Chapter 4

Development of a mCherry fluorescence based reporter assay
4.1 Introduction

Reporter assay is an invaluable tool in biological research to monitor the important cellular events involving gene functions (Barker et al., 1998, Carroll et al., 2010, Chalfie et al., 1994), protein-protein (Kanno et al., 2006), protein-DNA interactions (Carroll & James, 2009), protein localizations (Jiang et al., 2008), signal transduction pathways (Durocher et al., 2000) etc in vivo. In these assays, the amount of signal flow generated due to a cellular event induces the expression of a reporter gene. Hence the reporter gene assay represents a quantitative elucidation of the cellular event (Andreu et al., 2010). Two types of reporter assays are widely used: (i) Substrate-specific reporter assay: This type of reporter assay needs the addition of an external substrate for detection of interactions. like β-galactosidase (β-gal) assay (Timm et al., 1994), Chloramphenicol Acetylase (CAT) assay (Das Gupta et al., 1993), Alkaline Phosphatase (AP) assay, Catechol Deoxygenase (XylE) assay (Curcic et al., 1994) and Luciferase (Lux) assay (Jacobs et al., 1993, Riska et al., 1999). (ii) Bioluminescent protein based reporter assay: This type of reporter assay do not need addition of substrate for detection of promoter activity, rather the gene of interest is cloned in fusion to a fluorescent protein. Positive interaction between the promoter and the transcriptional factor leads to translational of the bioluminescent protein and the amount of this protein produced is a direct measurement of the interaction. Although mainly types of bioluminescent proteins are used, the most widely used is the Green Fluorescent Protein (GFP) (Chalfie et al., 1994) and its modified derivatives (Delagrave et al., 1995), Cyan Fluorescent Protein (CFP) (Shaner et al., 2004), Yellow Fluorescent Protein (YFP) (Shaner et al., 2004) etc.

To study the function of a transcriptional regulator by a reporter gene assay, the first step is to prepare a mutant strain either by removing the gene encoding the regulator protein to ensure no-expression of the regulator (knockout strain) (Dahl et al., 2003, Ehrト Schnappinger, 2006, Parish et al., 2003, Rickman et al., 2005) or by replacing the endogenous promoter element of the regulator gene by an inducible exogenous promoter to ensure minimum level of endogenous protein in absence of any inducer molecule (knockdown strain) (Guo et al., 2007, Kamionka et al., 2005). A plasmid borne exogenous reporter gene under the control of regulator protein is successively inserted into the mutant strains. As the expression of reporter gene depends on the amount of regulator protein, the assay with the mutant strains, lacking the expression of the regulator protein, would result in low level of expression of the reporter gene and serve as the background. However, when the regulator protein is produced in the mutant cells either by inserting a plasmid harbouring the same regulator gene (in knockout strain) or by supplying the inducer molecule (for knockdown
strain), the high level of expression of the reporter gene would interpret the successful manifestation of the regulator promoter interactions in vivo. These assays have been widely used to understand gene regulatory networks across an array of organisms. However, a very few successful endeavours involving the reporter gene assay have been made in Mycobacterium tuberculosis to study the function of its promoters or regulatory proteins. The wide manifestation of the reporter gene assay in M. tuberculosis is extremely tedious and time consuming because of the slow growing nature of the mycobacteria (Stahl & Urbane, 1990). Assuming the transcriptional apparatus of Mycobacterium smegmatis is similar to that of M. tuberculosis, attempts were made to develop the recombinant reporter gene assay to study the functions of M. tuberculosis promoters in M. semgmatis (Curcic et al., 1994, Das Gupta et al., 1993, Timm et al., 1994). These attempts have enjoyed limited success (only 5-10% of the promoters in entire M. tuberculosis genome exhibit activity in M. smegmatis (Timm et al., 1994)), leaving a doubt on the validity of using the transcriptional machinery of M. smegmatis as a model machinery for M. tuberculosis.

mCherry, a fluorescent protein, was discovered by Roger Y Tsien in 2004. It is a monmeric protein isolated from slime mould, Discosoma species (Shaner et al., 2004). Since its discovery, mCherry have become a preferred choice over GFP as a marker. The rationales for choosing mCherry as a marker for studying gene expression are as follows: (i) Unlike GFP, the fluorescent signal from mCherry has a greater half-life. It is extremely photostable. Bacterial colonies expressing mCherry can be seen red in colour for over a month. (ii) It is non-toxic to most of the strains of Escherichia coli. As a result, it does not interfere with the kinetics of transcription factor with its cognate promoter. (iii) It performs excellent in fusion clones to determine interactions of transcriptional factors with their cognate promoters. Since most of the reporter assays in the recent times have made use of mCherry as a marker for studying gene regulation, I have chosen the same molecule to develop a reporter assay using transcriptional machinery of M. tuberculosis in E. coli.

Taking the advantage of M. tuberculosis RNA polymerase assembly in E. coli (Chapter 2), I have developed a mCherry reporter assay that has enabled us to monitor the interaction of M. tuberculosis RNA polymerase with its promoter in vivo. The assays involve three-plasmid expression system in E. coli, wherein, the two plasmids will be responsible for M. tuberculosis (Mtb) RNAP production and the third plasmid will harbour the mCherry reporter gene expression cassette under the control of either a sigma factor or a transcriptional regulator dependent promoter element. This reporter assay has been validated for the principal sigma factor $\sigma^A$, for an alternative sigma factor, $\sigma^E$ and for a transcriptional regulator, CRP (cyclic AMP receptor protein) (Bai et al., 2005) from M. tuberculosis.
Figure 4.1: Basic principle of mCherry fluorescence based reporter assay:

Left Figure: *E. coli* cell containing pFPV-mCherry, in which Mtb σA specific promoter *sinP3* is inserted. In absence of Mtb RNAP, the native polymerase will not be able to bind to the promoter and as a result, there will be no mCherry expression.

Right Figure: *E. coli* cell in which the recombinant Mtb RNAP holo is produced from the dual plasmids. The individual subunits of RNAP will ensemble in cell and bind to *sinP3* to initiate transcription from its cognate promoter. The fusion mRNA when translated produces mCherry protein.
4.2 Results

4.2.1 Reporter assay with $\sigma^A$-RNAP holo: Strategy

Using this dual plasmid expression system in *E. coli* (discussed in details in Chapter-2), I have developed a recombinant reporter assay in *E. coli* (Ec) that enabled us to monitor the interactions of Mtb RNAP with its cognate promoter in vivo. For this assay, I have used a three plasmid expression system in *E. coli*: the dual plasmid system for in vivo expression of Mtb RNAP holo enzyme and a third plasmid for Mtb $\sigma^A$ promoter dependent mCherry expression (Figure 4.1, left panel). In the third plasmid, I have introduced a Mtb $\sigma^A$ dependent promoter element sinP3 (Jacques et al., 2006) or rrnB1 (Verma et al., 1999) at the promoter region of mCherry structural gene in pFPV-mCherry vector (Addgene, USA) (Carroll et al., 2010). In principle, when Mtb RNAP holo is expressed in *E. coli*, it would bind to the sinP3 (or rrnB1) promoter, and induce mCherry expression. Since the assay is being performed in *E. coli*, there is a possibility that the endogenous Ec RNAP holo enzyme, or Ec RNAP core enzyme in association with Mtb $\sigma^A$, or Mtb RNAP core in association with Ec $\sigma^70$ induce mCherry expression from the $\sigma^A$ dependent promoter. To test the above possibilities, I have performed in vitro transcription assays with Mtb promoters and all above three RNAP or RNAP combinations (Figure 4.1, right panel). The results show that neither Ec RNAP holo enzyme, nor Ec RNAP core enzyme in association with Mtb $\sigma^A$, and Mtb RNAP core in association with Ec $\sigma^70$ can produce transcript from the Mtb promoters, whereas the Mtb RNAP is able to initiate transcription from these promoters. The results indicate that the *E. coli* RNAP, and/or $\sigma^70$ would not interfere with the reporter assay.

4.2.2 Assay results: Reporter assay with $\sigma^A$-RNAP holo-sinP3

As expected, when pFPV mCherry-sinP3 plasmid was introduced in *E. coli* cells, I have observed a low amount of mCherry expression which sets the background level, suggesting non-interaction of Ec RNAP with sinP3 promoter. Similarly, when the Mtb RNAP core was expressed in *E. coli* containing pFPV mCherry-sinP3 plasmid, I have observed a 2 fold increase in the fluorescence signal than the background control, suggesting non-specific induction of mCherry expression by Mtb RNAP core. However, when Mtb RNAP holo was expressed in *E. coli* containing pFPV mCherry-sinP3 plasmid hrs, I have observed a 10 fold increase in the fluorescence signal over the control (Figure 4.2c, left panel). In all the above events, the mCherry fluorescence was monitored by growing the cells at 37°C for 6 hrs. When the growth temperature of the above system was lowered from 37°C to 16°C (for 16 hrs), I have observed a 16 fold increase in the fluorescence signal than the control (Figure 4.2c, right panel). This is in line with our expectation that the yield of RNAP holo increases at lower temperature resulting in higher induction of mCherry expression. It is important to
note that the growth conditions of assays mentioned above (37°C for 6 hrs or 16°C for 16 hrs after induction) were the conditions that provided the best signal to background ratio (Figure 4.3). To exclude the possibility of association of Mtb $\sigma^A$ with Ec RNAP core to induce the mCherry expression \textit{in vivo}, I have performed the above assay by introducing a plasmid containing the gene for Mtb $\sigma^A$ in the mCherry expression system. In this assay, the Mtb $\sigma^A$ along with \textit{E. coli} RNAP core could induce the mCherry expression to some extent, comparable to the mCherry expression by Mtb RNAP core and close to the background level.

\textbf{Figure 4.2: Recombinant \textit{in vivo} Mtb reporter assay using Mtb $\sigma^A$ and its specific promoter \textit{sinP3}:}

(a) Strategy for recombinant Mtb promoter activity assay: three plasmid expression system, dual plasmid (first two plasmids) for expression of Mtb RNAP holo and the third plasmid containing a $\sigma^A$ dependent promoter element \textit{sinP3} for expression of mCherry. All three plasmids were transformed in \textit{E. coli} BL21 (DE3) cells and then the cells were grown at 37°C up to OD (at 595 nm) 0.4 and induced with 0.5 mM IPTG at different growth conditions.

(b) \textit{In vitro} transcription assay to assess the ability of Mtb RNAP to initiate transcription on Mtb $\sigma^A$ dependent promoter fragment \textit{sinP3} were used. The results showed that the Mtb RNAP core was unable to produce transcript from \textit{E. coli} promoter either with Mtb $\sigma^A$ or with \textit{E. coli} $\sigma^70$. Lane 1: control = Mtb RNAP core + \textit{sinP3}, Lane 2: Mtb RNAP core +Mtb $\sigma^A$+\textit{sinP3}, Lane 3: Mtb RNAP core + \textit{E.coli} $\sigma^70$+\textit{sinP3}, Lane 4: \textit{E. coli} RNAP core+\textit{E.coli} $\sigma^70$+\textit{sinP3}, Lane 5: \textit{E. coli} RNAP core+ Mtb $\sigma^A$ + \textit{sinP3}

(c) Results for recombinant Mtb reporter assays: mCherry fluorescence of \textit{E. coli} cells; control: pFPV-mCherry-\textit{sinP3} plasmid; RNAP core: pFPV-mCherry-\textit{sinP3} plasmid along with dual plasmid for RNAP core expression (pAcYc Duet \textit{rpoA} + pCOLA Duet \textit{rpoB-rpoC}); RNAP holo: All three plasmids
σA: pFPV-mCherry-sinP3 plasmid along with plasmid for σA expression (pAcYc Duet-sigA). The data represents the mean of three replicates and error bars represent standard deviations. Left panel: cells were induced with 0.5 mM IPTG at 37°C for 6 hrs. Right panel: cells were induced with 0.5 mM IPTG at 16°C for 16 hrs.

**Figure 4.3:** *In vivo* recombinant Mtb reporter assay: Dependence of mCherry expression as a function of *E. coli* growth:

(a) Fluorescent intensity of mCherry was measured at regular intervals and plotted as a function of OD. Fluorescent intensity gets saturated at OD (595) = 1.5. Red Line= pFPVmCherry sinP3, Green Line= RNAP core + pFPVmCherry sinP3, Pink line= Mtb σA + pFPVmCherry sinP3, Blue line= RNAP-holo + pFPVmCherry sinP3. Assays were conducted at 37°C for 6 hours.

(b) Same as (a), assays were conducted at 16°C for 16 hours. The data represents the mean of three replicates and error bars represent standard deviations.

I have observed increased mCherry fluorescence when the assay was conducted at 16°C compared to 37°C. In order to determine its plausible reason, Fluorescence Activated Cell Sorting (FACS) was conducted with cell cultures induced at both the temperature. mCherry fluorescence from *E. coli* cells containing pFPV-mCherry sinP3 was set as a benchmark for background fluorescence (Figure 4.4). About 40% - 50% of *E. coli* cells expressing RNAP holo at 37°C showed mCherry fluorescence above the background level, whereas at 16°C, the proportion of cells increased to 80% - 90%. There was no significant difference in the mean fluorescence from individual cell at both the temperatures of expression. About 10% - 15% cells expressing Mtb RNAP core contributed to mCherry fluorescence.
Aliquots of cells from above assay were scanned at mCherry (610 nm) and Cy7 (760 nm) fluorescence channels; first panel: control: pFPV-mCherry-\textit{sinP3} plasmid; second panel: control: pFPV-mCherry-\textit{sinP3} plasmid along with Mtb RNAP core; third panel: all three plasmids as shown above, protein induction at 37°C; fourth panel: all three plasmids, protein induction at 16°C. Number of fluorescent cells in the fourth panel is approximately twice than that of third panel.

4.2.3 Reporter assay with $\sigma^A$-RNAP holo-\textit{rrnB1}

When the reporter assay was conducted with another Mtb $\sigma^A$ specific promoter \textit{rrnB1}, the results followed the same trend as that of \textit{sinP3} (Figure 4.5). As expected, when pFPV mCherry-\textit{rrnB1} plasmid was introduced in \textit{E. coli} cells, I have observed a low amount of mCherry expression which sets the background level, suggesting non-interaction of Ec RNAP with \textit{rrnB1} promoter. Similarly, when the Mtb RNAP core was expressed in \textit{E. coli} containing pFPV mCherry-\textit{rrnB1} plasmid, I have observed a 2 fold increase in the fluorescence signal than the background control, suggesting non-specific induction of mCherry expression by Mtb RNAP core. However, when Mtb RNAP holo was expressed in \textit{E. coli} containing pFPV mCherry-\textit{rrnB1} plasmid for 6 hrs, I have observed a 12 fold increase in the fluorescence signal over the control (Figure 4.5c, left panel). In all the above events, the mCherry fluorescence was monitored by growing the cells at 37°C for 6 hrs. When the growth temperature of the above system was lowered from 37°C to 16°C (for 16 hrs), I have observed a 16 fold increase in the fluorescence signal than the control (Figure 4.5c, right panel).
Figure 4.5: Recombinant *in vivo* Mtb reporter assay using Mtb σ^A^ and its specific promoter *rrnB1*:

(a) Strategy for recombinant Mtb promoter activity assay: same as Figure 1 except the third plasmid containing a σ^A^ dependent promoter element *rrnB1* for expression of mCherry. All three plasmids were transformed in *E. coli* BL21 (DE3) cells and then the cells were grown at 37°C up to OD (at 595 nm) 0.4 and induced with 0.5 mM IPTG at different growth conditions. (b) Cloning of *rrnB1* in pFPV-mCherry: Lane1: pFPV-mCherry-*rrnB1* digested with *KpnI* & *BamHI*, Lane 2: undigested plasmid, Lane 3: 1 kb DNA ladder. (c) Results for recombinant Mtb reporter assays: mCherry fluorescence of *E. coli* cells; control: pFPV-mCherry-*sinP3* plasmid; RNAP core: pFPV-mCherry-*sinP3* plasmid along with dual plasmid for RNAP core expression (pAcYc Duet *rpoA* + pCOLA Duet *rpoB-rpoC*); RNAP holo: All three plasmids (in Figure 5a); σ^A^: pFPV-mCherry-*sinP3* plasmid along with plasmid for σ^A^ expression (pAcYc Duet-*sigA*). The data represents the mean of three replicates and error bars represent standard deviations. Left panel: cells were induced with 0.5 mM IPTG at 37°C for 6 hrs. Right panel: cells were induced with 0.5 mM IPTG at 16°C for 16 hrs.
4.2.4 Reporter assay with $\sigma^E$-RNAP holo

In order to test whether the reporter assay could be used to monitor the interaction of alternative sigma factors with cognate promoter, I have conducted the assay with a representative Mtb alternative sigma factor, $\sigma^E$ (Figure 4.6) on its promoter $\text{sigBpr}$ (Song et al., 2008). When pFPVmCherry-$\text{sigBpr}$ plasmid was introduced either in $E.\ coli$ cells or $E.\ coli$ cells expressing Mtb RNAP core, I have observed the mCherry expression at a background level, suggesting non-interaction of either Ec RNAP or Mtb RNAP core with $\text{sigBpr}$ promoter. However, when Mtb RNAP-$\sigma^E$ holo was expressed in $E.\ coli$ containing pFPVmCherry-$\text{sigBpr}$ plasmid at 16°C for 12 hrs, I have observed a 4 fold increase in the fluorescence signal than the control (Figure 4.6b). The results indicate that the assay could be used with the alternative sigma factor.

**Figure 4.6: Recombinant in vivo Mtb reporter assay using Mtb $\sigma^E$ and its specific promoter-\text{sigBpr}.**

(a) Strategy for recombinant Mtb promoter activity assay: same as figure 2 (a) except $\text{sigA}$ is replaced by $\text{sigE}$ in dual plasmid system and $\text{sinP3}$ is preplaced by $\text{sigBpr}$ in pFPVmCherry. (b) Results for recombinant Mtb reporter assays: mCherry fluorescence of $E.\ coli$ cells; control: pFPV-mCherry-\text{sigBpr} plasmid; RNAP core: pFPV-mCherry-\text{sigBpr} plasmid along with dual plasmid for RNAP core expression; RNAP-$\sigma^E$ holo: All three plasmids (in Figure 4.6a); $\sigma^E$: pFPV-mCherry-\text{sigBpr} plasmid along with plasmid for $\sigma^E$ expression (pAcYc Duet-$\text{sigE}$). Cells were induced with 0.5 mM IPTG at 16°C for 16 hrs. The data represents the mean of three replicates and error bars represent standard deviations.
4.2.5 Reporter assay with the Mtb transcriptional regulator CRP

In order to access whether the reporter assay could be used to monitor the interaction of Mtb transcriptional regulator, I have performed the assay with a representative Mtb transcriptional activator, CRP (cAMP Receptor Protein) (Figure 4.7). For this assay, I have cloned Mtb CRP dependent promoter WhiB1 (Smith et al., 2010, Stapleton et al., 2010) to generate pFPV-WhiB1-mCherry, in which Mtb CRP was subsequently cloned under an inducible promoter to finally generate pFPV-WhiB1-mCherry-CRP (Figure 4.7a). I have observed background fluorescence when pFPV-WhiB1-mCherry or pFPV-WhiB1-mCherry-CRP alone is expressed in *E. coli* indicating non interference of *E. coli* RNAP holo alone or in combination with Ec CRP or Mtb CRP on Mtb CRP dependent promoter (WhiB1). Similarly in absence of Mtb CRP even Mtb RNAP holo could not induce mCherry expression suggesting non interaction of Mtb RNAP holo with Ec CRP (Figure 4.7d). This result was further validated by the *in vitro* transcription results that showed Ec CRP was unable to induce transcription from the Mtb WhiB1 promoter in presence of either Mtb RNAP of Ec RNAP (Figure 4.8). When BL21 (DE3) cells containing pFPV-WhiB1-mCherry-CRP expressed Mtb RNAP holo at 16°C for 12 hours, I have observed a 5 fold increase in the fluorescence signal than the signal obtained with RNAP holo (Figure 4.7d). The results show that Mtb CRP is able to regulate its cognate promoter only in presence of Mtb RNAP, not in presence of Ec RNAP holo.

![Figure 4.7: Recombinant in vivo Mtb reporter assay using Mtb CRP and its specific promoter WhiB1:](image)

Strategy for recombinant Mtb promoter activity assay: three plasmid expression systems, dual plasmid (first two plasmids) for expression of Mtb RNAP holo (same as figure 4.1) and the third plasmid containing Mtb CRP dependent promoter element WhiB1 for expression of mCherry.

a. In pFPV- mCherry, WhiB1 was inserted in frame with mCherry.

b. In pFPV-WhiB1-mCherry, Mtb CRP was inserted under the control of T7 promoter (pFPV-WhiB1-mCherry).

d. Results for recombinant Mtb reporter assays: mCherry fluorescence of E. coli cells; control: pFPV-WhiB1-mCherry plasmid; Mtb CRP only: pFPV-WhiB1-mCherry-CRP; Mtb RNAP holo only: plasmids combination as shown in figure 4.7a upper panel; Mtb CRP and Mtb RNAP holo: plasmids combination as shown in figure 4.7a lower panel. Cells were induced with 0.5 mM IPTG at 16°C for 16 hrs. The data represents the mean of three replicates and error bars represent standard deviations.

Figure 4.8: In vitro transcriptional activity assay to study the interactions of Mtb and E. coli RNAP and CRP on WhiB1 promoter:

100 nM Mtb RNAP holo enzyme was incubated with 200 and 400 nM of Mtb CRP. Run-off transcripts of 125 nucleotides (nt) were produced using the WhiB1 promoter DNA fragments. Lane 1: Mtb RNAP holo + WhiB1, Lane 2: Mtb RNAP holo + WhiB1 + 400 nM Mtb CRP +400µM cAMP, Lane 3: Mtb RNAP holo + WhiB1 + 400 nM E. coli CRP +400 µM cAMP, Lane 4: E. coli RNAP holo + WhiB1+ 400 nM E. coli CRP +400µM cAMP, Lane 5: E. coli RNAP holo + WhiB1+ 400 nM Mtb CRP +400µM cAMP. The data represents the mean of three replicates and error bars represent standard deviations.
4.3 Discussion

Reporter assay remains an important tool in biological research to monitor the functions of transcriptional factors in a biological network across an array of organisms (Jiang et al., 2008) (Barker et al., 1998, Dye et al., 2002, Lucas & Lee, 2000, Mansson et al., 2007a, Mansson et al., 2007b). It is used as a tool to understand the role of different transcriptional factors by dissecting out their interactions with the cognate promoters in the response to various environmental cues and thereby uncover the processes that govern the pathogenicity of the bacteria. (Jacobs et al., 1991, Collins et al., 1998, Curcic et al., 1994, Das Gupta et al., 1993, Sarkis et al., 1995, Wood & DeLuca, 1987, Barker et al., 1998, Chalfie et al., 1994). The development of a reporter assay in Mycobacterium tuberculosis has been limited by the preparation of knockout or knockdown variants of the cell due to inherent difficulty of handling the pathogenic bacteria along with its intrinsic slow growth rate. Assuming the transcriptional apparatus of Mycobacterium smegmatis is similar to that of M. tuberculosis, attempts were made to develop the recombinant reporter gene assay [substrate specific reporter assays like β-galactosidase (lacZ) (Timm et al., 1994), chloramphenicol acetylase (CAT) (Das Gupta et al., 1993), catechol deoxygenase (XylE) (Curcic et al., 1994), luciferase (Lux) (Jacobs et al., 1993, Riska et al., 1999) or with the bioluminescent marker based reporter assay like green fluorescent protein (GFP) (Kremer et al., 1995, Collins et al., 1998, Chalfie et al., 1994) and its modified derivatives (Delagrave et al., 1995, Heim et al., 1994)] to study the functions of M. tuberculosis promoters in M. smegmatis (Curcic et al., 1994, Das Gupta et al., 1993, Timm et al., 1994). These attempts have enjoyed limited success (only 5-10 % of the promoters in entire M. tuberculosis genome exhibit activity in M. smegmatis (Timm et al., 1994), leaving a doubt on the validity of using the transcriptional machinery of M. smegmatis as a model machinery for M. tuberculosis to identify and measure the relative strength Mycobacterium tuberculosis promoter either in E. coli or in M. smegmatis.

Here, I have developed a novel reporter assay to monitor the activity of Mtb transcriptional factors or Mtb promoters in vivo in E. coli. The assay employs recombinant Mtb RNAP for the expression of the reporter gene. The Mtb RNAP was assembled in E. coli by using a dual plasmid expression system, co-expressing all Mtb RNAP subunits in the host (Banerjee R). The assay is then developed by introducing a third plasmid containing a Mtb promoter dependent mCherry expression system in E. coli, in addition to the dual plasmids for the Mtb RNAP holo enzyme expression. In this assay, I have observed a significant increase in mCherry expression only when the Mtb holo enzyme is produced within the host, not in the presence of Mtb RNAP core or Mtb σ^A alone or host RNAP. This reporter assay has been further validated for a representative alternative sigma factor and a transcriptional regulator from M. tuberculosis, σ^E and CRP respectively. I have observed an increase in fluorescence intensity with the decrease in the temperature of RNAP expression (37⁰C to 16⁰C) while conducting the reporter assay with Mtb σ^A dependent promoter sinP3. Similar trend was
also observed in case of *rrnB1* promoter. There are can be two possible reasons leading to higher fluorescence intensity at lower temperature of induction: Each cell is producing greater fluorescence at lower temperature of induction, which cumulatively gives rise to the enhanced fluorescence, or greater proportions of cells are contributing to the fluorescence at lower temperature of induction without any change in the mean fluorescence from individual cell. In order to understand, which one of the above mechanisms is responsible for higher fluorescence intensity, Fluorescence Activated Cell Sorting (FACS) was conducted. Our results suggest that at lower temperature of induction, greater proportion of cells are contributing to the fluorescence than at higher temperature of induction, without any change in the mean of fluorescence from individual cells. This may be due to the fact that lowering the temperature increases the amount of recombinant protein inside the cell and decreases the stochasticity of its interactions with their cognate promoter which ultimately translates into their fluorescent signals.

*In vitro* transcription assays were conducted for determination of cross reactivity between *E. coli* and *M. tuberculosis* transcriptional machinery on the sets of promoter used for the *in vivo* reporter assay. In all the promoters, I have observed that neither *E. coli* RNAP holo nor *E. coli* RNAP alone or in association with *M. tuberculosis* sigma factors or transcriptional regulators could initiate transcription from these sets of promoters. This result along with the result that revealed the lack of *E. coli* RNAP subunit contamination in the recombinant *M. tuberculosis* RNAP, which was expressed in *E. coli*, lead us to believe the non significant interaction occurs among transcriptional machinery of *Mycobacterium tuberculosis* and *E. coli*. The greater fluorescence intensity (12-16 fold compared to control) observed in case of *M. tuberculosis* σ^A^ dependent promoters (*sinP3* and *rrnB1*) compared to σ^E^ dependent promoter (4 fold compared to control) could be attributed to lower binding affinity of alternative sigma factor σ^E^ to its cognate promoter (*sigBpr*), compared to the primary sigma factor σ^A^ of *M. tuberculosis*. Previous studies suggest that the primary sigma factor can associate (higher binding affinity relative to the alternative sigma factors) with RNAP core to initiate the process of transcription from its cognate promoter. Therefore, I propose that this novel recombinant reporter assay could be used to assess the ability of RNAP to interact with any Mtb σ factor and/or transcriptional regulator in order to activate transcription from Mtb promoter *in vivo*. The major advantage of this recombinant method is that these interactions could be monitored without handling the pathogenic *M. tuberculosis* bacteria. So this method nullifies the tedium generation of *M. tuberculosis* knockout strains in which the concerned promoter elements or the transcriptional regulators including σ are deleted (Dahl et al., 2003, Ehrt & Schnappinger, 2006, Parish et al., 2003, Rickman et al., 2005). In addition, the *in vivo* assays involving functional analysis of transcriptional regulators in the knockout host could be mis-represented due to cross-talk among the regulators having overlapping functions. Anticipating there are no significant functional overlaps between *E. coli* transcriptional regulators and their counterparts in *M. tuberculosis*, as observed in case of Mtb CRP, this recombinant assay would be free from the influence of other interfering Mtb transcriptional
regulators. On the other hand, the recombinant assay being performed in *E. coli* would be much less time consuming than in *M. tuberculosis*. Therefore, I envisage that this assay could be further used to screen/identify promoter elements specific to a particular Mtb σ factor or transcriptional regulator by inserting 200-300 bps random Mtb genomic DNA fragments in the promoter region of mCherry structural gene (in place of *sinP3* promoter element in Figure 1). If a DNA fragment contains a promoter element for the σ factor (or transcriptional regulator) and is in frame with the mCherry structural gene, the mCherry expression would result in a fluorescent bacterial colony. The DNA sequence of the promoter region of mCherry gene in the fluorescent bacteria would identify the promoter element (work in progress).

Overall, the recombinant reporter assay would be useful to establish the interaction of Mtb RNAP with its cognate promoters in association with any sigma factors and/or transcriptional regulators and thus understand the mechanism of gene regulation by these factors *in vivo*. Although the assay has been developed to monitor the function of transcriptional regulators of *M. tuberculosis*, the approach could be expanded for other bacterial systems. This novel reporter assay is the first example where the host transcriptional machinery was not used for the expression of the reporter gene. Since the assay employs the recombinant transcriptional machinery, the technique could be used to develop synthetic circuits for a biological pathway of other bacteria.
4.4 Materials and Methods

4.4.1 Cloning strategies

The genes encoding different RNAP subunits were amplified either from genomic DNA of *M. tuberculosis* H37Rv (kind gift from ATCC, USA) or from or plasmids (kind gift from Dr Rodrigue, University de Sherbrooke, Canada) (Jacques *et al.*, 2006) encoding the same gene using suitable primers (*Table 6*) and cloned in different Duet vectors using appropriate enzymes (*Table 7*). *sigA* and *sigE* were amplified from genomic DNA and cloned in pAcYc Duet (*Table 7*). Mtb CRP (Rv3676) was amplified from genomic DNA of *M. tuberculosis,* cloned first in pET28a and subsequently in pFPVmCherry (*Table 7*). *sinP3* promoter DNA was amplified from synthetic oligonucleotide template and cloned in pFPVmCherry (*Table 6,7*). *rrnB1, sigBpr* and *WhiB1*were amplified from genomic DNA of *M. tuberculosis* H37Rv (*Table 6,7*) and cloned in pBluescript II SK (+) by blunt end ligation and subsequently in pFPV mCherry (*Table 6*).

Table 6: Oligonucleotides used in the study:

<table>
<thead>
<tr>
<th>Name of the primer</th>
<th>DNA sequences</th>
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<tbody>
<tr>
<td><strong>pFPV-mCherry</strong></td>
<td>GAATTCCAGCCAGAAGTCATACCGTA</td>
</tr>
<tr>
<td><strong>sinP3 fwd</strong></td>
<td>CCGAAATTAATACGACTCACTATAGGGG</td>
</tr>
<tr>
<td><strong>sinP3 rev</strong></td>
<td>TTAAAGCTTTTACCTCGCTCGGCGG</td>
</tr>
<tr>
<td><strong>rrnB1 fwd</strong></td>
<td>TCTGGTACCTCGTGAGAGACCTGGTAGTC</td>
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<tr>
<td><strong>rrnB1 rev</strong></td>
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</tr>
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<td><strong>sigBpr fwd</strong></td>
<td>CGTCTGTGGCCGGCG</td>
</tr>
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<td><strong>sigBpr rev</strong></td>
<td>CCGTGTTGGCTGACATCGG</td>
</tr>
<tr>
<td><strong>BSII- WhiB1 fwd</strong></td>
<td>GCCTCTAGAGCAAGAAAAGCGATCTG</td>
</tr>
<tr>
<td><strong>BSII- WhiB1 rev</strong></td>
<td>GCAAGCTTGCCCTTGCTCCGGAACATC</td>
</tr>
<tr>
<td><strong>ET 28a-Mtb CRP fwd</strong></td>
<td>CATCCGGAATTCGTTGAGACGATCCCTGGCC</td>
</tr>
<tr>
<td><strong>ET 28a-Mtb CRP rev</strong></td>
<td>CATCATACTCGAGCAGTGTCGCTCGTCG</td>
</tr>
<tr>
<td><strong>ET 28a-E. coli CRP fwd</strong></td>
<td>CGAACCAGTATGCTGCTTGCAAAAAAGGCA</td>
</tr>
<tr>
<td><strong>ET 28a-E. coli CRP rev</strong></td>
<td>GCGTGCTGACAGAGACGAGTCGCTCCTGGG</td>
</tr>
<tr>
<td><strong>pFPV mCherry-CRP fwd</strong></td>
<td>CCCGCGAAATTAATACGACTCACTATAGGG</td>
</tr>
<tr>
<td><strong>pFPV mCherry-CRP rev</strong></td>
<td>TTAAGCTTTTACCTCGCTCGCGG</td>
</tr>
</tbody>
</table>
Table 7: Cloning strategies:

<table>
<thead>
<tr>
<th>Name of the plasmid of gene</th>
<th>Restriction enzymes used and cloning strategies</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCOLA rpoB-rpoC</td>
<td>rpoB-Ncol &amp; BamHI, rpoC-Ndel &amp; EcoRV</td>
<td>pET 16b-rpoB</td>
</tr>
<tr>
<td>pAcYc Duet rpoA-rpoZ</td>
<td>rpoA- Ncol &amp; BamHI; rpoZ- Ndel &amp; KpnI</td>
<td>Genomic DNA</td>
</tr>
<tr>
<td>pAcYc Duet rpoA- sigA</td>
<td>sigA- EcoRI &amp; NotI; rpoA- Ndel &amp; KpnI</td>
<td>Genomic DNA</td>
</tr>
<tr>
<td>pFPV-mcherry-sinp3</td>
<td>sinp3- EcoRI &amp; BamHI</td>
<td>Synthetic DNA</td>
</tr>
<tr>
<td>pFPV-mcherry-rrnb1</td>
<td>rrrnb1- KpnI &amp; BamHI</td>
<td>Genomic DNA</td>
</tr>
<tr>
<td>pFPV-mcherry-sigBpr</td>
<td>sigBpr- KpnI &amp; BamHI</td>
<td>Genomic DNA</td>
</tr>
<tr>
<td>pFPV-mcherry-WhiB1</td>
<td>WhiB1- KpnI &amp; BamHI</td>
<td>Genomic DNA</td>
</tr>
<tr>
<td>pFPV-mcherry-WhiB1-CRP</td>
<td>Rv3676- EcoRV &amp; Hind III</td>
<td>Genomic DNA</td>
</tr>
<tr>
<td>pAcYc Duet rpoA-sigE</td>
<td>rpoA:Ncol &amp; BamHI; sigE: Ndel &amp; KpnI</td>
<td>Genomic DNA</td>
</tr>
<tr>
<td>pET 28 a- Mtb CRP</td>
<td>Rv3676- EcoRI &amp; XhoI</td>
<td>Genomic DNA</td>
</tr>
<tr>
<td>pET 28 a- E. coli CRP</td>
<td>CRP- Ndel &amp; BamHI</td>
<td>Genomic DNA</td>
</tr>
<tr>
<td>pAcYc Duet- sigA</td>
<td>sigA- EcoRI &amp; NotI</td>
<td>Genomic DNA</td>
</tr>
<tr>
<td>pBluescript II Sk(+)WhiB1</td>
<td></td>
<td>Genomic DNA</td>
</tr>
<tr>
<td>pBluescript II Sk(+)rrnb1</td>
<td></td>
<td>Genomic DNA</td>
</tr>
<tr>
<td>pBluescript II Sk(+)sigBpr</td>
<td></td>
<td>Genomic DNA</td>
</tr>
</tbody>
</table>

4.4.2 Purification of proteins

4.4.2.1 Purification of M. tuberculosis RNAP and E. coli RNAP

In vivo assembled Mtb RNAP core, Mtb RNAP sigA holo and E. coli RNAP was purified essentially as discussed in details in chapter 1.

4.4.2.2 Purification of M. tuberculosis CRP

E. coli BL21 (DE3) containing pET28a-Rv3676 was grown in LB medium (containing Kanamycin, at a final concentration of 50 µg/ml) at 37°C, till OD_{600nm} reaches 0.6. Protein production was induced by addition of IPTG to a final concentration of 0.2mM followed by further growing them at 37°C, till OD_{600nm} reaches 1.5. Cells were harvested by centrifugation at 5000g for 20 min at 4°C. Cells were resuspended in resuspension buffer [50mM Tris-HCl,
(pH 7.9), 200mM NaCl, 10% Glycerol and 1mM PMSF). Cells were lysed by sonication (5-8 pulses, each pulse of 30 sec with 5 min interval between the pulses), supernatant was cleared by centrifugation at 10,000g for 30 min at 4°C and was loaded into Ni-NTA beads pre-
equilibrated with the resuspension buffer. The resin was next washed with 30 CV of resuspension buffer and 10 CV of resuspension buffer containing 20 and 40mM imidazole respectively. Protein was eluted out with 10ml resuspension buffer containing 250mM imidazole. The elution fractions containing the desired proteins were verified by running them in 10% SDS-PAGE and coomassie staining, pooled and subjected to anion exchange chromatography by Mono Q HR 10/10 column (GE Lifesciences, USA). The column was washed with 16 ml of buffer E {20mM Tris-HCl (pH 7.9), 200mM NaCl, 5% glycerol 0.1mM EDTA and 0.1mM DTT}. Protein was eluted out by setting a 160ml linear gradient between buffer E and buffer H [20mM Tris-HCl (pH 7.9), 1000mM NaCl, 5% glycerol 0.1mM EDTA and 0.1mM DTT]. The elution fractions containing the desired proteins were verified by running them on 10% SDS-PAGE and coomassie staining. Elution fractions containing Mtb CRP, free of any contamination (purity>95%) were concentrated in protein concentrator (GE Life sciences) having a molecular weight cut off (MWCO) of 9 KDa at 3000g, 4°C, to a final volume of 100 µl. It was thoroughly mixed with equal volume of pre-chilled 100% glycerol and stored in small aliquots at -80°C.

4.4.2.3 Purification of E. coli CRP

E. coli BL21 (DE3) containing pET28a-Ec CRP was grown in LB medium (containing Kanamycin, at a final concentration of 50 µg/ml) at 37°C, till OD₆₀₀nm reaches 0.6. Protein production was induced by addition of IPTG to a final concentration of 0.2mM. The cells were further grown at 37°C, till OD₆₀₀nm reaches 1.5. Cells were harvested by centrifugation at 5000g for 20 min at 4°C. Protein was purified essentially following the same protocol for purification of M. tuberculosis CRP (Section 4.4.2.2).

4.4.3 Preparations of templates for in vitro transcription assay

WhiB1 was amplified from pBluescript II SK (+)-WhiB1 using the primer pBSII-WhiB1 forward and pBSII-WhiB1reverse (Table 6). The cycle for amplification is as follows: 94°C for 2 min, 94°C for 30 sec, 55°C for 1 min, 72°C for 40 sec, and 72°C for 7 min. The total number cycles for amplification was 40.

sigBpr was amplified from pBluescript II SK (+)-SigBpr using the primer pBSII-SigBpr forward and pBSII-SigBpr reverse (Table 6). The cycle for amplification is as follows: 94°C for 2 min, 94°C for 30 sec, 55°C for 1 min, 72°C for 40 sec, and 72°C for 7 min. The total number cycles for amplification was 40.
E. coli CRP promoter was amplified from pBluescript II SK (+)-SigBpr using the primer pBSII-SigBpr forward and pBSII-SigBpr reverse (Table 6). The cycle for amplification is as follows: 94°C for 2 min, 94°C for 30 sec, 55°C for 1 min, 72°C for 40 sec, and 72°C for 7 min. The total number cycles for amplification was 40.

### 4.4.4 In vitro transcription assays with E. coli and Mycobacterium tuberculosis RNAP and principle Sigma Factors

In order to validate interactions between M. tuberculosis RNAP and E. coli sigma factors and vice versa, in vitro transcription assay was conducted with Mtb and E. coli RNAP core along with their cognate and interchanged sigma factors. sinP3 was used as promoter DNA. 100nM E. coli RNAP core was incubated either with 200nM of E. coli σ70 or with principal sigma factor of Mycobacterium tuberculosis: σA at 25°C for 30 min for RNAP holo formation. Open complex formation was initiated by addition of 40nM Mtb σA specific promoter (sinP3) to the above RNAP holo complex and incubated for 20 min at 37°C. For control, DNA was added to RNAP core instead of RNAP holo complex. Non specific RNAP-DNA complex formation was inhibited by addition of 1 μl heparin (0.5 mg/ml) to the reaction. RNA synthesis was conducted essentially similar to protocol described in section 2.4.7.1 of Chapter 2. Similarly, 100nM Mtb RNAP was incubated either with 200nM of its principle sigma factor σA or E. coli σ70. After holoenzyme formation, 40 nM sinP3 was added to both the holo polymerases (Mtb RNAP + σA or Mtb RNAP + σ70) and incubated for 20 min at 37°C. RNA synthesis was conducted essentially similar to protocol described in section 2.4.7.1.

### 4.4.5 In vitro transcription assay with between E. coli and Mycobacterium tuberculosis RNAP and cAMP Receptor Protein (CRP)

100nM E. coli or Mtb RNAP holo was incubated either with 400nM of E. coli CRP or with Mycobacterium tuberculosis CRP at 37°C for 20 min for RNAP holo-CRP formation. Open complex formation was initiated either by addition of 40nM of Mtb CRP specific promoter (WhiB1) to the above RNAP holo complex and incubated for 20 min at 37°C. For control DNA was added to RNAP holo instead of RNAP holo-CRP complex. Non specific RNAP-DNA complex formation was inhibited by addition of 1 μl heparin (0.5 mg/ml) to the reaction. RNA synthesis was carried out as described in section 2.4.7.1 of Chapter 2.

### 4.4.6 In vivo recombinant reporter assays

#### 4.4.6.1 Assays with Mtb σ specific promoter sinP3

For in vivo Mtb reporter assay, E. coli BL21 (DE3) strains were transformed with three plasmids as in figure 4.2. Co-transformed cells were grown in 50 ml LB broth at 37°C with respective antibiotics (Chloramphenicol 35μg/ml, Ampicillin 100μg/ml and Kanamycin
50μg/ml) up to OD (at 595nm) 0.4, induced by addition of 0.5 mM IPTG and were further grown at 37°C for 6 hours or at 16°C for 16 hours (till OD_{595nm} = 1.5). For control, the assays were performed with E. coli BL21 (DE3) strains harbouring the dual plasmid (pAcYc Duet rpoA-rpoZ + pCOLA Duet rpoB-rpoC) for RNAP core expression or plasmid pAcYc Duet-sigA for σ^{A} expression along with pFPVmcherry-sinP3 or pFPVmcherry-sinP3 alone. pAcYc Duet rpoA-sigA + pCOLA Duet rpoB-rpoC along with pFPVmcherry-sinP3 plasmid are used for Mtb holo expression. To optimize the bacterial growth duration at which the fluorescence signal to background ratio is highest, I have monitored the OD and fluorescence intensities of the bacterial cultures at regular intervals. 100 μl aliquots of the cultures were taken out and its O.D (at 595 nm) and fluorescence intensities (Excitation wavelength: 532nM, Emission Wavelength: 610nM) were measured at each interval. The normalized fluorescence intensity (fluorescence intensity/OD) data were plotted against nearest single decimal O.D (for example X is the fluorescence intensity of bacterial culture of O.D 0.37. So the normalized fluorescence intensity is X x 0.4/0.37 was plotted against O.D 0.4). The assays were repeated thrice and the mCherry expression kinetic curves were plotted (Figure 4.3). The duration of bacterial growth at which the optimum fluorescence signal to background ratio were obtained, were observed either at 37°C for 6 hours or at 16°C for 16 hours

4.4.6.2 Assays with Mtb σ^{E} specific promoter rrnB1

For in vivo Mtb reporter assay, E. coli BL21 (DE3) strains were transformed with three plasmids as in figure 4.5. Co-transformed cells were grown in 50 ml LB broth at 37°C with respective antibiotics (Chloramphenicol 35μg/ml, Ampicillin 100μg/ml and Kanamycin 50μg/ml) up to OD (at 595nm) 0.4, induced by addition of 0.5mM IPTG and were further grown at 37°C for 6 hours or at 16°C for 16 hours (till OD_{595nm} = 1.5). For control, the assays were performed with E. coli BL21 (DE3) strains harbouring the dual plasmid (pAcYc Duet rpoA-rpoZ + pCOLA Duet rpoB-rpoC) for RNAP core expression or plasmid pAcYc Duet-sigA for σ^{A} expression along with pFPVmcherry-rrnB1 or pFPVmcherry-rrnB1 alone. pAcYc Duet rpoA-sigA + pCOLA Duet rpoB-rpoC along with pFPVmcherry-rrnB1 plasmids were used for Mtb holo expression. The fluorescence intensity and O.D were monitored and quantified as described in above section (Figure 4.5).

4.4.6.3 Assays with Mtb σ^{E} specific promoter sigBpr

For in vivo Mtb reporter assay, E. coli BL21 (DE3) strains were transformed with three plasmids as in figure 4.6. Co-transformed cells were grown in 50 ml LB broth at 37°C with respective antibiotics (Chloramphenicol 35μg/ml, Ampicillin 100μg/ml and Kanamycin 50μg/ml) up to OD (at 595nm) 0.4, induced by addition of 0.5mM IPTG and were further grown at 37°C for 24 hours (till OD_{595nm} = 1.5). For control, the assays were performed with E. coli BL21 (DE3) strains harbouring the dual plasmid (pAcYc Duet rpoA-rpoZ + pCOLA Duet rpoB-rpoC) for RNAP core expression or plasmid pAcYc Duet-sigE for σ^{E} expression along with pFPVmcherry-sigBpr or pFPVmcherry-sigBpr alone. pAcYc Duet rpoA-sigE +
pCOLA Duet rpoB-rpoC along with pFPVmcherry-sigBpr plasmids were used for Mtb sigE holo expression. The fluorescence intensity and O.D were monitored and quantified as described in above section (Figure 4.6).

4.4.6.4 Assays with Mtb CRP

The assays with CRP and CRP specific promoter were conducted essentially as above method using the plasmids shown in Figure 4.7. Co-transformed cells were grown in 50 ml LB broth at 37°C with respective antibiotics (Chloramphenicol 35µg/ml, Ampicillin 100µg/ml and Kanamycin 50µg/ml) up to OD (at 595nm) 0.4, induced by addition of 0.5mM IPTG and were further grown at 16°C for 16 hours (till OD 595nm = 1.5). For control, the assays were performed with E. coli BL21 (DE3) strains harbouring the dual plasmid (pAcYc Duet rpoA-sigA + pCOLA Duet rpoB-rpoC) for RNAP holo expression without Mtb CRP along with pFPVmcherry-WhiB1 plasmid for mCherry expression or pFPVmcherry-WhiB1-CRP plasmid alone. For expression of Mtb CRP, pFPVmcherry-WhiB1-CRP was co-expressed along with pAcYc Duet rpoA-sigA + pCOLA Duet rpoB-rpoC. The fluorescence intensity and O.D were monitored and quantified as described in above section (Figure 4.7).

4.4.7 Fluorescence Activated Cell Sorting (FACS) analysis of the cells from the reporter assay

For Fluorescence Activated Cell Sorting (FACS) of in vivo Mtb reporter assay, E. coli BL21 (DE3) strains were transformed with three plasmids as in figure 4.2 (pCOLA Duet rpoB-rpoC + pAcYc Duet rpoA-sigA + pFPV mCherry-sinP3). Co-transformed cells were grown in 50 ml LB broth at 37°C with respective antibiotics (Chloramphenicol 35µg/ml, Ampicillin 100 µg/ml, Kanamycin 50µg/ml) up to OD (at 595nm) 0.4, induced by addition of 0.5mM IPTG and were further grown at 37°C for 6 hours or at 16°C for 16 hours (till OD 595nm = 1.5). The fluorescence intensities was produced by the following combination of plasmids, Control: pFPV mCherry-sinP3; RNAP core: pCOLA Duet rpoB-rpoC + pAcYc Duet rpoA-rpoZ + pFPV mCherry-sinP3; RNAP holo: pCOLA Duet rpoB-rpoC + pAcYc Duet rpoA-sigA + pFPV mCherry-sinP3. Aliquots of 2 ml cell cultures from the above reporter assays were harvested at 5000g for 5 min at 4°C. The pellets were dissolved in 1 ml PBS (pH 7.5) and transferred to FACS tubes (BD Biosciences, USA). Total of 20,000 cells were measured for each sample and every sample had three replicates. Data were acquired on FACS-ARIA (BD BioSciences, USA) and analysed using the FACS-Diva software.


