An Accurate Method for the Qualitative Detection and Quantification of Mycobacterial Promoter Activity

Saurabh Mishra, Deepak Anand, Namperumalsamy Vijayarangan, and Parthasarathi Ajitkumar*

Department of Microbiology and Cell Biology, Indian Institute of Science, Bangalore – 560012, Karnataka, India

Abstract: The present study was designed to determine the half-life of $gfp_m^{2+}$ mRNA, which encodes mycobacterial codon-optimised highly fluorescent GFPm$^{2+}$ protein, and to find out whether mycobacterial promoter activity can be quantitated more accurately using the mRNA levels of the reporter gene, $gfp_m^{2+}$, than the fluorescence intensity of the GFPm$^{2+}$ protein. Quantitative PCR of $gfp_m^{2+}$ mRNA in the pulse-chased samples of the rifampicin-treated Mycobacterium smegmatis/gfpm$^{2+}$ transformant showed the half-life of $gfp_m^{2+}$ mRNA to be 4.081 min. The levels of the $gfp_m^{2+}$ mRNA and the fluorescence intensity of the GFPm$^{2+}$ protein, which were expressed by the promoters of Mycobacterium tuberculosis cell division gene, ftsZ (MtftsZ), were determined using quantitative PCR and fluorescence spectrophotometry, respectively. The data revealed that quantification of mycobacterial promoter activity by determining the $gfp_m^{2+}$ mRNA levels is more accurate and statistically significant than the measurement of GFPm$^{2+}$ fluorescence intensity, especially for weak promoters.

Keywords: $gfp_m^{2+}$, half-life, promoter activity, mycobacteria, quantitative PCR, fluorescence intensity.

INTRODUCTION

Drug-resistant strains of tubercle bacilli and opportunist infection of HIV patients by tubercle bacilli have necessitated identification of novel drug targets that are vital to the bacilli. This has prompted the study of the regulation of expression of a large number of genes of Mycobacterium tuberculosis, the causative agent of tuberculosis, in terms of cloned promoter activity in M. tuberculosis, and in the surrogate Mycobacterium smegmatis, a saprophyte used for the study of mycobacterial biology. The activity of the promoters is usually determined by quantifying the levels or activity of the reporter proteins, such as β-galactosidase (lacZ) [1, 2], chloramphenicol acetyl transferase (cat) [3], catechol 2,3-dioxygenase (xyE) [4], bacterial luciferase (lux) [5], and variants of green fluorescent protein (gfp) [6-9]. However, the levels and activity of these reporter proteins are dependent on the transcriptional, translational, and stability regulations, with the possibility of making measurements erroneous [10, 11]. Secondly, when promoter activity needs to be quantitated during prolonged durations and dormant conditions, GFP and LacZ, which are stable reporter proteins [12, 13], would persist throughout the duration of the experiment, making measurements erroneous. Although GFP proteins of short half-life are available [14], they are derivatives of GFP MUT2 of lower intensity, making detection and quantification of weak promoter activity difficult.

A direct measurement of the levels of the reporter gene mRNA would alleviate these concerns, provided the mRNA has short half-life. Further, a fluorescent reporter protein of very high intensity, and hence sensitivity, would facilitate initial qualitative noninvasive detection of promoter activity. In view of these facts, the present study was designed to determine: (i). the half-life of mRNA of the reporter gene, $gfp_m^{2+}$ [15], which encodes mycobacterial codon-optimised GFPm$^{2+}$ protein of very high fluorescence intensity, and (ii). the accuracy and statistical reliability of the measurement of promoter activity by quantifying the levels of $gfp_m^{2+}$ mRNA, in comparison to the quantification of fluorescence intensity of GFPm$^{2+}$ protein, in mycobacterial cells.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Mycobacterium smegmatis mc$^2$155, which was used for the transformation with promoter constructs and transcription pulse chase experiments, was cultured in Middlebrook 7H9 (Difco) liquid medium containing 0.2% glycerol and 0.05% Tween-80, hygromycin (50 μg/ml) till OD$_{600}$ nm 0.6 (mid-log phase). Escherichia coli JM109, which was used for the propagation of the plasmid constructs, was cultured in Luria-Bertani medium containing hygromycin (150 μg/ml) at 170 rpm at 37°C.

Plasmid Constructs

The E. coli-mycobacterial shuttle vector, pMN406 [8], contains $gfp_m^{2+}$ gene, which encodes the GFPm$^{2+}$ variant possessing improved solubility characteristics, of high fluorescence intensity and twice more fluorescent than eGFP, and stable in expression in slow- and fast-growing mycobacteria.
by snap-chilling on ice for 5 min. Respective primers cDNA DNase I-treated RNA on formaldehyde agarose gel. Integrity of the RNA was rechecked by loading 500 ng of RT-PCR primers (mycgfp2+-RT-r and mycgfp2+-RT-f; Ta-

Rifampicin Treatment, RNA Extraction, and DNasel Treatment

Mid-log phase M. smegmatis transformants containing pMN406 were grown to mid-log phase and treated with rifampicin (500 µM/ml), as described [16]. Cells were washed with 0.5% Tween-80 solution, and RNA isolation was carried out using hot-phenol method, as described [8], but with slight modifications. Cells were harvested at 0, 5, 10, 15, and 20 min post-addition of rifampicin and washed with 0.5% Tween-80 solution Cell pellets were transferred to 1.5 ml capacity microfuge tube and were repeatedly chilled in liquid nitrogen and crushed using micro pestle. Cells were lysed in 30 mM Tris-HCl, pH 7.4, containing 100 mM NaCl, 5 mM EDTA, 1% SDS, 0.1 M β-mercaptoethanol, and 5 mM vanadyl ribonucleoside complexes (VRC). Lysates were incubated on ice for 10 min and then extracted with 65°C pre-

Real Time PCR

Real Time PCR was carried out using DyNamo SYBR Green qPCR kit (Finnzymes). The reaction mix contained 1x master mix, 1x of ROX dye, 0.5 µM each of forward and reverse primers, 1 µl of cDNA, and water to make up the reaction volume to 10 µl. Real Time PCR was performed for 40 cycles in ABI Prism (Real Time PCR System by Applied Biosystems) and the data were analysed using 7000 SDS (Version 2.3) software (Applied Biosystems). The Real Time PCR Ct values for 16S rRNA, obtained using specific primers (Table 1), were used for normalisation of Ct values for gfp+ mRNA.

Fluorescence Intensity Measurements

Fluorescence measurements were carried out in Fluoromax 4, HORIBA JOBIN YVON Fluorescence spectrophotometer, as described earlier [7]. In brief, different transformants were inoculated from glycerol stock and grown till O.D.600 = 0.6. These were again sub-cultured and grown to 0.6 O.D500 nm. In order to avoid clump formation, which is prevalent in mycobacterial cultures, 1 ml of culture was withdrawn and passed through a syringe containing 24 gauge needle. These cultures were then diluted 1:10 by adding 100 µl of culture in 900 µl of phosphate-buffered saline. GFP fluorescence was measured by using 490 nm excitation filter (slit width 10 nm) and 520 nm emission filter (slit width 10 nm). Fluorescence value of the buffer alone was deducted from the fluorescence values of GFPm+ expression from the different constructs, and the deducted values were plotted.

Statistical Analysis

Fold activities of different promoter constructs were measured using fluorescence spectrophotometer and real time PCR from three independent biological triplicates. Promoter activities within a given method (i.e. fluorescence spectrophotometry or real time PCR) were compared and two-sided P-values were obtained by paired t-tests. To com-

| Table 1. Oligonucleotide Primers used in the Study |
|-----------------------------------------------|
| mycgfp2-RT-f 5' atgtcgaagggcgaggagctgttcaccggc 3' |
| mycgfp2-RT-r 5' gaagcactggagcagctgtagtccagggggtg 3' |
| Ms-16SrRNA-RTf 5' gcgtgtgtacaagggcgcgg 3' |
| Ms-16SrRNA-RTTr 5' cggtaaattcatgccctattgctc 3' |

Statistical Analysis

Fold activities of different promoter constructs were measured using fluorescence spectrophotometer and real time PCR from three independent biological triplicates. Promoter activities within a given method (i.e. fluorescence spectrophotometry or real time PCR) were compared and two-sided P-values were obtained by paired t-tests. To com-

Statistical Analysis

Fold activities of different promoter constructs were measured using fluorescence spectrophotometer and real time PCR from three independent biological triplicates. Promoter activities within a given method (i.e. fluorescence spectrophotometry or real time PCR) were compared and two-sided P-values were obtained by paired t-tests. To com-

Statistical Analysis

Fold activities of different promoter constructs were measured using fluorescence spectrophotometer and real time PCR from three independent biological triplicates. Promoter activities within a given method (i.e. fluorescence spectrophotometry or real time PCR) were compared and two-sided P-values were obtained by paired t-tests. To com-
pare fluorescence intensity-based and real time PCR-based quantitation, unpaired t-tests were applied to obtain two-sided P-values.

RESULTS AND DISCUSSION

Determination of \( gfpm^{2+} \) mRNA Half-life

By plotting the levels of \( gfpm^{2+} \) mRNA remaining in the pulse-chased samples against time, the half-life of \( gfpm^{2+} \) mRNA was calculated, as first order exponential decay kinetics [18], as follows (Fig. 1A, B). Bacterial mRNA degradation follows first order exponential decay kinetics. Therefore, half-life of mRNA can be represented by the equation, \( t_{1/2} = \frac{0.693}{k} \), where \( k \) = the rate constant for mRNA decay (i.e., percent change over time). The value of \( k \) can be determined by plotting the concentration of mRNA over time and determining the slope of a best-fit line (slope = \( k \)). The value of rate constant, \( k \) for \( gfpm^{2+} \) mRNA degradation was found to be 0.1698. Thus, \( t_{1/2} \), the half-life was found to be 0.693/0.1698 = ~ 4.1 min. Rate constant was calculated using Graphpad Prism software using the following equation for one phase decay: \( Y = (Y_0 - \text{Plateau})*\exp(-K*X) + \text{Plateau} \), where \( X \) = Time, \( Y \) = mRNA levels, starting at \( Y_0 \) and decaying (with one phase) down to plateau; \( Y_0 \) and plateau have same units as \( Y \); \( K \) = Rate constant equal to the reciprocal of the X-axis units (Fig. 1A, B).

Quantification of Promoter Activity

Subsequent to determining the half-life of \( gfpm^{2+} \) mRNA to be low, the promoters of the cytokinetic gene, \( ftsZ \), of \( M.tuberculosis \) (MtftsZ), the activities of which were determined earlier in the laboratory [9], were used for quantitating promoter activity based on the \( gfpm^{2+} \) mRNA levels and GFP-m protein fluorescence intensity. The promoter region, Q1-K1, encompasses five promoters (P1, P2, P3, P4, and P6), while K2-K1 carries two promoters (P1 and P2), of MtftsZ [9] (Fig. 1C). These promoter regions were cloned in pMN406 [8], in place of the constitutive promoter, \( P_{imyc} \), as described [9]. pMN406 and pMN406-\( \Delta P_{imyc} \) (devoid of \( P_{imyc} \)) were used as the positive control and negative control, respectively. The activity of Q1-K1, K2-K1, \( P_{imyc} \), and of \( \Delta P_{imyc} \), were determined by quantitating the levels of \( gfpm^{2+} \) mRNA in the respective \( M.smegmatis \) mc²155 transformant, using real time PCR on \( gfpm^{2+} \) cDNA, as described [17]. With the \( gfpm^{2+} \) mRNA levels expressed from \( P_{imyc} \) (positive control) taken to be 1, the levels of \( gfpm^{2+} \) mRNA, from Q1-K1 was marginally (1.1-fold) higher (Fig. 2A). Whereas, the levels of \( gfpm^{2+} \) mRNA from K2-K1 region were 2.5-fold less than that of the positive control and 2.75-fold less than that from Q1-K1 (Fig. 2A). pMN406-\( \Delta P_{imyc} \) showed negligible expression. In parallel, the fluorescence intensity of GFP-m protein was determined in the \( M.smegmatis \) transforms, using fluorescence spectrophotometry, as described [7]. The fluorescence intensity of GFP-m was expressed from \( P_{imyc} \) was 4.5 \times 10^6 units (Fig. 2B), while that expressed from Q1-K1 accounted for 50% of the positive control. The level of expression from K2-K1 was 50% of the expression from Q1-K1 (Fig. 2B). \( \Delta P_{imyc} \) showed basal level of fluorescence.

Fig. (1). The one phase decay curve (A) and the mRNA quantification curve (B) from the pulse-chase experiment. The half-life of \( gfpm^{2+} \) mRNA was calculated from the equation, \( t_{1/2} = \frac{0.693}{k} \), where \( k \) = the rate constant for mRNA decay (i.e., percent change over time), as described in the text. (C). Organisation of the Q1-K1 region containing all the 5 promoters (P1, P2, P3, P4, and P6) and of K2-K1 containing P1 and P2 promoters of MtftsZ.
Fig. (2). Quantification of the activity of the MtfsZ promoters using the levels of reporter mRNA and fluorescence intensity of the reporter protein. (A). Real time PCR quantification of the gfpm$_{2+}$ reporter mRNA levels. (B). Fluorescence spectrophotometric quantification of the fluorescence intensity of the GFP$_{m2+}$ reporter protein. Significant differences in the activity between the promoters under each method are indicated in terms of P-values in the text.

Fold activities of different promoter constructs, which were measured using fluorescence spectrophotometer and real-time PCR from three independent biological triplicates, were used for statistical analysis. Promoter activities within a given method (i.e. fluorescence spectrophotometry or real-time PCR) were compared and two-sided P-values were obtained by paired t-tests. In order to compare fluorescence intensity-based and real-time PCR-based quantification, unpaired t-tests were applied to obtain two-sided P-values. Statistical analysis of the promoter activities obtained from gfpm$_{2+}$ mRNA quantification showed significant differences between K2-K1 and ΔPimyc (P value = 0.0108) (Fig. 2A). On the contrary, GFP$_{m2+}$ fluorescence from K2-K1 showed no significant difference from the negative control, ΔPimyc (P value = 0.1759) (Fig. 2B). Similarly, GFP$_{m2+}$ fluorescence from Q1-K1 showed only 0.4-0.5 fold activity (P value = 0.001), in comparison to the positive control, Pimyc (Fig. 2B). On the contrary, qPCR showed that promoter activities of Q1-K1 and Pimyc were comparable (Fig. 2A), as reported earlier [8]. Thus, for the determination of mycobacterial promoter activity, gfpm$_{2+}$ mRNA quantification is accurate, statistically significant, and therefore reliable, as compared to GFP$_{m2+}$ fluorescence intensity quantification, especially for regions of weak promoter activity, such as the K2-K1 region. The low half-life of gfpm$_{2+}$ mRNA justifies the quantification of the mRNA levels for promoter activity.

Although the half-life of mRNA of GFPm [6] was 2.5 min [19], it was not used in this study as it gives lower fluorescence, compared to GFP$_{m2+}$ [15], and hence not suited for quick qualitative detection of activity, especially of weak promoters. Similarly, the short half-life GFP variants [14] were not used, as they are derivatives of GFPMUT2, which is only 2-fold higher in fluorescence intensity than GFPMUT3 [20]. On the other hand, GFP$_{m2+}$ has 10-fold higher fluorescence intensity than GFPMUT3 and double the fluorescence of eGFP, with improved solubility characteristics and stable expression in slow- and fast-growing mycobacteria [15]. Although lacZ mRNA half-life is only 90 sec [21], lacZ mRNA was not used for quantification, as GFP$_{m2+}$ has the advantage of initial quick, non-invasive, and substrate-independent qualitative detection of promoter activity, over LacZ, using epifluorescence microscopy, fluorimetry, and flow cytometry [22], prior to the quantification of mRNA levels. Taken together, the present study offers a means for the sensitive, substrate-independent, quick, qualitative detection, and accurate and statistically significant quantification of mycobacterial promoter activity.
CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

ACKNOWLEDGEMENTS

This work was supported by a research grant (37(1361)/09/EMR-II) from CSIR and by Centre of Excellence in Tuberculosis Research part-grant from DBT. SM was a CSIR Senior Research Fellow. The infrastructural support from the DBT to the Indian Institute of Science, and from UGC-CAS, and DST-FIST to the Microbiology and Cell Biology Department, are acknowledged. The authors declare that they do not have any competing or conflicting interests.

REFERENCES

[1] Timm J, Lim EM, Gieque B. Escherichia coli-Mycobacteria shuttle vectors for operon and gene fusions to lacZ: the pJEM series. J Bacteriol 1994; 176: 6749-53.
[2] Rowland B, Purkayastha A, Monserrat C, Casart Y, Takiff H, McDonough KA. Fluorescence-based detection of lacZ reporter gene expression in intact and viable bacteria including Mycobacterium species. FEMS Microbiol Lett 1999; 179: 317-25.
[3] DasGupta SK, Bashyam MD, Tyagi AK. Cloning and assessment of mycobacterial promoters by using a plasmid shuttle vector. J Bacteriol 1993; 175: 5186-92.
[4] Cur ric R, Dhansyuthapani S, De retic V. Gene expression in mycobacteria: transcriptional fusions based on xylE and analysis of the promoter region of the response regulator mtrA from Mycobacterium species. Mol Microbiol 1994; 13: 1057-64.
[5] Roberts EA, Clark A, Friedman RL. Bacterial luciferase is naturally destabilised in Mycobacterium tuberculosis and can be used to monitor changes in gene expression. FEMS Microbiol Lett 2005; 243: 243-49.
[6] Barker LP, Brooks DM, Small PL. The identification of Mycobacterium marinum genes differentially expressed in macrophage phagosomes using promoter fusions to green fluorescent protein. Mol Microbiol 1998; 29: 1167-77.
[7] Cowley SC, Av-Gay Y. Monitoring promoter activity and protein localisation in Mycobacterium spp. using green fluorescent protein. Gene 2001; 264: 225-31.

Received: October 05, 2012 Revised: November 07, 2012 Accepted: November 20, 2012