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Analysis of the Genes Encoding the Antigen 85 Complex and MPT51 from *Mycobacterium avium*

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The components of the fibronectin-binding antigen 85 complex (85A, 85B, and 85C) and the related protein MPB/MPT51 are major secreted proteins in *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG. The *fbpA*, *fbpC*, and *mpt51* genes encoding 85A, 85C, and MPT51, respectively, were isolated from *Mycobacterium avium* and sequenced in this study. The structures of these genes, and that of the *fbpB* gene encoding the 85B protein, were conserved in these three species. The secreted amounts of 85A, 85B, 85C, and MPB/MPT51 were compared for *M. tuberculosis*, BCG, and *M. avium*. These four proteins were found in large amounts in the culture filtrates from *M. tuberculosis* and BCG. In contrast, in the culture filtrate from *M. avium*, 85B and MPT51 were abundant whereas 85A and 85C were hardly found, in spite of the presence of the encoding genes. The difference in the secretion amounts might be regulated at the transcription level. These facts might reflect host immunopathogenesis, the protective immunities against infections, and the drug susceptibilities of these organisms.

The intracellular pathogens *Mycobacterium avium* and *Mycobacterium intracellulare*, collectively referred to as the *M. avium* complex (MAC), were rarely identified as causes of severe infections in humans until recently (7, 46). However, it has recently been recognized that MAC is the major bacterial killer of elderly individuals and AIDS patients (6, 27). To control MAC infection is difficult because of the resistance of MAC to conventional antibiotics and antituberculosis drugs. Immunity to mycobacteria requires the activation of specifically sensitized T lymphocytes and macrophages (3, 33). Studies using the gamma interferon (IFN-γ) gene and IFN-γ receptor gene knockout mice have shown that IFN-γ plays an essential role in protective cellular immunity to mycobacterial infection (9, 20).

The mycobacterial antigen 85 complex (reviewed in reference 43) is one of the powerful inducers of IFN-γ synthesis on host cells (17, 18) and has the ability to bind to fibronectin (1). This complex is produced in large amounts (20 to 30% [wt/wt] of all of secreted proteins) by *Mycobacterium tuberculosis* either extracellularly in culture or intracellularly in human mononuclear phagocytes (22). As suggested by this fact, the antigen 85 complex may play a very important role in the physiology of mycobacterial cells. Indeed, this complex possesses mycolic acid transferase activity in cell wall synthesis (4). Recently, it was shown that the antigen 85 complex had immunoprotective potential against *M. tuberculosis* (15, 16). Also, the relationship between the antigen 85 complex and the antimycobacterial action of isonicotinic acid hydrazide (INH) was demonstrated (13). Exposure to INH induced the expression of two components of the antigen 85 complex, and it was suggested that the amounts of these components and susceptibility to INH were related.

The antigen 85 complex consists of three structurally related components (A, B [α antigen], and C) encoded by three genes (*fbpA*, *fbpB*, and *fbpC*, respectively) located at separate loci in the mycobacterial genome (8, 36). *M. tuberculosis* and *Mycobacterium bovis* BCG produce all three components in large amounts. Recently, another secreted protein, termed MPB/MPT51 (mycobacterial protein secreted from BCG and mycobacterial protein secreted from *M. tuberculosis*), was demonstrated to cross-react with the three components of the antigen 85 complex (28, 30, 45) and to have extensive primary structure similarity with those components (30). In addition, another protein, the 33-kDa antigen, which cross-reacted with each of the three components of the antigen 85 complex and with the MPB/MPT51 protein, was found (30).

The *fbpB* gene was first cloned from BCG (26). The genes encoding the components of the antigen 85 complex were recently cloned from several species (5, 8, 11, 21, 23, 25, 26, 30, 31, 36, 41). However, sequencing of the entire *fbpA* gene had been carried out with only *M. tuberculosis* and BCG (5, 11, 30). Sequencing of the entire *fbpC* gene had been carried out with only *M. tuberculosis*, BCG, and *Mycobacterium leprae* (8, 23, 41).

Wiker et al. observed components which immunologically reacted with antisera to BCG antigen 85 complex in a culture filtrate of *M. avium* by enzyme-linked immunosorbent assay (44). Drowart et al. demonstrated the existence of one component of the antigen 85 complex in *M. avium* by isoelectric focusing followed by Western blotting (12). But it is still unclear whether there are three components of the antigen 85 complex in *M. avium*.

In this study, we demonstrate the amounts of the components of the antigen 85 complex and MPT51 in culture filtrate of *M. avium* by two-dimensional electrophoresis (2D-E). We also describe the isolation of the *fbpA*, *fbpC*, and *mpt51* genes from *M. avium.*

MATERIALS AND METHODS

Bacterial strains and plasmids. *M. avium* ATCC 15769 (serotype 1) and BCG strain Tokyo were grown in the wholly synthetic Sauton medium (39). *Escherichia coli* K-12 strain XL1-Blue (Stratagene, La Jolla, Calif.) was used as a host for plasmids pUC19, pBluescript-II SK+, and pBluescript-II SK− (Stratagene) and their derivatives. *E. coli* XL1-Blue was grown on Luria-Bertani medium (38).

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2D-E. The 4-week-old culture supernatants of *M. avium* and of BCG were concentrated by ammonium sulfate precipitation (80% saturation), and the salt was removed by dialysis. The concentrated 5-week-old culture filtrate of *M. tuberculosis* was obtained from S. Nagai. Protein concentrate (40 μg) was analyzed by 2D-E, initially by isoelectric focusing in the Immobiline DryStrip (Pharmacia, Uppsala, Sweden) in the pH range of 4.0 to 7.0 by using the Multiphor II System (Pharmacia) and then by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 12.5% separating gel (29). The molecular mass was calibrated with standard proteins (Pharmacia). Gels were stained with Coomassie brilliant blue (CBB) and with $AgNO_3$ or transblotted onto Immobilon (Millipore, Bedford, Mass.) membranes. Western blotting was carried out as described previously (26). Polyclonal antibodies raised against the *M. tuberculosis* α antigen (T-α [85B]) and *M. tuberculosis* MPT51 (T-MPT51) (30) were used. The analysis of the stained gel was carried out with the aid of a computerized 2D-E gel analysis system from PDI (Huntington Station, N.Y.) supporting the PDQUEST software.

Determination of amino acid sequences. The specimen for amino acid analysis was obtained by cutting out a spot from a polyvinylidene difluoride membrane (Millipore). The proteins in the 2D-E gel were blotted electrophoretically onto the polyvinylidene difluoride membrane by using 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS; Sigma, St. Louis, Mo.)–NaOH buffer, pH 11, by the method of Matsudaira (24) and were detected by staining with CBB. The membrane pieces of the spots were submitted for determination of the amino-terminal sequence by automatic Edman degradation with an Applied Biosystems 477A gas-phase sequencer (Applied Biosystems, Foster City, Calif.).

DNA technology. Unless otherwise stated, standard procedures were used for the preparation and handling of DNA (38).

Preparation of probes. As nucleotide probes, two parts of the *fbpB* gene of BCG were used. One was a 0.7-kbp *Pst*I fragment containing the N-terminal end of the gene (probe A) and the other was a 0.3-kbp *Xho*I-*Pst*I fragment containing the C-terminal end of that gene (probe B) (26). Probes were labeled with an ECL direct nucleic acid labeling system according to the manufacturer's instructions (Amersham, Buckinghamshire, England).

DNA cloning. Cloning of the *fbpA* and *fbpC* genes of *M. avium* was carried out as described previously (31). *Sph*I-digested fragments of *M. avium* DNA were separated by electrophoresis in a 1% agarose gel. The DNA fragments with lengths of 4.9 and 3.6 kbp, which gave clear bands hybridized with both probes (31), were cloned into the *Sph*I site of pUC19. The ligated products were used to transform *E. coli* XL1-Blue. Ampicillin-resistant transformants were screened by the colony hybridization technique with the same probes.

DNA sequencing. A 2.0-kbp *Bam*HI fragment from pAASp49 (a positive clone containing the 4.9-kbp *Sph*I-digested fragment of *M. avium*) was subcloned into the *Bam*HI site of pBluescript-II SK− (pAAB20). A 2.3-kbp *Sma*I fragment from pAASp36 (a positive clone containing the 3.6-kbp *Sph*I-digested fragment of *M. avium*) was subcloned into the *Sma*I site of pBluescript-II SK− (pAAS23). The DNA sequences of the inserted DNA of pAAB20 and pAAS23 for both of the complementary DNA strands were determined by an automated DNA sequencer (Applied Biosystems) with a dye primer cycle sequencing kit (Applied Biosystems). Sequencing templates were prepared by subcloning.

Nucleotide sequence accession numbers. The sequence data will appear in the GSDB, DDBJ, EMBL, and NCBI nucleotide sequence databases with the accession no. D78144 and D87323.

RESULTS

Antigen 85 complex and MPT51 in culture filtrate from *M. avium*. To analyze the components of the antigen 85 complex and MPT51 in the culture filtrate from *M. avium*, we separated proteins in Sauton medium, after a 4-week culturing of *M. avium*, by 2D-E (Fig. 1A) and reacted them with anti-T-α (85B) and anti-T-MPT51 sera. The two reactions revealed similar patterns (Fig. 1B and C).

Next we tried to determine the amino acid sequences of these cross-reacted proteins. We cut out protein spots from 2D-E blots following CBB staining and submitted them to microsequence analysis. Among cross-reacted proteins, only the N-terminal amino acid sequences of two major proteins could be determined. One was 1-AGYESLMVPSAAMGR DI-17 and the other was 1-FSRPGLPVEYLQVPSAGMGR-20 for the proteins a and b, respectively, shown in Fig. 1B and C. The former sequence was similar to the N-terminal amino acid sequences of T-MPT51 and MPB51. The latter was similar to the N-terminal amino acid sequences of the components of the mycobacterial antigen 85 complex. We could not determine amino acid sequences of other proteins that reacted with anti-T-α and anti-T-MPT51 sera because of their small amounts.

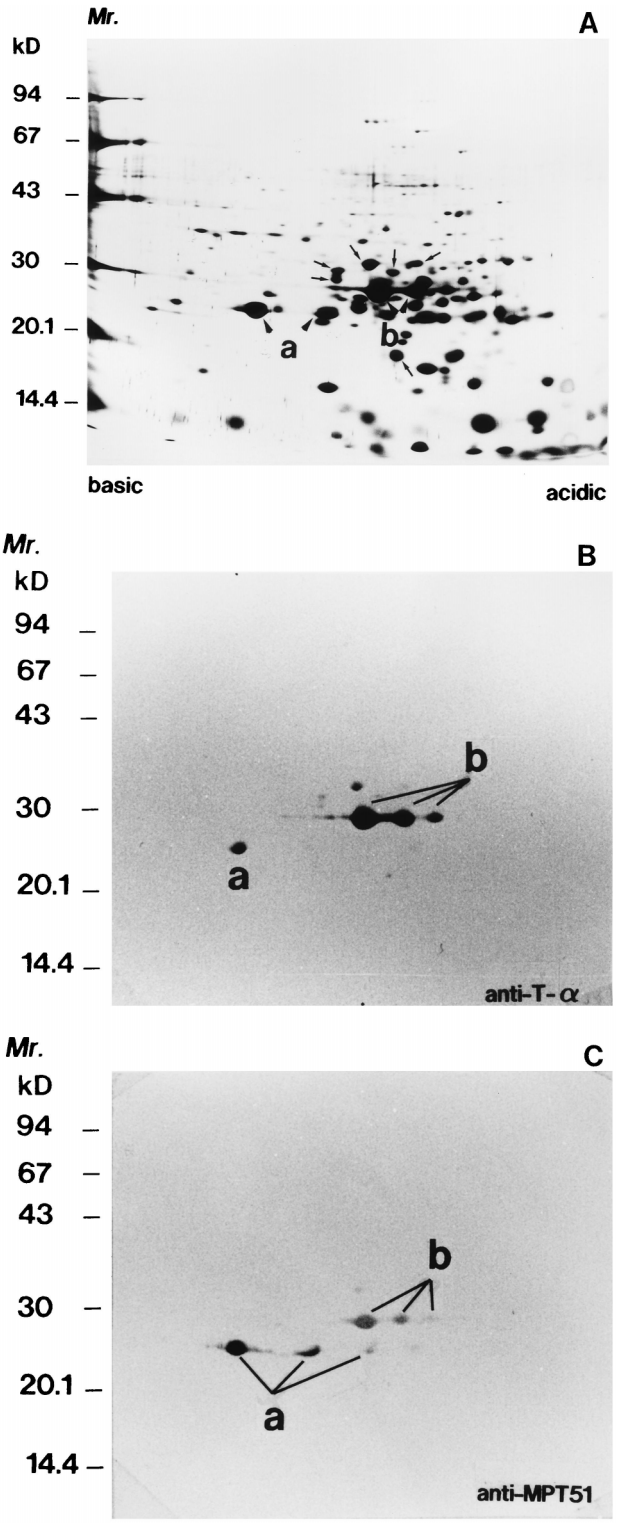

FIG. 1. Analysis by 2D-E and Western blotting of 4-week-old culture filtrate of *M. avium*. The gel was stained with CBB and $AgNO_3$ (A). Membranes were tested against anti-T-α (B) and anti-T-MPT51 (C). The spots that reacted with anti-T-α and anti-T-MPT51 are indicated by arrows in panel A. *Mr.*, molecular mass markers; a, protein a; b, protein b.

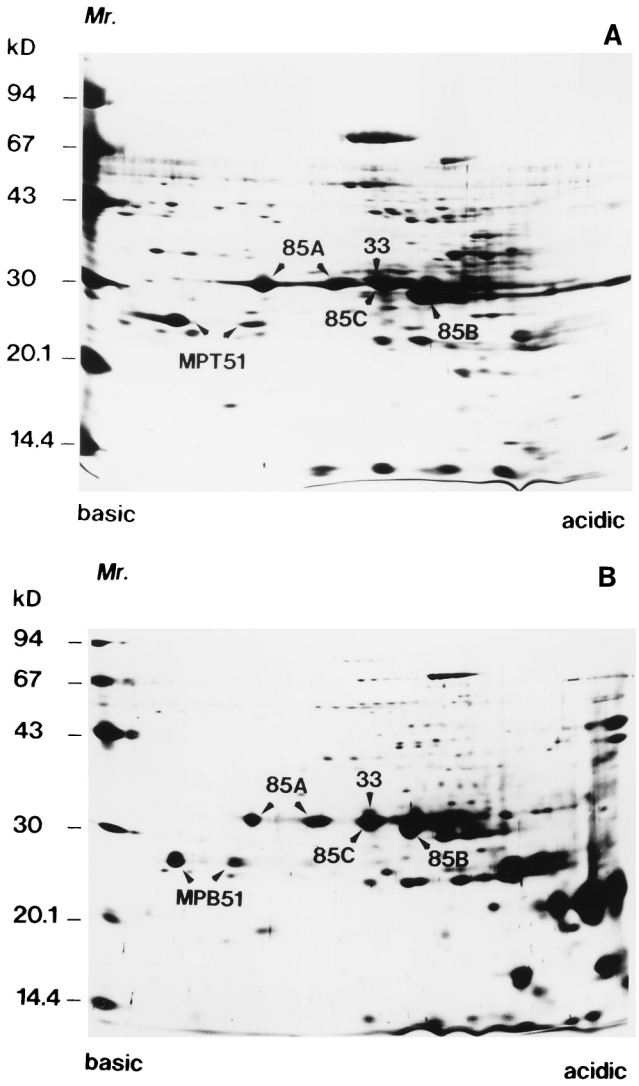

FIG. 2. Analysis by 2D-E of 5-week-old culture filtrate of *M. tuberculosis* (A) and of 3-week-old culture filtrate of BCG (B). The components of the antigen 85 complex and MPB/MPT51 are indicated. The 33-kDa protein that reacted with anti-T-α and anti-T-MPT51 (30) is also indicated. *Mr.*, molecular mass markers.

These results suggested, surprisingly, that only one protein among the three components of the antigen 85 complex and MPT51 was produced extracellularly in large amounts by *M. avium*.

This result was strikingly different from the results obtained when the culture filtrates of *M. tuberculosis* and BCG were used. *M. tuberculosis* and BCG produced large amounts of all three components of the antigen 85 complex and MPB/MPT51 in culture medium (Fig. 2). *M. tuberculosis* and BCG also produced the 33-kDa antigen that cross-reacted with both the antigen 85 complex and MPB/MPT51 (30), but we could not identify this antigen in *M. avium* culture filtrate.

DNA sequence of the *fbpA* and *fbpC* genes of *M. avium*. Two positive clones, obtained by the colony hybridization technique, were designated pAASp49 and pAASp36. They contained the mycobacterial 4.9-kbp DNA fragment and the mycobacterial 3.6-kbp DNA fragment, respectively. After subcloning according to the procedure described in Materials and Methods, the nucleotide sequences of the 2.0-kbp inserted DNA of pAAB20 (a derivative of pAASp49) and the 2.3-kbp inserted DNA of pAAS23 (a derivative of pAASp36) were determined. The nucleotide sequence of pAAB20 (Fig. 3) contained an open reading frame (ORF), ORFA, of 348 codons, starting with an ATG codon at position 590, which was preceded by a plausible ribosome binding site (AGGAAG). As expected, the deduced amino acid sequence for ORFA was homologous to the sequence of the previously sequenced mycobacterial antigen 85 complex, and the closest identity was observed with the 85A proteins of *M. tuberculosis* and BCG rather than with the 85B protein of *M. avium*. We therefore identified ORFA as the *fbpA* gene from *M. avium*. The predicted molecular mass of the 347 amino acids of the *M. avium* 85A protein is 36,093.55 Da. The N-terminal amino acid sequence of the mycobacterial antigen 85 complex was found to be identical to that deduced from the nucleotide sequence beginning with the TTC codon at position 719. There was a signal peptidase recognition sequence, A-G-A, just before the N-terminal region of the mature protein (42). This amino acid sequence included a 43-residue putative signal peptide and the

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GGATCCGGGGCTACCTGGACGCGCAGTGGGTCGCCGAGGCCGTCTCGGCGCTGCAGTGCC      60
CGGACACCCGGGCGGTGCTGGCCTCGGTGCGGGCCCCGATGACCCCGCACCGGTTCCTGT     120
CCAACGTGCTGCACTCCTTCCAATTCACCGGCTACCGGATCGATCGGGTGCCGCGCTACG     180
AGCTCGCCCGCTGCGGGCTGCCGGTGCCGGAGGAAGCGCCACCGCCGCCGCGCGCGAGTGAG     240
CGCCGCCGCAGAAGAATTTTCGGCCGATCCGCCACCGGTCGGGCCGCGGCCTCCGCGACAA     300
ATTGGCAGCAACATCACATCTGACCCCCGACCAGCTCGCCACGGCGGTCAAGCACATGCG     360
CCGACGTCGACTTTTGTACACGTGTTGGATGTGAAAACCGCTGGGTTCCAGGGCCGCTCG     420
GCCGCAGGCGGGCTCGCCTCGATGCGACCGAACGGCCACCGGTGTGGTTGACTACACGAG     480
CACTCCTGCGCTTCATGCCATCGGTAGGCGCAGGCGGTTCGAACCAATCAAGAAAGCGGC     540
CGAGCGGACGAACGCGCCGGAACGCCGCAAGAAGGATGAGGAAGCAAGGATGACGCTTGT     600
                                          fbpA   M   T   L   V
CGACAGATTGCGCGGCGCCGTGGCGGGGATGCCGCGCCGGCTCGTGGTGGGGGCCGCTGG     660
  D   R   L   R   G   A   V   A   G   M   P   R   R   L   V   V   G   A   A   G
GGCGGCGCTGCTCTCGGGCCTGATTGGCGCCGTCGGGGGCTCGGCGACCGCCGGGGCCTT     720
  A   A   L   L   S   G   L   I   G   A   V   G   G   S   A   T   A   G   A   F
CTCGCGCCCCGGTCTGCCGGTGGAGTACCTGCAGGTTCCCTCCGCCGCCATGGGACGGGA     780
  S   R   P   G   L   P   V   E   Y   L   Q   V   P   S   A   A   M   G   R   D
CATCAAGGTCCAGTTCCAAAGCGGTGGGGCCAACTCGCCCGCGTTGTACCTGCTCGACGG     840
  I   K   V   Q   F   Q   S   G   G   A   N   S   P   A   L   Y   L   L   D   G
GATGCGCGCGCAAGACGACTTCAACGGCTGGGACATCAACACCCCGGCGTTCGAGTGGTA     900
  M   R   A   Q   D   D   F   N   G   W   D   I   N   T   P   A   F   E   W   Y
CAACCAGTCGGGCATCTCGGTCGCCATGCCGGTCGGCGGCCAGTCCAGCTTCTACTCCGA     960
  N   Q   S   G   I   S   V   A   M   P   V   G   G   Q   S   S   F   Y   S   D
CTGGTACAAGCCCGCCTGCGGCAAGGCCGGCTGCACCACCTACAAGTGGGAGACCTTCCT    1020
  W   Y   K   P   A   C   G   K   A   G   C   T   T   Y   K   W   E   T   F   L
GACCAGCGAGCTGCCGCAGTACCTGTCGGCGCAGAAGCAGGTCAAGCCGACCGGCAGCGG    1080
  T   S   E   L   P   Q   Y   L   S   A   Q   K   Q   V   K   P   T   G   S   G
TGTCGTCGGGCTGTCGATGGCCGGCTCCTCGGCGCTGATCCTGGCCGCCTACCACCCCGA    1140
  V   V   G   L   S   M   A   G   S   S   A   L   I   L   A   A   Y   H   P   D
CCAGTTCGTCTACGCCGGCTCGCTGTCGGCGCTGCTGGACCCGTCGCAGGGCATGGGCCC    1200
  Q   F   V   Y   A   G   S   L   S   A   L   L   D   P   S   Q   G   M   G   P
GTCGCTGATCGGGCTGGCCATGGGTGACGCCGGTGGCTACAAGGCCGCCGACATGTGGGG    1260
  S   L   I   G   L   A   M   G   D   A   G   G   Y   K   A   A   D   M   W   G
TCCGAAGGAGGACCCGGCCTGGGCCCGCAACGACCCGTCGCTGCAGGTCGGCAAGCTGGT    1320
  P   K   E   D   P   A   W   A   R   N   D   P   S   L   Q   V   G   K   L   V
CGCGAACAACACCCGGATCTGGGTGTACTGCGGCAACGGCAAGCCGTCCGACCTCGGTGG    1380
  A   N   N   T   R   I   W   V   Y   C   G   N   G   K   P   S   D   L   G   G
CGACAACCTGCCCGCCAAGTTCCTGGAGGGCTTCGTGCGGACGTCCAACCTGAAGTTCCA    1440
  D   N   L   P   A   K   F   L   E   G   F   V   R   T   S   N   L   K   F   Q
GGACGCCTACAACGGCGCCGGTGGCCACAACGCGGTGTGGAACTTCGACGCCAACGGCAC    1500
  D   A   Y   N   G   A   G   G   H   N   A   V   W   N   F   D   A   N   G   T
CCACGACTGGCCCTACTGGGGCGCGCAGCTGCAGGCGATGAAGCCTGACCTGCAGTCGGT    1560
  H   D   W   P   Y   W   G   A   Q   L   Q   A   M   K   P   D   L   Q   S   V
GCTGGGCGCCACCCCGGGTGCCGGTCCGGCCACGGCCGCGGCCACCAACGCCGGGAACGG    1620
  L   G   A   T   P   G   A   G   P   A   T   A   A   A   T   N   A   G   N   G
CCAGGGCACCTAACCAATACCGAGCATGACGCCGGCGGCGGGGAAACCCTTCGGGGCTCCC    1680
  Q   G   T   *
GCCGCCGGTCGTTTCGCGACGGTGTGACGAGTTTCACTACCCGGCGCGCGGGGGAACTAC    1740

GGCCCTGGCTAATGTGCTCTCAGCGACTAGACGATGGAGGGTGGACATGACGGTCGTGCG    1800
                                          mpt51  M   T   V   V   R
GGGTGTGTCGGCGCTTCTTCGGGTGTTCTGCATCGCCATGCTGGCGGCGGGGCTGGGCGT    1860
  G   V   S   A   L   L   R   V   F   C   I   A   M   L   A   A   G   L   G   V
GGCGTTGCAGCCCGCCGCGGTCACCGGCGCGGCCCGGGCCGCGGGGTACGAGAGCCTGAT    1920
  A   L   Q   P   A   A   V   T   G   A   A   R   A   A   G   Y   E   S   L   M
GGTGCCGTCGGCGGCGATGGGCCGCGACATCCCGGTGGCCTTCCTGGCCGGCGGCCCGCA    1980
  V   P   S   A   A   M   G   R   D   I   P   V   A   F   L   A   G   G   P   H
TGC                                                                    1983
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FIG. 3. Complete nucleotide sequence of the inserted DNA of pAAB20. The first ORF, ORFA, corresponds to the *M. avium fbpA* gene; the second ORF, ORFB, corresponds to the *M. avium mpb51* gene. The predicted amino acid sequences for the ORFs are shown in one-letter code, and the asterisk indicates the stop codon. The signal sequences are shown in italics. Underlined nucleotides indicate putative Shine-Dalgarno sequences. The palindrome is indicated by wedges.

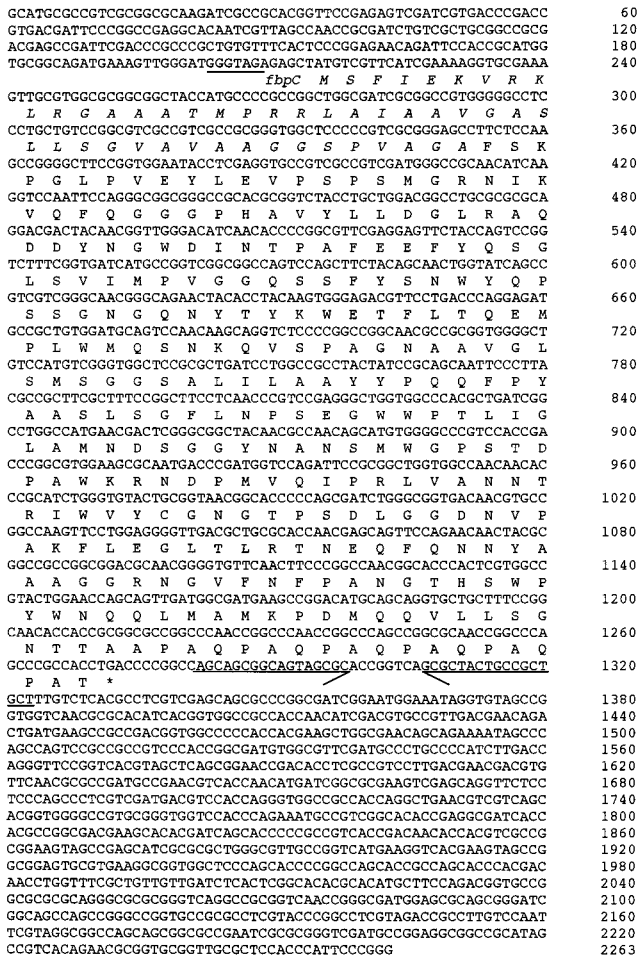

FIG. 4. Complete nucleotide sequence of the inserted DNA of pAAS23, showing ORFC of the *M. avium fbpC* gene. The predicted amino acid sequence for ORFC is shown in one-letter code, and the asterisk indicates the stop codon. The signal sequence is shown in italics. Underlined nucleotides indicate a putative Shine-Dalgarno sequence. The palindrome is indicated by wedges.

304-amino-acid mature protein. Its theoretical molecular mass was calculated to be 32,106.07 Da, and its pI was calculated to be 5.01. A 17-bp inverted repeat was seen immediately downstream of the stop codon of the *fbpA* gene.

A partial ORF (ORFB) was detected downstream from the *fbpA* gene in pAAB20. A putative ribosome-binding site (GGAGG) was located 7 nucleotides upstream of the in-frame ATG start codon at position 1787 for ORFB. The deduced amino acid sequence of ORFB was very similar to the sequence of the MPB/MPT51 proteins from BCG and *M. leprae* (61 and 67%, respectively). The N-terminal amino acid sequence of MPT51 was found to be identical to that deduced from the nucleotide sequence beginning with the GCG codon at position 1901. We therefore identified ORFB as the *mpt51* gene from *M. avium*. There was a signal peptidase recognition sequence, A-R-A, just before the N-terminal region of the mature protein (42). This amino acid sequence included a 38-residue putative signal peptide.

The nucleotide sequence of pAAS23 (Fig. 4) contained an ORF, ORFC, of 353 codons, starting with an ATG codon at position 215, which was preceded by a plausible ribosome-binding site (GGGTAG). As expected, the deduced amino acid sequence of ORFC was homologous to the sequence of the previously sequenced mycobacterial antigen 85 complex and the closest identity was observed with the 85C proteins of *M. tuberculosis* and BCG rather than with the 85A and 85B proteins of *M. avium*. We therefore identified ORFC as the *fbpC* gene from *M. avium*. The predicted molecular mass of the 352 amino acids of the *M. avium* 85C protein is 37,754.41 Da. The N-terminal amino acid sequence of the mycobacterial antigen 85 complex was found to be identical to that deduced from the nucleotide sequence beginning with the TTC codon at position 353. There was a signal peptidase recognition sequence, A-G-A, just before the N-terminal region of the mature protein (42). This amino acid sequence included a 46-residue putative signal peptide and the 307-amino-acid mature protein. Its theoretical molecular mass was calculated to be 33,321.40 Da, and its pI was calculated to be 4.83. A 17-bp inverted repeat was seen immediately downstream of the stop codon of the *fbpC* gene.

Sequence similarities of 85A, 85C, and MPT51 from *M. avium* with components of the antigen 85 complex and MPB/MPT51. 85A from *M. avium* showed 62 to 82% and 34 to 46% homology at the amino acid level to other mycobacterial components of the antigen 85 complex and mycobacterial MPB/MPT51, respectively. 85C from *M. avium* showed 60 to 83% and 38 to 39% homology at the amino acid level to other mycobacterial components of the antigen 85 complex and mycobacterial MPB/MPT51, respectively. MPT51 from *M. avium* showed 32 to 46% and 64 to 67% homology at the amino acid level to other mycobacterial components of the antigen 85 complex and mycobacterial MPB/MPT51, respectively. However, comparison of partial N-terminal sequences of mature proteins revealed that the MPT51 protein from *M. avium* had 85 and 92% homology with the BCG MPB51 and the *M. leprae* MPT51 proteins, respectively. No other proteins, except for PS1 from *Corynebacterium glutamicum* (19), with significant homologies to the 85A or the MPT51 protein from *M. avium* were detected in the entire SwissProt-National Biomedical Research Foundation data bank.

Comparison of amino acid sequences of proteins a and b and deduced amino acid sequences for antigen 85 complex genes and the *mpt51* gene. To determine the dominant proteins which we sequenced, we compared the N-terminal amino acid sequences of protein a and protein b and the putative N-terminal amino acid sequences of the components of the antigen 85 complex and MPT51 deduced from nucleotide sequences. The results are shown in Fig. 5. The N-terminal

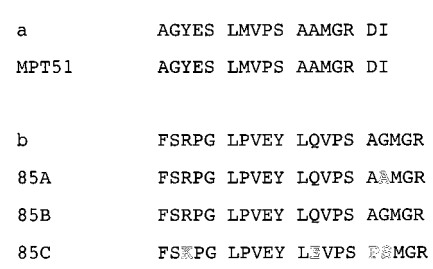

FIG. 5. Homology between the N-terminal amino acid sequences determined for the isolated proteins a and b from the *M. avium* culture filtrate and the N-terminal amino acid sequences deduced from *M. avium fbpA*, *fbpB*, *fbpC*, and *mpt51*. Upper rows, comparison of the N-terminal amino acid sequence of protein a and the N-terminal amino acid sequence deduced from the *mpt51* gene; lower rows, comparison of the N-terminal amino acid sequence of protein b and the N-terminal amino acid sequences deduced from the *fbpA*, *fbpB*, and *fbpC* genes. Amino acid differences from protein b are indicated by outlined characters.

amino acid sequence of protein a was completely identical to the putative N-terminal amino acid sequence of MPT51. The N-terminal amino acid sequence of protein b was completely identical to the putative N-terminal amino acid sequence of 85B. The N-terminal amino acid sequence of protein b was also very similar to but distinct from the putative N-terminal amino acid sequences of 85A and 85C: the Gly residue at position 17 in protein b was Ala in 85A; Arg, Gln, Ala, and Gly at positions 3, 12, 16, and 17, respectively, in protein b were Lys, Glu, Pro, and Ser, respectively, in 85C. We therefore identified protein a as MPT51 and protein b as 85B. From these results, it was suggested that *M. avium* mainly produced 85B and MPT51.

DISCUSSION

It is well known that live attenuated mycobacterial vaccines are more efficient than killed vaccines in providing protection against a challenge with virulent mycobacteria (32). Therefore, antigens secreted by live mycobacteria are expected to be more protective than the other cellular components (34). Among these secreted antigens, the α antigen (85B) and its related proteins, 85A, 85C, and MPB/MPT51, predominated in the culture filtrates of *M. tuberculosis* and BCG. These antigens were demonstrated to provide protective immunity against tuberculosis in animal models (15, 16). From an immunological point of view, it was very important which component(s) was predominantly expressed from mycobacterial cells, because the immunological activities of these antigens were not identical. Nagai et al. (28) reported that *M. tuberculosis* 85B had delayed-type hypersensitivity activity in guinea pigs sensitized with heat-killed *M. tuberculosis* but *M. tuberculosis* 85A did not. A similar explanation was stated by other investigators (10, 37). There is no information on 85C yet.

The 85A/85B/85C/MPT51 ratio in *M. tuberculosis* culture filtrate was 2/3/1/2 in this study. This ratio in BCG culture filtrate was similar to that in *M. tuberculosis* culture filtrate. This ratio did not conflict with a recent report (14). The 85A/85B/85C ratio was remarkably similar to the ratio of the corresponding mRNAs, suggesting that expression of these components was regulated at the transcriptional level (14). There is no information on MPT51 yet.

In *M. avium* culture filtrate, 85B and MPT51 predominated but 85A and 85C existed in small amounts or scarcely under the conditions used in this study. A possibility still remains that secretion of these moieties could occur under different conditions. The ratio of 85B to MPT51 was 5/4 and the ratio of 85A or 85C to 85B was $<1/10$ if 85A and/or 85C existed in the culture filtrate. This phenomenon was not specific for this strain. We investigated the culture filtrate of two other strains, *M. avium* P55 (serotype 4) and *M. intracellulare* P42 (serotype 12). Similar observations were obtained in both cases (data not shown). Though we showed the result for the 4-week-old *M. avium* culture filtrate, these ratios did not vary throughout the culture period, as shown for *M. tuberculosis* (2) and BCG (30) (data not shown). The phenomenon of small amounts of 85A and 85C in *M. leprae* was demonstrated with whole-cell lysate and polyclonal antisera against each component of the BCG antigen 85 complex (35).

Although the antigen 85 complex was shown to be involved in mycolic acid metabolism (4), the physiological role of the MPB/MPT51 protein is not clear yet. But the amounts of the three components of the antigen 85 complex and the MPB/MPT51 protein suggested that they might play similar but not identical functions in mycobacterial cells, and the difference between the ratios of these compounds in *M. tuberculosis* or BCG and *M. avium* may cause the difference in the cell wall components of these organisms. If true, it might be anticipated that the differences in the structure of the cell wall and resistance against antimycobacterial agents may occur. Exposure to INH, which is believed to attack certain enzymes involved in the biosynthetic pathways of mycolic acids, induced the expression of two components of the antigen 85 complex (13). Their molecular weights suggested that these proteins corresponded to 85A and 85C. The antimycobacterial agent ethionamide, structurally related to INH and believed to affect the same pathway, induced a similar response, but other types of agents did not. This induction was not detected in INH-resistant strains of *M. tuberculosis*. These facts let us imagine that there may be a relationship between a low susceptibility to INH and a low production of 85A and 85C in *M. avium*. And there is the possibility that the immune response against *M. avium* and BCG is reflected by different secretion patterns for the antigen 85 complex and MPB/MPT51.

In *M. tuberculosis*, BCG, *M. leprae*, and *M. avium*, three components of the antigen 85 complex, A, B, and C, are encoded by three nonclustered genes, *fbpA*, *fbpB*, and *fbpC*, respectively. Recently, it was demonstrated that another extracellular protein, designated MPB/MPT51, was closely related to the antigen 85 complex by Western blotting development. In these organisms, the *mpb*/*mpt51* gene was indeed mapped at a downstream region of *fbpA* and it was suggested that the *mpb*/*mpt51* and the *fbpA* genes were localized within the same operon. Despite the head-to-tail association of these genes, large amounts of the product of *mpt51* were observed whereas only very small amounts of the product of *fbpA* were observed. This suggested that the *mpb*/*mpt51* and the *fbpA* genes were not cotranscribed as an operon. And a 17-bp palindrome found in the intergenic region might play a role as a transcriptional terminator and not as a transcriptional attenuator. Also, MPB/MPT51 might play an important role and be essential in mycobacterial cells. Involvement of MPB/MPT51 in mycolic acid metabolism is one possibility.

Our results clearly indicated that there are entire *fbpA*, *fbpB*, *fbpC*, and *mpt51* genes in *M. avium* and the constructions of these genes are very similar to those of the same genes in *M. tuberculosis* and BCG but the expression levels of these genes are very different between *M. avium* and both *M. tuberculosis* and BCG. To answer the question of why this difference occurred, we must investigate insights into the regulation mechanisms of these genes, and this investigation is now in progress.

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