EXPERIMENTAL and MOLECULAR MEDICINE, Vol. 36, No. 6, 510-514, December 2004

The upstream sequence of *Mycobacterium leprae* 18-kDa gene confers transcription repression activity in orientation-independent manner

Hyun Bae Kim[1] and Ji Chang You[1,2]

[1]Department of Pathology
School of Medicine, The Catholic University of Korea
Seoul 137-701, Korea
[2]Corresponding author: Tel, 82-2-590-1318;
Fax, 82-2-595-2241; E-mail, jiyou@catholic.ac.kr

Accepted 30 October 2004

Abbreviations: BCG, Bacille Calmette-Guerin; hsp, heat shock protein; LB broth, Luria-Bertani broth; *M. bovis*, *Mycobacterium bovis*; *M. leprae*, *Mycobacterium leprae*; *M. smegmatis*; *Mycobacterium smegmatis*; ONPG, *O*-nitrophenyl-β-D-galactopyranoside

Abstract

In order to understand the role of the upstream region of the *Mycobacterium leprae* 18-kDa gene on the gene regulation, the region was divided into two at the -50 position from the first start codon of the gene and their effect on transcription was examined by using a *LacZ* transcriptional reporter gene assay. The presence of each of these two regions conferred transcription repression not only on its cognate *M. lepraerae* 18-kDa gene promoter, but also on a heterologous promoter such as the *Mycobacterium bovis* BCG hsp65 gene promoter. Moreover, it was found that these regions could confer transcription repression activity in both cases in an orientation-independent manner. Thus, these results indicate that the upstream region of the *M. leprae* 18-kDa gene harbors transcription repression responsive element(s) acting as an operator and can be further divided into two separately functional regions, suggesting a bipartite structure of the element(s). The identification of transcription repression activity of the upstream region in the *M. leprae* 18-kDa gene will contribute greatly for the understanding of the 18-kDa gene regulation mechanism, and provide also useful information for the manipulation of mycobacterium gene expression.

Keywords: gene expression regulation; genes, bacterial; *M. leprae*; promoter regions

Introduction

Mycobacterium leprae, the causative agent of leprosy, remains one of the major pathogenic bacteria causing worldwide health problems particularly in developing countries. *M. leprae* cannot be cultivated *in vitro* however, it survives and proliferates within host macrophage cells by escaping its bactericidal activities, although the exact mechanism is still elusive. In order to understand the immunopathological mechanism of the pathogen and to develop effective vaccine candidates, many molecular biological studies have been undertaken to identify and characterize the immunodominant antigenic proteins of *M. leprae* (Hunter *et al*., 1990; Rivoire *et al*., 1994; Pessolani and Brennan, 1996). A number of genes for these antigens identified have also been cloned and sequenced (Thole *et al*., 1995). Among them, an 18-kDa antigen, a member of the heat shock proteins, is known to be specific to *M. leprae*. The 18-kDa gene is specifically activated during intracellular growth and might be involved in the survival of *M. leprae* within the macrophages (Dellagostin *et al*., 1995).

A number of bacterial genes that are activated upon entering host cells are important for their survival and pathogenesis. Thus, identification of the *M. leprae*-specific 18-kDa gene activation mechanism would be important for a better understanding of the molecular biology and pathogenesis of *M. leprae*. However, the detailed mechanism(s) is not known.

Recently, we have found using a deletion mapping analysis that the upstream region of the 18-kDa gene contains a sequence that is responsible for transcriptional repression, whose deletion resulted in remarkable transcription activation in a transcription reporter gene assay. In this study, we report that the upstream region can be further divided into two separately functional regions that could operate as transcription repression responsive elements in an orientation-independent manner. The results suggest that the transcription repression responsive element in the 18-kDa gene could act as an operator and might be composed of a bipartite element.

Materials and Methods

Materials

The Middlebrook 7H9 broth was purchased from Difco Laboratories (Detroit). Tween-80 was obtained from Showa Co. (Japan). *O*-nitrophenyl-β-D-galactopyranoside (ONPG) was from Sigma Chemical Co. (St. Louis). Restriction endonucleases were purchased from either Roche (Mannheim, Germany) or New England Biolabs (Beverly). All other materials were reagent grade and were obtained from commercial sources.

Cell culture

Mycobacterium smegmatis mc^2 155 were grown in Middlebrook 7H9 broth containing 0.2% glycerol and 0.05% Tween-80 at 37°C. *E. coli* DH10B was used as a host for plasmids and were cultured in a Luria-Bertani (LB) broth. For the strains carrying the drug resistance genes, kanamycin and ampicillin were used at 40 μg/ml and 50 μg/ml, respectively.

Transformation of *M. smegmatis*

M. smegmatis mc^2 155 competent cells were transformed as described by Snapper *et al.* (1990), with a few modification. Briefly, 100 μl of the mycobacterial competent cells were mixed with plasmid DNAs and transformed in 0.4 cm-gap electroporation cuvette using a Gene Pulser (Bio-Rad) set at 2.5 kV, 25 μF and 1,000 Ω. After electroporation, the cells were immediately diluted with 0.5 ml of the appropriate growth medium and incubated at 37°C for 1 h before plating on an LB agar containing the appropriate antibiotics. The cells were then incubated at 37°C for 3 days to select transformants.

Vector construction

The pM4-OF vector series [pM4-OF and pM4-OF(-)] were constructed in the followed steps: (I) 60-bp synthetic oligomer corresponding the regulatory element (OF region) of the *M. leprae* 18-kDa gene was digested with *Xba*I and cloned into the corresponding restriction sites of pBluescript SK(-) to yield pSK(-)-3G DNA vector series. Because same cloning site (*Xba*I) was used, both forward and reverse orientation were existed, reversed orientation was named as (-). (II) a *Sal*I-*Bam*HI fragment containing the promoter region of the *M. leprae* 18-kDa gene was isolated from the pM4GAL vector, which do not contain the regulatory sequence. This fragment was cloned into the *Sal*I-*Bam*HI site of the abovementioned pSK(-)-3G DNA vector series. The resulting plasmid, pSK(-)-M4-3G, contained both the promoter of the *M. leprae* 18-kDa gene and the OF regulatory sequence. (III) The pSK(-)-M4-3G vector was digested with *Sal*I and *Not*I, the fragment containing promoter region and regulatory sequence was then isolated and blunt-ended. (IV) The resulting fragment was blunt-ligated into the *Xba*I site of the pM0GAL vector.

A pM4-OB vector series was constructed to identify additional regulatory element in the upstream region of the *M. leprae* 18-kDa gene. The construction procedure was the same as described above except that 3G DNA (OF region) was replaced by the OB region, which was generated by the amplification of a 56-bp fragment using the primers PG3-1 (5'-ACTCGCATG-CTCTAGAGCTATATA-3') and PG3-2 (5'-ACTCGCATG-CTC TAGATGTGGTCAC-3') and pUS935 vector (Dellagostin *et al.*, 1995).

Construction of vectors containing heterologous promoter

To construct the reporter vectors containing a heterologous promoter, a pB3 vector series, derivative vectors such as pM0GAL-BCG, pSK(-)-3G DNA, and SK(-)-(BCG+3G), was made. The steps for the construction of the reporter vector (pB3 vector series) were: (I) The hsp65 promoter region was amplified by using the primers hsp1 (5'-GGGTC TAGACGGTG-ACCACAACCACGCG-3') and hsp2 (5'-GGGTCTAG-ACGCGTCCGGATCG GGGATG-3') from the *M. bovis* BCG genomic DNA. The hsp65 promoters were inserted into the *Xba*I site of the plasmid pM0GAL, which contained the promoterless-*LacZ* gene and the kanamycin resistance gene, to construct the pM0GAL-BCG. (II) the 383-bp of *Sal*I-*Bam*HI fragment containing *M. bovis* BCG hsp65 promoter from pM0GAL-BCG were replaced with the corresponding portion of the above-mentioned pSK(-)-3G DNA vector series. The resulting plasmid, pSK(-)-(BCG+3G), contains both the *M. bovis* BCG hsp65 promoter and the regulatory sequence. (III) 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 [pB3 and pB3(-)].

β-galactosidase assay

For the β-galactosidase activity assays, the *M. smegmatis* transformants were grown in a Middlebrook 7H9 broth containing 40 μg/ml 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 previously described method (Miller, 1992), and the assay conditions are as follows. After the *M. smegmatis* transformants were grown to the late exponential phase, 0.5 ml of the

bacterial suspension were mixed with 0.5 ml of the Z buffer 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 μl of chloroform and 10 μl of 0.1% SDS to each assay mixture. The tubes were vortexed for 1 min and placed in a 28°C water bath for 5 min. The reaction was started by adding 100 μl of ONPG (4 mg/ml) to each tube, and the tubes were shaken for a few seconds. After a sufficient yellow color had developed, the reaction was quenched by adding 200 μl of 1 M Na_2CO_3 and then centrifuged at 13,000 rpm for 5 min. For each tube, the optical density was read at 420 nm and 550 nm. The β-galactosidase units were calculated as follows.

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

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

Results and Discussion

Identification of transcriptional repression activity by the upstream region in the *M. leprae* 18-kDa gene

M. leprae 18-kDa gene contains approximately 80 base pair long sequences located upstream of the first translation initiation codon of the gene, as depicted in Figure 1. Previously, we observed that a deletion of this upstream sequence resulted in a remarkable transcription activation in a transcription fusion assay using *LacZ* as the reporter gene (shown as in the case of pM4GAL), suggesting that 18-kDa gene expression might be controlled by the transcriptional repression responsive element(s) harbored in the upstream sequence. From our examination of the upstream sequence of the 18-kDa gene reported previously (Booth *et al*., 1988), it contains a putative palindrome sequence with possibly a dyad symmetry (CTATgTAG $(N)_{10}$ CTATaTAG), which could be separable at around -50 position of the upstream sequence. To prove the putative transcription repression responsiveness in the upstream region of the 18-kDa gene, the region was divided into two regions; -81 to -50 and -52 to -2 position of the upstream sequence, and separately isolated and placed under the immediate downstream of the Pribnow box sequence of the 18-kDa gene harbored in the pM4GAL vector, yielding the pM4-OF and pM4-OB vectors respectively. The β-galactosidase activity of these resulting constructs as well as the control vectors were then determined after transformation into the *M. smegmatis* mc^2 155 strain, which is a fast-growing mycobacterium widely used in various molecular genetic studies of mycobacteria, as a surrogate host of the uncultivable *M. leprae*. In both pM4-OF and pM4-OB

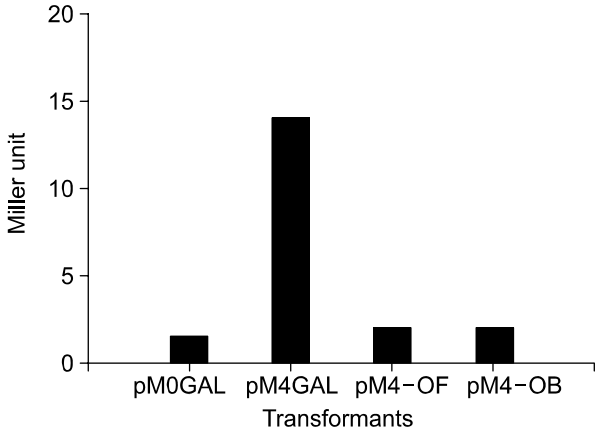

Figure 2. The effect of the first and second half region of the *M. leprae* 18-kDa gene upstream sequence on *LacZ* transcription reporter gene expression. The β-galactosidase specific activities of each of the *M. smegmatis* transformants with the indicated vector constructs were determined as described in Materials and Methods. Shown are the results of the assay at 4 h incubation. pM0GAL; promoterless-*LacZ* expression reporter vector as a negative control, pM4GAL; a vector in which *LacZ* expression is driven by the *M. leprae* 18-kDa gene promoter sequence (-231 to -81 sequence of the gene) but without having an upstream region of the gene. pM4-OF; a reporter vector in which *LacZ* expression is driven by the *M. leprae* 18-kDa gene promoter sequence plus the first half (-81 to -50 region) of the 18-kDa gene upstream sequence. pM4-OB; a vector in which *LacZ* expression is driven by the *M. leprae* 18-kDa gene promoter sequence plus the second half (-52 to -2 region) of the 18-kDa gene upstream sequence.

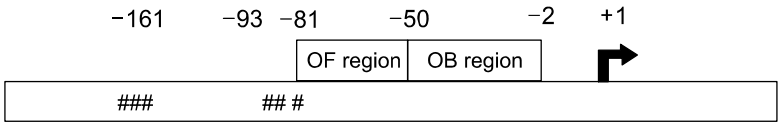

Figure 1. A schematic representation of the *M. leprae* 18-kDa gene organization. Shown are the 80 base-pair long upstream region from the downstream of the first pribnow box sequence to the first translation initiation codon of the gene and the region for the first half (OF region) and second half (OB region) examined in this study. The symbols for # indicate three possible Pribnow box sequences of the gene (Booth *et al*., 1988).

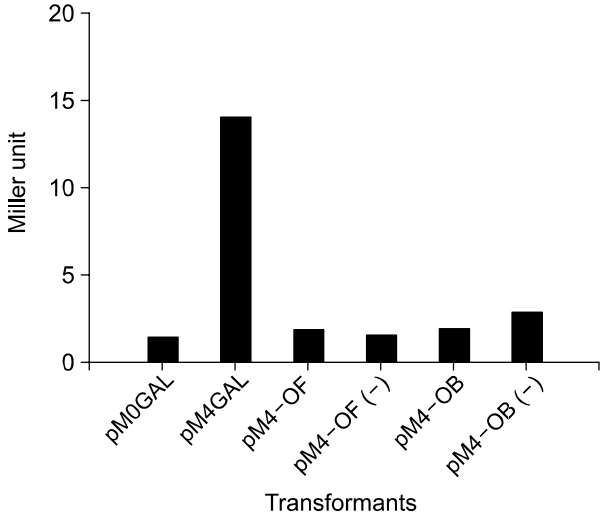

Figure 3. Orientation-independent transcription repression by the first and second half region of the *M. leprae* 18-kDa gene upstream sequence. The determination of β-galactosidase activity and all of the vector constructs used are as described in the legend of Figure 1, except that the pM4-OF(-) contains a *M. leprae* 18-kDa gene promoter sequence plus the first half (-81 to -50 region) of the 18-kDa gene upstream sequence in a reversed orientation, and the pM4-OB(-) vector contains both 18-kDa gene promoter region and the second half (-52 to -2 region) of the 18-kDa gene upstream sequence in a reversed orientation. The results are the average values of the β-galactosidase activity from two independent experiments.

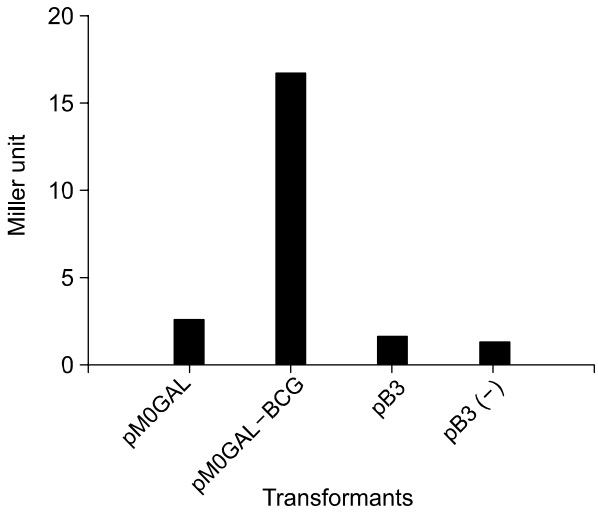

Figure 4. Heterologous transcription repression conferred by the 18-kDa gene upstream sequence. The regulatory element between -81 and -50 in the *M. leprae* 18-kDa upstream region represses the heterologous *M. bovis* BCG hsp65 promoter activity in an orientation-independent manner. pM0GAL-BCG; a reporter vector in which *Lac*Z expression is driven by the *M. bovis* BCG hsp65 promoter sequence. pB3 and pB3(-); reporter vectors in which *Lac*Z expression is driven by the M. bovis BCG hsp65 promoter sequence plus the first half (-81 to -50 region) of the 18-kDa gene upstream sequence in the forward- and reverse-oriented, respectively. The β-alactosidase specific activities with each of the indicated vectors were determined at 4 h incubation as described in Materials and Methods. The results are the average values of the β-galactosidase activity from two independent experiments.

cases, as it was previously found with a reporter vector containing the 80-bp full-length upstream sequence, their reporter gene activities was found to be suppressed dramatically from pM4GAL level to almost the same level of pM0GAL which is a promoterless-*LacZ* vector as a negative control vector (Figure 2). The results indicate that not only the region of the -81 to -50 but also the -52 to -2 position of the upstream sequence could confer equally transcriptional repression activity. Therefore, the transcription repression responsive element(s) in the upstream region of the *M. leprae* 18-kDa gene are most likely spanned around the -50 position of the upstream sequence, but they could be functionally separable at the point.

The transcription repression responsive element is able to function in an orientation-independent manner

The transcription repression responsive element such as an operator is known to act in orientation-independent manner in many cases (Zhang-Keck *et al*., 1991; Powell *et al*., 1992; Schafer *et al*., 2004), The effect of the two regions placed in a reverse orientation on the transcriptional repression activity was tested. The pM4-OF(-) and the pM4-OB(-) vector, in which the region of -81 to -50 and of -52 to -2 of the upstream sequence in the pM4-OF and the pM4-OB vector were reversed, were constructed and analyzed their β-galactosidase expression level. As shown in Figure 3, the transcriptional repression level with either pM4-OF (forward oriented) or pM4-OF(-) (reverse oriented) was equally repressed compared with that of pM4GAL. The level of β-galactosidase activity with the pM4-OB vector series, namely the pM4-OB vector and pM4-OB(-) vector containing the other element (-52 to -2) again in either the forward- or reversed-orientation respectively, were also found to be equally reduced. Taken together, these results demonstrate that the transcription regulatory elements in the upstream region of the 18-kDa gene could confer transcription repression activity in an orientation-independent manner.

The effect of the transcription regulatory element in the upstream sequence of the *M. leprae* 18-kDa gene on the heterologous promoter

To prove further the transcriptional repression activity of the transcription regulatory element(s) in the up-

stream sequence of the *M. leprae* 18-kDa gene, we examined if the element(s) could also confer transcription repression on another heterologous promoter (Cherrington *et al*., 1991; Biegalke, 1998; Wang *et al*., 2002). β-galactosidase reporter vector driven by a *M. bovis* BCG hsp65 gene promoter was constructed and named as pM0GAL-BCG, as well as pB3 and pB3(-), a derivatives of pM0GAL-BCG, in which the -81 to -50 upstream sequence of the *M. leprae* 18-kDa gene was placed immediately downstream of the promoter sequence of *M. bovis* BCG hsp65 gene in forward- and reversed-orientation, respectively. Measurement of the β-galactosidase activities with these vectors revealed again that the upstream sequence of the *M. leprae* 18-kDa gene in either orientation could effectively suppress the *M. bovis* BCG hsp65 gene promoter activity (compare pB3 series to pM0GAL-BCG in Figure 4). The same analysis with the other transcription regulatory element in the -52 to -2 sequence of the 18-kDa gene upstream region showed the same effect (data not shown). In conclusion, these results demonstrate that the transcription repression element in the upstream sequence of the *M. leprae* 18-kDa gene could also function in the heterologous promoter and it could act in an orientation-independent manner like an operator element for transcriptional repression. Identification of the transcription repression activity of the upstream region in the *M. leprae* 18-kDa gene will be important and contribute for the detailed understanding of the 18-kDa gene regulation mechanism as well as provide useful information for the manipulation of mycobacterium gene expression in future.

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