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Heterologous Regulation of BCG hsp65 Promoter by *M. leprae* 18 kDa Transcription Repression Responsive Element

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Abstract

Among a number of antigens characterized in *M. leprae*, an etiological agent of Leprosy, the 18 kDa antigen, is unique to *M. leprae*. We have previously determined a sequence specific element in the 18 kDa gene of *M. leprae*, which confers transcriptional repression. In this report, we have examined if the element could be applied to genes other than the 18 kDa gene of *M. leprae*. To identify the roles of the regulatory sequence in heterologous promoter, we have constructed pB3 vector series, which contains BCG hsp65 promoter and the *M. leprae* 18 kDa transcription repression responsive element in tandem using *LacZ* gene as a reporter gene. Cloning of hsp65 promoters of *M. bovis* BCG or *M. smegmatis* in front of *LacZ* gene resulted in normal β-galactosidase activity as expected. However, when the sequence element was placed between the promoter and the *LacZ* gene, β-galactosidase activity was reduced 10-fold less. Also we have examined with pB3(-) vector, that harbors the transcription repression responsive element in a reversed orientation, the β-galactosidase activity was found to be similar to pB3(+) vector. Thus, these results further confirm that *M. leprae* 18 kDa transcription repression responsive element could regulate BCG hsp65 heterologous promoter and that the element could act as an operator for the transcription of mycobacteria.

Keywords: heterologous promoter, *Mycobacterium leprae*, transcriptional repression regulatory element

Introduction

The genera *Mycobacterium* is one of the largest bacterial genia and includes the pathogenic species *Mycobacterium leprae* and *Mycobacterium* tuberculosis (Mulder *et al.*, 1999). 18 kDa antigen is one of the various antigens found in *M. leprae*, it is specific-antigen in *M.leprae* and may be involved in survival of *M. leprae* with in macrophage during infection (Dellagostin *et al.*, 1995). It has been suggested that the expression of 18kDa gene might be regulated by sequence-specific transcriptional repressor that binds to the regulatory sequence.

Recently, there became much researches about the genetic elements that contribute to the control of gene expression in mycobacteria. Since many mycobacterial promoters and transcriptional initiation have been studied, consensus promoter sequences have been proposed. However, many of these promoters are specifically regulated (Ramesh *et al.*, 1995; Bashyam *et al.*, 1996). Some of the mycobacterial promoters resemble the typical *E. coli* consensus promoter and function in this organism, but most have a higher G+C content and differ from the *E. coli* consensus promoters. It has been shown that these promoters function more efficiently in Streptomyces than in *E. coli* (Kieser *et al.*, 1986). In general mycobacteria have G+C-rich genomes. It is known that, in bacteria, the overall G+C contents of the genome affects the choice of translation initiation and termination, and the promoter recognition sites for RNA polymerase (Nakayama *et al.*, 1989; Ohama *et al.*, 1987). A study of mycobacterial promoters is necessary for not only understanding about the mycobacterial transcriptional machinery but also a better understanding of the genetic basis for the observed phenomena of gene regulation in various mycobacteria.

We have previously found a sequence specific element in the 18 kDa gene of *M. leprae*, which confers transcriptional repression for the gene. In this report, we have examined if the element could be applied to genes other than the 18 kDa gene of *M. leprae*. To do so, we have investigated the gene expression control of hsp65 promoters of *M. bovis* BCG or *M. smegmatis* by the regulatory sequence of *M. leprae* 18 kDa gene.

Materials and Methods

Bacterial strains and media

E. coli DH10B was grown in Luria-Bertani (LB) media. Transformed *E. coli* were selected on LB media with 100 $\mu g/ml$ of ampicillin or 40 $\mu g/ml$ kanamycin. *M. smegmatis* mc^2 155, which was used to propagate vectors for mycobacterium, was grown in Middlebrook 7H9 broth

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containing 0.2% glycerol and 0.05% Tween-80 at 37℃ shaking incubator. Transformed mycobacteria were selected on LB agar containing 40 ㎍/㎖ kanamycin.

Transformation of *M. smegmatis*

M. smegmatis mc²155 competent cells were transformed by electroporation methods using 10%(v/v) glycerol (Jacobs *et al.*, 1991). For electroporation, the Gene Pulser (Bio-Rad) was set at 2.5 ㎸ and 25 ㎌, and the pulse controller resistance was set at 1000Ω. Recombinant DNAs were then added to 100 ㎕ of mycobacterial cells in 0.4 ㎝ electrode gap cuvette. After electroporation, diluted into 0.5 ㎖ of broth, incubated for 1 hr at 37℃ with shaking before plating and then the cell were plated on LB agar containing 40 ㎍/㎖ kanamycin and incubated at 37℃ for 3 days.

PCR amplification

Genomic DNA were purified from *M. bovis* BCG and *M. smegmatis* mc² 155 as described by Jacobs *et al.* (1991). PCR amplification of the mycobacterial hsp65 promoter sequences was performed using *M. bovis* BCG and *M. smegmatis* mc² 155 genomic DNA as the template with the 5' -primer (hsp1 ; 5' -GGG TCT AGA CGG TGA CCA CAA CCA CGC G-3' and 3' -primer (hsp2 ; 5' -GGG TCT AGA CGC GTC CGG ATC GGG GAT G-3') resulting 383-bp in length. PCR was performed in 50 ㎕ reaction mixture containing 10 mM Tris-HCl (pH8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM dNTP, 1 U of Taq polymerase, 10 pmol of each set of primers, and about 100 ng of template DNA. The amplification mixture were subjected to 5 min at 94℃ and 35 cycles of 1 min at 94℃, 1 min at 60℃ and 1 min at 72℃, and then 10 min at 72℃. PCR products were used for cloning by purifying with GeneClean Kit.

Vector construction

Vector pM0GAL-BCG and pM0GAL-MS were constructed by cloning the 383-bp *Xba* I - *Xba* I fragment from PCR product, which were amplified by using *M. bovis* BCG and *M. smegmatis* genomic DNA respectively, into the *Xba* I site of pM0GAL vector containing the promoterless-*LacZ* gene and kanamycin resistance gene (Fig. 1).

Another derivatives, 60-bp synthetic oligomer corresponding the regulatory element of the *M. leprae* 18 kDa gene was digested with *Xbal* and cloned into the corresponding restriction sites of pBluescript SK(-) to yield pSK(-)-3G DNA vectors. Because same cloning site (*Xba* I) was used, both forward and reverse orientation were existed, it was named as (+) and (-) respectively. A 383-bp *Sal* I-*Bam*H I fragment containing the BCG hsp65 promoter from pM0GAL-BCG was replaced with same sites in pSK(-)-3G DNA vector series to produce the pSK(-)-(BCG+3G). A 451-bp *Sal* I-*Not* I fragment from pSK(-)-(BCG+3G) vector was generated blunt-end by klenow enzyme and then cloned into blunt-ended *Xba* I site of pM0GAL vector to construct the pB3 vector series (Fig. 2). These constructed vectors contain both *M. bovis* BCG hsp65 promoter region and regulatory sequence of *M. leprae* 18 kDa gene.

β-galactosidase assay

For the β-galactosidase activity assays, *M. smegmatis* transformants were grown in Middlebrook 7H9 broth containing 40 ㎍/㎖ kanamycin, 0.2% glycerol and 0.05% Tween-80 at 37° C with aeration by shaking. The β-galactosidase activity assays were performed according to the Miller' method (Miller, 1972), and the assay condition was as follows.

After *M. smegmatis* transformants were grown to late exponential phase, 0.5 ㎖ of bacterial suspension were mixed with 0.5 ㎖ of Z buffer (6 mM NaH_2PO_4, 10 mM KCl, 50 mM β-mercaptoethanol, 1 mM $MgSO_4$), the cells were lysed by adding both 20 ㎕ of chloroform and 10 ㎕ of 0.1% SDS to each assay mixture. Vortex the tubes for 1 min and place the tubes in a 28° C water bath for 5 min. The reaction was started by adding 100 ㎕ of ONPG(4 ㎎/㎖) to each tube, and shake the tubes for a few seconds. After sufficient yellow color has developed, stop the reaction by adding 200 ㎕ of 1 M Na_2CO_3 and then centrifuge at 13,000 rpm for 5 min. For each tube, the optical density was read at 420 nm and at 550 nm. β-galactosidase units was calculated as following

$$\frac{1000 \times (OD_{420} - 1.75 \times OD_{550})}{t \times v \times OD_{600}} = units\ of\ \beta\text{-}galactosidase$$

[t = the time of the reaction in minute and v = the volume of culture used in the assay (㎖)]

Results

Construction of reporter vectors driven by heterologous promoters

In order to determine whether the transcriptional activation could be induced by heterologous promoters, we have constructed the pM0GAL-BCG and pM0GAL-MS vectors. PCR amplification was used to amplify the hsp65 promoter region from *M. bovis* BCG and *M. smegmatis* mc²155. These promoters region were cloned as a 382-bp in length *Xba* I - *Xba* I fragment into the upstream of *LacZ* gene of pM0GAL vector, which contained promoterless-*LacZ* gene and kanamycin resistance gene. The resulting vectors

were name as pM0GAL-BCG and the pM0GAL-MS vector, respectively (Fig. 1). Using these constructed pM0GAL-BCG and pM0GAL-MS vectors, we have investigated the transcriptional activity by heterologous promoters. We have used the promoterless pM0GAL vector and non-regulated pM4GAL vector as controls to compare the β-galactosidase activity with derivative vectors.

In order to identify whether the regulatory element of *M. leprae* 18 kDa gene could confer transcriptional repression in other heterologous promoter, we have also constructed pB3 vector series (Fig. 2). pB3 vector series contains BCG hsp65 promoter region from PCR-amplification, the *M. leprae* 18 kDa transcription repression responsive element from synthesized oligomers, and *LacZ* gene from pM0GAL vector in tandem. pB3(+) vector and pB3(-) vector, containing differently-oriented fragment of the regulatory sequence, were also tested for β-galactosidase activity to examine the effect by orientation of the regulatory sequence.

Expression of β-galactosidase gene in different promoters

To investigate the transcriptional activation by hsp65 promoter, which originated from *M. bovis* BCG and *M. smegmatis*, pM0GAL-BCG vector and pM0GAL-MS vector were electroporated into *M. smegmatis* and tested for β-galactosidase activity. pM0GAL vector showed the background β-galactosidase activity but pM0GAL-BCG and pM0GAL-MS vectors showed high promoter activities and these activities were corresponded about 60-80% to activity of pM4GAL vector (Fig. 3). In these results, we suggested that BCG hsp65 promoter, which originated from other strain, was recognized by transcription apparatus of *M. smegmatis*.

Effects of regulatory sequence on hsp65 promoter activity

To examine the effects of the regulator sequence of *M. leprae* 18 kDa gene on heterologous promoters and different strains, two vectors were constructed and named pB3(+) and pB3(-) vectors, which contained hsp65 promoter, regulatory sequence of *M. leprae*, and *LacZ* gene in tandem. The constructs were assayed the β-galactosidase activity in *M. smegmatis*. Insertion of regulatory sequence to the pM0GAL-BCG resulted in an 10-fold reduction in activity than pM0GAL-BCG vector in *M. smegmatis* and reached to the background β-galactosidase activity like pM0GAL (Fig. 4). Also, we have found the similar activity with pB3(-) vector, which contained the regulatory sequence in a reversed orientation, as have seen with pB3(+) vector. The results indicate that the orientation of regulatory element is not critical.

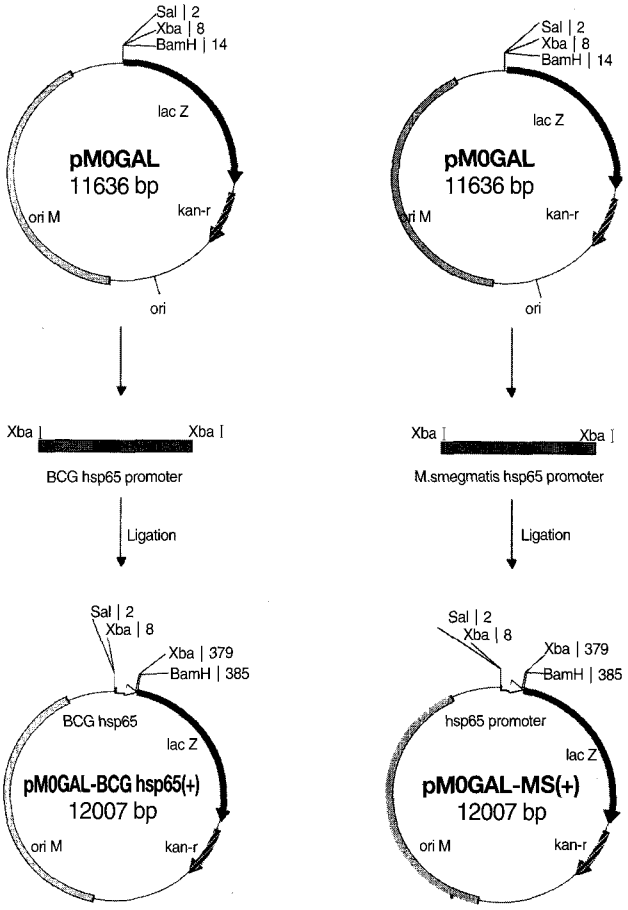

Fig. 1. Construction of pM0GAL-BCG and pM0GAL-MS vectors. The β-galactosidase gene in pM0GAL is promoterless. The hsp65 promoter region (371-bp) amplified from genomic DNA was ligated into *Xba* I site of pM0GAL vector and eliminate the constructed vector that promoter region was ligated in a revered orientation.

These results indicate clearly that regulatory sequence of *M. leprae* 18 kDa gene can also affect the transcription not only by homologous promoter but also by heterologous promoter and regulatory sequence of *M. leprae* 18 kDa gene can regulate the expression in different strains because *M. smegmatis* strain used for mycobacterial host.

Discussion

We have studied about heterologous promoter activity and mycobacterial transcription repression by regulatory element using the mycobacterial promoterless vector, which contained the β-galactosidase reporter gene. Using

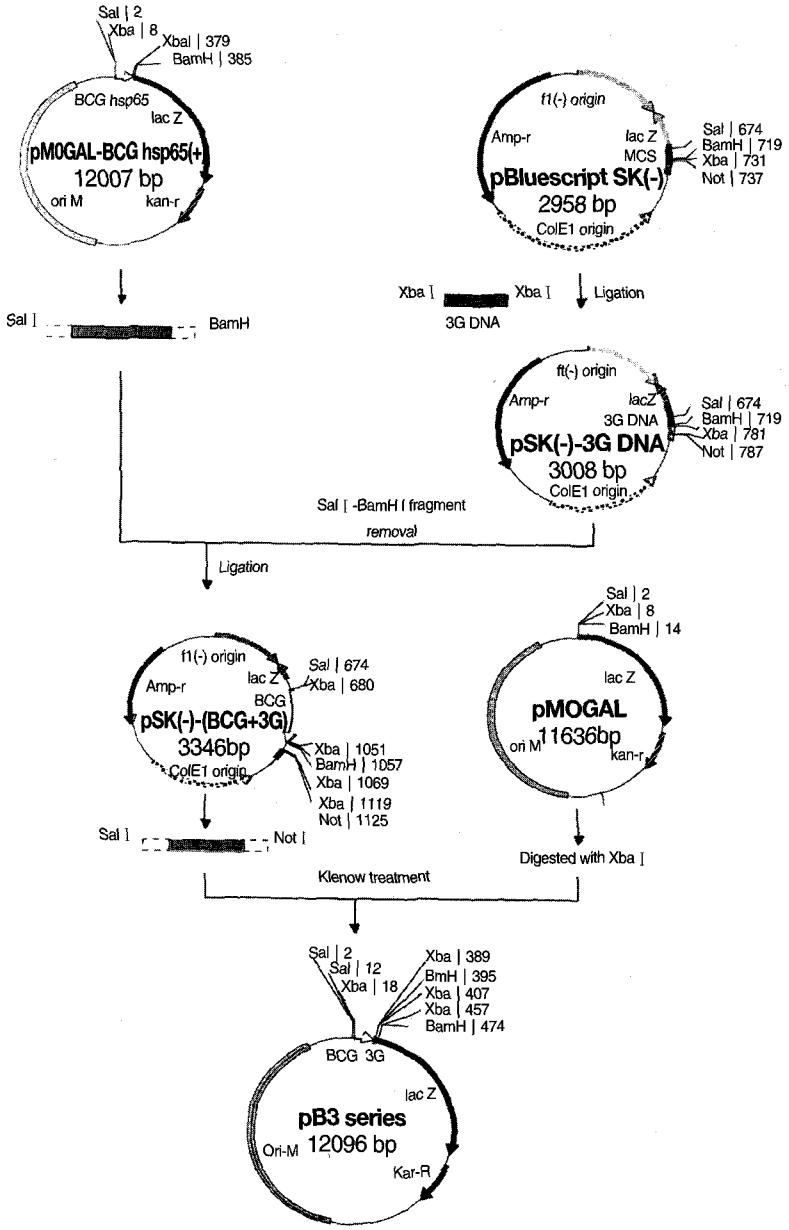

Fig. 2. Construction of pB3 vector series. Strategy of pM0GAL-BCG vector construction is mentioned in Fig. 1. Vector pSK(-)-3G DNA was constructed by cloning 50-bp regulatory sequence from synthesized oligomers into *Xba* I site of pBluescript SK(-). A 383-bp fragment containing the BCG promoter was digested with *Sal* I and *Bam*H I from pM0GAL-BCG vector and then replaced with corresponding site of pSK(-)-3G DNA vector to yield pSK(-)-(BCG+3G) vector. A 451-bp *Sal* I and *Not* I fragment containing hsp65 promoter region and regulatory sequence from pSK(-)-(BCG+3G) vector was generated blunt-end by klenow enzyme and then cloned into blunt-ended *Xba* I site of pM0GAL vector to yield pB3 vector series.

the pM0GAL-BCG and pM0GAL-MS vector (Fig. 1), we achieved promoter activity analysis in *M. smegmatis* cell. Both vectors have displayed the increased β-galactosidase activity compare to pM0GAL used as control vector (Fig 3). These results indicate that transcription is activated by BCG hsp65 promoter as heterologous promoter in *M. smegmatis*.

In general, these results suggest that transcription

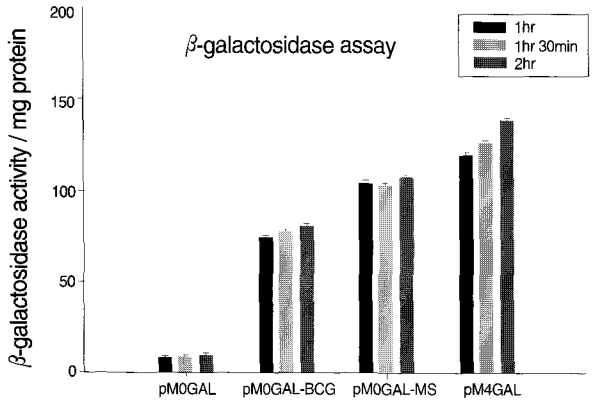

Fig. 3. Comparison of strength of *M. bovis* BCG hsp65 promoters and *M. smegmatis* hsp65 promoters in *M. smegmatis*. Promoter strengths were determined by β-galactosidase assays in *M. smegmatis* transformants during extracellular growth. Data were presented as an average of two or three experiments.

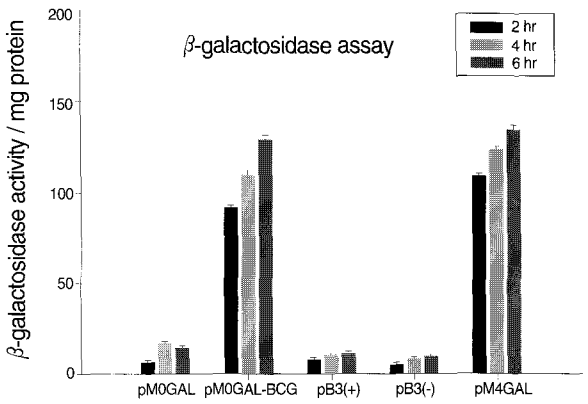

Fig. 4. Regulation of *M. bovis BCG* hsp65 promoter by *M. leprae* 18 kDa regulatory element in *M. smegmatis*. β-galactosidase activity per mg protein was measured in *M. smegmatis* transformants during extracellular growth. Data were presented as an average of two or three experiments. The sequence of the regulatory element is : 5' -CTAGATCGACCAGTGCTATATCAA ATCTATGTAGTCAGGAACAGCATGCT-3' (+ orientation).

signals are conserved among the mycobacteria. It has been demonstrated that the efficiency and specificity of transcriptional recognition is conserved in fast-growing *M. smegmatis* and slowly-growing *M. tuberculosis* and *M. bovis* BCG, since the promoters examined exhibited similar activities and utilized the same transcription start sites in these hosts (Bashyam *et al.*, 1996). Therefore, *M. smegmatis* may be used as a surrogate host for mycobacterial expression study because it is fast growing, nonpathogenic, and more easily transformable than other mycobacterial species (Hosson *et al.*, 1990). Although the recognition of promoter sequences appears to be conserved among the mycobacteria, there may be small differences in the transcription machinery between slowly growing bacteria and fast growing bacteria (Timm *et al.*, 1994).

Many prokaryotic genes are regulated by environmental conditions or by growth phase. These regulations usually involve the binding of either a repressor or activator protein to sequences within or upstream of promoter.

It is known that *M. leprae* 18 kDa promoter exhibits low levels of expression in bacterial culture, but shows high levels of expression in macrophage (Dellagostin *et al.*, 1995). The expression of 18 kDa gene is regulated by a sequence-specific transcription repression responsive element and its cognate repressor, which binds to the element. In this study, to investigate the heterologous transcriptional repression activity of this element, we have constructed pB3 vector series contained BCG hsp65 promoter and regulatory element and then performed the β-galactosidase assay (Fig. 2). In these vectors, β-galactosidase activity was reduced 10-fold less than pM0GAL vector, which contained BCG hsp65 promoter alone and showed similar β-galactosidase activity both pB3(+) vector and pB3(-) vectors which harbors the transcription repression responsive element in a reversed orientation (Fig. 4).

From these results, we could conclude that the regulator element of *M. leprae* 18 kDa gene could regulate the heterologous BCG hsp65 promoter and regulatory element may function regardless of orientation.

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