Identification of a novel mycobacterial transcriptional regulator and its involvement in growth rate dependence and stringent control

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Abstract

A novel transcriptional regulator has been identified in the 400-bp upstream region of the guaA gene of Mycobacterium tuberculosis H37Rv that promotes the expression of lacZ gene in Mycobacterium smegmatis mc2-155 and M. tuberculosis H37Rv but not in Escherichia coli DH5α. PCR-mediated deletion mutagenesis and cloning identified a 120-bp fragment upstream from the guaA gene to be the actual regulator. Primer extension analysis mapped the transcription start site to be the first ‘G’ residue of the translation start codon GTG of the guaA gene. Electrophoretic mobility shift assay showed strong binding of M. smegmatis RNA polymerase holoenzyme to the 400-bp fragment that expresses lacZ in mycobacterial species and a weak binding to the 280-bp fragment that expresses only in E. coli DH5α. Both promoter recombinants revealed varied response in the presence of purine nucleotides and exhibited down-regulation when subjected to amino acid starvation.

Keywords: Promoter; Transcriptional regulator; Stringent response; Induction

1. Introduction

Slow growth rate, poor transformation efficiencies and lack of suitable plasmid vectors are the major constraints in understanding mycobacterial molecular biology. Compared to other bacterial systems, mycobacteria have a low transcription rate, which is also manifested in a very low content of RNA per unit of DNA, and the total RNA varies only 2-fold between stationary phase cultures and actively growing cells [1]. Studies reveal that the problem not only lies in the transcriptional activity of RNA polymerase but also in the structures and functions of the promoter regions. A number of mycobacterial promoters have been studied [2] and several consensus sequences have been proposed [3]. Many of these promoters are not typical and specifically regulated in vivo.

De novo purine biosynthesis is essential for infectivity, growth and virulence of many bacteria and in mammals [4]. Guanosine monophosphate (GMP) synthetase of Mycobacterium tuberculosis H37Rv coded by guaA gene is a key enzyme in the purine biosynthetic pathway catalyzing the conversion of xanthosine monophosphate to guanosine monophosphate. Studies on the transcriptional machinery of this gene could determine its functional significance. In this study, we have analyzed the promoter region of the guaA gene and identified a transcriptional regulator in the upstream region of the gene. We have also shown that this transcriptional regulator influences the induction of transcription of the gene differentially in Escherichia coli and Mycobacterium.

Virulent mycobacteria must adapt to adverse conditions encountered during an infectious process. This means that the amounts of certain bacterial proteins must be increased in response to the changing environment and that of others lowered. Hence, studies on the transcriptional machinery of the mycobacterial genes gain importance. In several bacteria including E. coli, the number of ribosomes varies linearly with growth rate over a range of conditions. This phenomenon, termed as growth rate dependent control (GRDC) of ribosome synthesis, serves to maintain the cellular pool of ribosomes at a level commen-
surate with the requirement of the cell for protein synthesis [5]. The guaA promoter of E. coli [6] as well as the rrn promoter of M. tuberculosis [7] have been found to be involved in GRDC and stringent response, which leads to long-term survival of the organisms even in stress conditions. In Bacillus subtilis, the genes encoding the purine synthetic enzymes are subject to dual control by adenine and guanine compounds [16]. Nutrient deprivation, in the form of amino acid and carbohydrate depletion, is also likely to coincide with the formation of intact granulomas in a process thought to be essential for curtailing growth of the microorganism. The exact mechanism by which GRDC is attained is still under active investigation, although evidence implies a role for ppGpp and pppGpp [8] and translational linked events [9]. We have attempted to study whether the guaA promoter of mycobacteria has any role in stringent response and GRDC.

2. Materials and methods

2.1. Strains and plasmids

A 9.3-kb promoter probe vector pJEM13 [10], which has a lacZ reporter gene coding for β-galactosidase enzyme, was used for cloning purposes. Both E. coli DH5α and Mycobacterium smegmatis mc²155 (Table 1) recombinant strains were maintained in Luria–Bertani (LB)–kanamycin (25 μg ml⁻¹) agar plates. M. tuberculosis H37Rv recombinants were maintained in Middlebrook 7H9 broth and 7H10 agar with kanamycin (25 μg ml⁻¹) for selection of recombinants.

2.2. Construction of deletion derivatives

PCR deletions of the 400-bp upstream region were carried out using four upstream primers (KK1–KK4) and one downstream primer (KK5) (Table 1). The amplified fragments of lengths 370 bp, 280 bp, 170 bp and 100 bp cloned in pJEM13 and transformed into E. coli DH5α, M. smegmatis mc²155 and M. tuberculosis H37Rv were designated pKAM1, pKAM2, pKAM3 and pKAM4, respectively. The initial 120 bp from the 5’-end of the 400 bp was also amplified and cloned in pJEM13.

2.3. Primer extension analysis

Total RNA (50 μg) isolated from recombinants pSKV22, pKAM2 and pJEM13 was used with primers VAS2, SAP12, KK5 and KK6. Briefly, the RNA was annealed with ϕ·32P-labeled primer at 60°C for 20 min. The annealed mixtures were subjected to primer extension with 200 U of MMLV reverse transcriptase (USB) at 37°C for 1 h. The products were analyzed on a 6% polyacrylamide–7 M urea gel alongside a sequencing ladder generated with the same primer and the corresponding recombinant.

2.4. β-Galactosidase assay

The assay for β-galactosidase was performed as described by Miller [11]. All the promoter recombinants obtained in this study were subjected to this enzyme assay to check for the expression of the reporter gene. The assay was carried out at 28°C using o-nitrophenyl β-D-galactopyranoside (ONPG) as substrate and the enzyme activity,
expressed in terms of Miller units, was detected by measuring the optical density at 420 nm.

2.5. Electrophoretic mobility shift assay (EMSA)

EMSA was performed with both pSKV22 and pKAM2 clones using RNA polymerase (RNAP) isolated from M. smegmatis. Two hundred and fifty nanograms of the digested fragments were labeled with \( \alpha^{32}\text{P} \)dATP and used for binding with E. coli RNAP (USB) and M. smegmatis RNAP. The reaction mixture was incubated for 15 min at 37°C in the presence of 5×RNA polymerase binding buffer, 1% BSA and 0.1% DTT and run on a 4% non-denaturing PAGE at a constant voltage of 200 V after adding 5 µl of stop solution. For footprinting, the samples of the binding reactions were treated with 0.1 U of DNase I for 30 s and the samples were run on a 6% urea–PAGE gel along with a G+A ladder. After the run the gel was dried and autoradiographed.

2.6. Regulation experiments

For regulation experiments, one representative clone expressing in each of the hosts was taken. The clones selected were pKAM2 that expressed in DH5α and pSKV22 that expressed in mc2155 cells. These experiments were performed as described by Davies and Drabble [6].

2.6.1. Addition of nucleotides

The cultures were grown in LB-kanamycin and M-63 minimal medium [11] containing glucose (4 mg ml\(^{-1}\)) and casamino acids (40 µg ml\(^{-1}\)) in the presence of different concentrations of adenine and guanine, viz. 20, 40 and 60 µg ml\(^{-1}\), and β-galactosidase activity was determined.

2.6.2. Amino acid starvation

The cultures were grown in a minimal medium deprived of isoleucine and assayed for the enzyme activity as described previously. Cultures grown in the presence of all amino acids were processed simultaneously as control.

3. Results and discussion

In the present study, we have identified a transcriptional regulator in the upstream region of the guaA gene of M. tuberculosis H37Rv that is responsible for repression of promoter activity in E. coli. The same regulator behaves as an activator in M. smegmatis and M. tuberculosis H37Rv. We have also found the transcription start site of the gene and analyzed the involvement of the promoter in stringent and growth rate dependent control.

3.1. Identification and transcript mapping of the guaA promoter

The 400-bp upstream region of the guaA gene of M. tuberculosis in pJEM13, named pSKV22, was found to promote expression of the lacZ gene in M. smegmatis mc\(^{155}\) and M. tuberculosis H37Rv but not in E. coli DH5α. It is known that many mycobacterial promoters do not function well in E. coli [12]. The promoter activity of this clone was 253–275 Miller units in M. smegmatis mc\(^{155}\) and 754–810 Miller units in M. tuberculosis H37Rv (Fig. 1). The reason for the reduced expression of M. tuberculosis promoter in M. smegmatis remains unclear, but may reflect differences in transcription machinery amongst mycobacterial species or the existence of some repressive elements within M. smegmatis [13].

MMLV reverse transcriptase was used to identify the transcriptional start site of the promoter with primer VAS2, located 178 bp downstream from the coding region...
of the lacZ and a single transcription start site was mapped to the first G residue of the GTG translation start codon. The putative −10 and the −35 regions have been identified along with a potential GC-rich discriminator region and a DnaA-protein binding region.

3.2. PCR mutagenesis of the promoter

Four deletions were made by the PCR cloning method on the 400-bp upstream region to analyze the promoter. Of the four deleted upstream fragments, pKAM1 showed expression in M. smegmatis mc2155 and M. tuberculosis H37Rv similar to pSKV22 with an enzyme level of 264–276 and 745–815 Miller units, while pKAM3 and pKAM4 did not promote lacZ expression in either E. coli DH5α or M. smegmatis mc2155. The clone, pKAM2, on the other hand, promoted high level expression in E. coli DH5α and had a very strong promoter activity of 20,134–22,954 Miller units (Fig. 1). The clone pKAM2 was sequenced and no mutations were observed in the 280-bp sequence. As the high level expression of pKAM2 may be related to the multi-copy nature of the vector, we compared the expression of pKAM2 with other promoter clones in the same multi-copy vector isolated in our laboratory and a few commercial multi-copy number vectors (pST BLUE-1 and pGEM4Z) harboring T7 promoter driving the lacZ gene. As observed earlier pKAM2 had a higher expression

![Fig. 3. Nucleotide sequence of the guaA promoter region. The transcription start site is mapped to the first G residue of the GTG translation start codon. The putative −10 and the −35 regions have been identified along with a potential GC-rich discriminator region and a DnaA-protein binding region.](image-url)

![Fig. 4. Electrophoretic mobility shift assay and footprinting assay were performed using M. smegmatis RNAP and E. coli RNAP (USB). The mobility shift assay (a) was carried out using 400 bp and 280 bp. Lanes C and C indicate controls with labeled probes 400 bp and 280 bp respectively. Lanes T1 and T2 indicate binding assays performed with M. smegmatis and E. coli RNAP with the 400-bp fragment. Lanes T1+ and T2+ indicate binding assay, performed with M. smegmatis and E. coli RNAP with the 280-bp fragment. Footprinting (b) was performed by DNase I treatment on the enzyme bound fractions of the mobility shift reaction. Lane S indicates the G+A ladder of the 400-bp region, lanes D1 and D2 indicate the DNase I treated RNAP bound fraction of the 400-bp fragment in duplicates and lane C indicates the DNase I treated 400-bp fragment alone.](image-url)
level when compared to other multi-copy number vectors, indicating that pKAM2 is a specific regulator that helps in high level expression. The 5'-120-bp region alone did not promote expression either in E. coli DH5α or M. smegmatis mc2155 [15].

3.3. Specificity of M. smegmatis RNAP

To substantiate the result of the differential expression of the clones pSKV22 and pKAM2, EMSA and in vitro transcription assay were carried out. Both E. coli and M. smegmatis RNA polymerases were found to bind to the promoter fragments (Fig. 4a). The MgCl2 and KCl concentrations were found to influence the binding of the polymerases. M. smegmatis RNAP bound strongly to the promoter using reaction buffer without MgCl2. Neither enzyme bound to a non-promoter fragment (5'-120-bp region), indicating the specificity of the enzymes. However, the binding of M. smegmatis RNAP with pKAM2 was very weak when compared to pSKV22, but there was no difference in the strength of binding of E. coli RNAP to the two promoter clones. As the mobility shift assay does not reveal the functional status of the promoter clones, the in vitro transcription assay was performed. M. smegmatis RNAP was able to transcribe the pSKV22 clone in vitro while no transcription was observed with pKAM2 as observed by the scintillation counts (data not shown). These results confirm the activity of the RNAP in vitro as well as the expression of pSKV22 in mycobacterial species alone. Further, the footprinting assay revealed the protected region to lie between the 3'-7 and 3'-38 region of the promoter fragment supporting the observation of the binding assay (Fig. 4b).

3.4. Regulation of the promoter recombinants

We have presented experimental evidence both for stringent control and for ‘growth rate dependent’ regulation of the guaA promoter of M. tuberculosis by studying the effect of addition of nucleotides and depletion of amino acids on pSKV22 and pKAM2. Earlier studies had shown that the levels of some of the enzymes involved in the synthesis of IMP are repressed by the addition of purines to the medium. Guanine was found to induce the pSKV22

| Table 2 |
| Effect of isoleucine starvation on pKAM2 and pSKV22 clones |

<table>
<thead>
<tr>
<th></th>
<th>pKAM2 (E. coli)</th>
<th>pSKV22 (M. smegmatis)</th>
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<tbody>
<tr>
<td></td>
<td>OD&lt;sub&gt;600&lt;/sub&gt;</td>
<td>β-Gal (Miller units)</td>
</tr>
<tr>
<td>Control (cultures with isoleucine)</td>
<td>0.355</td>
<td>20134-22954</td>
</tr>
<tr>
<td>Test (cultures without isoleucine)</td>
<td>0.340</td>
<td>6800-7430</td>
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</table>

The cultures were grown in M-63 medium with all amino acids except isoleucine till the OD<sub>600</sub> reached 0.3-0.4 and were assayed for β-galactosidase enzyme. Cultures with all amino acids were processed similarly and served as control.
clone in *M. smegmatis* mc²155 and in *M. tuberculosis* H37Rv significantly, whereas it was found to decrease the promoter activity of pKAM2 in *E. coli* DH5α by 20–40% (Fig. 5a,b). A similar pattern of results was also observed with adenine induction. To check the specificity of guanine induction on pSKV22, the same experiment was repeated with the other promoter clones driving the expression of the lacZ gene isolated in our laboratory, and no induction was observed (Fig. 5c). Also, IPTG did not exert any induction on either pSKV22 or pKAM2, indicating that the promoter clones could be induced specifically by guanine and not with any other common inducers. Isoleucine starvation caused a 60% and 40% decrease in enzyme activity of pKAM2 and pSKV22 respectively (Table 2). These findings are supported by earlier reports where a 75% fall in the enzyme activity was exerted by the guaA operon of *E. coli* under isoleucine starved conditions [6] and a similar decrease was reported for the P1 promoters of the *rrn* operon [7]. The addition of isoleucine after 6 h to the deleted clone pKAM2 in *E. coli* DH5α increased the growth but the enzyme activity remained low, confirming the repression to be ‘growth rate dependent’ and the addition of isoleucine to the pSKV22 clone in *M. smegmatis* mc²155 did not increase the growth nor the enzyme activity further, confirming the ‘growth rate independent’ regulation of the promoter clone. Further, depletion of other amino acids did not have any effect on the promoter clones. Further studies in relation to the accumulation of some of the second messengers like hyperphosphorylated guanine residues and the σ factors responsible for the expression of the promoter might throw light on the regulatory mechanisms of the guaA promoter of *M. tuberculosis* and its survival under stress conditions.

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