Cloning and Sequence Analysis of the 10 kDa Antigen Gene of *Mycobacterium tuberculosis*

By Paul N. Baird, Lucinda M. C. Hall and Anthony R. M. Coates*

Department of Medical Microbiology, The London Hospital Medical College, Turner Street, London E1 2AD, UK

(Received 19 September 1988; revised 14 November 1988; accepted 13 December 1988)

The gene encoding a major protein antigen of *Mycobacterium tuberculosis* has been cloned and sequenced using oligonucleotide probes derived from the N-terminal sequence of the analogous protein from *Mycobacterium bovis* BCG. The gene analysis revealed a sequence encoding a protein of 99 amino acid residues, with a molecular mass of 10.7 kDa. Computer prediction suggests that the protein contains three T-cell-determined epitopes (of which one has been demonstrated experimentally) and three B-cell-determined epitopes. The protein sequence was homologous to two prokaryote heat-shock proteins and the gene possessed heat-shock-like promoter sequences upstream of the initiation codon. A hairpin loop identified in the upstream sequence may also be important in regulation of the gene.

INTRODUCTION

Tuberculosis which is caused by *Mycobacterium tuberculosis* is still a major cause of mortality and morbidity. The World Health Organization estimates that there are three million deaths from the disease annually, mainly in developing countries (IUAT/WHO Study Group, 1982). Protection against tuberculosis in adolescents has been achieved in some countries by vaccination with *Mycobacterium bovis* Bacillus Calmette Guérin (BCG) (Hart & Sutherland, 1977), although it does not seem to affect the prevalence of the disease in a country (Sutherland, 1981). In other countries, for example India, BCG vaccination has proved ineffective (Tuberculosis Prevention Trial, Madras, 1980). A further problem with the BCG vaccine is that it can cause fatal disseminated disease, although this is very rare (Lotte et al., 1978). If protective antigens of *Mycobacterium* species could be identified, the inadequacies of BCG vaccination could be better understood. As yet, it is unknown which antigens of any mycobacterial species induce immune resistance.

Immunity against *M. tuberculosis* is T cell mediated (Lefford, 1975; Hahn & Kaufman, 1981). Thus an important criterion in seeking protective antigens should be that they induce T-cell-mediated immunity. The best-characterized group of *M. tuberculosis* antigens that bear epitopes recognized by T lymphocytes (T cell epitopes) (Kingston et al., 1987; Young et al., 1986; Kadival et al., 1987; Oftung et al., 1987) are those defined by the ‘TB’ series of monoclonal antibodies (mAbs), (Coates et al., 1981). The gene for one of these antigens, of molecular mass 65 kDa, has been cloned (Young et al., 1985) and sequenced (Shinnick, 1987): the antigen appears to be a heat-shock protein.

Apart from those antigens defined by the ‘TB’ series of mAbs, a 10 kDa antigen from *M. bovis* BCG has been purified with the mAb SA12 and its N-terminal sequence of 20 amino acids determined (Minden et al., 1984). This purified 10 kDa antigen induces T-cell-mediated delayed-type hypersensitivity in guinea-pigs vaccinated with *M. bovis* BCG (Minden et al.,...
1984). A cross-reacting antigen of 10 kDa is also present in *M. tuberculosis* (Minden et al., 1984) and is detected in immunoblots by sera from patients with pulmonary tuberculosis (Coates et al., 1989). Sera from tuberculosis patients compete with mAb SA12 for this antigen (A. Guy, personal communication). Taking these factors into account, this antigen may play a role in protection against tuberculosis.

In this paper we report the cloning and nucleotide sequence determination of the gene encoding the 10 kDa antigen of *M. tuberculosis*, and compare the gene flanking sequences with those of other mycobacterial genes.

**METHODS**

*Bacteria and bacteriophage strains. M. tuberculosis* H37Rv, obtained from the Trudeau Institute (Saranac Lake, New York, USA), was used for all mycobacterial preparations. *Escherichia coli* strain JM105 was the host organism for transformation. M13 phages mp18 and mp19 (Norrander et al., 1983) were used for cloning and the determination of the nucleotide sequence.

*Cultures. M. tuberculosis* H37Rv was grown on Middlebrook's 7H11 (Difco) agar plates for 6–8 weeks at 37 °C, or until large colonies were visible. *E. coli* JM105 was cultured on 2 × YT plates and in 2 × YT broth (Amersham M13 Cloning and Sequencing Handbook). Clones from sublibraries were taken from overnight plates as duplicate plaque lifts onto nylon filters, the phage lysed and DNA bound to the filters (Maniatis et al., 1982) was conducted until pure plaques containing DNA complementary to the oligonucleotide probe were obtained.

*Restriction enzymes.* These were obtained from Northumbria Biologicaal or Boehringer Mannheim (BCL) and were used according to the manufacturer's instructions.

*Restriction mapping.* A restriction map of the 2.25 kb *SalI* fragment using both single and double digests of *BamHI*, *SalI*, *XhoI*, EcoRI, Smal, PstI, HindIII, KpnI, and *TaqI* was constructed.

*Oligonucleotide preparations.* A 60mer DNA oligonucleotide probe based on the N-terminal amino acid sequence of *M. bovis* BCG (Minden et al., 1984) was constructed; this probe should recognize the 5' end of the 10 kDa antigen gene of *M. tuberculosis*. Three synthetic primers were made to various regions of the sequence, primer 1 (5' GACACCGCCAAGGAGAAGCC 3') and primer 2 (5' GGCCTCTTCTTGCCGTTCA 3') and primer 3 (5' AGGCTAGCTCCGTAACCGATG 3') (Fig. 3a). Probe and primers were synthesized on a New Brunswick Cyclone DNA synthesizer using phosphoramidite chemistry at the Department of Medical Microbiology, London Hospital Medical College, and used without further purification.

*Labelling of probe.* The 60mer oligonucleotide used was 5' end-labelled using T4 polynucleotide kinase (Pharmacia) and [γ-32P]ATP (Amersham) to a specific activity of between 10⁶ and 10⁷ c.p.m. (μg DNA)⁻¹. Unincorporated [γ-32P]ATP was removed by the use of a Sephadex G-50 column equilibrated in TE pH 8.0, as described by Maniatis et al. (1982).

*Agarose gel electrophoresis, hybridization and autoradiography.* Electrophoresis of *M. tuberculosis* H37Rv was performed on 1% (w/v) horizontal agarose gels in Tris/borate/EDTA buffer, pH 8.0 (Maniatis et al., 1982). Several tracks of each digest were run and one track from each digest was then transferred to a nylon membrane (Hybond-N; Amersham) by the method of Southern (1975). Filters were then prehybridized and probed at 52 °C in 6 × SSC, 0.5% SDS, 5 × Denhardt's solution and 100 μg denatured salmon sperm DNA ml⁻¹ according to Maniatis et al. (1982) 1 × SSC is 0.15 M-NaCl, 0.015 M-trisodium citrate, pH 7.0, 1 × Denhardt's solution is 0.02% Ficoll (Pharmacia), 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin (fraction V). Filters were then washed twice in 2 × SSC, followed by more washes in 2 × SSC/0.1% SDS, for 15 min each, at 52 °C. Kodak X-AR5 film was exposed to the dried filters overnight with a CAWO intensifying screen at −70 °C.

Regions of homology located on the Southern blot after probing were then used to identify the corresponding regions remaining in the tracks of the agarose gel. These regions were excised from the agarose, electroeluted and purified on DE-52 according to the method of Maniatis et al. (1982).

*Cloning and DNA sequencing.* The regions containing the 600 bp *Sau3A* fragment and the 2.25 kb DNA *SalI* fragment, purified as described above, were ligated with either a *BamHI*-digested or a *SalI*-digested M13mp18 bacteriophage respectively and used to transform *E. coli* JM105 according to the Amersham M13 Cloning and Sequencing Handbook. Clones from sublibraries were taken from overnight plates as duplicate plaque lifts onto nylon filters, the phage lysed and DNA bound to the filters (Maniatis et al., 1982). Positive clones were identified by hybridization with the labelled 60mer DNA probe as described above. Where necessary, secondary screening was conducted until pure plaques containing DNA complementary to the oligonucleotide probe were obtained. Phage DNA from positive plaques was purified from 1.5 ml cultures and sequenced by the chain-termination method (Sanger et al., 1977), using either Klenow polymerase (BCL) or Sequenase (United States Biochemical
Gene for a mycobacterial 10 kDa antigen

**RESULTS AND DISCUSSION**

Cloning of the 10 kDa antigen gene

A 10 kDa antigen which is shared by *M. tuberculosis* and *M. bovis* BCG stimulates T-cell-mediated immunity (Minden *et al.*, 1984) and is recognized by sera from tuberculosis patients (Coates *et al.* 1989). This suggests that the antigen may be important in the generation of protective immunity against tuberculosis. With the objective of selecting the *M. tuberculosis* 10 kDa antigen gene, a 60mer DNA probe corresponding to the N-terminal amino acid sequence of the *M. bovis* BCG 10 kDa antigen was made. In designing this probe the potential redundancy of the probe was reduced to eight sequences (Fig. 1c) by observing the codon preference of the *M. tuberculosis* 65 kDa antigen gene (Shinnick, 1987) and taking into account the high G+C ratio of *M. tuberculosis* (Bradley, 1973). It was anticipated that the probe would detect the *M. tuberculosis* gene since there is antigenic cross-reactivity of the 10 kDa proteins between the two species (Minden *et al.*, 1984) and previous studies have demonstrated DNA sequence similarity between *M. bovis* BCG and *M. tuberculosis* (Collins & De Lisle, 1985; Imaeda, 1985). It was subsequently established that the probe varied from the *M. tuberculosis* sequence in seven positions (Fig. 1c, d).

Southern transfers of restricted *M. tuberculosis* genomic DNA were probed with the 60mer oligonucleotide. A 0·6 kb Sau3A fragment and a 2·25 kb SalI fragment (Fig. 2) were excised from untransferred tracks of the gel, purified and cloned directly into M13 bacteriophage (Wei & Surzycki, 1986). Phages containing the fragment of interest were detected in a plaque lift assay. The clone containing the 0·6 kb Sau3A fragment was sequenced using an M13 universal primer. This fragment contained the 5' end of the 10 kDa gene encoding the N-terminal part of the protein, but was truncated at the 3' end by a BamHI site in the coding region (Fig. 3a). The sequence of the gene and adjoining regions was completed on the 2·25 kb SalI fragment using three synthetic primers (Fig. 3a).

General features of the coding region

The coding region of the gene (Fig. 3b) is predicted to begin with the initiation codon GTG at position 1 and end with the termination codon TAG, 300 bp later (Fig. 3b). The sequence should encode 99 amino acids, starting with alanine, corresponding to a protein of 10673 Da; this is in good agreement with previous estimates of 10 kDa (Minden *et al.*, 1984) and 12 kDa (Engers *et
al., 1986). Homology was found between this protein and two heat-shock proteins (Baird et al., 1988), the groES protein of E. coli (Hemmingsen et al., 1988) and the htpA gene product of Coxiella burnetii (Vodkin & Williams, 1988). This finding suggests that 10 kDa heat-shock proteins are widely conserved amongst bacteria. Whilst the biological significance of such proteins in M. tuberculosis is still unknown, it has been shown in other organisms, including C. burnetii, that stress or heat will induce their production (Vodkin & Williams, 1988). As a potential candidate for a vaccine, highly conserved proteins might protect against a range of bacterial infections. However, the implication that the 65 kDa antigen of mycobacteria is involved in autoimmune arthritis (Res et al., 1988) should be borne in mind when considering heat-shock proteins as vaccines.
Gene for a mycobacterial 10 kDa antigen

(a)

(b)

TACCCGTTCGCTATGACGGGACGTCGTGACCACCCGCTTGGCGAATGACCTGGTACCGACACGCTGGCAA -223

CCAGAAGCAAGGGGCGCCCT _ G _ A _ GTGTCAGCACTCTCATGTATAGAGT~CTAGATGGCAA -163

TCGGCTAACCCTGCGTGCGCAACCCGCGACGACGCGGCGAGCGGACGAGATGCGAAG -103

TCACCTGGTAATTGGACGTTTCGCGACGCCCCGGGACCACCCGCACTTCGGTGCCGCC -43

ValAlaLysValAsnIle 5
GAGCGTCCGGGCTGATCAAAATAGTGGAGGCTCCAATCGTGGCGAAGGTGAACATC 18

LysProLeuGluAspLysIleLeuValGlnAlaAsnGluAlaGluThrThrAlaSer 25
AAGCCACTCGAGAGAACACTTCGTGACGGCAACGAGGCGGAGCGTGTGCCGTCGC 78

GlyLeuValIleProAspThrAlaLysGluLysProGlnGluGlyThrValValAlaVal 45
GGTCTGGTCAATTCTGACGCCGCGACGACGGGCCGAGGGCGGACGAATGCCG~A 138

GlyProGlyArgTrpAspGluAspGlyLysArgIleProLeuAspValAlaGluGly 65
GGCCCTG GCCCGGAGACGGGAAACGAGGCGGAGATCCCGCTGTAGGGAGGCT 198

AspThrValIleTyrSerLysTyrGlyThrGluIleLysTyrAsnGlyGluGluTyr 85
GACACCGTCATCTAGCAAGTAGACGGGGAGGCGGATCCACGCTGAGAG 258

LeuIleLeuSerAlaArgAspValLeuAlaValValSer*** 99
CTGATCCGTGACGGGACGCTGCTGCGGCTGTTCCCAAGTACGAGCGTGTCCGG 318

(300)

CCC GGCGATCCGGGCTTCCACGCTGATTTCCGGGGCAGTCGTACGACTACG 378

CTGCCTAGAGA 389
The first 20 amino acids of the protein are identical to the N-terminal sequence from *M. bovis* BCG (Minden et al., 1984) except for a glutamic acid to glutamine change at amino acid 15. Our sequence is also in agreement with the first 15 amino acids of an N-terminal amino acid sequence described by Patarroyo et al. (1986), for an *M. tuberculosis* antigen (MTP-13), but from position 16 the sequences diverge.

**Prediction of T and B cell epitopes**

The method of Rothbard & Taylor (1988) predicted three possible T cell sites located at amino acid positions 6–9 (Lys-Pro-Leu-Glu), 11–15 (Lys-Ile-Leu-Val-Gln) and 57–64 (Arg-Ile-Pro-Leu-Asp-Val-Ala-Glu) (Fig. 3b). The latter site is composed of two overlapping motifs: 57–61 (Arg-Ile-Pro-Leu-Asp) and 61–64 (Asp-Val-Ala-Glu), (Fig. 3b). The algorithm used identifies the core of any potential T cell epitope, but it should be noted that the complete T cell site is normally 8–12 residues in length. Rothbard & Taylor (1988) include threonine as a hydrophobic residue in T cell epitope prediction, whereas Kyte & Doolittle (1982) assigned this residue a hydrophobicity value of -0.7, which suggests that it is not hydrophobic. If threonine was included in the hydrophobic core, then another motif at amino acid positions 31–34 (Asp-Thr-Ala-Lys) would also be predicted. No epitopes could be defined using the modified DeLisi & Berzofsky (1985) algorithm. The predicted T cell epitopes at amino acid positions 6–9 and 11–15 overlap with a synthetic peptide (BCG-a) (Minden et al., 1986) which elicited a T-cell-mediated response in the skin of BCG-vaccinated guinea pigs. This peptide comprised the first 13 amino acids of the 10 kDa antigen of *M. bovis* BCG and was antigenic only when polymerized.

The method of Hopp & Woods (1981) predicted three B cell epitope sites at amino acid positions 6–11 (Lys-Pro-Leu-Glu-Asp-Lys), 34–39 (Lys-Glu-Lys-Pro-Gln-Glu), and 51–56 (Asp-Glu-Asp-Gly-Glu-Lys) (Fig. 3b), of which 51–56 is the most hydrophilic. Minden et al. (1986) showed that a synthetic peptide derived from the N-terminal 13 amino acids of the 10 kDa antigen which overlaps with our predicted 6–11 site could elicit an antibody response in rabbits. This suggests that the N-terminus of the protein possesses a B cell epitope.

**Features of the non-coding region**

Sequences upstream of the proposed initiation codon at +1 (Fig. 3b) were compared with sequences involved in initiation of transcription and translation in *E. coli*. At position −14, there is a perfect match with the *E. coli* ribosome-binding site GGAGG (Shine & Dalgarno, 1974). Three sequence motifs at positions beginning −191, −182, and −180 have four bases out of six homologous with the −10 or TATAAT box sequence of *E. coli* (Pribrnow, 1975). At position −203, there is a three-out-of-three base homology with the highly conserved TTG of the −35 sequence found in *E. coli* (Hawley & McClure, 1983). This comparison suggests that the start of transcription should occur around 170 bp upstream of the initiation codon.

Examination of the upstream sequences of three other mycobacterial genes encoding the 65 kDa antigen from different species (Fig. 4) shows that each has a potential RNA polymerase binding site at 160–190 bp before the proposed initiation codon at a similar distance to that of the 10 kDa antigen gene. A second set of −35- and −10-like sequences occurs 26 bp upstream of the predicted coding region in these 65 kDa antigen genes, but not in the 10 kDa antigen gene (Fig. 4). The 65 kDa antigen, like the 10 kDa antigen, has homology to heat-shock proteins of other organisms (Young et al., 1988), so it is of interest to compare the upstream sequences of these genes. In *E. coli* heat-shock genes, a relatively long 5′ leader is also found in the *groE* operon, whilst the *dnaK* operon gene has three sites for initiation of transcription, at 115, 40 and 19 nucleotides upstream of the ATG (Gowing et al., 1985). However, a consensus established for the RNA polymerase binding site of heat shock promoters in *E. coli* differs from non-heat-shock promoters, particularly in the −10 region (Cowing et al., 1985). A match of five bases out of eight with this −10 consensus is present at the proposed RNA polymerase binding site of the *M. tuberculosis* 10 kDa antigen gene (Fig. 5). To determine the significance of such consensus sequences in mycobacteria, transcription studies are clearly required.

As well as the similarities to *E. coli* upstream sequences, the mycobacterial genes have other shared sequence elements. Firstly, a pentamer of GATCC is found, which lies close to the
Homology to RNA polymerase binding site

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<th>Homology to RNA polymerase binding site</th>
<th>GATCC SD</th>
<th>Met</th>
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<tr>
<td><strong>M. tuberculosis</strong> 10 kDa</td>
<td>TTGAGT-17-TAGAGT-----------------------</td>
<td>---148---</td>
<td>---9---</td>
</tr>
<tr>
<td><strong>M. tuberculosis</strong> 65 kDa*</td>
<td>TTGCAC-18-AAGAAT-133-TTGCCG-17-TTTTCA-4-GATCC-GAGG-12-ATG</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>M. bovis</strong> 64 kDa†</td>
<td>TTGCAC-18-AAGAAT-133-TTGCCG-18-TTTTCA-4-GATCC-GAGG-12-ATG</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>M. leprae</strong> 65 kDa‡</td>
<td>TTGCAC-23-AATGATCC-165-TTGTG-17-CTTTCA-9-GAGG-12-ATG</td>
<td></td>
<td></td>
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<tr>
<td><strong>E. coli consensus</strong>§</td>
<td>TTGACA-15-21-TATAAT-------------------</td>
<td>GAAGG-12-18-ATG</td>
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Fig. 5. Comparison of the upstream heat-shock promoter sequence of the 10 kDa antigen gene of *M. tuberculosis* with the homologous *groES* gene promoter and heat-shock consensus sequence. *Hemmingsen et al. (1988). †Cowing et al. (1985).

Fig. 6. Comparison of upstream hairpin loop structures located 5' to the gene: (1) *M. leprae* 65 kDa, (2) *M. tuberculosis* 10 kDa, (3) *M. tuberculosis* 65 kDa and *M. bovis* 64 kDa (same sequence). Numbering at the apex of the loop indicates central position. Numbering on stems is in relation to the stem of (1).
Shine-Dalgarno sequence in the 10 kDa and 65 kDa antigen genes of *M. tuberculosis*, the 64 kDa antigen gene of *M. bovis* BCG, and further upstream in the 65 kDa antigen gene of *M. leprae* (Fig. 4). Secondly, in all of these genes potential hairpin loop structures sharing a common stem sequence were found 5' to the start of the coding regions (Fig. 6). Such conservation in sequence and structure may suggest a regulatory role for these elements.

Downstream of the 10 kDa antigen gene in the 3' flanking region, an additional 88 bases have been sequenced. An inverted repeat occurs at positions 316 and 350 which is G/C rich and is likely to be involved in transcription termination (Rosenberg & Court, 1979).

In conclusion, this paper describes for the first time the entire DNA sequence of the gene encoding the 10 kDa antigen of *M. tuberculosis*. The derived protein sequence contains one known T cell epitope and an additional predicted T cell site. It is not known whether this antigen will be protective, but knowledge of the sequence will enable large quantities of either synthetic or recombinant antigen to be produced for studies in animals.

We thank Dr P. Minden for BCG-a and MAB SA12, Mr J. Cookson (Hill Centre, London Hospital Medical College) for the computer searches of T cell epitopes and American Cyanamid for financial support.

REFERENCES


