Transcription and autoregulation of the *Rv3134c-devR-devS* operon of *Mycobacterium tuberculosis*

Gargi Bagchi,[†] Santosh Chauhan,[†] Deepak Sharma
and Jaya Sivaswami Tyagi

Correspondence

Jaya S. Tyagi
jstyagi@aiims.ac.in

Department of Biotechnology, All India Institute of Medical Sciences, Ansari Nagar, New Delhi 110029, India

DevR is a transcriptional regulator that mediates the genetic response of *Mycobacterium tuberculosis* to oxygen limitation and nitric oxide exposure. *devR* is co-transcribed along with *devS*, which encodes its cognate sensor kinase, and an upstream gene, *Rv3134c*. The transcriptional activity of this operon was characterized by primer extension, transcriptional fusion and electrophoretic mobility shift assays (EMSAs) under aerobic conditions. Transcription start points (Tsps) were detected upstream of both *Rv3134c* and *devR*, and the major transcript was derived from upstream of *Rv3134c*. Sequences with similarity to sigma factor consensus elements and to DevR-binding motifs were detected in the vicinity of the Tsps by *in silico* analysis. EMSAs with promoter regions and DevR protein showed that DevR binds to its own promoters in a sequence-specific manner with differing affinities. Consistent with the primer extension and EMSA data, *Rv3134c* promoters, and not *devR* promoters, were determined to be the principal promoters of this operon using reporter assays performed in *Mycobacterium smegmatis* and *Escherichia coli*. Furthermore, DevR modulated the activity of both *devR* and *Rv3134c* promoters. From these findings it is inferred that the *Rv3134c-devR-devS* operon is transcribed from multiple promoters and is autoregulated.

Received 8 July 2005

Revised 24 August 2005

Accepted 21 September 2005

INTRODUCTION

devR (*Rv3133c/dosR*) encodes a response regulator of *Mycobacterium tuberculosis* that was identified earlier in our laboratory through a screen for candidate virulence genes (Dasgupta *et al.*, 2000; Kinger & Tyagi, 1993). The importance of this two-component system in pathogenesis became evident from animal experiments, which showed that disruption or deletion of *devR* affected the virulence status of *M. tuberculosis* (Malhotra *et al.*, 2004; Parish *et al.*, 2003). Two-component systems play a central role in the adaptive response of bacteria to environmental stresses. Deciphering their properties and transcriptional regulation is expected to enhance our understanding of *M. tuberculosis* adaptation within host tissues. Of the two-component systems in *M. tuberculosis*, only four, namely, *senX3-regX3* (Himpens *et al.*, 2000), *trcR-trcS* (Haydel *et al.*, 2002), *prfA-prfB* (Ewann *et al.*, 2004) and *mprA* (He & Zahrt, 2005) have been subjected to transcription regulatory studies. Autoregulation was a common feature in all four systems as deduced from studies in surrogate mycobacterial and non-mycobacterial hosts. Downstream of *devR* (*Rv3133c/dosR*) maps *devS* (*Rv3132c/dosS*), which encodes a sensor kinase.

DevR-DevS is a bona fide two-component system of *M. tuberculosis* (Roberts *et al.*, 2004; Saini *et al.*, 2004). The *devR-devS* locus is preceded by the *Rv3134c* gene, predicted to encode an Ala-Val-rich protein containing a universal stress protein (USP) domain (Nystrom & Neidhardt, 1992, 1994). We showed earlier that *Rv3134c*, *devR* and *devS* are co-transcribed (Dasgupta *et al.*, 2000).

DevR mediates the genetic response of *M. tuberculosis* to oxygen limitation (Park *et al.*, 2003) and is implicated in the bacterium's response to nitric oxide exposure (Voskuil *et al.*, 2003), two conditions encountered by bacteria *in vivo* and thought to be associated with latent tuberculosis (Nathan & Shiloh, 2000; Wayne & Sohaskey, 2001). Analysis of the transcriptional regulation of *devR-devS* is required to define the function of these genes in establishing and maintaining tuberculosis latency in human beings. To begin to study the regulation of this system, we characterized the transcription of the *Rv3134c-devR-devS* locus under aerobic conditions. We show here that there are two regions with promoter activity in this operon that map upstream of *Rv3134c* and *devR*, respectively, and transcription occurs from upstream of both genes to produce transcripts under basal conditions. Sequence analysis indicates the presence of sequences with similarity to consensus sites for sigma factors and DevR-binding sites in this region. DevR protein bound specifically to the promoter regions of this operon and regulated *devR*

[†]These authors contributed equally to the work.

Abbreviations: EMSA, electrophoretic mobility shift assay; GST, glutathione S-transferase; Tsp, transcription start point.

and *Rv3134c* promoter activity in *Mycobacterium smegmatis* and *Escherichia coli*. Our findings suggest the potential for complex regulation of the *Rv3134c-devR-devS* operon under inducing conditions.

METHODS

Plasmids, bacterial strains and culture conditions. The plasmids used in this study are listed in Table 1. *M. tuberculosis* H37Rv was cultured in Middlebrook 7H9 medium, at 37 °C under aerobic shaking conditions, to an OD₅₉₀ of 0.4–0.6, after which the cells were pelleted at 4 °C and processed further for RNA isolation. *M. smegmatis* mc²155 (a kind gift from Dr W. R. Jacobs, New York, USA) was used for reporter assay studies and was cultured in Dubos-Tween-Albumin broth (DTA). *E. coli* was routinely grown in LB medium, and in 2 × YT medium for protein overexpression. Recombinant GST-DevR was produced in *E. coli* BL23(DE3). Antibiotics, when required, were used at the concentrations indicated: ampicillin at 100 µg ml⁻¹; kanamycin at 50 µg ml⁻¹ for *E. coli* and 25 µg ml⁻¹ for *M. smegmatis*. All the cloning steps were carried out in *E. coli* XL-1 Blue (Stratagene) as described by Sambrook & Russell (2001).

RNA isolation. RNA was isolated from *M. tuberculosis* culture pellets using a slightly modified version of the RNeasy Mini protocol for isolation of total RNA from yeast (RNeasy Mini kit, Qiagen). Briefly, the pellet was resuspended in an appropriate volume of the lysis buffer provided in the kit. The cells were subjected to bead beating with 0.1 mm zirconium silicate beads in a mini Bead beater (Biospec) with three pulses of 30 s each. The beads and cell debris were removed by centrifugation at 2000 r.p.m. for 3 min at 4 °C. The supernatant was collected and processed according to the manufacturer's guidelines. To remove residual quantities of DNA in the RNA preparation, the RNA was incubated at 37 °C for 1 h with 100 units of DNase in the presence of DNase buffer, extracted with phenol/chloroform and precipitated with ethanol overnight. The precipitated RNA was recovered by centrifugation at 18 000 r.p.m. for 30 min at 4 °C and then resuspended in minimum volume of 0.1 mM EDTA. The A₂₆₀ of 1 µl samples was checked to assess the yield.

Primer extension. Primer extension was carried out using an appropriate gene-specific reverse primer and ~10 µg RNA from exponential-phase cultures of *M. tuberculosis* H37Rv. Briefly, the ³²P end-labelled primer was annealed to RNA at 60 or 65 °C for 45 min and cDNA was synthesized at 37 °C for 60 min using 50 units Stratascript Reverse Transcriptase as described by the manufacturer. cDNA was recovered by ethanol precipitation, resuspended in 4 µl of 40 µg RNase ml⁻¹ and incubated at room temperature for 3 min; the reaction was stopped by adding formamide gel loading buffer.

The sequencing reaction was performed with plasmid pDP2, which contained 1.45 kb of *devR* upstream region cloned into pDK16 (Bagchi *et al.*, 2003), as template and oligonucleotide R3 or R9c primers and the fmol^R DNA sequencing system (Promega) to map the transcription start points (Tsps) upstream of *Rv3134c* and *devR*. To map the Tsp upstream of *devS*, the sequencing ladder was obtained using plasmid pJT53.34 (Kinger & Tyagi, 1993) and oligonucleotide Srev3. The sequences of the primers used for primer extension are listed in Table 2 and their positions are indicated in Fig. 1(a). The sequencing reactions were set up according to the manufacturer's guidelines and were analysed on a 6% denaturing polyacrylamide gel along with the primer extension products. Results are representative of at least triplicate experiments with two to four individual RNA preparations.

Reporter plasmid constructions. The plasmid pFPV27 (Valdivia *et al.*, 1996) containing a promoterless *gfp* reporter gene was used to construct the transcriptional fusions. DNA fragments corresponding to the *Rv3134c* and *devR* upstream regions and containing two Tsps each (see Fig. 1a) were amplified using specific oligonucleotides (Table 2). The amplified DNAs were A-tailed, cloned into pGEMT-Easy (Promega), digested with *EcoRI* and cloned into the *EcoRI* site upstream of the promoterless *gfp* of pFPV27. Plasmid pSC1 was constructed with primers R7 and R9c. Plasmid pSC3 was constructed with primers 53R1 and R3 (Tables 1 and 2).

DevR autoregulation was also assessed in *E. coli* DH5α using a two-plasmid system. For this, DNA fragments corresponding to the *Rv3134c* and *devR* upstream regions (the same as those used for *M. smegmatis* experiments) were cloned upstream of the promoterless *lacZ* gene in pDK16 (A. K. Tyagi & D. K. Kaushal, unpublished) to generate pSC5 and pSC6, respectively (Table 2, Fig. 1a). Plasmid pDK16

Table 1. Plasmids

Plasmid	Description	Source or reference
pGEMT-Easy	<i>E. coli</i> cloning vector	Promega
pGEX4T1	<i>E. coli</i> expression vector (N-terminal GST tag)	Amersham Biosciences
pFPV27	<i>E. coli</i> -mycobacteria shuttle plasmid containing promoterless <i>gfp</i> , Km ^r	Valdivia <i>et al.</i> (1996)
pDK16	<i>E. coli</i> -mycobacteria shuttle integrating plasmid carrying promoterless <i>lacZ</i> , Km ^r	A.K. Tyagi & D.K. Kaushal, unpublished
pSCDevR	<i>devR</i> coding region in pGEX4T1 to overexpress DevR with GST N-terminal tag	This study
pDSR217P	pDSR217 (Saini <i>et al.</i> , 2004) modified by replacing the pMB1 origin of replication with the p15A origin of replication to make it compatible with pDK16	This study
pSC1	pFPV27 containing <i>Rv3134c</i> promoter (R7–R9c fragment) upstream of <i>gfp</i>	This study
pSC2	pSC1 containing <i>M. tuberculosis devR</i> and 327 bp upstream sequences	This study
pSC3	pFPV27 containing <i>devR</i> promoter (53R1–R3 fragment) upstream of <i>gfp</i>	This study
pSC4	pSC3 containing <i>M. tuberculosis devR</i> and 327 bp upstream sequences	This study
pSC5	pDK16 containing <i>Rv3134c</i> promoter (R7–R9c fragment) upstream of <i>lacZ</i>	This study
pSC6	pDK16 containing <i>devR</i> promoter (53R1–R3 fragment) upstream of <i>lacZ</i>	This study

Table 2. Oligonucleotide primers

Primer or primer pair, and use	Sequence (5'–3')	Product size (bp)
Mapping of Tsp by primer extension		
R9C	CTCATCGACCGCCCAACG	
R3	GACCTTTACCACCAGGGCACC	
Srev3	CGCTGTGACCACGAGCATGG	
EMSA, GFP, LacZ assays		
R7/	CGGTGGTAAGAACGCGTAGTCC/	698
R9c	CTCATCGACCGCCCAACG	
53R1/	GTCAGCGCGTTGTGCGGGAG/	402
R3	GACCTTTACCACCAGGGCACC	
DevR overexpression		
devRBamf/	TGGGAGTGGATCCCTGGTGGTAAAGG/	
devRBamr	CGAGGATCCCTGTTGTCATGGTCCAT	

contains a multiple cloning site upstream of *lacZ*, a kanamycin-resistance cassette, the L5 *att-int* region for integration into the mycobacterial genome and a *ColE1* origin for replication in *E. coli*.

Plasmids pSC5 and pSC6 were transformed along with the DevR expression construct pDSR217P (see below) into *E. coli* and selected on LB agar plates containing kanamycin and ampicillin. The cultures were

(b)

Promoter (Sigma factor)	-35	Spacer	-10	Spacer	Tsp
P2 _{Rv3134c} (SigA)	cTcACA	17	TATtCT	5	T
P1 _{Rv3134c} (SigC)	CGatAT	20	gGTtCG	9	G
P2 _{devR}	–	–	cACGcT	12	G
P1 _{devR} (SigC)	CGtcAT	16	aGcCGG	5	C, T
P2 _{Rv3134c} (<i>E. coli</i> σ^{70})	cTcACA	17	TATtCT		?
SigA consensus	TTGACW	17	TATAMT		
SigC consensus	SSSAAT	16–20	CGTSSS		
<i>E. coli</i> σ^{70} consensus	TTGACA	16–18	TATAAT		

(c)

DevR motif	Sequence	Score	Reference
1	acCtGGACgAgccaCCCGtg	5.50	This study
2	aTaaGGACTAAcGgCCctcA	11.91	Park <i>et al.</i> (2003); Florczyk <i>et al.</i> (2003)
3	gTGGGGACcAAcGcCCctgg	12.69	Park <i>et al.</i> (2003); Florczyk <i>et al.</i> (2003)
4	acGGGatgTATccgCCCCAg	5.71	This study
5	ggtcGGcCTTAtGcCCCGtg	6.41	This study
6	TgCGGGTggATcGggCCatc	4.35	This study
DevR consensus motif	TTSGGGACTWWAGTCCCSAA WRGGGACNTTNGNCCCYN		Park <i>et al.</i> (2003) Florczyk <i>et al.</i> (2003)

Fig. 1. Organization of the *Rv3134c-devR-devS* operon in the *M. tuberculosis* genome. (a) Horizontal dashed arrows indicate the position of primers used for primer extension. The major Tsp is indicated by a thick arrow and the minor ones by thin arrows. The regions/fragments, along with their respective primers, used for EMSAs and for constructing the *gfp* and *lacZ* reporter plasmids are shown. Vertical rectangles (numbered as 1, 2, etc.) indicate the putative DevR-binding motifs. (b) Potential matches to the -10 and -35 sigma consensus sequences identified upstream of the Tsp are indicated. The SigA, SigC and *E. coli* σ^{70} consensus motifs are from Manganelli *et al.* (2004) and Lewin (1997). (c) Sequences of the putative DevR-binding motifs are tabulated with their scores along with the consensus motifs. [In (b) and (c), lower-case letters indicate disagreement with motif consensus; W, A/T; M, A/C; S, C/G; R, A/G; Y, C/T].

grown overnight with shaking at 37 °C, diluted 100-fold into fresh LB medium, grown to an OD₅₉₅ of 0.6–0.7 and induced with 0.1 mM IPTG for 3 h. β -Galactosidase assay was performed as described by Miller (1972). The mean activity of three independent experiments is reported.

Construction of plasmids expressing DevR. *devR* was amplified from *M. tuberculosis* DNA using devRBamf and devRBamr primers (Table 2, *Bam*HI sites are underlined), digested with *Bam*HI and cloned into the *Bam*HI site of pGEX-4T1 (Amersham), generating pSCDevR. Plasmids pSC2 and pSC4, which expressed *M. tuberculosis* DevR at a physiological level in *M. smegmatis*, were constructed by digesting pSC1 and pSC3 at their unique *Nhe*I site and inserting the *devR* gene along with 327 bp of upstream sequences derived from pDSdevR (Malhotra *et al.*, 2004).

For *E. coli lacZ* reporter assays, DevR was overexpressed from pDSR217P, generated from pDSR217 (Saini *et al.*, 2004) by replacing the pMB1 origin of replication in the latter with a p15A origin of replication derived from pGP1-2 (Tabor & Richardson, 1985). In this way pDSR217P was compatible with pDK16 in *E. coli* cells carrying both plasmids.

DevR purification. DevR was overexpressed as an N-terminally GST-tagged 49.2 kDa fusion protein from pSCDevR in *E. coli* BL23(DE3). Briefly, 600 ml of culture was grown at 37 °C to an OD₅₉₅ of 1.0 and induced at 25 °C for 5 h with 0.1 mM IPTG. The induced cells were harvested by centrifugation, resuspended in phosphate-buffered saline (pH 7.4) containing protease inhibitor cocktail (Roche) and sonicated (duty cycle 70, three pulses of 2 min each, Branson Sonifier 450, USA). Briefly, DevR was purified by affinity chromatography using 2 ml GST.Bind.Resin (Novagen) previously equilibrated with wash/bind buffer (43 mM Na₂HPO₄, 14.7 mM KH₂PO₄, 1.37 M NaCl and 27 mM KCl pH 7.3). The lysate was mixed thoroughly with the resin and incubated at room temperature for 30 min. The flowthrough was collected and then the column was washed with 30–35 bed volumes of the same buffer. Bound DevR was eluted from the column with GST elution buffer (10 mM reduced glutathione dissolved in 50 mM Tris/HCl pH 8.0). Protein-containing fractions were pooled, dialysed against buffer containing 50 mM Tris/HCl pH 8.0, 50 mM NaCl, 50 % (v/v) glycerol and 0.1 mM DTT and stored at –20 °C. The concentration of the dialysed protein was determined by the bicinchoninic acid method (Pierce) using BSA as standard. Purified GST-DevR (henceforth referred to as DevR) was used for the EMSA experiments (see below).

Electrophoretic mobility shift assays (EMSAs). Purified recombinant DevR was used to assess protein binding to two promoter fragments derived from the *Rv3134c-devR-devS* operon. The radiolabelled promoter DNA fragments for these assays were generated by PCR from *M. tuberculosis* DNA using primers that were end-labelled with [γ -³²P]ATP (specific activity ~3000–5000 Ci mmol^{–1}, ~110–185 TBq mmol^{–1}; BRIT, India) using T4 polynucleotide kinase. PCR products were purified from the gel before use in EMSAs. The primers and probes that were used are listed in Table 2. In standard reactions, DevR protein was incubated with binding buffer containing 25 mM Tris/HCl (pH 8.0), 0.5 mM EDTA, 20 mM KCl, 6 mM MgCl₂, 5 % glycerol, 0.1 mM DTT and 1 μ g poly (dI-dC) for 20 min on ice. Three nanograms of ³²P-labelled DNA was added in a final reaction volume of 20 μ l and incubated on ice for another 30 min. DNA–protein complexes were separated on a 5 % non-denaturing polyacrylamide gel by electrophoresis in 0.25 $\times$ TBE at 150 V at 4–8 °C and the DNA–protein complexes were visualized by autoradiography.

***M. smegmatis* protein extract preparation and immunoblot analysis.** Lysates were prepared from exponential-phase and stationary-phase cultures of recombinant *M. smegmatis* carrying

pFPV27 vector, pSC1 and pSC2 as described by Saini *et al.* (2004). Lysates containing 5 μ g protein were electrophoresed on 12.5 % SDS-PAGE gels and subjected to immunoblotting using 1:4000 dilution of high-titre polyclonal anti-DevR sera as described by Saini *et al.* (2004).

Measurement of GFP fluorescence. *M. smegmatis* cultures carrying the various recombinant *gfp* constructs (pSC series) and pFPV27 vector were grown in DTA containing kanamycin (starting OD₅₉₅ 0.01) under aerobic conditions at 37 °C until they attained stationary phase (60–70 h). Samples were taken from mid-exponential and stationary-phase cultures and GFP fluorescence was assessed in a spectrofluorimeter using an excitation wavelength of 483 nm and an emission wavelength of 515 nm (Molecular Devices).

The inserts of all reporter constructs and the DevR expression plasmid were verified for accuracy by DNA sequencing.

RESULTS

The *devR-devS* genes are arranged contiguously on the complementary strand in the *M. tuberculosis* genome and their coding regions overlap by 4 bp (Cole *et al.*, 1998). They form an operon along with the *Rv3134c* gene, which lies 27 bp upstream of *devR* and encodes a putative 28 kDa protein (Dasgupta *et al.*, 2000). A 584 bp intergenic sequence is present between *Rv3134c* and the divergently transcribed *Rv3135* gene, which encodes a PPE protein (Cole *et al.*, 1998; Dasgupta & Tyagi, 1998). The upstream and intergenic regions are shown in Fig. 1(a).

The *Rv3134c-devR-devS* operon is transcribed from several promoters

Two primer extension products were obtained using primer R9c and *M. tuberculosis* RNA from mid-exponential-phase cells grown under aerobic conditions. These included a prominent product, T1_{*Rv3134c*} at –240 and a minor Tsp, T2_{*Rv3134c*} at –286 (Fig. 2a, b). Since multiple promoters are common in *M. tuberculosis*, and *devR* upstream sequences supported DevR expression in *devR* mutant strains complemented with DevR (Malhotra *et al.*, 2004; Parish *et al.*, 2003), we searched for additional Tsps upstream of *devR* using primer R3. Two primer extension products, T2_{*devR*} and T1_{*devR*}, were obtained upstream of *devR* at –273 and –120/–119 (Fig. 2c, d). Product T1_{*devR*} corresponded to a doublet. We also searched for Tsps upstream of *devS* using primer Srev3 and no signal was detected (data not shown). Annealing of primers to RNA at 65 °C gave similar primer extension products. These data show that the *Rv3134c-devR-devS* operon is transcribed from Tsps located upstream of both *Rv3134c* and *devR* and that the major transcript originated upstream of *Rv3134c*. The location of various Tsps in relation to the coding regions is indicated in Fig. 1(a). The four primer extension products are likely to represent individual Tsps driven by their independent promoters. It cannot however be ruled out that the proximal Tsps, T1_{*devR*} and T1_{*Rv3134c*} are produced by processing from the distal Tsps, T2_{*devR*} and T2_{*Rv3134c*} respectively. In the absence of specific data on transcript processing, we consider each of the Tsps to be independently generated.

Fig. 2. Primer extension analysis of the *Rv3134c-devR-devS* operon. Primer extension was performed with RNA isolated from exponential-phase cultures of *M. tuberculosis* using the indicated primers. Products (Mtb) were resolved on a 6% denaturing polyacrylamide gel along with a sequencing ladder of the corresponding region, obtained using the same primer. T1_{Rv3134c} and T2_{Rv3134c} represent the Tsps upstream of *Rv3134c*; T1_{devR} and T2_{devR} represent the Tsps upstream of *devR*.

In silico analysis of the promoter region

The identification of several primer extension products raised the possibility of defining the promoter elements involved in transcription initiation at this locus. Regions upstream of the experimentally determined Tsps were searched for matches to the consensus elements proposed for various *M. tuberculosis* sigma factors (Calamita *et al.*, 2005; Manganelli *et al.*, 2004; Raman *et al.*, 2004) using MyPatternFinder (<http://www.imtech.res.in/raghava/MyPatternFinder>; D. Sharma and others, unpublished data). Perfect matches or those with one mismatch to the consensus -10 or -35 promoter elements were not found, so we allowed two mismatches in the bipartite elements and a spacing of 5–9 bp between the -10 hexamer motif and the Tsps. Sequences similar but not identical to consensus motifs corresponding to SigA- and SigC-binding sites were located upstream from the Tsps (Fig. 1b). Both SigA and SigC sigma factors are expressed during exponential growth (Hu & Coates, 2001; Manganelli *et al.*, 1999), i.e. when RNA was isolated for primer extension analysis. Only a putative -10 promoter element was detected 12 bp upstream of T2_{devR} (Bashyam *et al.*, 1996); relaxation in the spacing (from the Tsp) was allowed in this case since no sigma or -10 consensus motif was present at the normal position.

Six copies of the DevR consensus motif 5'-TTSGGGACT-WWAGTCCCSAA-3' (Park *et al.*, 2003) or 5'-WRGGGACNTTNGNCCCYN-3' (Florczyk *et al.*, 2003) were also identified in the region encompassing the *devR* upstream region, *Rv3134c* coding sequences and *Rv3134c* upstream region (see Fig. 1) using MyPatternFinder, suggesting that DevR could bind at multiple sites in this region. Motifs 2 and 3 displayed >80% identity with that described by Florczyk *et al.* (2003). To detect motifs similar to that proposed by Park *et al.* (2003), scores were calculated as described and motifs with scores >4 are indicated in Fig. 1(c). These putative binding sites varied in their location with respect to the Tsps or sigma factor consensus elements; they were

arranged upstream as well as downstream of Tsps or in translated and untranslated regions (Fig. 1c).

DevR autoregulation

For functional characterization of the *Rv3134c-devR-devS* regulatory region, we constructed plasmids pSC1 and pSC3 (Fig. 1a) containing transcriptional fusions between *gfp* and DNA sequences. Each plasmid carried two Tsps (and two promoters) and putative DevR-binding sequences. The plasmids were electroporated into *M. smegmatis* and the levels of *gfp* expression were compared over a 60–70 h period that encompassed the exponential and stationary phases of growth. *M. smegmatis* has been used frequently to study the activity of *M. tuberculosis* promoters (Himpens *et al.*, 2000; Sala *et al.*, 2003; Verma *et al.*, 1999) because the transcriptional machinery is well conserved between the two organisms (Bashyam *et al.*, 1996). Further, the *M. tuberculosis* *Rv3134c-devR* promoter region was shown earlier to be active in *M. smegmatis* (Bagchi *et al.*, 2003). The data indicated that the *Rv3134c* promoters fragment (*Rv3134c_{pro}*) carried the major promoters, which were notably stronger than the *devR* promoters (*devR_{pro}*). *Rv3134c_{pro}* remained active throughout and GFP fluorescence increased as the cells advanced into the stationary phase. The increase with time in GFP fluorescence from the *Rv3134c* promoters indicates that they are induced during stationary phase. The high activities of the *Rv3134c* promoters correlated with the strength of the Tsp signals detected upstream of *Rv3134c* by primer extension (Table 3, Fig. 2). The precise contribution of each Tsp to the total transcript pool awaits further assessment of the individual promoters.

Many *M. tuberculosis* response regulators are subjected to autoregulation. We next assessed *devR* autoregulation under conditions that better reflected physiological conditions. For this, *devR* was placed under the control of its own promoter located within 327 bp of upstream sequences in

Table 3. Measurement of promoter activity using GFP fluorescence

Plasmid carried by <i>M. smegmatis</i>	GFP fluorescence at:	
	Mid-exponential phase	Stationary phase
pSC1	398.8 ± 35.1	1207.5 ± 45.6
pSC2 (expressing <i>M. tb</i> DevR)	259.5 ± 19.6	732.8 ± 10.5
pSC3	18.2 ± 3.1	22 ± 0.3
pSC4 (expressing <i>M. tb</i> DevR)	33.7 ± 4.7	70.7 ± 2.4

plasmids pSC2 and pSC4 as described in Methods. These plasmids were introduced into *M. smegmatis* and the expression of *M. tuberculosis* DevR protein was confirmed by Western blotting using anti-DevR polyclonal antibodies (Fig. 3). DevR expression under aerobic conditions resulted in a 35–40 % reduction in GFP fluorescence from the *Rv3134c* promoters, suggesting that DevR negatively regulates their activity. In contrast, DevR expression caused a very modest rise in the activity of *devR* promoters (Table 3).

Since *M. smegmatis* has its own DevR-DevS system, we examined the effect of exogenously supplied *M. tuberculosis* DevR on promoter activity in *E. coli*, which lacks *devR-devS* genes. Through similar studies on the *trcR* promoter in *E. coli*, Haydel *et al.* (2002) concluded that TrcR was involved in transcriptional autoactivation. Plasmids pSC5 and pSC6, containing the *Rv3134c* promoters and *devR* promoters respectively (Table 1, Fig. 1a), were transformed into *E. coli* and LacZ was measured in exponential-phase aerated cultures. The *Rv3134c* promoters were well recognized in *E. coli* while the *devR* promoters did not support detectable *lacZ* activity. In the presence of DevR expressed from the *trc* promoter upon induction, the *Rv3134c* promoter was repressed by ~68 %. The mean activity of the *Rv3134c* promoters in the absence and presence of DevR was 533 and

172 Miller units, respectively. The *Rv3134c* promoter was repressed to a greater extent in *E. coli* than in *M. smegmatis* (68 % vs 35–40 %). A possible reason for this difference could be that DevR was expressed at significantly higher levels under inducing conditions in *E. coli* than in *M. smegmatis*, where DevR was expressed from its upstream promoter, which is rather weak. The *devR* promoters did not exhibit any activity in either the absence or presence of DevR, suggesting that they are not recognized in *E. coli*, as reported earlier for many *M. tuberculosis* promoters (Das Gupta *et al.*, 1993). We conclude on the basis of these experiments conducted in *M. smegmatis* and *E. coli* that DevR represses the activity of the *Rv3134c* promoters under aerobic conditions.

DevR binds to DNA fragments containing DevR boxes in the *Rv3134c-devR-devS* operon

DevR was shown to bind to a conserved DNA motif located upstream of the *acr* gene of *M. tuberculosis* (Park *et al.*, 2003). In view of the modulation of promoter activity by DevR in *gfp* reporter assays and the detection of putative DevR-binding motifs by computer analysis, we evaluated DevR binding to sequences of the *Rv3134c-devR-devS* operon. Two DNA fragments containing the *Rv3134c* and *devR* promoters were analysed. The *Rv3134c_{pro}* fragment was generated by PCR using primers R7 and R9c and it contained T1_{*Rv3134c*} and T2_{*Rv3134c*} Tsps and four putative DevR-binding sites (Fig. 1a). Purified DevR bound to radiolabelled *Rv3134c_{pro}* promoter probe DNA and retarded its mobility. The extent of binding and DNA retardation increased with the protein concentration used, and nearly complete DNA binding was observed with 25 µM protein (Fig. 4a). This suggests that additional DevR was bound, perhaps to the multiple DevR-binding sites present in the fragment, when increasing amounts were added in the reaction. The *devR_{pro}* promoter probe, which encompassed the two *devR* upstream Tsps and one potential DevR-binding site, was also analysed in EMSAs. Compared to the *Rv3134c_{pro}* probe, DevR retarded very low amounts of the *devR_{pro}* fragment at equivalent protein concentrations (Fig. 5a). Binding to both the promoter probes could be specifically competed with a 100-fold excess of the corresponding unlabelled DNA. In assays containing a 100-fold excess of poly(dI-dC), specific binding to both the promoter fragments was retained (Figs 4b and 5b). Purified GST protein failed to bind DNA (not shown), indicating that

Fig. 3. Western blot analysis of *M. smegmatis* lysates expressing *M. tuberculosis* DevR. DevR protein of *M. tuberculosis* (arrowed) was detected in exponential- and stationary-phase *M. smegmatis* cultures using anti-DevR antibody. Lane 1, purified DevR protein with 6 × His tag (Saini *et al.*, 2004); lanes 2 and 3, lysates of exponential-phase *M. smegmatis* cultures carrying pSC2 and pSC1, respectively; lanes 4 and 5, lysates of stationary-phase *M. smegmatis* cultures carrying pSC2 and pSC1, respectively. The immunoreactive protein of smaller size is likely to be derived from intact DevR protein (upper band).

Fig. 4. DevR binds strongly to the *Rv3134c* promoter region. The promoter-containing fragment was generated by PCR amplification using primers R7 and R9c. (a) EMSA was performed in the absence of DevR (lane 1) or in the presence of 5, 12.5 and 25 μ M DevR (lanes 2–4). (b) Competition was performed with DevR (5 μ M) with no competitor (lane 6), with 300 ng (100 $\times$) of self-competitor (lane 7) or 300 ng (100 $\times$) of non-specific competitor poly(dI-dC) (lane 8); lane 5, labelled DNA only.

DevR in the recombinant fusion protein bound to the promoter probes in a sequence-specific manner. Also, DevR failed to bind to other fragments from this operon that mapped inside the *devR* gene and lacked putative DevR-binding sites (not shown). The differential affinities to DevR of the two promoter fragments were confirmed through reciprocal competition experiments where DevR was incubated (a) with labelled *Rv3134c_{pro}* in the presence of an

excess of unlabelled *devR_{pro}*, or (b) labelled *devR_{pro}* in the presence of an excess of unlabelled *Rv3134c_{pro}*. In reaction (a), binding to *Rv3134c_{pro}* was competed by 20-fold excess of *devR_{pro}* DNA while in the latter reaction, ≤ 5 -fold excess of unlabelled *Rv3134c_{pro}* DNA was sufficient to compete for binding with DevR (not shown).

DISCUSSION

Here we investigated the transcriptional activity of the *devR-devS-Rv3134c* operon under aerobic conditions in *M. tuberculosis* cells at the mid-exponential phase of growth. This operon is transcribed from two regions that lie upstream of, and also within the first gene, *Rv3134c*. One Tsp that mapped upstream of *Rv3134c* was more efficiently utilized than all others in aerated cultures. Tsp usage in *M. tuberculosis* correlated with promoter activity in *M. smegmatis* under aerobic conditions and binding efficiency of DevR to the promoter fragments *in vitro*.

Many two-component systems are transcribed from more than one promoter: for example *phoPQ* in *Salmonella typhimurium* (Soncini *et al.*, 1995), *bvgAS* of *Bordetella pertussis* (Scarlato *et al.*, 1990) and *phoPR* in *Bacillus subtilis* (Paul *et al.*, 2004). This strategy allows for differential usage and regulation of promoters under diverse environmental conditions to facilitate bacterial adaptation. The potential for regulation of the *Rv3134c-devR-devS* operon by more than one sigma factor was suggested since the Tsps were preceded by matches, albeit inexact, to the consensus elements for SigA and SigC. Intriguingly, an exact copy of the consensus motif proposed for the primary housekeeping sigma factor SigA (Manganelli *et al.*, 2004) was not found in the entire *M. tuberculosis* genome (D. Sharma and others, unpublished data). This suggests that there exists considerable flexibility in promoter recognition and a search for promoter sequences must necessarily accommodate mismatches in sequence or spacing of the bipartite elements.

The requirement for DevR during induction of *Rv3134c-devR-devS* indicates autoregulation of the operon during hypoxia (Park *et al.*, 2003). In the present study, it was noted that the *Rv3134c* and *devR* promoters exhibited striking differences in their activities and their modulation by DevR under aerobic conditions. DevR activated the weak *devR* promoters and repressed the stronger *Rv3134c* promoters. The repressing effect of DevR on *Rv3134c* promoters was observed in both *M. smegmatis* and *E. coli*. Interestingly, -35 and -10 motifs having 4/6 identity with the *E. coli* σ^{70} consensus sequences exactly overlapped the putative *M. tuberculosis* SigA consensus element predicted upstream of Tsp T2_{*Rv3134c*} (Fig. 1b). The differential regulation of *devR* and *Rv3134c* promoters could be attributed to differences in their affinity for DevR. The presence of four potential protein-binding sites in the *Rv3134c_{pro}* promoter fragment could explain the stronger binding of DevR with the *Rv3134c_{pro}* in comparison to the *devR_{pro}* fragment, which contained only one putative binding element. When the

Fig. 5. DevR binds weakly to the *devR* promoter region. The promoter-containing fragment was generated by PCR using primers 53R1 and R3. (a) EMSA was performed in the absence of DevR (lane 1) or in the presence of 5, 12.5 and 25 μ M DevR (lanes 2–4). (b) Competition was performed with DevR (12.5 μ M) with no competitor (lane 6) or with 300 ng (100 $\times$) of self-competitor (lane 7) and 300 ng (100 $\times$) of non-specific competitor poly(dI-dC) (lane 8); lane 5, labelled DNA only.

two promoters were assessed separately, the *Rv3134c* promoters were repressed while the *devR* promoters were activated in the presence of DevR. The overall effect of DevR in the context of the intact operon awaits evaluation in *M. tuberculosis*. Further experiments in *M. tuberculosis* under inducing conditions (hypoxia or nitric oxide exposure, for example) will help delineate the role of DevS sensor kinase and DevR itself in regulating the activity of this operon.

ACKNOWLEDGEMENTS

This work was financially supported by a grant to J.S.T. from the Indian Council of Medical Research, New Delhi. We thank Dr L. Ramakrishnan for the generous gift of plasmid pFPV27. Santosh Chauhan and Deepak Sharma are grateful to CSIR for Junior and Senior Research Fellowships, respectively. We are thankful to our past and present laboratory members, in particular Deepak Saini and Kohinoor Kaur, for helpful discussions.

REFERENCES

- Bagchi, G., Mayuri & Tyagi, J. S. (2003). Hypoxia-responsive expression of *Mycobacterium tuberculosis* *Rv3134c* and *devR* promoters in *Mycobacterium smegmatis*. *Microbiology* **149**, 2303–2305.
- Bashyam, M. D., Kaushal, D., Dasgupta, S. K. & Tyagi, A. K. (1996). A study of mycobacterial transcriptional apparatus: identification of novel features in promoter elements. *J Bacteriol* **178**, 4847–4853.
- Calamita, H., Ko, C., Tyagi, S., Yoshimatsu, T., Morrison, N. E. & Bishai, W. R. (2005). The *Mycobacterium tuberculosis* SigD sigma factor controls the expression of ribosome-associated gene products in stationary phase and is required for full virulence. *Cell Microbiol* **7**, 233–244.
- Cole, S. T., Brosch, R., Parkhill, J. & 39 other authors (1998). Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* **393**, 537–544.
- Das Gupta, S. K., Bashyam, M. D. & Tyagi, A. K. (1993). Cloning and assessment of mycobacterial promoters by using a plasmid shuttle vector. *J Bacteriol* **175**, 5186–5192.
- Dasgupta, N. & Tyagi, J. S. (1998). Identification of a restriction fragment length polymorphism associated with a deletion that maps in a transcriptionally active open-reading frame, *orfX*, in *Mycobacterium tuberculosis* Erdman. *Tuber Lung Dis* **79**, 75–81.
- Dasgupta, N., Kapur, V., Singh, K. K., Das, T. K., Sachdeva, S., Jyothsri, K. & Tyagi, J. S. (2000). Characterization of a two-component system, *devR-devS*, of *Mycobacterium tuberculosis*. *Tuber Lung Dis* **80**, 141–159.
- Ewann, F., Locht, C. & Supply, P. (2004). Intracellular autoregulation of the *Mycobacterium tuberculosis* PrrA response regulator. *Microbiology* **150**, 241–246.
- Florczyk, M. A., McCue, L. A., Purkayastha, A., Currenti, E., Wolin, M. J. & McDonough, K. A. (2003). A family of *acr*-coregulated *Mycobacterium tuberculosis* genes shares a common DNA motif and requires *Rv3133c* (*dosR* or *devR*) for expression. *Infect Immun* **71**, 5332–5343.
- Haydel, S. E., Benjamin, W. H., Jr, Dunlap, N. E. & Clark-Curtiss, J. E. (2002). Expression, autoregulation, and DNA binding properties of the *Mycobacterium tuberculosis* TrcR response regulator. *J Bacteriol* **184**, 2192–2203.
- He, H. & Zahrt, T. C. (2005). Identification and characterization of a regulatory sequence recognized by *Mycobacterium tuberculosis* persistence regulator MprA. *J Bacteriol* **187**, 202–212.
- Himpens, S., Locht, C. & Supply, P. (2000). Molecular characterization of the mycobacterial SenX3-RegX3 two-component system: evidence for autoregulation. *Microbiology* **146**, 3091–3098.
- Hu, Y. & Coates, A. R. (2001). Increased levels of *sigJ* mRNA in late stationary phase cultures of *Mycobacterium tuberculosis* detected by DNA array hybridisation. *FEMS Microbiol Lett* **202**, 59–65.
- Kinger, A. K. & Tyagi, J. S. (1993). Identification and cloning of genes differentially expressed in the virulent strain of *Mycobacterium tuberculosis*. *Gene* **131**, 113–117.
- Lewin, B. (1997). Transcription. In *Genes VI*, pp. 287–334. New York: Oxford University Press.
- Malhotra, V., Sharma, D., Ramanathan, V. D. & 8 other authors (2004). Disruption of response regulator gene, *devR*, leads to attenuation in virulence of *Mycobacterium tuberculosis*. *FEMS Microbiol Lett* **231**, 237–245.
- Manganelli, R., Dubnau, E., Tyagi, S., Kramer, F. R. & Smith, I. (1999). Differential expression of 10 sigma factor genes in *Mycobacterium tuberculosis*. *Mol Microbiol* **31**, 715–724.
- Manganelli, R., Provvedi, R., Rodrigue, S., Beaucher, J., Gaudreau, L. & Smith, I. (2004). σ factors and global gene regulation in *Mycobacterium tuberculosis*. *J Bacteriol* **186**, 895–902.
- Miller, J. H. (1972). *Experiments in Molecular Genetics*, pp. 352–355. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Nathan, C. & Shiloh, M. U. (2000). Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. *Proc Natl Acad Sci U S A* **97**, 8841–8848.
- Nystrom, T. & Neidhardt, F. C. (1992). Cloning, mapping and nucleotide sequencing of a gene encoding a universal stress protein in *Escherichia coli*. *Mol Microbiol* **6**, 3187–3198.
- Nystrom, T. & Neidhardt, F. C. (1994). Expression and role of the universal stress protein, UspA, of *Escherichia coli* during growth arrest. *Mol Microbiol* **11**, 537–544.
- Parish, T., Smith, D. A., Kendall, S., Casali, N., Bancroft, G. J. & Stoker, N. G. (2003). Deletion of two-component regulatory systems increases the virulence of *Mycobacterium tuberculosis*. *Infect Immun* **71**, 1134–1140.
- Park, H. D., Guinn, K. M., Harrell, M. I., Liao, R., Voskuil, M. I., Tompa, M., Schoolnik, G. K. & Sherman, D. R. (2003). *Rv3133c/dosR* is a transcription factor that mediates the hypoxic response of *Mycobacterium tuberculosis*. *Mol Microbiol* **48**, 833–843.
- Paul, S., Birkey, S., Liu, W. & Hulet, F. M. (2004). Autoinduction of *Bacillus subtilis* *phoPR* operon transcription results from enhanced transcription from $E\sigma^A$ - and $E\sigma^E$ -responsive promoters by phosphorylated PhoP. *J Bacteriol* **186**, 4262–4275.
- Raman, S., Hazra, R., Dascher, C. C. & Husson, R. N. (2004). Transcription regulation by the *Mycobacterium tuberculosis* alternative sigma factor SigD and its role in virulence. *J Bacteriol* **186**, 6605–6616.
- Roberts, D. M., Liao, R. P., Wisedchaisri, G., Hol, W. G. & Sherman, D. R. (2004). Two sensor kinases contribute to the hypoxic response of *Mycobacterium tuberculosis*. *J Biol Chem* **279**, 23082–23087.
- Saini, D. K., Malhotra, V., Dey, D., Pant, N., Das, T. K. & Tyagi, J. S. (2004). DevR-DevS is a bona fide two-component system of *Mycobacterium tuberculosis* that is hypoxia-responsive in the absence of the DNA-binding domain of DevR. *Microbiology* **150**, 865–875.
- Sala, C., Forti, F., Di Florio, E., Canneva, F., Milano, A., Riccardi, G. & Ghisotti, D. (2003). *Mycobacterium tuberculosis* FurA autoregulates its own expression. *J Bacteriol* **185**, 5357–5362.

- Sambrook, J. & Russell, D. W. (2001).** *Molecular Cloning: a Laboratory Manual*, 3rd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Scarlato, V., Prugnola, A., Arico, B. & Rappuoli, R. (1990).** Positive transcriptional feedback at the *bvg* locus controls expression of virulence factors in *Bordetella pertussis*. *Proc Natl Acad Sci U S A* **87**, 6753–6757.
- Soncini, F. C., Vescovi, E. G. & Groisman, E. A. (1995).** Transcriptional autoregulation of the *Salmonella typhimurium* *phoPQ* operon. *J Bacteriol* **177**, 4364–4371.
- Tabor, S. & Richardson, C. C. (1985).** A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. *Proc Natl Acad Sci U S A* **82**, 1074–1078.
- Valdivia, R. H., Hromockyj, A. E., Monack, D., Ramakrishnan, L. & Falkow, S. (1996).** Applications for green fluorescent protein (GFP) in the study of host-pathogen interactions. *Gene* **173**, 47–52.
- Verma, A., Sampla, A. K. & Tyagi, J. S. (1999).** *Mycobacterium tuberculosis* *rrn* promoters: differential usage and growth rate-dependent control. *J Bacteriol* **181**, 4326–4333.
- Voskuil, M. I., Schnappinger, D., Visconti, K. C., Harrell, M. I., Dolganov, G. M., Sherman, D. R. & Schoolnik, G. K. (2003).** Inhibition of respiration by nitric oxide induces a *Mycobacterium tuberculosis* dormancy program. *J Exp Med* **198**, 705–713.
- Wayne, L. G. & Sohaskey, C. D. (2001).** Nonreplicating persistence of *Mycobacterium tuberculosis*. *Annu Rev Microbiol* **55**, 139–163.