

Molecular and Immunological Characterization of the Highly Conserved Antigen 84 from *Mycobacterium tuberculosis* and *Mycobacterium leprae*

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Crossed immunoelectrophoresis (CIE) has been used to develop a reference system for classifying mycobacterial antigens. The subsequent use of specific antibodies allowed further determination of antigens by molecular weight. The monoclonal antibody F126-2, originally raised against a 34-kDa antigen of *Mycobacterium kansasii*, reacted with antigen 84 (Ag84) in the CIE reference system for *Mycobacterium bovis* BCG and *Mycobacterium tuberculosis*. To characterize Ag84, we screened a λ gt11 gene library from *M. tuberculosis* with antibody F126-2 and identified the encoding gene. The corresponding *Mycobacterium leprae* Ag84 gene was subsequently selected from a cosmid library, using the *M. tuberculosis* gene as a probe. Both genes were expressed as 34-kDa proteins in *Escherichia coli*, and the recombinant proteins indeed corresponded to Ag84 in the CIE reference system. The derived amino acid sequences of the *M. tuberculosis* and *M. leprae* proteins showed 85% identity, which indicates that Ag84 constitutes a group of highly conserved mycobacterial antigens. Antibodies of almost 60% of lepromatous leprosy patients responded to Ag84, indicating that the protein is highly immunogenic following infection in multibacillary leprosy.

The introduction of DNA expression libraries has greatly enhanced the molecular and immunological investigation of individual mycobacterial antigens (29, 30). A large number of mycobacterial proteins are able to induce an immune response, and various recombinant mycobacterial antigens have been identified on the basis of their immunogenic properties. Sera from tuberculosis patients have been used to identify recombinant *Mycobacterium tuberculosis* antigens which have potentials in serological diagnosis of the disease (9). Similarly, sera from leprosy patients have been used to select and to identify immunorelevant recombinant antigens from *Mycobacterium leprae* (16, 18). Furthermore, polyclonal and monoclonal antibodies (MAbs) against various mycobacterial antigens have been raised and used to select recombinant antigens from expression libraries of *M. tuberculosis* and *M. leprae* (for a review, see reference 28). Numerous mycobacterial proteins are currently under investigation. Young and colleagues have prepared a compiled list of these mycobacterial proteins with respect to their molecular sizes, biological functions, and immunological properties (28).

To classify the mycobacterial proteins, Closs et al. developed a reference system based on the reactivities of the proteins with hyperimmune antisera as detected by crossed immunoelectrophoresis (CIE) (3). With this method, more than 50 distinct antigen peaks were identified, and a system of nomenclature based on CIE was established (3, 5, 7, 24-26).

In this study, the mycobacterial antigen 84 (Ag84) as defined

by CIE was investigated. The *M. tuberculosis* gene encoding Ag84 was selected from a λ gt11 expression library, using MAb F126-2. This MAb was originally raised against a *Mycobacterium kansasii* 34-kDa antigen (22) and reacted with Ag84 in the CIE reference system for *Mycobacterium bovis* BCG and *M. tuberculosis*. On the basis of the DNA homology between the mycobacterial Ag84 genes, the corresponding *M. leprae* gene was selected from a cosmid library. The mycobacterial Ag84 genes were characterized, and the immunological properties of the recombinant mycobacterial proteins were investigated, using sera from leprosy and tuberculosis patients.

MATERIALS AND METHODS

Recombinant DNA libraries, bacterial strains, and plasmids. The λ gt11 expression library of *M. tuberculosis* was a gift from R. A. Young (29). *Escherichia coli* Y1090 was used as a host to screen the *M. tuberculosis* λ gt11 gene library. The pHCT9 cosmid gene library of *M. leprae* was provided by J. E. Clark-Curtiss (2). *E. coli* DH5 α F' was used for plasmid-cloning purposes (13). Relevant properties of the recombinant strains and plasmids used in this study are summarized in Table 1. The recombinant plasmids pPH5253 and pPH5254 were derived from pPH5250 by exonuclease III-mung bean nuclease deletion mutagenesis (Stratagene, La Jolla, Calif.), using the restriction enzymes *Kpn*I and *Hind*III. Recombinant plasmid pPH5253 Δ M/S was obtained from pPH5253 by deletion of the *MamI-Sma*I fragment (13). The *M. bovis* BCG Copenhagen vaccine strain 1331 and *M. tuberculosis* H37Rv (ATCC 27294) were used for antigen preparation. BCG culture fluid, containing secreted and cytoplasmic proteins due to cell lysis, was obtained after 2 weeks of culture in Sauton broth. *M. tuberculosis* H37Rv was grown for 3 weeks in Sauton broth.

Screening of the *M. tuberculosis* and *M. leprae* genomic libraries. The λ gt11 gene library of *M. tuberculosis* was screened by plaque blot analysis as described by Young and coworkers (30). MAb F126-2 (see below) was used to select a recombinant phage expressing Ag84 of *M. tuberculosis*. The pHCT9 gene library of *M. leprae* was screened by Southern blot hybridization analysis. The *M. tuberculosis* 2.0-kb *Eco*RI DNA insert of plasmid pPH5250, containing the *M. tuberculosis* Ag84 gene, was used as a probe to screen the *M. leprae* recombinant cosmids (14, 19). The DNA probe was labeled with [α -³²P]dCTP, using the multiprimer DNA labeling system (Amersham).

DNA sequencing. Sequence analysis of the Ag84 genes from *M. tuberculosis* and *M. leprae* was performed by the chain termination method (17). The M13

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TABLE 1. *E. coli* strains and plasmids used in this study

<i>E. coli</i> strain/plasmid	Relevant properties of the plasmid
EC1/pBlue KS ⁺ ^a	
MT5250/pPH5250	pBlue KS ⁺ , containing the 2.0-kb <i>EcoRI</i> <i>M. tuberculosis</i> DNA fragment from recombinant phage λPH5211; entire Ag84 gene is present.
MT5270/pPH5270	pBlue KS ⁺ , containing the 2.0-kb <i>EcoRI</i> fragment of pPH5250 in opposite orientation.
MT5253/pPH5253	Deletion mutant of pPH5250; the entire Ag84 gene is present.
MT5253ΔM/S/pPH5253ΔM/S	Deletion mutant of pPH5253; the entire Ag84 gene is present.
MT5254/pPH5254	Deletion mutant of pPH5250; the N-terminal part of the Ag84 gene is absent.
ML1527/pBL1527	pPH79, containing a 35-kb DNA fragment of <i>M. leprae</i> .
ML1000/pPF1000	pBlue KS ⁺ , containing a 3.5-kb <i>Bam</i> HI fragment of pBL1527; the entire Ag84 gene of <i>M. leprae</i> is present.
ML1020/pPF1020	pBlue KS ⁺ , containing the 3.5-kb <i>Bam</i> HI fragment of pPF1000 in opposite orientation.
ML2000/pPF2000	pBlue KS ⁺ , containing a 1.9-kb <i>Cla</i> I fragment of pBL1527; the entire Ag84 gene of <i>M. leprae</i> is present.
ML2020/pPF2020	pBlue KS ⁺ , containing the 1.9-kb <i>Cla</i> I fragment of pPF2000 in opposite orientation.

^a Stratagene.

forward primer (5' TGTA AACGACGGCCAGT) and the M13 reverse primer (5' CAGGAAACAGCTATGACC) were used to initiate the sequence analysis of the mycobacterial DNA inserts of plasmids pPH5250 and pPF2000, respectively. Oligonucleotide primers based on the sequence information of the mycobacterial DNA inserts were synthesized (Pharmacia LKB, Roosendaal, The Netherlands) to proceed with double-strand sequencing.

Antibodies. Polyvalent anti-BCG immunoglobulin was kindly provided by Dako Immunoglobulins, Copenhagen, Denmark (code B124, lot 063). Monospecific, polyclonal rabbit antibody to a 34-kDa protein of *M. bovis* was a gift from M. E. Patarroyo, Bogota, Colombia. MAb F126-2, originally raised against an *M. kansasii* 34-kDa protein antigen (22), was provided by A. H. J. Kolk. The monospecific polyclonal rabbit antibody K57 was obtained by immunization with purified recombinant *M. bovis* BCG 65-kDa heat shock protein.

CIE. CIE with an intermediate gel was performed on glass plates (5 by 7 cm) as described in detail previously (3, 5). The method of Axelsen (1) was used to identify the specificity of monospecific precipitating antibodies. These antibodies were incorporated into the intermediate gel, and the effect on the CIE pattern was investigated. The specificity of MAb F126-2 was characterized as described by Wiker and Harboe (23).

Sera. Leprosy sera were obtained from patients who were clinically examined by the All Africa Leprosy Rehabilitation and Training Centre. The Armauer Hansen Research Institute performed the histopathological studies on clinical specimens from these patients. Cases were classified as lepromatous (LL and BL; *n* = 22) or tuberculoid (BT and TT; *n* = 11) leprosy based on clinical and histopathological criteria. Tuberculosis sera (*n* = 20) were obtained from Dutch patients with pulmonary tuberculosis. Laboratory diagnosis of tuberculosis was confirmed by culturing *M. tuberculosis* from sputum samples. Control sera (*n* = 10) were obtained from healthy Ethiopians working at the Armauer Hansen Research Institute.

SDS-PAGE and immunoblotting. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed with bacterial sonic extracts (20 μg of total protein in each lane), using a 5% (wt/vol) stacking gel and an 8 to 18% gradient gel (Excel Gel SDS; Pharmacia LKB Biotechnology, Uppsala, Sweden) in a 2117-001 Multiphor II electrophoresis unit (Pharmacia LKB). The proteins were transferred onto nitrocellulose membranes by Western blotting (immunoblotting) (13). Antigen bands were detected by using MAb F126-2 diluted 1:5,000 and peroxidase-labeled F(ab')₂ sheep anti-mouse immunoglobulin (Amersham) diluted 1:2,000, with diaminobenzidine as the substrate. Rainbow protein molecular weight markers (Amersham) were used as standards. Western blot analysis was also used to demonstrate the presence of antibodies to Ag84 in patient sera. For each serum to be tested, SDS-PAGE was performed in two lanes, one containing a sonic extract of the *E. coli* recombinant ML2000 expressing the *M. leprae* Ag84 gene and the other containing a sonic extract of *E. coli* EC1 as a negative control. The proteins were transferred onto nitrocellulose membranes. Prior to Western blot analysis, the sera were absorbed with *E. coli* EC1 sonic extract to remove antibodies reacting with *E. coli*. For this purpose, *E. coli* EC1 was grown overnight in liquid LB medium at 37°C in the presence of ampicillin (13). The bacteria were spun down and washed twice with Tris-HCl-buffered saline (pH 7.0), and a 10-fold concentrated *E. coli* suspension was prepared in the same buffer. The bacteria were sonicated on ice five times for 30 s, using an MSE 100-W ultrasonic disintegrator. To absorb from the sera antibodies that react with *E. coli*, 75 μl of serum diluted 1:100 was mixed with 75 μl of *E. coli* total sonic extract containing soluble proteins and insoluble components. Incubation for 1 h at room temperature with careful and repeated mixing was followed by incubation at 4°C overnight. The tubes were spun for 10 min at 12,000 rpm in an Eppendorf centrifuge. The supernatant, providing a final dilution of serum of 1:200, was used for Western blot experiments. Antibody binding was detected by using peroxidase-labeled F(ab')₂ sheep anti-human immunoglobulin (Dako) diluted 1:2,000 and diaminobenzidine as the substrate. The intensity of the 34-kDa Ag84 band in the sonic extract containing the

recombinant plasmid was compared directly with the appearance of the negative control at the same position. The different degrees of positive reactions were graded visually as ++, +, or (+), and negative reactions were graded as – when there was no difference between the two lanes. All reactions were judged blind, without knowledge of the origins of the individual sera.

Nucleotide sequence accession numbers. The sequences of the Ag84 genes of *M. tuberculosis* and *M. leprae* are present in the EMBL, GenBank, and DDBJ databases under accession numbers X77129 and X77128, respectively.

RESULTS

Selection of the Ag84 genes from *M. tuberculosis* and *M. leprae*. MAb F126-2, originally raised against a 34-kDa protein antigen from *M. kansasii* (22), cross-reacted with a 34-kDa protein band of *M. bovis* BCG and *M. tuberculosis* (Fig. 1, lanes 10 and 11). CIE demonstrated that MAb F126-2 reacted specifically with the mycobacterial Ag84 (see below). The *M. tuberculosis* Ag84 gene was selected from a λgt11 expression library (29), using MAb F126-2. About 10⁵ phage recombinants were screened by plaque blot analysis, and the recombinant clone λPH5211, recognized by MAb F126-2, was selected. Restriction enzyme analysis of λPH5211 showed the presence of a 2.0-kb *EcoRI* mycobacterial DNA insert. This insert was isolated and cloned into *lacZ* expression vector pBlue KS⁺, resulting in *E. coli* MT5250. MT5250 expressed Ag84 of molecular size comparable to that of Ag84 from *M. bovis* BCG and *M. tuberculosis* (Fig. 1, lanes 2, 10, and 11).

The presence of an Ag84-homologous 34-kDa protein in *M.*

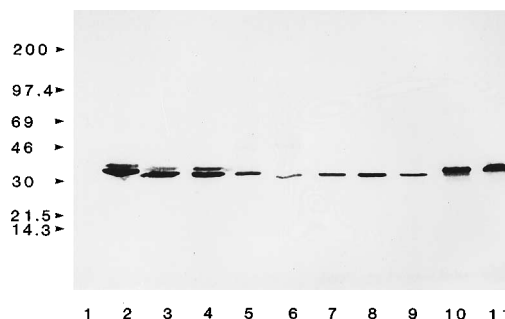


FIG. 1. Western blot analysis of the expression of the Ag84 genes of *M. tuberculosis* and *M. leprae* by *E. coli*. Lanes: 1, sonic extract of *E. coli* control strain EC1; 2 to 5, *E. coli* sonic extracts of MT5250, MT5253, MT5253ΔM/S, and MT5270, respectively, expressing recombinant Ag84 of *M. tuberculosis*; 6 to 9, *E. coli* sonic extracts of ML1000, ML1020, ML2000, and ML2020, respectively, expressing recombinant Ag84 of *M. leprae*; 10, culture fluid of *M. bovis* BCG Copenhagen containing secreted as well as cytoplasmic proteins; 11, sonic extract of *M. tuberculosis*. MAb F126-2 was used to detect Ag84 expression. Sizes are indicated in kilodaltons.

leprae that was recognized by MAb F126-2 has been established by Kolk and coworkers (12). To select the homologous gene of *M. leprae*, the pHC79 cosmid gene library was screened. Seventy-nine recombinant cosmids containing different *M. leprae* DNA fragments, as determined by restriction pattern comparison, were screened by Southern blot hybridization analysis. The 2.0-kb *EcoRI* insert of recombinant plasmid pPH5250, containing the Ag84 gene of *M. tuberculosis* and flanking sequences, was used as a probe. Recombinant cosmid pBL1527 was found to hybridize strongly with the Ag84 gene probe (data not shown). Southern blot analysis of *Bam*HI and *Cla*I digests of recombinant cosmid pBL1527 demonstrated that the probe hybridized with a 3.5-kb *Bam*HI fragment and a 1.9-kb *Cla*I fragment (data not shown). Both the 3.5-kb *Bam*HI fragment and the 1.9-kb *Cla*I fragment were cloned into pBlue KS+, resulting in the recombinant plasmids pPF1000 and pPF2000, respectively. Ag84 of *M. leprae* was expressed as a 34-kDa protein by both *E. coli* recombinants (Fig. 1, lanes 6 and 8).

Molecular characterization of the Ag84 genes from *M. tuberculosis* and *M. leprae*. The recombinant plasmid pPH5250 was used to determine the DNA sequence of the Ag84 gene from *M. tuberculosis*. M13 reverse primer sequencing to initiate the sequence analysis was followed by the double-strand sequencing of 1,465 bp of the 2.0-kb mycobacterial *EcoRI* insert downstream of the M13 reverse primer site (Fig. 2). A large open reading frame (ORF) was observed. This ORF was located at positions 125 to 931, with a potential methionine start codon at position 152. The molecular mass of the putative protein of 260 amino acids was 28 kDa. In the sequenced part of recombinant pPH5250, no other ORFs of significant size were detected. Analysis of the region upstream of the putative start codon revealed the presence of a potential mycobacterial Shine-Dalgarno (SD) sequence (5' AGGGGAC) which shares significant homology with the putative SD sequence of the *M. bovis* BCG Ag85B (BCG α) gene (5' AGGGGCA) (4). Furthermore, the *M. tuberculosis* Ag84 gene and the *M. bovis* BCG Ag85B gene share a 5-bp distance between the SD sequence and the methionine start codon.

Deletion mutagenesis of recombinant plasmid pPH5250 was performed to confirm the location of the Ag84 gene within the sequenced 1,465 bp of recombinant pPH5250. The deletion mutant pPH5253 was derived from pPH5250 and lacked the first 64 bp, as depicted in Fig. 2. Western blot analysis showed that expression of the recombinant Ag84 gene was not affected by the deletion (Fig. 1, lane 3). The pPH5250-derived deletion mutant pPH5254, which lacked the first 175 bp (Fig. 2), did not express Ag84 (data not shown), suggesting that the gene is affected by the deletion. This observation is consistent with the deduced location of the recombinant Ag84 gene, since the putative promoter region and the first eight codons of the Ag84 gene are missing in recombinant pPH5254. Deletion mutant pPH5253 Δ M/S was derived from pPH5253 by a *Mam*I-*Sma*I deletion, resulting in the exclusive presence of the DNA fragment covering positions 65 to 1028 (Fig. 2). Western blot analysis showed the expression of Ag84 by this deletion mutant (Fig. 1, lane 4). Since the predicted putative Ag84 gene is the only large ORF which is present in recombinant pPH5253 Δ M/S, it is evident that this ORF harbors the Ag84 gene of *M. tuberculosis*.

To characterize the Ag84 gene from *M. leprae*, recombinant pPF2000, containing a 1.9-kb mycobacterial *Cla*I insert, was entirely analyzed by DNA sequencing. A single large ORF was located at positions 781 to 1647, with a potential methionine start codon at position 850 (Fig. 3). The molecular mass of the putative protein of 266 amino acids is 29 kDa. This protein

<i>EcoRI</i>	gaatccc	GTTTAAATTTGGTCTTATTACTTAGTCCAATCGGCCAACCGCCGAGTCTGGT	60
	r>	pPH5253; pPH5253 Δ M/S	
	GTGACAGGATGGACGGTAGTTGCACCCACGGCTACCACTCCGTTCTACACTTCAGATCGT	120	
	r>	pPH5254	
	TTGACCGTCCAGGAAGCTCGAGGGGACAAACAATGCGCGCTTACACTGCCGACGTCCACAA	180	
	SD	M P L T P A D V H N	10
	TGTGGCGTTTCAGTAAGCCGCTATCGGCAACGTTGGTACAACGAAGTAGGTTGACGCG	240	
	V A F S K P P I G K R G Y N E D E V D A	30	
	CTTCTCGACTGGTGGAAAACGAGCTGACCCGCTGATCGAAGAGAACTCCGATCTGCG	300	
	F L D L V E N E L T R L I E E N S D L R	50	
	TCAGAGGATCAACGAGCTGGATCAAGAGCTCGCGCGGGCGCGGTGCCGGTTCACGCC	360	
	Q R I N E L D Q E L A A G A R E D A G V T	70	
	GGAGCCACCCAGGCAATCCCGGCTATGAGCCGCAACCGGGCAAGCCGGCCGGCGGC	420	
	Q A T Q A I P A Y E P E P G K P A P A A	90	
	GGTCTCGCGGGGATGAACGAGGAACAGGCGCTGAAGCGGGCGGAGTGTGAGTCTGGC	480	
	V S A G M N E E Q A L K A R A R E V T A D L A	110	
	CCAAGACACCGCCGACCGGCTTACAACACCGCCAAAGCCGAGTCGGACAAGATGCTGCC	540	
	Q D T A D R L T N T A K A E S D K M L A	130	
	CGATCCCGTCCCAATCGCGAGCAGATCTCGGTGAGGCCGACACCCGCGCAGCCAC	600	
	D A R A N A E I L G A A G G A R H T A D L T	150	
	GGTCCCGAGCCCGCCAGCGTGCCTGATGCCATGTCGGCGATGCCCAATCCCGATCCGA	660	
	V A E A R Q R A D A M L A D A Q S R S E	170	
	GGCCAGTTGCGCCAGGCGCAGGAGAAGGCCGATGCCTTACAGCCGATGCGGAAACGCAA	720	
	A Q L R Q A Q E K A D A L Q A D A E R K	190	
	GCATTCGAGATCATGGGAACCATCAACCAGCAGCGCGGCTTGAAGGCCCGCTCGA	780	
	H S E I M G T I N Q Q R A V L E G R L E	210	
	GCAGTCCGCTACCTTCGAACGTGAGTACCGCACCAGGCTCAAGACTTACTTGAATCGCA	840	
	Q L R T F E R E Y R T R L K T Y L E S Q	230	
	GCTGGGAACTCGCCAGCGTGGATCGCGCGCGCGTTCGATTCCAATGCGGATGCCCG	900	
	L E E L G Q R G S A A P V D S N A D A G	250	
	TGGCTTCGATCAATCAACCGGGGCAAAACTAGCCACCAGGCGTCCGGACACCGCCCA	960	
	G F D Q F N R G K N 260		
	GTTTTCGAGTTCGGCAGCAGATCCTGGTAGTTGGGCAGTCCGCTGACCGGTTGGAG	1020	
	pPH5253 Δ M/S		
	GATGACCGATGCTGATCATTGCGCTGGTCTTGGCCCTGATTGGGCTCTGGCCTTGGTGT	1080	
	TCGCGGTGCTCACAGCAACCAGCTAGTGGCTGGGTATGATCGGGCCAGCGTGTCTGG	1140	
	GTGTGGCGTTCGTGATCGTTCGATGCGTTGCGGAGAACCAGCAAGGTGGCGCGACGAAG	1200	
	CTGATGGGGCTGGGAAACCGGTGTGCGGAGGAAGCCGACGCTCGACTACCCGAGGAAG	1260	
	CCCCGAGGAGACCAAGCCGTCGACGCGGCTCATCGGCAGTGGAGGCCATCGGAGG	1320	
	AGGCCAGCGAAGCCAGGAGTCCGCGGTATCGCGGACCGAAGCGACGACGAGCCCA	1380	
	AGTAGGGTCTGTCAGCGACCCGACTCGGTAGGGCGCTCGTGGGAGGTTATCGGGTGGTT	1440	
	TGTCGCCAGCTCCGGATCTAGACC	1465	

FIG. 2. DNA sequence of the Ag84 gene and flanking regions from *M. tuberculosis*. The translation product, the putative SD sequence, and the deletion mutants pPH5253, pPH5253 Δ M/S, and pPH5254 are also depicted.

showed 85% identity with Ag84 from *M. tuberculosis*. The lowest degree of homology between the proteins was observed in the central part of the protein, which in Ag84 of *M. leprae* contained six additional amino acids (data not shown). The *M. tuberculosis* and *M. leprae* Ag84 genes were 81% identical. Furthermore, the *M. leprae* Ag84 gene shared the putative mycobacterial SD sequence (5' AGGGGAC) and its distance of 5 bp from the methionine start codon with the *M. tuberculosis* Ag84 gene.

No significant homology of the *M. tuberculosis* and *M. leprae* DNA sequences and their encoding Ag84 proteins was observed with other sequences present in the National Center for Biotechnology Information data library (release January 1995).

Mycobacterial Ag84 gene expression by *E. coli*. Western blot analysis of the *E. coli* recombinants pPH5250, pPH5253, and pPH5253 Δ M/S, containing the *M. tuberculosis* Ag84 gene, showed the expression of Ag84. A major protein band of 34 kDa and a minor band of 36 kDa were observed (Fig. 1, lanes 2 to 4). As determined by DNA sequencing (Fig. 2), the *M. tuberculosis* Ag84 gene present in pPH5250 was located in the

*Cla*I
ATCGATTTGGTGTGCGATGGACAATGCTGACGCTAAACGCCTGGTCGATTTTCGCCGCGGGC 60
 TTGGCCCTTTGGTGTGCGTGGATCCCTTCGACAAGGTCGCAACCAAGGTGTTCTGCTGTGCG 120
 CCAGCCGACGTCGACGTATCCCCTGAGGAGCGTCGCCGGATCGCCGAAACCGGCTTTTAC 180
 GCCTACCAATAGATCGGTTTATAATCAGATCCACCGCAACATATGGGTCCGCTCCGACC 240
 TCCGAGTCGATTTCTGGCGGTAAACCGTACGTGTAGGCGGTGGTGGTATCTTCATTTGGT 300
 AAGTCCGGGCTTTAGTTTGGCGTGTGTTTATCAGATCCCTGGATTGGCGGTGTTGCTCTT 360
 TTGGTGTGCTGATCGCCCGGGTTCGTCGAGTTCATACGTTCAATTTAGCCGTCGACTG 420
 GCGCCCCAACGGTGTCACTGTTGTGATCTTGGAGACTATCATGTGATCACCGATCCGCC 480
 GGTGAAGCTGTTGCGGTTGATTCGCCAACTCACCATTTGGCGGGTCCGCTTCGACTTGTG 540
 CATCATGGTGTGCTTGGTGGTTCGTTTATAGGCATGCAATTTGGCTTTAAGCGCTGCGGC 600
 TTAAGCCATGCCCGACGCCCGGGGAGGAGCGAGGTTGCGCCACGTTTGGCGACGGT 660
 TTGCTCCGTTGATTTCAAATCTAAGGGTTGGGATTAATTTGGCCTTACAGTTTGAATTC 720
 TTTACTTAATTTATTAGATAACGGCAACAGCCGGTCTGGTGTGACAGGATGGACGGTAG 780
 TTGCGCATGGCTACCGCGCTTCTACACTTTCCAATAAATTTGACAAGGGACTTGAGG 840
GGACGAACAATGCCGCTTACACCGAGCCGACGTCACCAACGTTGGCGTTCAGTAAGCCGCC 900
 SD M P L T P A D V H N V A F S K P P 17
 ATCGGCAAGCGTGGTTATAACGAAGATGAGGTGACGCTTCTCGACCTGGTGTGAGAAC 960
 I G K R G Y N E D E V D A G P V I A D L V E N 37
 GAGCTGACTCAGTCTATTGAAGAGAATTCGACCTACGCCAGCGGATCGAGGAGTGGAT 1020
 E L T Q L I E E N S D L R Q R I E E L D 57
 CATGAATAGCTGCGGGTGGCGTACTGGTGTGCGCCGTTATCGCCGTGCAGCCCACT 1080
 H E L A A G E L A M K A T R V L S L A Q P T 77
 CAGGCGCTTCCACTTTGCAACCCGAAGTACTGTCTGCCAAGCAGGCGCCAGTGGCAGCT 1140
 Q A L S T F E P E L V S A K Q A P V A A 97
 GTTCCGAGACGCCGAAGAATTTGGCGATGAAGGCTACTAGGGTGTGAGCCTGGCCGAG 1200
 V A E T A E E L A M K A T R V L S L A Q 117
 GATACCGCTGACCGCTCACCAGTACCGCTAAGGTCGAATCGGACAAGATGTTGGCCGAT 1260
 D T A D Q L T S T A K V E S D K M L A D 137
 GCGCGTGTCAATGCCGACCAAAATCTCGCGAGGCTCGCCTCACGGCGGAGGCTACCGTC 1320
 A R V N A D Q I L G E A R L V I A E A T V 157
 GCCGAAGCCAGCAACGCCGCGGATGTTGGCCGACGCCAACTCGCTCTGAGGTC 1380
 A E A Q Q R A D A M L A D A Q T R S E V 177
 CAGTCCGTCAGCCGAGGAGAGGCGGACGCCCTCGAGCCGGAAGCCGCGCTAAGCAT 1440
 Q S R Q A Q E K A D A L Q A E A E R K H 197
 TCTGAGATTTGGGAGCCATCAGCCAGCAGCGCGTGTGGAAAGCCGACTCGAGCAG 1500
 S E I M G A I S Q Q R T V L E G R L E Q 217
 CTACGTACCTTCGAACGCGAGTATCGGACCCGCTCAAGACCTATCTGGAATCTCAGCTT 1560
 L R T F E R E Y R T R L K T Y L E S Q L 237
 GAGGAATCTCGGTGAGCGTGGTCCGCTGACCGGTTGATTCCTCAATGCGGATGCTGGTGA 1620
 E E L G Q R G S A A P V D S N A D A G G 257
 TTCGACCAATCAATCGGGTAACTAAGCTGAGAAAGTGTGCCGACTCCACGCTCGAC 1680
 P D Q F N R G N N 266
*Cla*I
 GTCCGGGAACACCACTGATCGAT 1884

FIG. 3. DNA sequence of the mycobacterial DNA insert of pPF2000, containing the Ag84 gene and flanking regions from *M. leprae*. The translation product, the putative SD sequence, and the mutant pPF1000 are also depicted.

lacZ orientation. To investigate the influence of *lacZ* on the expression of Ag84, the 2.0-kb *Eco*RI fragment from pPH5250 was cloned in opposite orientation in pBlue KS+, resulting in recombinant plasmid pPH5270. As shown in lane 5 of Fig. 1, Ag84 was also expressed by the pPH5270-containing *E. coli* recombinant MT5270. This finding indicates that the recombinant Ag84 of *M. tuberculosis* is expressed independently from the *lacZ* gene promoter. To investigate the mode of expression of the recombinant *M. leprae* Ag84, clones pPF1000 and pPF2000 were further analyzed. DNA sequencing of pPF1000 and pPF2000 demonstrated that the Ag84 genes in both recombinants were located in the same orientation as the *E. coli lacZ* gene. The 3.5-kb mycobacterial *Bam*HI insert of pPF1000

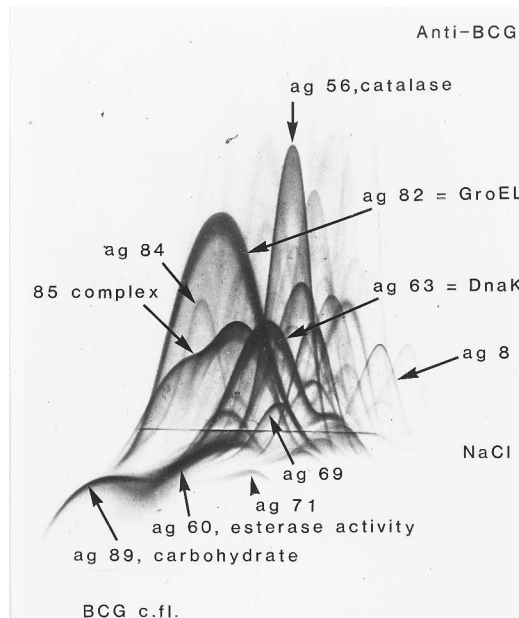


FIG. 4. CIE of an *M. bovis* BCG antigen preparation containing secreted as well as cytoplasmic proteins. The top gel contains polyvalent anti-BCG immunoglobulin. Individual mycobacterial antigens are indicated with numbers and, when known, by functional characteristics. c.fl., culture fluid.

and the 1.9-kb *Cla*I insert of pPF2000 were subcloned in pBlue KS+ in the orientation opposite that of *lacZ*, resulting in pPF1020 and pPF2020, respectively. The *E. coli* recombinants ML1020 and ML2020, in which the Ag84 gene was located in the orientation opposite that of *lacZ*, both showed expression of Ag84 (Fig. 1, lanes 7 and 9). This result indicates that the *M. leprae* recombinant Ag84 is also expressed independently from the *E. coli lacZ* gene.

Characterization of Ag84 from *M. tuberculosis* and *M. leprae* by CIE. The complex CIE pattern of an *M. bovis* BCG antigen preparation containing secreted as well as cytoplasmic proteins is shown in Fig. 4. Individual antigen precipitates are indicated with their CIE antigen codes and, when known, by functional characteristics. Ag84 gives a distinct, characteristic precipitate. Both Mab F126-2 and the monospecific, polyclonal rabbit antibody to a 34-kDa protein of *M. bovis* affected the precipitate line of Ag84 (data not shown). This result indicates that Mab F126-2 and the polyclonal rabbit antibody react with Ag84. CIE results of the recombinant Ag84 of *M. tuberculosis* and *M. leprae* are shown in Fig. 5. The *E. coli* control strain EC1 gave a single precipitate line with polyvalent anti-BCG in the top gel (Fig. 5A), while the corresponding sonic extracts of the recombinant ML1000 and deletion mutant MT5253ΔM/S, expressing Ag84 of *M. leprae* and *M. tuberculosis*, respectively, showed two peaks (Fig. 5B and C). Note that the additional peaks caused by Ag84 have distinctly different positions due to lower mobility of the *M. tuberculosis* antigen during first-dimension electrophoresis. The faster-moving protein which gave a precipitate in all four plates is the 65-kDa heat shock protein. Incorporation of monospecific antibody to the *E. coli* 65-kDa heat shock protein of *M. bovis* BCG in the intermediate gel resulted in retention of the protein (Fig. 5D). The position of the precipitate was lowered, with both legs extending through the intermediate gel.

Immunogenicity of Ag84 after infection. The presence of antibodies to Ag84 in serum from patients with leprosy or

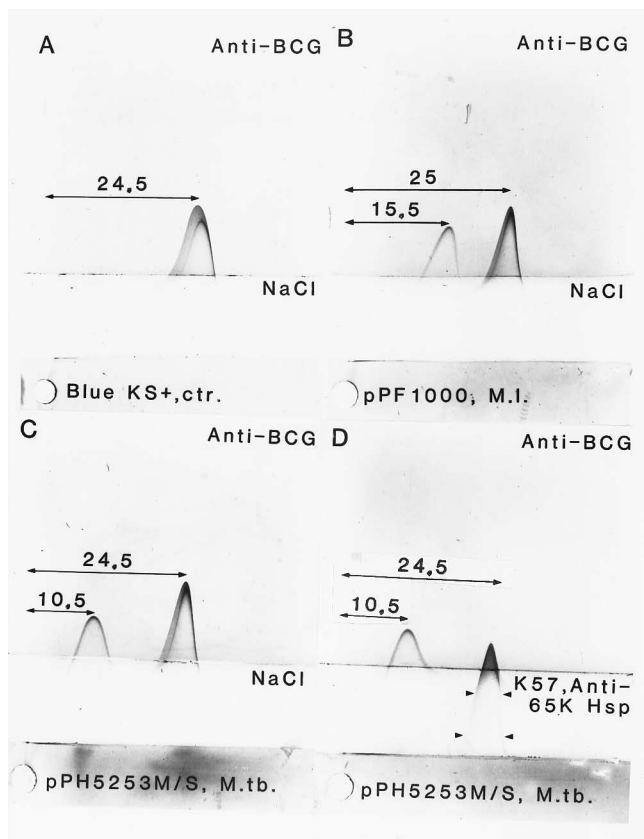


FIG. 5. CIE of sonic extracts of the *E. coli* control strain EC1 (Blue KS+, ctr.) (A) and the recombinants ML1000 (pPF1000, M.I.) and MT5253ΔM/S (pPH5253M/S, M.tb.) (B and C, respectively). The top gel contains polyvalent anti-BCG immunoglobulin in all four plates. Panels B and C demonstrate the different electrophoretic mobilities of Ag84 of *M. leprae* and *M. tuberculosis* during first-dimension electrophoresis. Panel D identifies the fast-moving precipitate appearing in all panels as the 65-kDa heat shock protein (Hsp) by addition monospecific polyclonal rabbit antibody to the 65-kDa heat shock protein in the intermediate gel.

tuberculosis was initially tested by using a double-antibody enzyme-linked immunosorbent assay (ELISA). The capture mAb was F126-2, the antigen was a sonic extract of *M. bovis* BCG, and human serum diluted 1:50 was used in the third layer. Extensive controls were used, including assays with all steps being identical while addition of antigen in the second step, indicating a marked tendency of binding of immunoglobulin to mAb F126-2 (data not shown), ELISA was considered unsuitable for the purpose. Tests using SDS-PAGE and immunoblotting were then performed. The results are illustrated in Fig. 6. The membrane to the left shows the reactivity of mAb F126-2 with the *M. leprae* Ag84 expressed by ML2000 (lane 1) and no reaction at this position with protein of the *E. coli* control strain EC1 (lane 2). Three sera from patients with lepromatous (LL and BL) leprosy with reactions recorded as ++, +, and -, respectively, are also shown. The results for serum samples from 20 tuberculosis patients, from 22 lepromatous and 11 tuberculoid leprosy patients, and from 10 healthy controls are summarized in Table 2. In a large proportion of individuals with lepromatous leprosy (almost 60%), antibodies to Ag84 were detected, indicating that Ag84 is highly immunogenic in these patients. In contrast, distinctly

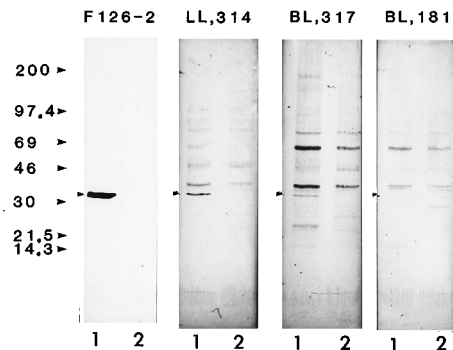


FIG. 6. Western blot analysis to demonstrate antibodies to Ag84 in serum from leprosy and tuberculosis patients. The membrane to the left shows the reactivity of mAb F126-2 with the *M. leprae* Ag84, present in the sonic extract of *E. coli* ML2000 (lane 1), and no reaction at this position with *E. coli* EC1 control sonic extract (lane 2). The reactions of sera from lepromatous leprosy patients LL314, BL317, and BL181, recorded as ++, +, and -, respectively, are shown. The position of Ag84 is indicated with arrowheads. Sizes are indicated in kilodaltons.

fewer and weaker positive reactions were observed with sera from tuberculoid leprosy and tuberculosis patients. Furthermore, all control sera were negative. Identical results were observed for the recombinant *M. tuberculosis* Ag84 (data not shown).

DISCUSSION

In this study, the *M. tuberculosis* gene encoding Ag84, as defined in the CIE reference system for *M. bovis* BCG (3) and *M. tuberculosis* (24), and its homologous gene in *M. leprae* were cloned and characterized. The location of the *M. tuberculosis* Ag84 gene on recombinant plasmid pPH5250 was identified by deletion mutagenesis. Deletion mutant MT5253ΔM/S, containing a single large mycobacterial ORF at positions 125 to 931, expressed Ag84 of *M. tuberculosis* (Fig. 2). Deletion of the N-terminal part of this ORF blocked the expression of Ag84. These findings strongly suggest that the observed large ORF harbors the *M. tuberculosis* Ag84 gene.

Ag84 of *M. leprae* showed 85% amino acid sequence identity with the *M. tuberculosis* homolog, demonstrating a high degree of evolutionary conservation. A similar degree of protein homology between *M. tuberculosis* and *M. leprae* has been observed for several other antigens, such as Ag85A (81%), Ag85B (82%), Ag85C (82%), and the heat shock proteins GroEL (87%) and GroES (91%) (15, 16). The promoter region contained a SD sequence, but no *E. coli*-like -35 transcription region was observed. Furthermore, the absence of a known signal sequence suggests that these mycobacterial antigens are not secreted. This observation is consistent with our

TABLE 2. Serological responses to Ag84 of tuberculosis patients, leprosy patients, and healthy controls

Serum	No. with indicated strength of reactivity				Total
	++	+	(+)	-	
Leprosy					
Lepromatous (LL, BL)	4	8	1	9	22
Tuberculoid (BT, TT)	0	1	3	7	11
Tuberculosis	1	0	0	19	20
Controls	0	0	0	10	10

finding that the *M. tuberculosis* Ag84 is not found in the medium after short-term culture to avoid bacterial lysis but is exclusively present in the cytoplasmic fraction (data not shown).

The *E. coli* recombinants containing the entire Ag84 gene of *M. tuberculosis* or *M. leprae* all expressed the mycobacterial antigen. The expression of recombinant Ag84 of both mycobacterial species occurred independently of the *lacZ* promoter, as Ag84 expression was observed in both orientations of the mycobacterial genes in the *lacZ*-containing vector (Fig. 1). Most of the mycobacterial genes identified so far are not expressed by *E. coli*, because the host does not recognize the mycobacterial promoter sequences (4). Those recombinant mycobacterial genes that are expressed in *E. coli* by their own promoter signals mostly belong to the heat shock protein families. The high degree of conservation of these genes in nature explains the expression by *E. coli* (10, 21, 27).

E. coli MT5250, MT5253, and MT5253ΔM/S, in which the *M. tuberculosis* Ag84 gene was located in the *lacZ* orientation, showed an additional minor protein band of 36 kDa that reacted with MAb F126-2. The additional band was absent in MT5270, in which the Ag84 gene was located in the orientation opposite that of *lacZ* (Fig. 1). The observation was repeatedly confirmed and suggests the expression of a fusion protein consisting of the N-terminal β-galactosidase part and Ag84. However, two stop codons are present upstream of the Ag84 gene at positions 77 and 122, which indicates that the presence of the double band cannot be explained by the expression of a β-galactosidase–Ag84 fusion protein. The double protein band remains unexplained.

The polyvalent anti-BCG antibody reacted strongly with the recombinant *M. tuberculosis* and *M. leprae* Ag84, giving a distinct precipitate in CIE with these proteins (Fig. 5). Figures 5B and C show distinctly different mobilities of the *M. leprae* and *M. tuberculosis* antigens during first-dimension electrophoresis. This can be explained by the difference in the deduced amino acid sequences of the two mycobacterial proteins. Comparison of the two sequences demonstrated that there is a net difference of four positively charged amino acids in the *M. tuberculosis* protein, and thus it is more basic during electrophoresis. This explains the lower mobility of Ag84 of *M. tuberculosis*. A similar difference in electrophoretic mobility during first-dimension electrophoresis in CIE has also been observed between antigen 1 of *M. leprae* and antigen 1 of *Mycobacterium smegmatis* (6).

The polyvalent anti-BCG antibody gave a strong precipitate with the sonic extract of *E. coli* control strain EC1 (Fig. 5A), indicating very strong cross-reactivity of this *E. coli* antigen in CIE. This precipitate was expected to correspond to the bacterial common antigen originally discovered as a protein antigen of *E. coli* that cross-reacted with an antigen present in more than 50 other bacterial species as defined by gel precipitation experiments (8, 11). This antigen was later shown to correspond to the 65-kDa heat shock protein of *M. tuberculosis*. The cross-reactivity of this protein in CIE can be explained by the prominent characteristic of heat shock proteins being highly conserved between different species (10, 21, 27). The identity of this *E. coli* protein was confirmed to be the 65-kDa heat shock protein by incorporation of an antibody to this protein in the intermediate gel (Fig. 5D).

The presence of antibodies to the mycobacterial Ag84 in serum from both tuberculosis and leprosy patients was tested, using SDS-PAGE and immunoblotting (Fig. 6 and Table 2). Antibodies to Ag84 were clearly demonstrated to be present in multibacillary lepromatous leprosy patients; some, but distinctly fewer and weaker, positive reactions were observed in

tuberculoid leprosy patients. Since almost 60% of the lepromatous leprosy patients responded to Ag84, the protein is considered to be highly immunogenic following infection in multibacillary leprosy. This feature has also been observed previously with various other mycobacterial antigens (18, 20) and reflects the lower stimulation of the B-cell system in paucibacillary leprosy because of the lower antigenic load compared with multibacillary leprosy. The minor proportion of tuberculosis patients reacting with Ag84 is probably also related to the low amount of mycobacterial protein antigen during *M. tuberculosis* infection. The observation that the Ag84 immunoreactive sera from tuberculosis and leprosy patients recognize both the *M. tuberculosis* and *M. leprae* antigens indicates that shared epitope(s) are recognized by these patients.

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