A *Mycobacterium tuberculosis* operon encoding ESAT-6 and a novel low-molecular-mass culture filtrate protein (CFP-10)

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The early secreted antigenic target 6 kDa protein (ESAT-6) is a potent T-cell protein antigen synthesized by *Mycobacterium tuberculosis*. Its corresponding gene (*esat-6*) is located in RD1, a 10 kb DNA region deleted in the attenuated tuberculosis vaccine strain *Mycobacterium bovis* BCG. The promoter region of *M. tuberculosis* *esat-6* was cloned and characterized. A new gene, designated *lhp* and cotranscribed with *esat-6*, was identified. Moreover, computer searches in the *M. tuberculosis* genome identified 13 genes related to the *lhp/esat-6* operon, defining a novel gene family. The transcription initiation sites of the *lhp/esat-6* operon were mapped using *M. tuberculosis* RNA. The corresponding promoter signals were not recognized in *Mycobacterium smegmatis*, in which transcription of *lhp/esat-6* is initiated at different locations. The *M. tuberculosis* *lhp* gene product was identified as CFP-10, a low-molecular-mass protein found in the short-term culture filtrate. These results show that the genes encoding CFP-10 and ESAT-6 are transcribed together in *M. tuberculosis* and that both code for small exported proteins.

Keywords: tuberculosis, *Mycobacterium tuberculosis*, operon, ESAT-6, CFP-10

INTRODUCTION

Tuberculosis is an infectious disease that causes the death of three million people each year. Three closely related mycobacteria, namely *Mycobacterium tuberculosis*, *Mycobacterium bovis* and *Mycobacterium africanum*, have been recognized as the aetiological agents of the disease in humans. The virulence of these pathogens is associated with their ability to parasitize and survive within phagocytic cells (Fenton & Vermeulen, 1996). Little is known about mechanisms governing mycobacterial gene expression during the intracellular growth stage. This issue is of prime importance as the intracellular stage of pathogenic mycobacteria can be viewed as an adaptative process, involving transcriptional regulatory mechanisms.

Mycobacterial genes affecting intracellular growth and

virulence are being actively sought (Collins *et al.*, 1995; Collins, 1996; Quinn *et al.*, 1996). An important contribution was recently provided by the molecular analysis of the genetic differences between virulent *M. bovis* and the attenuated vaccine strain *M. bovis* BCG. Using subtractive genomic hybridization, Maheiras *et al.* (1996) identified three regions of difference designated RD1–RD3. RD1 was detected in all strains of *M. tuberculosis* and *M. bovis* tested but is absent in all BCG substrains.

The *esat-6* gene (Sørensen *et al.*, 1995), encoding the early secreted antigenic target 6 kDa protein (ESAT-6), is found within RD1 where it was provisionally referred to as *orf1C* (Maheiras *et al.*, 1996). The ESAT-6 protein is a major T-cell antigen which has been purified from *M. tuberculosis* short-term culture filtrates (ST-CFs) (Sørensen *et al.*, 1995; Harboe *et al.*, 1996). Purified ESAT-6 stimulates the production of gamma interferon from mice memory immune T lymphocytes and may contribute to the development of antituberculous immunity (Andersen *et al.*, 1995). No function has yet been ascribed to ESAT-6 but its strong antigenic nature suggests that it is produced during tuberculosis infection.

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Abbreviation: ST-CF, short-term culture filtrate.

The GenBank accession number for the sequence reported in this paper is AF004671.

As a first step to elucidating the mechanisms controlling ESAT-6 expression and antigenicity, we identified and characterized its promoter region. We found that *esat-6* is cotranscribed with *lhp*, a new gene encoding a low-molecular-mass protein found in the *M. tuberculosis* ST-CF. Moreover, we present data suggesting that correct expression of *lhp/esat-6* in *M. tuberculosis* may require specific transcription factor(s).

METHODS

Bacterial strains and plasmids. *Escherichia coli* DH5 α was used as a host for cloning experiments. *Mycobacterium smegmatis* mc²155 (Snapper *et al.*, 1990), *M. bovis* BCG strain Pasteur 1173P2 and *M. tuberculosis* H37Rv (type strain; ATCC 27294) were used for characterization of promoter activity. The characteristics of the plasmids used in this study are listed in Table 1.

Media and culture conditions. *E. coli* was grown in solid or liquid Luria-Bertani (LB) medium as described by Sambrook *et al.* (1989). Mycobacterial strains were propagated in Middelbrook 7H9 broth (Difco) supplemented with albumin-dextrose complex (ADC) containing 0.05% Tween 80 (Sigma). When antibiotic selection was required, kanamycin (20 $\mu\text{g } \mu\text{l}^{-1}$) and ampicillin (100 $\mu\text{g } \mu\text{l}^{-1}$) were added to the

culture media. All bacterial strains were grown at 37 °C under agitation. For the determination of β -galactosidase activity, mycobacterial cultures (5 ml) were grown to an optical density of 1. Mycobacteria were then pelleted by centrifugation, resuspended in 500 μl phosphate-buffered saline (PBS; 0.145 M sodium chloride, 0.15 M sodium phosphate, pH 7.5) and sonicated three times for 1 min at 80 W.

DNA manipulations. Plasmid extraction, DNA cloning and restriction analysis were performed according to Sambrook *et al.* (1989). All the restriction enzymes used were purchased from Boehringer Mannheim. The oligonucleotides used are listed in Table 2. Construction of *lacZ* fusions was achieved using promoter-probe vectors of the pJEM series (Timm *et al.*, 1994) replicative in both *E. coli* and the mycobacteria. The 1.1 kb *Pst*I fragment from pIPX61 was blunted with T4 DNA polymerase and inserted into T4-blunted, *Sna*BI-digested pJEM13 and pJEM14. This resulted in pIPX15 and pIPX16 respectively. Oligonucleotide pairs OF1 and OB1, and OF1 and OB2, together with PE-1 and PE-4 were used for PCR amplification from pIPX61. Plasmids pIPX46, pIPX47 and pIPX18 were obtained by insertion of the corresponding *Bam*HI/*Asp*718-digested PCR fragments into pJEM13 and pJEM15 (see Table 1). Sequence of plasmid DNA was determined on both strands using a Taq Dye Deoxy Terminator Cycle sequencing kit (Applied Biosystems) in the presence of 10% DMSO. Sequencing reactions were separated

Table 1. Plasmids used in this study

Plasmids	Characteristics*	Source or reference
pBluescript II KS (+)	Cloning vector, Ap ^r	Stratagene
pAA249	1.8 kb <i>Eco</i> RI/ <i>Bam</i> HI fragment of <i>M. tuberculosis</i> H37Rv DNA in pBluescript II KS (+), contains <i>esat-6</i> , Ap ^r	Sørensen <i>et al.</i> (1995)
pIPX61	1.1 kb <i>Pst</i> I fragment of <i>M. tuberculosis</i> H37Rv DNA in pBluescript II KS (+), contains <i>lhp</i> and part of <i>esat-6</i> , Ap ^r	This study
pMCT6	IPTG-inducible <i>E. coli</i> expression vector derived from pET14b	Novagen
pJEM13	Promoter-probe vector used for creating translational fusions (phase +2), Km ^r	Timm <i>et al.</i> (1994)
pJEM14	Promoter-probe vector used for creating translational fusions (phase +3), Km ^r	Timm <i>et al.</i> (1994)
pJEM15	Promoter-probe vector used for creating transcriptional fusions, Km ^r	Timm <i>et al.</i> (1994)
pIPX15	1.1 kb <i>Pst</i> I fragment from pIPX61, blunted and inserted in pJEM13, Km ^r	This study
pIPX16	1.1 kb <i>Pst</i> I fragment from pIPX61, blunted and inserted in pJEM14, Km ^r	This study
pIPX18	PE1-PE2 PCR fragment cloned in the <i>Bam</i> HI/ <i>Kpn</i> I sites of pJEM15, Km ^r	This study
pIPX46	OF1-OB1 PCR fragment cloned in the <i>Bam</i> HI/ <i>Kpn</i> I sites of pJEM13, Km ^r	This study
pIPX47	OF1-OB2 PCR fragment cloned in the <i>Bam</i> HI/ <i>Kpn</i> I sites of pJEM13, Km ^r	This study

* Ap^r, confers resistance to ampicillin; Km^r, confers resistance to kanamycin.

Table 2. Oligonucleotide primers used in this study

Primer	DNA sequence	Position in pIPX61
OF1	5' GGGGGGATCCCAGGTGACGTCGTTGTTTCAGC 3'	7-27
OB1	5' GGGGGGTACACGGTGACGTCGTTGTTTCAGC 3'	1069-1051
OB2	5' GGGGGGTACCAACGGTGACGTCGTTGTTTCAGC 3'	1069-1051
PE-1	5' GGGGGGTACCGGGTGGCCGGGAAGTCTGTTG 3'	448-426
PE-4	5' GGGGGGATCCCTGCAGCAGGTGACGTCGTTG 3'	1-21
E64	5' CCCTGCAACGAACCTGCCGTCGACTCCACC 3'	646-617

on a DNA Analysis system model 373 (Applied Biosystems) as recommended by the manufacturer. DNA sequences were assembled and processed using Gene Navigator (Applied Biosystems) and DNA strider (CEA) software packages. The BLAST algorithm was used to search protein databases for similarity.

Amino acid sequencing. ST-CF was produced as previously described (Andersen *et al.*, 1991). Briefly, *M. tuberculosis* (8×10^6 c.f.u. ml⁻¹) was grown in modified Sauton medium (Sauton, 1912) on an orbital shaker for 7 d. The culture supernatants were sterile-filtrated and concentrated on an Amicon YM3 membrane. The CFP-10 protein was purified from ST-CF by preparative SDS-PAGE using the PrepCell system (Bio-Rad). A 1 ml fraction containing 8 mg ST-CF was applied on a matrix of 16% polyacrylamide and separation was performed under an electrical gradient for 22 h. Fractions (3 ml) were collected and analysed on silver-stained SDS-PAGE gels. A pool of fractions containing the CFP-10 protein was concentrated in the presence of 0.1% SDS in a Centricon-3 unit (Amicon) followed by acetone precipitation. The precipitate was redissolved in Tricine SDS-PAGE sample buffer and loaded on a precast 10–20% Tricine SDS-PAGE gel (Novex). After electrophoresis the gel was blotted to Problott PVDF membrane (Applied Biosystems) by semi-dry electroblotting in 10 mM CAPS, 10% methanol, pH 11. ST-CF was checked for the presence of heat-shock protein 65 and isocitrate dehydrogenase and levels lower than 1/100th of their corresponding cytoplasmic amount were detected. The PVDF membrane was stained with 0.1% Coomassie R-250 in 40% methanol, 1% acetic acid, and destained in 50% methanol. The band of interest was excised and subjected to N-terminal sequence analysis by automated Edman degradation using a Procise 494 sequencer (Applied Biosystems) as described by the manufacturer.

Expression and purification of recombinant CFP-10. The *hlp* gene was amplified by PCR and cloned into the pMCT6 expression vector digested with *Xma*I/*Bam*HI. Recombinant CFP-10 was produced in fusion with a stretch of eight histidines at its N terminus. Recombinant CFP-10 was purified by metal ion affinity chromatography using the Talon resin (Clontech), in the presence of 8 M urea, as described by the supplier. Fractions were collected and analysed by SDS-PAGE. Fractions containing recombinant CFP-10 were pooled and applied to a 1 ml Resource Q column (Pharmacia), in equilibration buffer (10 mM Tris/HCl, 3 M urea, pH 7.5) and eluted using a linear gradient of sodium chloride (0–1 M). Fractions containing recombinant CFP-10 were pooled and dialysed against 25 mM HEPES pH 8.5, concentrated by dialysis against PEG 20000 and dialysed once again against 25 mM HEPES pH 8.5.

Nucleic acid hybridization. Southern and Northern nucleic acid transfer and colony lifts were performed according to Sambrook *et al.* (1989). The DNA was immobilized by UV illumination at 365 nm for 3 min. Membranes were pre-hybridized at 65 °C for 2 h in Rapid-Hyb buffer (Amersham) supplemented with denatured *E. coli* DNA (0.25 mg ml⁻¹; Sigma). The labelled probe (10^6 c.p.m. ml⁻¹) was denatured, added to the membrane and incubated at 65 °C for 2 h. The membranes were washed under high-stringency conditions three times, each for 10 min at 65 °C with 2×, 1× and 0.1× SSC, respectively.

RNA extraction. Erlenmeyer flasks containing 100 ml 7H9/ADC broth were inoculated 1/50 (v/v) with a saturated preculture of *M. tuberculosis* Mt103 stored at 4 °C. The cells

were harvested between days 5 and 16 and D-cycloserine (1 mg ml⁻¹) was added 12 h before the extraction. Total RNA was extracted using a method described by Bashyam & Tyagi (1994). The best yields were obtained when extraction was performed at day 7 and provided about 1 µg total RNA (ml culture)⁻¹.

Primer extension analysis. The mapping of transcription start sites was performed with the E64 oligonucleotide as described by Berthet *et al.* (1995). Unphosphorylated E64 primer (15 pmol) was 5'-labelled using T4 polynucleotide kinase (New England Biolabs) in the presence of [γ -³²P]dATP (100 µCi; 3.7 MBq), and unincorporated radioactivity was eliminated on a SEP-PAK C18 column (Waters Millipore). RNA (10 µg) and labelled TSM-2 primer (0.1 pmol) were mixed in a volume of 7 µl containing 0.05 M Tris/HCl pH 8.3 and 0.1 M KCl. The reaction mixture was incubated for 1 min at 94 °C, 10 min at 65 °C and 15 min on ice. The annealing mix was adjusted to 12 µl by addition of 1 µl dATP, dGTP, dCTP, dTTP mix (2.5 mM each), 0.5 µl (6 U) RNasin (RNAguar; Pharmacia), 2.2 µl 5× reverse transcriptase buffer (0.25 M Tris/HCl pH 8.3, 0.2 M potassium chloride, 0.036 M magnesium acetate, 0.01 M DTT), 0.8 µl diethylpyrocarbonate (DEPC)-treated H₂O and 0.5 µl avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim). Reverse transcription was performed for 45 min at 42 °C and stopped by addition of 5 µl T7 sequencing kit stop buffer. Primer extension mixtures were subjected to electrophoresis in 6% polyacrylamide gels containing 8 M urea, in comparison with a sequence ladder of the *esat-6* gene generated using the E64 oligonucleotide, and analysed by autoradiography.

β-Galactosidase assay. β-Galactosidase activity was assayed in sonicated extracts of mycobacteria as described by Timm *et al.* (1994). Experiments were carried out in triplicate. Activity of β-galactosidase was calculated by the following formula: 1 unit = $200 \times \text{OD}_{420} \text{ (mg protein)}^{-1} \text{ min}^{-1}$. Soluble proteins in bacterial extracts were measured by the Bio-Rad colorimetric assay.

RESULTS AND DISCUSSION

Genetic organization upstream from the *M. tuberculosis* *esat-6* gene

To isolate the potential promoter region, we cloned the 1.1 kb DNA sequence upstream from the *M. tuberculosis* *esat-6* gene. A 150 bp DNA fragment covering the first half of the *esat-6* gene was obtained by digestion of the plasmid pAA249 with *Eco*RI/*Pst*I (see Fig. 1). This fragment, referred to as ESA-B, was radioactively labelled and used to probe a cosmid library of *M. tuberculosis* strain H37Rv by colony hybridization. A 1.1 kb *Pst*I restriction fragment shared by three strongly hybridizing cosmids was transferred to pBluescript II KS(+) to give pIPX61 (Fig. 1). Double-stranded DNA sequencing revealed perfect nucleotide identity between the 1069 bp insert of pIPX61 and its counterpart in *M. bovis* RD1 (Maheiras *et al.*, 1996). It included a 285 bp ORF preceded by a potential ribosome-binding site (AGAGA) in the same transcriptional orientation as *esat-6* (Fig. 2a). This ORF was designated *hlp* (L45 homologous protein) since its deduced product shared 40% peptide identity with the *M. leprae* L45 seroreactive

Fig. 1. Plasmid constructs and *lacZ* gene fusions used in this study. Stem-loops represent probable transcription terminators and open triangles indicate 18 bp tandem repeats upstream from *lhp*. The location of DNA probes (ESA-A and ESA-B) is indicated.

antigen (Fig. 2b). This *M. leprae* antigen is strongly recognized by sera from lepromatous leprosy patients (Satish *et al.*, 1990). The function of *lhp* is currently unknown. *lhp* is absent from the genome of *M. bovis* BCG and was not described in the *M. bovis* RD1 sequence published by Maheiras *et al.* (1996). However, *lhp* was recently identified and annotated Rv3874 during the *M. tuberculosis* sequencing project (Cole *et al.*, 1998).

lhp and *esat-6* define a novel gene family in *M. tuberculosis*

Several genes sharing weak similarities with *esat-6* have been previously identified during the *M. tuberculosis* genome sequencing project. Although these genes share less than 35% sequence similarity with each other, they were grouped in the *esat-6* gene family since they all potentially code for small polypeptides of about 100 amino acids (Cole *et al.*, 1998). In that context, we searched the *M. tuberculosis* genome database for the presence of sequences related to the predicted *lhp* gene product. As reported in Table 3, this allowed us to identify 15 genes potentially coding for proteins related to the *lhp* gene product and/or to ESAT-6. Sequence analysis indicated that *lhp* and *lhp*-related sequences

belong to and extend the *esat-6* gene family identified by Cole *et al.* (1998). As observed for *lhp* and *esat-6* (Fig. 2c), all these genes are organized in operon-like structures and are frequently preceded by genes encoding repetitive proteins of the PE and the PPE families. The significance of this finding is presently unknown but suggests that gene duplication events have occurred simultaneously for the *lhp/esat-6* and PPE/PE gene families.

lhp encodes the 10 kDa culture filtrate protein CFP-10

The ESAT-6 protein consists of 95 amino acids and was previously shown to be present in the *M. tuberculosis* ST-CF. Since *lhp* is next to *esat-6*, and potentially encodes a polypeptide of 100 amino acids, we investigated its eventual presence in the *M. tuberculosis* ST-CF. Low-molecular-mass ST-CF fractions were separated by preparative SDS-PAGE and submitted to systematic N-terminal sequencing. As shown in Fig. 3a, fraction number 4 yielded a peptide sequence matching almost perfectly (14/15) the N terminus deduced from the *M. tuberculosis* *lhp* gene sequence. This 10 kDa culture filtrate protein was referred to as CFP-10. To further characterize the *lhp* gene product, we over-expressed and purified recombinant CFP-10 in *E. coli*, in fusion with a stretch of eight histidines. Separation of recombinant CFP-10 by SDS-PAGE indicated an apparent molecular mass of 14 kDa (Fig. 3b), slightly higher than the apparent molecular mass of recombinant ESAT-6 (His6) (10 kDa). The difference in size between native and recombinant CFP-10 may be attributable to the presence of the histidine tag. Our results demonstrated that *M. tuberculosis* *lhp* is a gene and encodes a small polypeptide which, like ESAT-6, is found in the low-molecular-mass fraction of the ST-CF. In spite of the fact that no obvious export signal has so far been identified in the sequence of CFP-10, our data suggest this protein is released extracellularly during broth culture of *M. tuberculosis*, as already observed for ESAT-6. Since CFP-10 shares 40% sequence identity with the *M. leprae* L45 seroreactive antigen, we tested the reactivity of human tuberculous sera against recombinant CFP-10 in a Western blot assay. Twenty human sera were tested against both recombinant CFP-10 and recombinant Des, a potent *M. tuberculosis* humoral antigen (Jackson *et al.*, 1997). Although 20/20 sera reacted against Des, only 1/20 recognized CFP-10. This result indicates that CFP-10 may not be a major target of the humoral immune response during human tuberculosis.

Analysis of *lhp::lacZ* and *esat-6::lacZ* gene fusions

To investigate promoter activity, we constructed translational fusions between *esat-6*, *lhp* and the *lacZ* reporter gene. DNA fragments containing either *lhp* and *esat-6* or *lhp* alone were inserted into promoter-probe vectors of the pJEM series (Timm *et al.*, 1995), out- or in-frame with regard to *lacZ*. The resulting plasmids, named pIPX15 and pIPX16, and pIPX46 and pIPX47

Fig. 2. Gene arrangement upstream from the *M. tuberculosis* *esat-6* gene. (a) Nucleotide sequence and deduced amino acid sequence of *lhp*. A potential ribosome-binding site (RBS) upstream from the predicted start codon is underlined. Potential transcriptional start sites identified in *M. tuberculosis* (+1 Mt) and in *M. smegmatis* (+1 Ms) are indicated by triangles and deduced -10 regions are boxed. (b) Peptide sequence similarity between the predicted *M. tuberculosis* *lhp* gene product (Mtb CFP-10) and the *M. leprae* L45 seroreactive protein antigen (accession number X90946) (Mlep L45). Dots, double dots and vertical lines indicate structurally related, chemically related and identical amino acids, respectively. (c) Genetic organization upstream from the *lhp/esat-6* operon.

respectively (Fig. 1), were subsequently introduced by electroporation into *M. smegmatis* mc²155, *M. bovis* BCG Pasteur and *M. tuberculosis* H37Rv. β -Galactosidase activity was assayed in corresponding bacterial cell extracts and results are presented in Table 4. For the three mycobacterial species tested, strong β -galactosidase activities were detected in extracts of cells carrying in-frame fusions with *lacZ* (pIPX16 and pIPX47) but not in extracts of bacteria carrying out-of-frame *lacZ* constructs (pIPX15 and pIPX46). This demonstrated that (i) *lhp* was expressed and translated and (ii) there was mycobacterial promoter activity present in the 900 bp upstream from the *esat-6* start codon. The differences in levels of β -galactosidase activity produced from pIPX16 and pIPX47 may reflect differences in the stability of LacZ fusion proteins or corresponding messenger RNAs. Alternatively, it may

be attributable to differences in the efficiency of *lhp* and *esat-6* translation signals. An unusual structure [series of poly(A)] upstream from the *esat-6* ATG start codon and overlapping the predicted ribosome-binding site, may potentially lead to an enhanced translation of *esat-6::lacZ*.

lhp and *esat-6* are organized as an operon

To investigate whether *lhp* and *esat-6* belong to the same transcriptional unit, we performed Northern blotting hybridizations using total RNA extracted from *M. tuberculosis*. Hybridizations were carried out using radiolabelled DNA probes corresponding to either *lhp* (ESA-A) or *esat-6* (ESA-B) genes. With *M. tuberculosis* RNA, a single hybridization signal migrating at about 800 bp was detected for both the ESA-A and the ESA-B

Table 3. The *lhp/esat-6* gene family in *M. tuberculosis*

Gene name	Similarity to the <i>lhp</i> gene product CFP-10 (%)*	Similarity to ESAT-6 (%)*	Operon-like structure (accession number)	Genes directly upstream
Rv0287	32	25	Yes (AL021930)	PE/PPE
Rv0288	21	28		
Rv1036c	30	21	Yes (Z92539)	PE/PPE
Rv1037c	18	27		
Rv1792	28	NS	Yes (AL022021)	PPE/PE
Rv1793	21	26		
Rv3019c	19	32	Yes (AL021287)	PPE/PE
Rv3444c	25	28	Yes (Z95389)	Unknown
Rv3445c	27	26		
Rv3874 (<i>lhp</i>)	100	25	Yes (AL022120)	PE/PPE
Rv3875 (<i>esat-6</i>)	25	100		
Rv3890c	NS	27	Yes (Z94121)	PE/PPE
Rv3891c	21	NS		
Rv3904c	24	25	Yes (Z94121)	Unknown
Rv3905c	33	25		

* Determined using the GAP algorithm included in the Genetics Computer Group (GCG) package. NS, Not significant.

Fig. 3. Detection of *M. tuberculosis* CFP-10. (a) Protein content of the *M. tuberculosis* ST-CF analysed by SDS-PAGE and silver staining (first lane) and corresponding purified low-molecular-mass fractions (following lanes) analysed by SDS-PAGE. Fraction number 4 contained CFP-10, the N-terminal sequence of which is indicated (arrow). (b) Separation of recombinant ESAT-6 and CFP-10 under SDS-PAGE conditions. Lanes: 1, molecular mass markers; 2, recombinant ESAT-6; 3, recombinant CFP-10.

probes (Fig. 4, lanes 2 and 4). The specificity of the ESA-A and ESA-B probes was confirmed by the absence of detectable hybridization with total RNA extracted from *M. bovis* BCG (Fig. 4, lanes 1 and 3), which lack the *lhp* and *esat-6* genes. Moreover, comparable amounts of the *lhp/esat-6* transcript were detected in early (day 5) and

late (day 16) cultures (Fig. 4, lanes 5–8). Consequently, the *lhp/esat-6* transcript appears to be produced in *M. tuberculosis* from a constitutive promoter and/or is very stable. In view of its size, a mRNA transcript of 800 bp covers both *lhp* and *esat-6*. The presence of additional genes downstream of *esat-6* is unlikely since a structure

Table 4. Analysis of *lhp::lacZ* and *esat-6::lacZ* gene fusions

Fusion with <i>lacZ</i>	β -Galactosidase activity (units) detected in sonicated cell extracts of:		
	<i>M. smegmatis</i> mc ² 155	<i>M. bovis</i> BCG Pasteur	<i>M. tuberculosis</i> H37Rv
Promoter-less pJEM15	58 $\pm$ 10	31 $\pm$ 4	7 $\pm$ 4
Out-of-frame pIPX15	85 $\pm$ 21	17 $\pm$ 3	31 $\pm$ 6
pIPX46	77 $\pm$ 6	31 $\pm$ 5	34 $\pm$ 8
In-frame pIPX16	1789 $\pm$ 74	892 $\pm$ 150	817 $\pm$ 212
pIPX47	1010 $\pm$ 29	453 $\pm$ 67	306 $\pm$ 5
Transcriptional pIPX18	36 $\pm$ 4	170 $\pm$ 15	93 $\pm$ 14

Fig. 4. Analysis of the *lhp/esat-6* mRNA transcript. Total RNA was extracted from *M. bovis* BCG (lanes 1 and 3) and *M. tuberculosis* (lanes 2, 4 and 5–8) broth cultures on day 5 (lanes 1–5), day 9 (lane 6), day 13 (lane 7) and day 16 (lane 8). Total RNA (5 μ g) was separated on 1% agarose gel supplemented with formamide/formaldehyde and processed for Northern blotting as described by Sambrook *et al.* (1989). Hybridization was carried out using either the radiolabelled ESA-A (*lhp*) or ESA-B (*esat-6*) probes. Autoradiography was performed for 4 (lanes 1 and 2) or 24 (lanes 3–8) h.

similar to a Rho-independent transcription terminator is present 40 bp downstream from the *esat-6* stop codon.

Mapping of the *lhp/esat-6* promoter activity in *M. tuberculosis*

Primer extension experiments were performed to identify the 5' ends of the *lhp/esat-6* mRNA transcript. Total RNA was extracted from *M. tuberculosis* and also from *M. smegmatis* mc²155 transformed with pIPX16.

By successively walking upstream from the *esat-6* start codon, we identified one major and two secondary primer extension products in *M. tuberculosis* (see Fig. 5a). The corresponding sites in the *M. tuberculosis* genome may correspond to genuine transcription initiation sites. Alternatively, these extension products may result from 5' end processing of the RNA transcript or transcriptional stops of the reverse transcriptase enzyme used in the assay due to RNA secondary structures. This latter hypothesis is however unlikely since: (i) RNA were extensively denatured by heat before performing primer extension, and (ii) no obvious RNA secondary structures were detected by computer screening. The nucleotides corresponding to primer extension products are close together within a region of 30 bp, located about 430 nucleotides upstream from the *esat-6* ATG start codon. The position of the mapped 5' RNA ends identified in *M. tuberculosis* is consistent with an *lhp/esat-6* transcript of about 800 bp, as presented in Fig. 4. The major extension product (T1) is preceded by an (A + T)-rich region (TAATGA), which may correspond to the –10 hexamer motif identified in promoters of mycobacteria and other bacterial genera. At the corresponding –35 position, two tandem repeats of a 18 nucleotide motif, extending from positions –25 to –60, were identified (Fig. 5b). The significance of this organization is unknown but it may serve a regulatory function (Collado-Vides *et al.*, 1991).

The *lhp/esat-6* promoter activity differs in *M. smegmatis*

Primer extension mapping using *M. smegmatis* mc²155 (pIPX16) RNA yielded signals different from those identified in *M. tuberculosis* (Fig. 5c). The major extension product generated with *M. smegmatis* RNA (S1) corresponded to a nucleotide located immediately downstream from the predicted *lhp* ribosome-binding site (Fig. 2a). An explanation of this discrepancy may reside in a processing of the *lhp/esat-6* mRNA transcript

Fig. 5. Mapping of the *lhp/esat-6* promoter activity. (a) Primer extension mapping of the transcriptional start sites (T1, T2 and T3) in *M. tuberculosis*. Reverse transcription was performed as described by Berthet *et al.* (1995) using the E64 oligonucleotide with (lane 1) or without (lane 2) RNA. The DNA ladder was generated by sequencing pIPX61 with E64 using the T7 sequencing kit (Pharmacia Biotech). (b) Structural features of the *M. tuberculosis* *esat-6* promoter. (c) Primer extension mapping of the transcriptional start sites (S1 and S2) in *M. smegmatis* transformed with pIPX16. Experimental conditions were the same as described in (a).

occurring in *M. smegmatis*. Alternatively, our observation may suggest that the genuine *lhp/esat-6* promoter may not be recognized and that alternative transcription signals are used in *M. smegmatis*. To test this hypothesis, we constructed a transcriptional fusion with *lacZ* encompassing only the promoter region deduced from primer extension mapping in *M. tuberculosis*. The resulting plasmid (pIPX18) was electroporated into *M. smegmatis* mc²155, *M. bovis* BCG Pasteur and *M. tuberculosis* H37Rv and β -galactosidase activity was assayed. As reported in Table 4, β -galactosidase activity was detected in *M. bovis* BCG and *M. tuberculosis* protein extracts but not in the protein extract corresponding to *M. smegmatis* containing the pIPX18 fusion. The level of β -galactosidase activity corresponding to the pIPX18 construct was however five to eight times weaker than the one conferred by pIPX16 or pIPX47 in *M. bovis* BCG and *M. tuberculosis*. In this respect, the lack of a 5' RNA leader region in the pIPX18 construct may impair the stability of the *lacZ* transcript, leading consequently to an apparent decrease in the corresponding β -galactosidase activity. All together, these data suggest that correct expression of the *M. tuberculosis* *lhp/esat-6* operon may require factor(s) absent from or not functional in *M. smegmatis*.

Concluding remarks

In this study we mapped and characterized the promoter region controlling the synthesis of the *M. tuberculosis*

ESAT-6 antigen. Moreover, we obtained evidence showing that *esat-6* is cotranscribed with a novel gene designated *lhp*. The predicted *lhp* gene product shares sequence similarities with a protein antigen recognized by sera from lepromatous leprosy patients. However, recombinant CFP-10 produced in *E. coli* does not seem to be a major target of the humoral immune response during human tuberculosis. In this work, we have shown that like ESAT-6, CFP-10 is secreted in the ST-CF. Whether the *M. tuberculosis* CFP-10 protein is, as observed for ESAT-6, a target of the cellular immune response during tuberculosis remains now to be determined.

ESAT-6 and CFP-10 share the same messenger RNA transcript, have similar molecular masses and are both detected in the extracellular medium during broth culture of *M. tuberculosis*. This suggests that CFP-10 and ESAT-6 may interact with one another and/or serve a common function in *M. tuberculosis*. This process may not be essential for growth under axenic conditions since *lhp* and *esat-6* are deleted in *M. bovis* BCG. Alternatively, other members of the *lhp/esat-6* family may complement the loss of *lhp* and *esat-6* in *M. bovis* BCG. The contribution of *lhp* and *esat-6* to the intracellular multiplication and the virulence of *M. tuberculosis* will now be studied by means of allelic exchange, gene disruption and complementation.

The repetitive structure of the potential '-35' promoter region determined in *M. tuberculosis* has not been

reported in other mycobacterial promoters characterized so far (Kremer *et al.*, 1995; Bashyam *et al.*, 1996; Kenney *et al.*, 1996). A computer search through the *M. tuberculosis* DNA genome database failed to identify similar motifs in other locations. Such a promoter organization suggests that transcriptional regulator(s) may control the expression of *lhp/esat-6* in *M. tuberculosis*. Such a regulator should be present in both *M. tuberculosis* and *M. bovis* BCG since the pIPX16 construct confers a similar level of β -galactosidase in these bacteria. This hypothesis is also consistent with the finding that the *M. tuberculosis lhp/esat-6* promoter does not function in the fast-growing strain *M. smegmatis*. The search for proteins interacting specifically with the *lhp/esat-6* promoter region is currently under way. This work opens the way to the study of the role of CFP-10 and possible environmental modulation of *lhp/esat-6* expression during *M. tuberculosis* infection.

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