transcript at saturation level in both the cases remained the same. In the presence of 400 nM Rv1222, the saturation level of transcripts could not be reached at the maximum time point we tested, however, the amount was increasing with time. For Ec RNAP, the result obtained was similar to that with Mtb RNAP, except that, the time taken by Ec RNAP to produce saturation level transcripts in the presence of Rv1222 was less than that of Mtb RNAP (Figure 6C and D). Overall, the result indicates that Rv1222 reduces the amount of RNA synthesis by RNAP in a time-dependent manner and the reduction does not depend on promoter sequence and the amount of RNA synthesis decreases with the concentration of Rv1222 present.

All our in vitro data suggested that Rv1222 reduces the amount of RNA synthesis by RNAP. We therefore expected that the protein would also reduce transcription in vivo and that reduction would also not be specific for any particular promoter. To test this hypothesis we developed an in vivo reporter assay in E. coli involving two plasmids (38): one contained an mCherry expression cassette (48) under the control of lacCONS promoter, while the second compatible plasmid contained Rv1222 ORF under the control of an inducible promoter (Figure 6E). In the absence of Rv1222, RNAP would produce mCherry mRNA from the lacCONS promoter and subsequently express the mCherry protein. In the presence of Rv1222, the amount of promoter activity would be reduced as the protein slows down the mRNA synthesis by RNAP. As expected, we observed a significant decrease in the percentage of cells (14%) that produces mCherry fluorescence when Rv1222 was expressed, as compared to the percentage of cells (94%) that lacks Rv1222 expression (Figure 6F). On the other hand, when Rv1222ΔC was expressed, the percentage of cells that produce mCherry fluorescence was relatively unaffected (84%) and was close to that observed in the absence of Rv1222. This result further corroborates the fact that Rv1222ΔC loses its ability to inhibit transcription.

Rv1222 does not inhibit DNAP or T7 RNAP

Since we find that Rv1222 binds to DNA non-specifically, there is a possibility that the DNA, upon binding with Rv1222 randomly, presents a ‘bumpy road’ to molecule that moves along the DNA slowing down the movement of the molecule. As DNAP moves along DNA during DNA synthesis (49) like RNAP during RNA synthesis, Rv1222 bound DNA would slow down not only RNA synthesis but also DNA synthesis. We performed in vitro replication assay with E. coli DNAP (Klenow fragment) to investigate whether Rv1222 had any effect on DNA synthesis in a dose-dependent manner or on kinetics of DNA synthesis. The results showed that Rv1222 neither affected the DNA synthesis by DNAP (Figure 7A) nor the rate of DNA synthesis (Figure 7C). This is due the inability of Rv1222 to efficiently bind DNAP (Kd = 245 ± 78 nM, as compared to Kd for RNAP = 60 ± 10 nM, Figure 7B). Similarly, when in vitro transcription assay was performed with RNAP from bacteriophage T7, Rv1222 was unable to inhibit the polymerase (Figure 7D). The binding assay of Rv1222 to T7 RNAP revealed that the protein exhibits very little affinity toward this polymerase (Figure 7E), consistent with the fact that T7 RNAP does not share sequence homology with the bacterial RNAP. This observation suggests that the binding of Rv1222 to DNA could not be the sole reason for inhibition of transcription.

Rv1222 slows down the growth rate of bacteria

As Rv1222 reduces the level of mRNA synthesis in vivo, the protein would be responsible for reducing the amount of total mRNA in the cell. As transcription is one of the key cellular processes, any decrease in total mRNA level within the cell would be reflected in a slow growth of the cell. We investigated this hypothesis by introducing Rv1222 into M. smegmatis as well as in E. coli and monitored cell growth. The plasmid pLAM12 containing the Rv1222 gene under an acetamide inducible promoter was inserted in M. smegmatis as well as in E. coli, and monitored cell growth. When Rv1222 was expressed, the percentage of cell that did not have any expression of Rv1222 (Figure 8A). To investigate whether the reduced growth rate of the cell was due to reduction of the mRNA levels, we quantified the mRNA levels of the cell at different time points of growth. For this assay, we grew M. smegmatis in a media that contained 32P-sodium phosphate. Upon 32P-phosphate uptake by the cell, newly synthesized NTP would incorporate the radiolabelled phosphate, which subsequently would be incorporated into the RNA (50). In this assay, we intended to measure the in vivo level of RNA under different levels of Rv1222 in the cells. However, as the growth rate of the cell changed upon increase in Rv1222 expression, we estimated the relative amount of radiolabelled RNA per cell at each time point during the cell growth by normalizing it against the OD600. When Rv1222 was expressed, the level of total RNA per cell was significantly reduced (Figure 8B). Although from this assay we cannot rule out whether other possible biological pathways, if any, are involved in the reduction of growth rate by Rv1222, it is very likely that the observed slow growth rate of the cell in the presence of Rv1222 is due to reduction in the level of RNA synthesis. To monitor the effect of Rv1222 expression on the growth rate of E. coli, we inserted a plasmid containing an IPTG inducible rlv1222 gene into the bacteria. As IPTG concentration was increased, the growth rate of the bacteria is reduced. At 100 μM IPTG concentration the growth rate was reduced by ~75% upon expression of Rv1222 (Figure 8C). This reduction was changed to 20% upon expression of Rv1222ΔC (Figure 8D). This indicates that dele-
Rv1222 inhibits transcription in vitro (in a time-dependent manner) and in vivo. (A) In vitro transcription reactions were performed with 100 nM Mtbb RNAP and 50 nM sinP3 promoter DNA and 32P-labelled NTP. Rv1222 was added to the reaction mixture after open-complex formation, before addition of NTP. Heparin was added to the reaction mixtures to ensure single-round transcription. The reactions were stopped at the indicated times after the addition of NTP. (B) The intensities of the bands at different time points as obtained from the in vitro transcription assay with Mtbb RNAP were plotted against time. ––Rv1222, - - - - - 200 nM Rv1222, --- 400 nM Rv1222. (Data is representative of three independent experiments.) (C) Same as A for Ec RNAP. (D) Same as B for Ec RNAP. (E) Strategy for promoter activity assay: pAcYc Duet plasmid, pAcYc Rv1222 and pAcYc Rv1222ΔC were co-transformed with pFPVinCherry-lacCONS plasmid in E. coli BL21 (DE3) cells and grown at 37 °C up to OD (at 600 nm) 0.4, induced with 0.5 mM IPTG, and further grown for 14 h. (F) FACS data: cells were diluted to make up equal number of cells in each sample. Aliquots of cells from above assay were scanned at mCherry (610 nm) and Cy7 (760 nm) fluorescence channels; first panel (control): pFPVinCherry-lacCONS + pAcYcDuet; second panel: pFPVinCherry-lacCONS + pAcYc-Rv1222 Duet; third panel: pFPVinCherry-lacCONS + pAcYc-Rv1222ΔC Duet. The percentage of fluorescent cells for the panels –Rv1222, +Rv1222 and +Rv1222ΔC are 94%, 14% and 84%, respectively. Cy7 channel was used as reference at which the cells display minimum auto fluorescence.

DISCUSSION

Previously, Dona et al., using a combination of western blot assay and in vivo pulled down assay and also in vitro transcription assay, showed that Rv1222 binds to σE. In another work, Barik et al. showed that Rv1222 inhibits transcription from a σE-dependent promoter. These results led to the interpretation that Rv1222 functions as an anti-σE. Here, we show that Rv1222 exhibits a moderate binding affinity to σE and also inhibits transcription from a σE-dependent promoter, consistent with the previous observation. However, if Rv1222 was an anti-sigma factor for σE, it would inhibit the open-complex formation and would not inhibit transcription when added after open-complex formation. Our results show that Rv1222 does not affect the open-complex formation, rather inhibits transcription when added after open-complex formation. These observations argue the previous conclusion and indicate that the protein may not function as an anti-σE. We further show that Rv1222 inhibits transcription by RNAP core and RNAP holoenzymes of three different bacteria: E. coli, B. subtilis and M. tuberculosis, in association with their respective principal sigma factors. Therefore, our results unequivocally establish that inhibition of transcription by Rv1222 does not involve the binding of the protein to σE. This inhibition occurs at the RNA synthesis step including promoter escape and transcription elongation, but not at the open-complex formation step. The C-terminal tail of Rv1222 that contains positively charged residues is critical, possibly responsible for DNA binding. The protein derivative in which this tail is deleted fails to bind DNA and is consequently inefficient for transcription inhibition. Thus, binding of Rv1222 to RNAP alone cannot be the sole reason for the inhibition of transcription. On the other hand, Rv1222 does not bind to DNAP or T7 RNAP and does not inhibit these polymerases. Thus, the binding of Rv1222 on DNA also cannot be the sole reason for the observed inhibition of transcription by Rv1222. Therefore, inhibition of transcription requires simultaneous binding of Rv1222 to the RNAP core and to DNA. Based on our experimental data we propose a model in which Rv1222 anchors RNAP onto DNA and thereby restricts the translocation of RNAP along DNA during RNA synthesis. Our model predicts that Rv1222 does not com-
Figure 7. Rv1222 does not inhibit DNA synthesis. (A) In vitro replication assay: 0.5 U of Ec Klenow DNAP, Cy5-labelled primer and 65 base DNA fragments were used in the assay. Rv1222 was incubated with DNAP before the replication reactions were initiated with dNTP. Reactions were stopped after 1 min and products were separated on 12% Urea-PAGE. The gel was scanned on a Typhoon Trio+ at Cy5 channel. (B) Binding of 20 nM TMR labelled Rv1222 to Klenow polymerase. (C) Effect of Rv1222 on the kinetics of DNA replication: Same as A except the reactions were stopped at the indicated time interval. (D) In vitro transcription assay with T7 RNAP: 0.2 U of T7 RNAP was used with 100 nM of T7 promoter containing DNA fragment. (E) Binding of 20 nM TMR labelled Rv1222 to T7 RNAP by fluorescence anisotropy.

 completamente prevent transcription, but slows down the synthesis of mRNA by RNAP. This is a novel global mechanism for transcriptional regulation and is not restricted to any specific promoter. This is different from the mechanism by which HK022 Nun protein anchors the RNAP and completely prevents its translocation at specific DNA site (21).

When Rv1222 was overexpressed in M. smegmatis or E. coli, the growth rate of the bacteria is significantly reduced. The level of synthesis of mRNA in M. smegmatis is reduced when Rv1222 is expressed in the cell. As transcription is one of the essential steps in the bacterial growth cycle, it is plausible that slowing down of mRNA synthesis would result in a slow growth of the bacterium. Thus, our results indicate that Rv1222 could be a determinant for the growth rate of mycobacterium. However, to confirm whether Rv1222 is responsible for slow rate of RNA synthesis, further study is required involving generating a knock out mutant of M. tuberculosis strain that lacks the expression of Rv1222 (under progress). It is also possible that the level of Rv1222 increases when the bacteria enter the dormant stage, making the bacteria extremely slow growing consistent with the observations that the mRNA level of M. tuberculosis Rv1222 is upregulated in dormant state or under growth conditions leading to dormant state. On the other hand, the protein is degraded by CipC1P2 protease upon PknB-dependent phosphorylation (23). Thus, there may be a possibility that proteolysis of Rv1222 in dormant state may restore the bacteria in its active state. To test whether the level of Rv1222 is a possible determinant in transition of the bacteria from active to dormant state, further investigation is required to monitor the level of expression of the protein in the two different states, e.g. active and dormant state. We note that the
Rv1222 is present in various mycobacteria, e.g. *M. tuberculosis*, *M. leprae*, *M. bovis*, *M. marinum* and *M. smegmatis* and with high sequence homology. We hypothesize that Rv1222 in these mycobacteria function by a similar mechanism as the protein from *M. tuberculosis*.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

We thank R. Landick (UW-Madison), R. H. Ebright (Waksman Institute), S. Wig痱hweraraj (Imperial College London) for suggestions; R. Sur (University of Calcutta), A. B. Dutta and P. Parrack (Bose Institute) for critically reading the manuscript and comments.

CONFLICT OF INTEREST STATEMENT

None declared.

FUNDING

Department of Biotechnology, India Grant [BT/PR 5345/MED/29/648/2012] to JM; CSIR India Fellowships to P.R., R.B., and R.K.P. Funding for open access charge: Institutional support.

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