Recombinant Reporter Assay Using Transcriptional Machinery of *Mycobacterium tuberculosis*

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Development of an *in vivo* gene reporter assay to assess interactions among the components of the transcription machinery in *Mycobacterium tuberculosis* remains a challenge to scientists due to the tediousness of generation of mutant strains of the extremely slow-growing bacterium. We have developed a recombinant mCherry reporter assay that enables us to monitor the interactions of *Mycobacterium tuberculosis* transcriptional regulators with its promoters *in vivo* in *Escherichia coli*. The assay involves a three-plasmid expression system in *E. coli* wherein two plasmids are responsible for *M. tuberculosis* RNA polymerase (RNAP) production and the third plasmid harbors the mCherry reporter gene expression cassette under the control of either a σ factor or a transcriptional regulator-dependent promoter. We observed that the endogenous *E. coli* RNAP and σ factor do not interfere with the assay. By using the reporter assay, we found that the functional interaction of *M. tuberculosis* cyclic AMP receptor protein (CRP) occurs with its own RNA polymerase, not with the *E. coli* polymerase. Performing the recombinant reporter assay in *E. coli* is much faster than if performed in *M. tuberculosis* and avoids the hazard of handling the pathogenic bacterium. The approach could be expanded to develop reporter assays for other pathogenic and slow-growing bacterial systems.

Mycobacterium tuberculosis, the causative agent of tuberculosis (TB), is the second most frequent cause of death worldwide from a single infectious disease (1–3). The pathogen remains deadly because of its ability to adapt to the harsh environmental conditions it encounters in the course of a successful infection. The bacterium can survive the unfavorable environmental conditions by adopting alternative gene expression systems and employing important players from the pool of 13 σ factors and 180 transcriptional regulators (4). It is believed that a set of genes are expressed under a particular environmental cue to maintain the minimum cellular activities required for its survival under stress (5, 6). One or more σ factors along with transcriptional regulators are thought to be responsible for gene regulation under the particular stress conditions. Upon encountering a different stress condition, the bacterium may switch gene expression from one set of genes to another by altering the σ factors in the transcriptional machinery.

In order to understand *M. tuberculosis* regulation under different stress conditions, many researchers have performed chromatin immunoprecipitation (ChIP) assays, microarray analysis, or quantitative reverse transcription-PCR (7–10) to identify the regulons for several σ factors and transcriptional regulators. However, there is a need to develop rapid assays to validate the above-described findings by assessing the *in vivo* interactions of a transcriptional factor with its cognate promoters.

One of the ways to validate gene regulation by a σ factor and transcriptional regulator is to develop a reporter gene assay in *M. tuberculosis*. For such an assay, the first step would be to prepare a mutant strain either by removing the gene encoding the regulator protein to ensure loss of expression of the regulator (knockout strain) (11–14) or by replacing the endogenous promoter element of the regulator gene via an inducible exogenous promoter, to ensure a minimum level of endogenous protein in the absence of any inducer molecule (knockdown strain) (15, 16). However, the broad manifestations of reporter gene assays in *M. tuberculosis* would be extremely tedious and time-consuming because of the slow-growing nature of mycobacteria (17). As a consequence, very

few successful endeavors involving reporter gene assays have been made with *M. tuberculosis* to study the interactions of its promoters with regulatory proteins. Assuming that the transcriptional apparatus of *Mycobacterium smegmatis* is similar to that of *M. tuberculosis*, attempts were made to develop recombinant reporter gene assays to study the functions of *M. tuberculosis* promoters in *M. smegmatis* (18–22). However, functional orthologs of *M. tuberculosis* transcriptional regulators are often present in *M. smegmatis* (23–25). Therefore, study of the interactions of these regulators in *M. smegmatis* demands the generation of knockout strains for the regulators. This approach would be comparatively faster than performing the reporter assay in *M. tuberculosis*, but it would still be a time-consuming assay, as generation of knockout strains is involved. In this report, we present an alternative recombinant *in vivo* reporter assay which is less time-consuming and is devoid of the generation of a knockout strain. We describe an mCherry reporter assay in *E. coli* that has enabled us to monitor the interactions of *M. tuberculosis* σ factors and transcriptional regulators with its promoters *in vivo*. The assay employs recombinant *M. tuberculosis* RNA polymerase (RNAP) holo enzyme and a plasmid that harbors an mCherry reporter gene expression cassette under the control of either a σ factor or a transcriptional regulator-dependent promoter element.

MATERIALS AND METHODS

Cloning strategies. Cloning of the genes encoding different RNAP subunits in different Duet vectors, with use of appropriate enzymes, has been discussed by Banerjee et al. (26). *sigA* and *sigE* were amplified from genomic DNA and cloned in pAcYc Duet (see Table S2 in the supplemental material). *sinP3* promoter DNA was amplified from synthetic oligonucleotide template and cloned in pFPVmCherry (see Tables S1 and S2 in the supplemental material). *sigBpr* and *WhiB1*were amplified from genomic DNA of *M. tuberculosis* H37Rv (see Tables S1 and S2) and cloned in pBluescript II SK(+) by blunt end ligation and subsequently in pFPVmCherry (see Table S2). An *rrnA* DNA template, a kind gift from Jaya Tyagi (AIIMS, India) (21) was amplified and cloned in pFPVmCherry. The *M. tuberculosis* cyclic AMP (cAMP) receptor protein (CRP; Rv3676) was amplified from genomic DNA of *M. tuberculosis* cloned first in pET28a and subsequently in pFPVmCherry (see Table S2).

Purification of proteins. *In vivo*-assembled *M. tuberculosis* RNAP core and *M. tuberculosis* RNAP-σ^A holo were purified as described by Banerjee et al. (26). The *E. coli* RNAP core was purified as described by Mukhopadhyay et al. (27). *M. tuberculosis* CRP was purified essentially as described by Bai et al. (28), except that a different resuspension buffer (50 mM Tris-HCl [pH 7.9], 200 mM NaCl, 10% glycerol, and 1 mM phenylmethylsulfonyl fluoride) was used instead of sodium phosphate buffer.

In vitro transcription assays. To perform the transcription assay with *M. tuberculosis* RNAP holo with or without the ω-subunit, increasing concentrations (50, 100, and 200 nM) of RNAP holo were incubated with 40 nM DNA template (*sinP3* or *rrnA*) in 18 μl transcription reaction buffer (45 mM Tris-HCl [pH 8.0], 70 mM KCl, 1 mM dithiothreitol, 10% glycerol, 5 mM $MgCl_2$, and 1.5 mM $MnCl_2$) at 37°C for 20 min. One microliters of heparin (0.5 mg/ml) was added to the reaction mixture to inhibit nonspecific RNAP-DNA complex formation. RNA synthesis was initiated by the addition of 1 μl nucleoside triphosphate mix (final concentrations, 125 μM ATP, GTP, and UTP and 50 μM CTP containing 0.4 μCi [α-^{32}P]CTP) (26). Following incubation at 37°C for 30 min, the reactions were terminated by adding 5 μl FLB dye (80% formamide, 10 mM EDTA, 0.01% bromophenol blue, 0.01% xylene cyanol). Products were heated at 95°C for 5 min, chilled on ice, and resolved by urea-PAGE (29), analyzed with a storage phosphor scanner (Typhoon Trio+; GE Healthcare), and quantified using ImageQuant TL software.

The *in vitro* transcription assay with the *M. tuberculosis* σ^E-dependent promoter *sigBpr* was conducted following the same protocol described above, except that *M. tuberculosis* RNAP (σ^E) holo was used instead of RNAP (σ^A) holo.

To perform the *in vitro* transcription assay with the *M. tuberculosis* CRP-dependent promoter *WhiB1*, 400 nM *M. tuberculosis* CRP was incubated with increasing concentrations of RNAP holo (with or without the ω-subunit) for 20 min at 37°C in transcription buffer. RNA synthesis was initiated following the same protocol described above.

In vitro transcription assays using *M. tuberculosis* RNAP and σ^A, *E. coli* RNAP and σ^{70}, or hybrid RNAP holo enzymes formed by interchanging the sigma factors were conducted following the above-described protocol. Native or hybrid RNAP holo enzymes were formed by incubating RNAP and sigma factor for 20 min at 37°C. For conducting the *in vitro* transcription assay using the *WhiB1* promoter, *M. tuberculosis* RNAP and *E. coli* RNAP were incubated with either *E. coli* CRP or *M. tuberculosis* CRP for 20 min at 37°C prior to addition of DNA template.

In vivo recombinant reporter assays. (i) Reporter assay with σ^A-RNAP holo. *E. coli* BL21(DE3) was transformed with the three plasmids indicated in the figures (see Fig. 1A; see also Fig. S2 in the supplemental material). Cotransformed cells were grown in 50 ml LB broth at 37°C with appropriate antibiotics (chloramphenicol at 35 μg/ml, ampicillin at 100 μg/ml, kanamycin at 50 μg/ml) to an optical density at 595 nm (OD_{595}) of 0.4, induced by the addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and were further grown at 37°C for 6 h or at 16°C for 16 h (until the OD_{595} reached 1.5).

As a control, the assays were performed with *E. coli* BL21(DE3) har-

boring the dual plasmid (pAcYc Duet *rpoA-rpoZ* plus pCOLA Duet *rpoB-rpoC*) for RNAP core expression or plasmid pAcYc Duet-*sigA* for σ^A expression, along with pFPVmCherry-*sinP3* (or pFPVmCherry-*rrnA*) or pFPVmCherry-*sinP3* alone. pAcYc Duet *rpoA-sigA* plus pCOLA Duet *rpoB-rpoC* along with pFPVmCherry-*sinP3* were used for *M. tuberculosis* holo expression. Identical assays were conducted with pFPVmCherry-*rrnA* plasmid.

To optimize the bacterial growth duration at which the fluorescence signal versus background ratio was highest, we monitored the OD and fluorescence intensities of the bacterial cultures at regular intervals. Aliquots of 100 μl of the cultures were taken, and the OD (at 595 nm) and fluorescence intensities (excitation wavelength, 592 nm; emission wavelength, 610 nM) were measured at each interval. The normalized fluorescence intensity (fluorescence intensity/OD ratio) data were plotted against the nearest single-decimal OD value (for example, if X was the fluorescence intensity of a bacterial culture at an OD of 0.37, the normalized fluorescence intensity, which was calculated as [X × (0.4/0.37)], was plotted against an OD of 0.4). The assays were repeated twice, and the mCherry expression kinetic curves were plotted (see Fig. S1 in the supplemental material). The duration of bacterial growth at which the optimum fluorescence signal-to-background ratio was obtained was observed either at 37°C for 6 h or at 16°C for 16 h.

Assay with M. tuberculosis σ^E. The assays with σ^E and a σ^E-specific promoter were conducted essentially as described above, except that σ^A was replaced with σ^E and the *sinP3* promoter was replaced with *sigBpr*. The three-plasmid expression system for σ^E is shown in Fig. 2A, below.

Assay with M. tuberculosis CRP. The assay with CRP and a CRP-specific promoter were conducted according to the above-described method and using the plasmids shown in Fig. 3A and B, below. As control, the assays were performed with *E. coli* BL21(DE3) harboring the following plasmid(s): (i) pFPVmCherry-*WhiB1*, to test whether *E. coli* RNAP induces mCherry expression at the *WhiB1* promoter; (ii) pFPVmCherry-*WhiB1*-CRP, to test whether *E. coli* RNAP in the presence of *M. tuberculosis* CRP induces mCherry expression at the *WhiB1* promoter; (iii) pAcYc Duet *rpoA-sigA* plus pCOLA Duet *rpoB-rpoC* plus pFPVmCherry-*WhiB1*, to test whether *M. tuberculosis* RNAP induces mCherry expression at the *WhiB1* promoter; (iv) pAcYc Duet *rpoA-sigA* plus pCOLA Duet *rpoB-rpoC* plus pFPVmCherry-*WhiB1*-CRP, to test whether *M. tuberculosis* RNAP in the presence of *M. tuberculosis* CRP induces mCherry expression at the *WhiB1* promoter (see Fig. 3).

In vivo reporter assay in the presence of the ω-subunit. *E. coli* BL21(DE3) was transformed with two plasmids (pAcYc Duet *rpoA-sigA* plus pCOLA Duet *rpoB-rpoC* or pAcYc Duet *rpoA-sigE* plus pCOLA Duet *rpoB-rpoC*) harboring genes for the *M. tuberculosis* RNAP holo (σ^A or σ^E) without the ω-subunit (see Fig. 1A and 2A). These cotransformed cells were grown in 200 ml LB broth at 37°C with appropriate antibiotics (chloramphenicol at 35 μg/ml, kanamycin at 50 μg/ml) to an OD (at 595 nm) of 0.4. Cells were harvested at 5,000 × g for 10 min at 4°C. Cell pellets were resuspended in 20 ml of prechilled 100 mM $MgCl_2$ and incubated on ice for 30 min, followed by centrifugation at 5,000 × g for 10 min at 4°C. Cell pellets were resuspended in 50 ml of prechilled 100 mM $CaCl_2$ and incubated for 4 h on ice. Finally, cells were harvested at 5,000× g for 10 min at 4°C, resuspended in 3 ml of mixture containing prechilled 85 mM $CaCl_2$ and 15% absolute glycerol, and incubated for 12 h at −80°C to gain competency. These competent cells containing the above-described one of the two-plasmid combinations were further transformed either with the respective plasmid alone (pFPVmCherry-*sinP3*, pFPVmCherry-*rrnA*, pFPVmCherry-*sigBpr*, or pFPVmCherry-*WhiB1*-CRP) or with the respective plasmid along with pCDF 1b-*rpoZ*. These cotransformed cells, containing either three or four plasmids, were grown in 50 ml LB broth at 37°C with appropriate antibiotics (chloramphenicol at 35 μg/ml, ampicillin at 100 μg/ml, kanamycin at 50 μg/ml, and streptomycin at 50 μg/ml) up to an OD (at 595 nm) of 0.4, induced by the addition of 0.5 mM IPTG, and further grown at 16°C for 16 h (until the OD_{595} reached 1.5). mCherry fluorescence was determined by using a fluorescence scanner
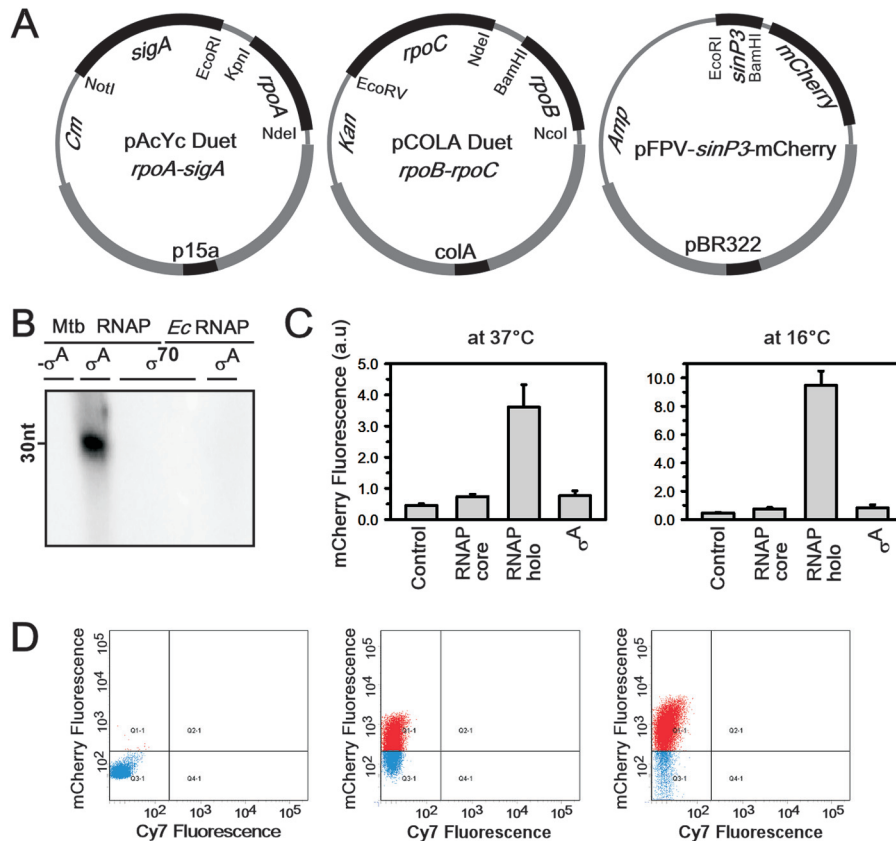
FIG 1 Recombinant *in vivo* *M. tuberculosis* reporter assay using *M. tuberculosis* σ[A] and its specific promoter, *sinP3*. (A) Strategy for use of the three-plasmid expression system, with dual plasmids (first two plasmids) for expression of *M. tuberculosis* RNAP holo and the third plasmid containing a σ[A]-dependent promoter element, *sinP3*, for expression of mCherry. All three plasmids were transformed in *E. coli* Bl21(DE3) cells grown at 37°C up to an OD (at 595 nm) of 0.4 and induced with 0.5 mM IPTG under different growth conditions. (B) Results of an *in vitro* transcription assay to assess the interactions between the *M. tuberculosis* RNAP core and *E. coli* σ[70] and between the *E. coli* RNAP core with *M. tuberculosis* σ[A]. *sinP3* was used as the DNA template. Runoff transcripts of 30 nucleotides (nt) were produced. Lane 1, *M. tuberculosis* (Mtb) RNAP core; lane 2, *M. tuberculosis* RNAP core plus *M. tuberculosis* σ[A]; lane 3, *M. tuberculosis* RNAP core plus *E. coli* (Ec) σ[70]; lane 4, *E. coli* RNAP plus *E. coli* σ[70]; lane 5, *E. coli* RNAP plus *M. tuberculosis* σ[A]. (C) Results for the recombinant *M. tuberculosis* reporter assays. The bars represent mCherry fluorescence of *E. coli* cells containing the pFPVmCherry-*sinP3* plasmid in the presence of the following: control, with no *M. tuberculosis* RNAP; *M. tuberculosis* RNAP core (pAcYc Duet *rpoA-rpoZ* plus pCOLA Duet *rpoB-rpoC*); *M. tuberculosis* RNAP holo (as in panel A); σ[A] (pAcYc Duet-*sigA*). The data represent means of three replicates, and error bars represent standard deviations. (Left) Cells were induced with 0.5 mM IPTG at 37°C for 6 h. (Right) Cells were induced with 0.5 mM IPTG at 16°C for 16 h. (D) FACS data. Aliquots of cells from the above-described assay were scanned for the mCherry (610 nm) and Cy7 (760 nm) fluorescence channels. (Left) Control, pFPVmCherry-*sinP3* plasmid; (middle) all three plasmids as described above, with protein induction at 37°C; (right) all three plasmids, with protein induction at 16°C. The number of fluorescent cells in the right panel is approximately twice than that in the middle panel.

(Typhoon Trio+; GE Healthcare) and quantified using ImageQuant TL software. The changes in mCherry fluorescence were determined based on the ratio of signal in the presence versus absence of *M. tuberculosis* RNAP.

To monitor the expression of *M. tuberculosis* RNAP with the ω-subunit in the above-described *in vivo* reporter assays, RNAP was purified from 50-ml *E. coli* cultures via Ni-nitrilotriacetic acid (NTA) chromatography following the protocol described by Banerjee et al. (26). The subunit composition of *M. tuberculosis* RNAP was analyzed by 10% SDS-PAGE followed by Coomassie staining. As a control, the identical method was followed for an assay in which *M. tuberculosis* RNAP was expressed without the ω-subunit.

FACS analysis of the cells from the reporter assay. Aliquots (2 ml) of cell cultures from the above-described reporter assays were harvested at 5,000 × *g* for 5 min at 4°C. The pellets were dissolved in 1 ml phosphate-buffered saline (PBS; pH 7.5) and transferred to fluorescence-activated cell sorting (FACS) tubes (BD Biosciences). A total of 20,000 cells were measured for each sample, and every sample had three replicates. Data were acquired on a FACS-ARIA apparatus (BD BioSciences) and analyzed using the FACS-Diva software.

RESULTS

Reporter assay with σ[A]-RNAP holo. Previously, we developed a method to assemble *M. tuberculosis* RNAP holo enzymes *in vivo* in *E. coli* by employing a dual-plasmid expression system (26). These methods yield large amounts of transcriptionally active enzymes, free of *E. coli* RNAP contamination. In order to develop a reporter assay for *M. tuberculosis* promoters in *E. coli* cells that express *M. tuberculosis* RNAP, we introduced a third plasmid that harbors an mCherry cassette under an *M. tuberculosis* σ[A]-dependent promoter element (Fig. 1A). We expected that the recombinant *M. tuberculosis* RNAP in association with σ[A] would induce mCherry expression from the *M. tuberculosis* σ[A]-dependent promoter element, resulting in fluorescent *E. coli* cells. On the other hand, the endogenous *E. coli* RNAP holo enzyme, the *E. coli* RNAP core enzyme in association with *M. tuberculosis* σ[A], or the *M. tuberculosis* RNAP core in association with *E. coli* σ[70] would not induce mCherry expression from the σ[A]-dependent promoter. To test
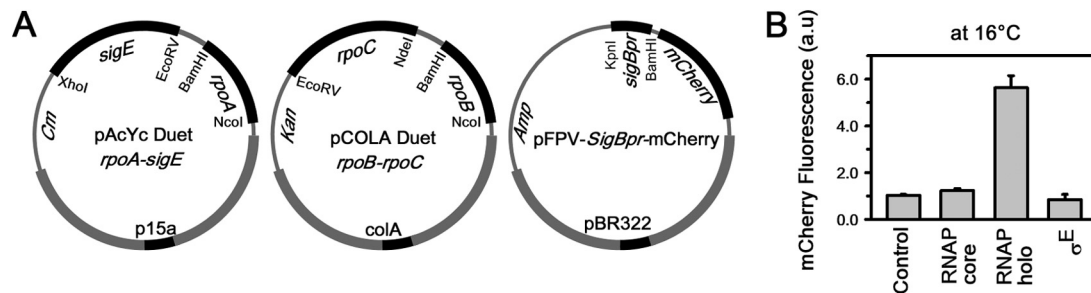
FIG 2 Recombinant *in vivo M. tuberculosis* reporter assay using *M. tuberculosis* σ^E and its specific promoter, *sigBpr*. (A) The strategy for this experiment was the same as that shown in Fig. 1A, except *sigA* was replaced by *sigE* in the dual-plasmid system and *sinP3* was preplaced by *sigBpr* in pFPVmCherry. (B) Results for recombinant *M. tuberculosis* reporter assays: The bars represent mCherry fluorescence of *E. coli* cells containing pFPVmCherry-*sigBpr* plasmid in the presence of the following: no *M. tuberculosis* RNAP (control); *M. tuberculosis* RNAP core (pAcYc Duet *rpoA* plus pCOLA Duet *rpoB-rpoC*); *M. tuberculosis* RNAP-*sigE* holo (as in panel A); σ^E (pAcYc Duet-*sigE*). The data represent means of three replicates, and error bars represent standard deviations.

the above-described possibilities, we first performed *in vitro* transcription assays with *M. tuberculosis* promoters and all of the above-described three RNAPs or RNAP combinations (Fig. 1B). The results showed that only the *M. tuberculosis* RNAP holo was able to initiate transcription from *M. tuberculosis* σ^A-specific promoter DNA fragments, indicating that *E. coli* RNAP and/or σ^{70} did not interfere with the reporter assay.

As expected, when the pFPVmCherry-*sinP3* plasmid was introduced into *E. coli* cells, we observed a small amount of mCherry expression. These data served as a control, set the background level, and indicated a lack of interaction of *E. coli* RNAP with the *sinP3* promoter *in vivo*. When the *M. tuberculosis* RNAP core was expressed in *E. coli* containing the pFPVmCherry-*sinP3* plasmid, we observed a 2-fold increase in the fluorescence signal over the background control, indicating nonspecific induction of mCherry expression by the *M. tuberculosis* RNAP core. However, when *M. tuberculosis* RNAP holo was expressed in the above-described assay system, we observed a 10-fold increase in the fluorescence signal (Fig. 1C, left panel). In all the above-described events, mCherry fluorescence was monitored by growing the cells at 37°C for 6 h. When the growth temperature of the above-described system was lowered from 37°C to 16°C (for 16 h), we observed a 16-fold increase in the fluorescence signal (Fig. 1C, right panel). This is in line with our previous report that the yield of RNAP holo assembly increases at the lower temperature, resulting in higher induction of mCherry expression (26). To exclude the possibility of association of *M. tuberculosis* σ^A with *E. coli* RNAP core to induce mCherry expression *in vivo*, we performed the above-described assay by introducing a plasmid containing the gene for *M. tuberculosis* σ^A into our mCherry expression system. In this assay, the *M. tuberculosis* σ^A along with *E. coli* RNAP core induced mCherry expression to some extent, comparable to mCherry expression by *M. tuberculosis* RNAP core and close to the background level. The growth conditions for the assays mentioned above (37°C for 6 h or 16°C for 16 h after induction) were the conditions that provided the best signal-to-background ratio (see Fig. S1 in the supplemental material). FACS analyses of the cells from the above-described assays also revealed that 40 to 50% of the cells were fluorescent at the mCherry channel (610 nm) when grown at 37°C, whereas 80 to 90% of the cells were fluorescent when grown at 16°C (Fig. 1D).

When the reporter assay was conducted with another *M. tuberculosis* σ^A-specific promoter *rrnA* (21), the results followed the

same trend as those with *sinP3* (see Fig. S2B in the supplemental material).

All of the above-described results indicated that the *E. coli* RNAP in association with either *E. coli* σ^{70} or with *M. tuberculosis* σ^A was not functional at *M. tuberculosis* promoters. Thus, the reporter assay would allow us to monitor the interactions with *M. tuberculosis* RNAP with its promoters *in vivo* in *E. coli*.

Reporter assay with σ^E-RNAP holo. In order to test whether the reporter assay could be used to monitor the interaction of alternative σ factors with their cognate promoters, we conducted the assay with a representative *M. tuberculosis* alternative σ factor, σ^E (Fig. 2) on its promoter, *sigBpr* (30). Using an *in vitro* transcription assay, we confirmed that RNAP-*sigE* holo was able to initiate transcription from the *sigBpr* promoter DNA fragment (see Fig. S3 in the supplemental material). When the pFPVmCherry-*sigBpr* plasmid was introduced into either *E. coli* cells or *E. coli* cells expressing *M. tuberculosis* RNAP core, we observed mCherry expression at a background level, as observed for the assay with σ^A. However, when *M. tuberculosis* RNAP-*sigE* holo was expressed in *E. coli* containing the pFPVmCherry-*sigBpr* plasmid at 16°C for 12 h, we observed a 6-fold increase in the fluorescence signal (Fig. 2B). These results indicated that the assay could be used with the alternative σ factors of *M. tuberculosis*.

Reporter assay with the *M. tuberculosis* transcriptional regulator CRP. In order to assess whether the reporter assay could be adapted to monitor the interactions of *M. tuberculosis* transcriptional regulators with their cognate promoters, we performed the assay with a representative *M. tuberculosis* transcriptional activator, CRP (Fig. 3) (28, 31). We chose *M. tuberculosis* CRP for the assay, as our *in vitro* transcription assay results indicated that *E. coli* CRP, although possessing structural and functional similarities with *M. tuberculosis* CRP, could not function with the *M. tuberculosis* transcriptional machinery (Fig. 3C). On the other hand, *M. tuberculosis* CRP was not functional in *E. coli* due to its inability to interact with the *E. coli* transcriptional machinery (28). Therefore, we expected that the endogenous CRP from *E. coli* would not interfere with the assay. As a control, we used the plasmid pFPV-*WhiB1*-mCherry, in which mCherry expression is under the control of an *M. tuberculosis* CRP-dependent promoter, *WhiB1* (31, 32) (Fig. 3A). The *M. tuberculosis* CRP gene was subsequently cloned under an inducible promoter and inserted into the above-described plasmid to generate pFPV-*WhiB1*-mCherry-CRP (Fig. 3B). When the assay was performed using either pFPV-
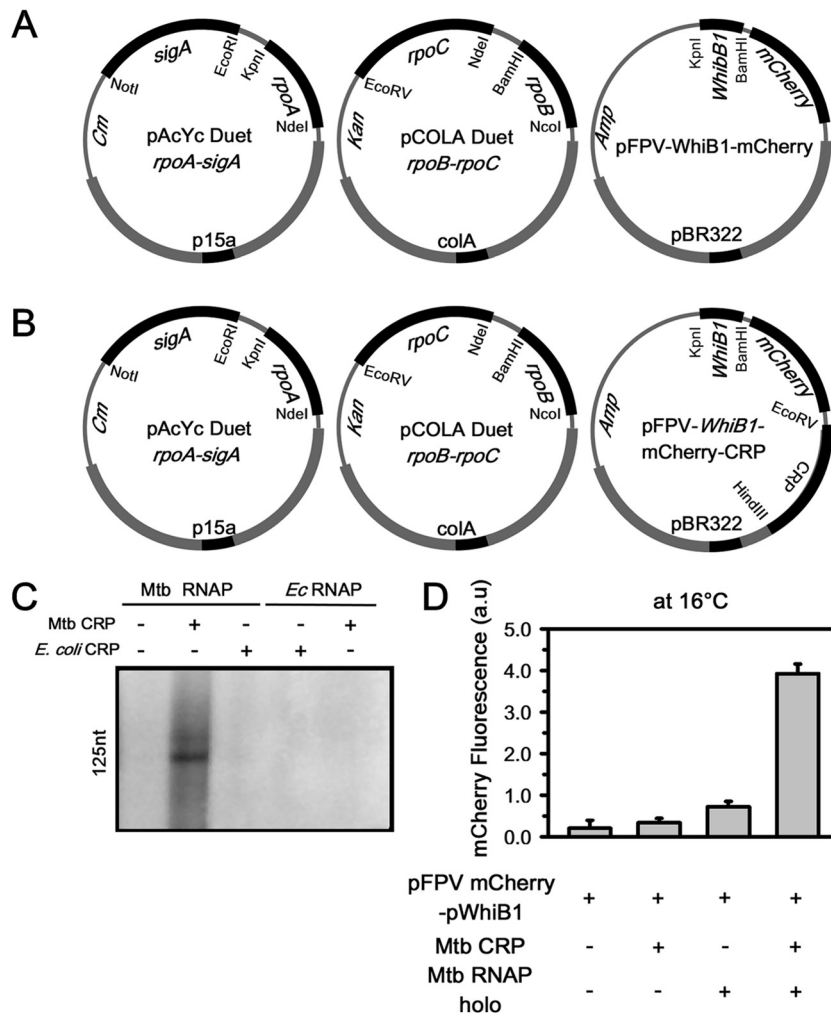
FIG 3 Recombinant *in vivo M. tuberculosis* reporter assay using *M. tuberculosis* CRP and its specific promoter, *WhiB1*. (A) Strategy for three-plasmid expression system, with dual plasmids (first two plasmids) for expression of *M. tuberculosis* RNAP holo (as in Fig. 1A) and the third plasmid containing *M. tuberculosis* CRP-dependent promoter element *WhiB1* for expression of mCherry. (B) In pFPV-*WhiB1*-mCherry, *M. tuberculosis* CRP was inserted under the control of the T7 promoter (pFPV-*WhiB1*-mCherry-CRP). (C) *In vitro* transcriptional activity assay to study the interactions of *M. tuberculosis* (Mtb) and *E. coli* (Ec) RNAP and CRP on *WhiB1* promoter DNA fragments. Runoff transcripts of 125 nucleotides (nt) were produced. Lane 1, *M. tuberculosis* RNAP holo; lane 2, *M. tuberculosis* RNAP holo plus 400 nM *M. tuberculosis* CRP plus 400 μM cAMP; lane 3, *M. tuberculosis* RNAP holo plus 400 nM *E. coli* CRP plus 400 μM cAMP; lane 4, *E. coli* RNAP holo plus 400 nM *E. coli* CRP plus 400 μM cAMP; lane 5, *E. coli* RNAP holo plus 400 nM *M. tuberculosis* CRP plus 400 μM cAMP. (D) Results for recombinant *M. tuberculosis* reporter assays. The bars represent mCherry fluorescence of *E. coli* cells containing the pFPVmCherry-*WhiB1* plasmid in the presence of the following; no *M. tuberculosis* RNAP holo or *M. tuberculosis* CRP; *M. tuberculosis* RNAP CRP (pFPV-*WhiB1*-mCherry-CRP); *M. tuberculosis* RNAP holo (all three plasmids in panel A); *M. tuberculosis* RNAP holo and *M. tuberculosis* CRP (all three plasmids in panel B). The data represent the means of three replicates, and error bars represent standard deviations.

WhiB1-mCherry or pFPV-*WhiB1*-mCherry-CRP, we observed a low level of fluorescent signal. The results indicated that *E. coli* RNAP holo alone or in combination with *E. coli* CRP or *M. tuberculosis* CRP could not induce mCherry expression on the *M. tuberculosis* CRP-dependent promoter (*WhiB1*). Similarly, in the absence of *M. tuberculosis* CRP, *M. tuberculosis* RNAP holo could not induce mCherry expression from the above-described promoter. When BL21(DE3) cells containing pFPV-*WhiB1*-mCherry-CRP expressed *M. tuberculosis* RNAP holo, we observed a 5-fold increase in the fluorescent signal over the signal obtained with RNAP holo (Fig. 3D). The results showed that *M. tuberculosis* CRP is able to regulate its cognate promoter only in the presence of *M. tuberculosis* RNAP.

Reporter assay in the presence of the RNAP ω-subunit. All

three of the above-described assays were performed with *M. tuberculosis* RNAP without the ω-subunit. Previous reports suggested that the ω-subunit of *M. tuberculosis* is essential for optimal growth of the bacterium (33) and that removal of the ω-subunit from the genome leads to diverse phenotypes (33, 34). Thus, the ω-subunit could play an important role in *M. tuberculosis* RNAP expression *in vivo* or could be essential for the function of some of the transcriptional regulators of *M. tuberculosis*, as in the case of *E. coli*, where the ω-subunit is essential for the function of ppGpp during the stringent response (35, 36). Therefore, we performed the above-described reporter assays by incorporating the ω-subunit in each of the above-described three cases. A plasmid containing the gene that encodes the ω-subunit, pCDF-*rpoZ* (26), was inserted into *E. coli* BL21(DE3) cells within the existing three-
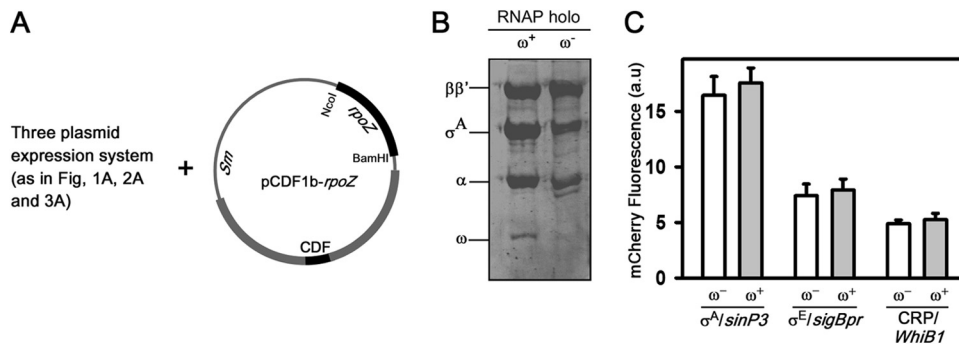
FIG 4 Reporter assay in the presence of the *M. tuberculosis* ω-subunit. (A) Strategy for the four-plasmid expression systems. Plasmid pCDF1b-*rpoZ* harboring genes for the *M. tuberculosis* ω-subunit was incorporated into *E. coli* harboring the three-plasmid expression system shown in Fig. 1A, 2A, and 3A. (B) SDS-PAGE analysis of expression of *M. tuberculosis* RNAP with and without the ω-subunit in an *in vivo* reporter assay. (C) Results for recombinant *M. tuberculosis* reporter assays in the presence (+) or absence (−) of the ω-subunit. Fold changes in mCherry fluorescence of *E. coli* BL21(DE3) cells with respect to cells without *M. tuberculosis* RNAP expression are shown. (Left) *M. tuberculosis* RNAP-σᴬ holo; the plasmid used was pFPVmCherry-*sinP3*. (Middle) *M. tuberculosis* RNAP-σᴱ holo; the plasmid used was pFPVmCherry-*sigBpr*. (Right) *M. tuberculosis* CRP and *M. tuberculosis* RNAP-σᴬ holo. The plasmid used was pFPVmCherry-*WhiB1*-CRP. The data represent the means of three replicates, and error bars represent standard deviations.

plasmid expression system (Fig. 4A). To make sure that ω was incorporated into *M. tuberculosis* RNAP, we purified Ni-NTA chromatography on RNAP from the *E. coli* cells harboring the above-described four-plasmid expression system, in which the σᴬ of *M. tuberculosis* RNAP was His tagged. The subunit composition of the affinity-purified RNAP by using SDS-PAGE and Coomassie staining confirmed the presence of ω in RNAP (Fig. 4B). The assay results suggested that *E. coli* cells can accommodate up to four compatible plasmids simultaneously. Comparing the data of the reporter assays in the presence and absence of ω in each of the above-described three cases (for σᴬ, σᴱ, and CRP), we observed no significant changes in the promoter activities (in the presence of ω, mCherry fluorescence increased by 5 to 10%, which was within error limit) (Fig. 4C). This result was consistent with our previous findings and also with the results of *in vitro* transcription assays (see Fig. S3 in the supplemental material) indicating that the ω-subunit of *M. tuberculosis* may not play any role in RNAP assembly or the transcriptional activity of the enzyme.

DISCUSSION

The reporter assay remains an important tool in biological research for monitoring the functions of transcriptional factors in a biological network (37–40). However, the development of a reporter assay in *M. tuberculosis* has its limitation, since the preparation of knockout or knockdown variants of the species is difficult because it requires handling of pathogenic bacteria that also have an intrinsically slow growth rate (11–14). Here, we have described a novel recombinant reporter assay that could provide a rapid and efficient avenue for monitoring the interactions among *M. tuberculosis* RNAP, σ factors, transcription factors, and their cognate promoters *in vivo* without the hazard of handling the pathogenic slow-growing bacterium.

The reporter assay has been validated for the principal σ factor, a representative alternative σ factor, a transcriptional regulator from *M. tuberculosis*, σᴱ, and CRP. Therefore, we propose that this novel recombinant reporter assay could be used to assess the ability of RNAP to interact with other *M. tuberculosis* σ factors and/or transcriptional regulators for transcriptional regulation at *M. tuberculosis* promoters *in vivo*. Using the assay with *M. tuberculosis* CRP, we have been able to show that the hypothesis made by Bai et

al. (28) is indeed true. Our assays showed that the functional interaction of *M. tuberculosis* CRP is restricted to *M. tuberculosis* RNAP, not with *E. coli* RNAP. The assay results in the presence of the ω-subunit support our previous finding that the ω-subunit may not be involved *in vivo* assembly of *M. tuberculosis* RNAP. However, the development of the assay to include incorporation of ω might be useful for monitoring the function of a transcriptional regulator(s) in cases where the presence of ω in RNAP is essential for the function of the regulator(s). The assay performed in *E. coli* may not show the same level of promoter activity as when performed in *M. tuberculosis*, due to different micromilieus within the two bacteria. Nonetheless, any level of promoter activity in this recombinant assay validates the interactions of the promoter with the transcriptional regulator. Also, performance of the recombinant assay in *E. coli* would be much less time-consuming than in *M. tuberculosis*. In addition, the *in vivo* assays involving functional analysis of transcriptional regulators in the knockout host might be often misrepresented due to cross talk among regulators that have overlapping functions. Anticipating that there are no significant functional overlaps between *E. coli* transcriptional regulators and their counterparts in *M. tuberculosis*, as observed by us with *M. tuberculosis* CRP, this recombinant assay would be free from the influence of other interfering *M. tuberculosis* transcriptional regulators. Although we cannot rule out the possibility of interference of the *E. coli* RNAP with *M. tuberculosis* promoters and transcriptional regulators, this interference could easily be detected in a control experiment where reporter assays are performed in the absence of *M. tuberculosis* RNAP or transcriptional regulators, as shown in the assays here.

We envisage that this assay could be further used to screen/identify promoter elements specific to a particular *M. tuberculosis* σ factor or transcriptional regulator by inserting 200 to 300 bp of random *M. tuberculosis* genomic DNA fragments in the promoter region of the mCherry structural gene (in place of the *sinP3* promoter element [Fig. 1]). If a DNA fragment contains a promoter element for the σ factor (or transcriptional regulator), mCherry expression will result in a fluorescent bacterial colony. The DNA sequence of the promoter region of the mCherry gene in fluorescent bacteria would identify the promoter element (this work is in progress).

Overall, the recombinant reporter assay would be useful in

establishing the interaction of *M. tuberculosis* RNAP with its cognate promoters in association with any σ factors and/or transcriptional regulators and thus help increase the understanding of the mechanism of gene regulation by these factors *in vivo*. Although the assay was developed to monitor the function of transcriptional regulators of *M. tuberculosis*, the approach could be expanded for other slow-growing pathogenic bacterial systems. This novel reporter assay is the first example in which the host transcriptional machinery was not used for expression of the reporter gene. Since the assay employs recombinant transcriptional machinery, the technique could be used to develop synthetic circuits to assess biological pathways of other bacteria.

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