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Homologs of *Mycobacterium leprae* 18-Kilodalton and *Mycobacterium tuberculosis* 19-Kilodalton Antigens in Other Mycobacteria

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Most of the antigens of *Mycobacterium leprae* and *M. tuberculosis* that have been identified are members of stress protein families, which are highly conserved throughout many diverse species. Of the *M. leprae* and *M. tuberculosis* antigens identified by monoclonal antibodies, all except the 18-kDa *M. leprae* antigen and the 19-kDa *M. tuberculosis* antigen are strongly cross-reactive between these two species and are coded within very similar genes. Studies of T cell reactivity against mycobacterial antigens have indicated that *M. tuberculosis* bears epitopes that are cross-reactive with the *M. leprae* 18-kDa antigen, but attempts to identify an 18-kDa antigen-like protein or protein coding sequence in *M. tuberculosis* have been unsuccessful. We have used a combination of low-stringency DNA hybridization and polymerase chain reaction techniques to identify, isolate, and sequence genes from *M. avium* and *M. intracellulare* that are very similar to the 18-kDa antigen gene of *M. leprae* and others that are homologs of the 19-kDa antigen gene of *M. tuberculosis*. Unlike *M. leprae*, which contains a single 18-kDa antigen gene, *M. avium* and *M. intracellulare* each have two 18-kDa antigen coding sequences. Although the *M. leprae*, *M. avium*, and *M. intracellulare* 18-kDa antigen genes are all very similar to one another, as are the *M. tuberculosis*, *M. avium*, and *M. intracellulare* 19-kDa antigen genes, we have been unable to detect any 18-kDa antigen-like coding sequences in DNA from *M. tuberculosis*.

Over the last 10 years, considerable effort has been directed towards identifying and characterizing antigens of mycobacteria. During this process, a small set of immunodominant antigens that appear to be highly conserved among the mycobacteria has emerged, and many of them represent mycobacterial heat shock or stress proteins (6, 32–34). For example, the 70-, 65-, 28-, and 14-kDa protein antigens of mycobacteria are members, respectively, of the Hsp70-DnaK, Hsp65-GroEL, superoxide dismutase, and GroES eukaryotic and prokaryotic stress protein families, with which they share between 30 and 60% amino acid sequence identity (4, 12, 26–28). The 18-kDa antigen of *Mycobacterium leprae* also appears to be a member of the low-molecular-weight heat shock protein family and bears 30% sequence identity to the soybean Hsp18 family of molecules (22). However, unlike the other mycobacterial antigens, which are highly cross-reactive with other mycobacteria, the 18-kDa antigen has not been detected in any other species except *M. leprae* and *M. habana* (16). The 18-kDa antigen-specific monoclonal antibody L5 cross-reacts with an 18-kDa molecule from cultivable *M. habana*, and when *M. habana* was subjected to heat shock, expression of the protein was significantly increased (16). *M. habana* is now considered to be *M. simiae* serovar 1, and a recent DNA hybridization analysis of mycobacterial DNA with an *M. leprae* 18-kDa antigen gene-derived probe has revealed that, as well as *M. simiae*, *M. intracellulare*, *M. kansasii*, *M. terrae*, *M. avium*, *M. scrofulaceum*, *M. gordonae*, and *M. chelonei* appear to possess homologous sequences (20).

Although most of the immunodominant mycobacterial antigens were originally identified by use of monoclonal antibodies directed against mycobacterial extracts, some contain epitopes that are antigenic for T cells and that may be involved in the development of immunity to mycobacterial infection (9, 10, 14, 19, 21, 23, 30). We have been particularly interested in the *M. leprae* 18-kDa antigen because of its apparent absence from *M. tuberculosis* and because it appears to be a good stimulator of $CD4^+$ T cell responses. For example, nearly half of the *M. leprae*-specific T cell clones from volunteers immunized with killed *M. leprae* were stimulated to proliferate by lysates containing an epitope of the 18-kDa antigen (21). Furthermore, cultured T cells from control subjects (putative noncontacts) responded poorly to antigens from sonicates of *M. leprae* that had been separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, blotted onto nitrocellulose, and then added to cultures as antigen-bearing nitrocellulose particles. In contrast, T cells from leprosy contacts responded variably to a wide range of antigens and most frequently (23%) to the 18-kDa fraction (11).

Given the high degree of homology and cross-reactivity among the other mycobacterial antigens, the question arises as to whether T cells specific for the *M. leprae* 18-kDa antigen also cross-react with other mycobacteria. Results obtained by four groups of investigators indicated that they do. Lymph node cells from mice immunized with purified recombinant *M. leprae* 18-kDa antigen responded in culture

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to challenge with either *M. leprae* or *M. tuberculosis* (14). Protection of mice against infection with *M. leprae* could be achieved by immunizing them with *M. habana* (16). Fifty percent of untreated tuberculoid leprosy patients and 93% of long-term leprosy contacts responded to a recombinant 18-kDa fusion protein. Moreover, 70% of *M. bovis* BCG-vaccinated European donors also responded, and 18-kDa antigen-responsive T cell lines could be isolated from the blood lymphocytes of a BCG-vaccinated donor (9). Forty seven percent of patients with tuberculosis in Nepal had serum antibodies against *M. leprae* 18-kDa protein (25).

Collectively, these results strongly suggest either that an 18-kDa counterpart exists in *M. tuberculosis* or that epitopes found on the *M. leprae* 18-kDa antigen cross-react with similar epitopes on unrelated *M. tuberculosis* antigens. In this paper, we report on endeavors to detect 18-kDa antigen homologs in *M. tuberculosis* and other mycobacteria.

MATERIALS AND METHODS

Mycobacterial isolates and purification of mycobacterial DNA. *M. intracellulare* serotypes 18 and 19 and *M. avium* serotype 2 bacteria were grown and DNAs were prepared from them as previously described (20).

Antibodies. Monoclonal antibody L5, which recognizes a determinant on the *M. leprae* 18-kDa protein (10), was obtained from A. Basten, Sydney, New South Wales, Australia. Polyclonal antibodies raised in rabbits against purified recombinant (*Escherichia coli*-derived) 18-kDa protein and in mice against synthetic 20-mer peptides derived from the 18-kDa protein sequence were previously described (10).

DNA manipulation, sequencing, and gene library construction. Mycobacterial DNA was digested with restriction endonucleases, fractionated by agarose gel electrophoresis, ligated into plasmid or bacteriophage vectors, and transformed into *E. coli* (strain DH5α for plasmids and strain JM101 for M13 bacteriophage) by standard molecular biological techniques (2). Restriction endonucleases, DNA ligase, and DNA polymerase were obtained from New England Biolabs, Beverly, Mass. DNA fragments for subcloning were isolated from agarose gels by use of Geneclean (Bio 101, Inc., La Jolla, Calif.). For Southern blot analysis, DNA fragments were transferred from agarose gels to GeneScreen Plus (New England Nuclear, Wilmington, Del.) or Immobilon-P (Millipore Corp., Bedford, Mass.) nylon membranes by standard alkaline capillary transfer methods (24). Gene libraries were constructed from *M. avium* serovar 2 and *M. intracellulare* serovar 19 (Darden) DNAs as follows. Purified DNA (2 μg) was digested with 20 U of either *Eco*RI or *Pst*I for 2 h at 37°C and then purified by phenol-chloroform extraction and ethanol precipitation. DNA digests were ligated into *Eco*RI-cut or *Pst*I-cut pUC19 plasmid vectors and transformed into *E. coli* DH5α. Colonies were replicated on Hybond-C Extra nitrocellulose membranes (Amersham, Buckinghamshire, England) and lysed, and DNA was denatured, air dried, and baked at 80°C for 2 h in vacuo. After being washed, the filters were probed at a low stringency with a ^{32}P-labelled *M. leprae* 18-kDa antigen gene probe, washed again, and autoradiographed (see below).

DNA fragments for sequencing were subcloned into M13mp series, M13BM20, or M13BM21 vectors (Boehringer GmbH, Mannheim, Germany), and single-stranded recombinant DNA was prepared and sequenced by use of an Applied Biosystems model 373A automated sequencer. Sequences of DNA fragments were compared, assembled into complete coding sequences, and translated by use of the University of Wisconsin Genetics Computer Group sequence analysis software package (7). For all deduced amino acid sequences described in this paper, both strands of the DNA were completely sequenced.

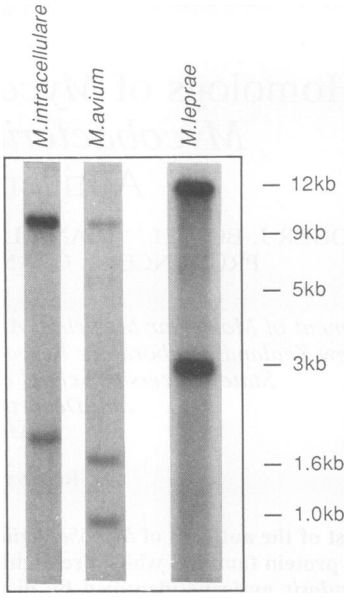

FIG. 1. Detection of 18-kDa antigen gene-like sequences in *M. avium* and *M. intracellulare*. *M. leprae*, *M. avium* serovar 2, and *M. intracellulare* serovar 19 (Darden) DNAs (2 μg) were digested with *Eco*RI, fractionated on a 1% agarose gel, Southern blotted onto a nylon membrane, probed with a ^{32}P-labelled *M. leprae* 18-kDa antigen gene probe at a low stringency, and autoradiographed.

Probe preparation and hybridization. A 624-bp *Asp*718-*Bam*HI DNA fragment containing the complete *M. leprae* 18-kDa antigen coding sequence (GenBank accession number M19058) was isolated from plasmid PML3 (5), and a 610-bp *Eco*RI fragment containing the complete *M. tuberculosis* 19-kDa antigen coding sequence (GenBank accession number X07945) was isolated from plasmid pTB147APT (1). Following purification from agarose gels by the Geneclean method, these fragments were labelled with [^{32}P]dCTP (Amersham) by use of a random priming DNA labelling system (GIBCO BRL Life Technologies, Gaithersburg, Md.). Southern blot or colony hybridization was performed under low-stringency conditions with this probe as follows. Membranes were hybridized at 60°C for 48 h in hybridization solution (1 M NaCl, 10% dextran sulfate, 1% SDS, 100 μg of denatured salmon sperm DNA per ml) containing 15×10^6 cpm of the ^{32}P-labelled probe per ml, washed for 1 h at 60°C in 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) with 0.1% SDS, and then autoradiographed overnight at −70°C with X-Omat XAR-5 film (Eastman Kodak Co., Rochester, N.Y.) and two Cronex Lightning-Plus intensifying screens (Dupont, Wilmington, Del.).

PCR analysis. For amplification of 19-kDa antigen gene fragments from mycobacteria (see Fig. 5), 250 pg of mycobacterial DNA in 39 μl of H_2O was boiled for 5 min, rapidly cooled on ice, and then mixed with 5 μl of 10× polymerase chain reaction (PCR) buffer (15)–1 μl of deoxynucleotide triphosphate (dNTP) mixture (15 mM each dNTP)–2.5 μl of each 25-mer primer (20 pmol/μl)–1 μl of *Taq* polymerase (2 U/μl; Amersham) in a 0.5-ml microcentrifuge tube. Mineral

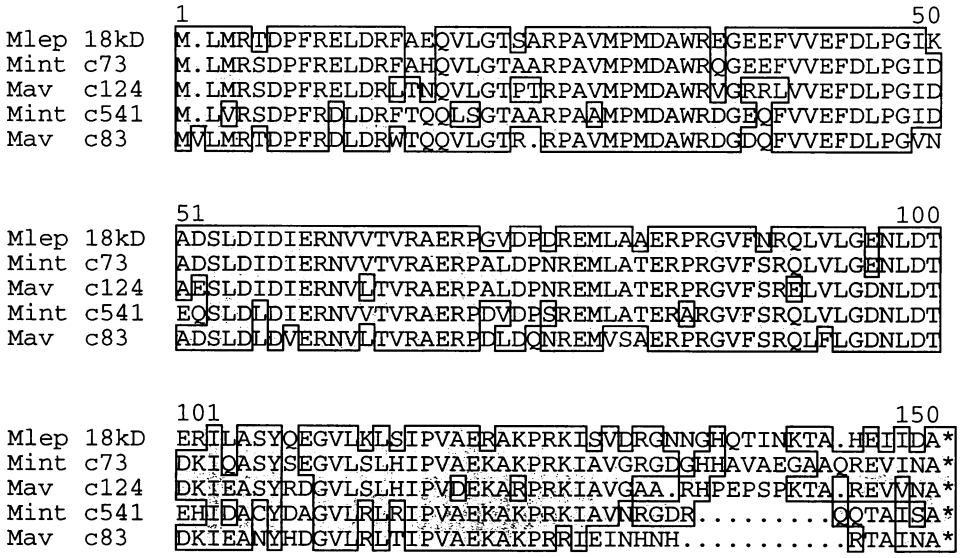

FIG. 2. Comparison of 18-kDa proteins from *M. leprae*, *M. avium*, and *M. intracellulare*. Shown are deduced amino acid sequences of genes coding for 18-kDa protein-like proteins isolated from *M. avium* and *M. intracellulare* plasmid libraries with an *M. leprae* 18-kDa antigen gene probe. Predicted molecular weights for the proteins are as follows: *M. leprae* (Mlep 18kD), 16,607; MintC73, 16,508; MavC124, 16,543; MintC541, 15,748; and MavC83, 15,948. With respect to the *M. leprae* 18-kDa protein sequence, the *M. avium* and *M. intracellulare* sequences display the following percentages of amino acid similarity (identity): MintC73, 88 (79); MavC124, 84 (69); MintC541, 85 (71); and MavC83, 88 (69).

oil (100 μl) was added to each reaction mixture, which was then subjected to 40 cycles of 94°C for 2 min, 37°C for 2 min, and 72°C for 3 min in a Perkin-Elmer Cetus DNA thermal cycler.

Attempts to amplify 18-kDa antigen gene-like fragments from *M. tuberculosis* DNA by use of degenerate primers (see Fig. 7) were carried out as follows. Purified DNA (200 ng) in 82.2 μl of H_2O was mixed with 10 μl of 10× PCR buffer (15)–1.3 μl of dNTP mixture–3 μl of each degenerate primer (15 pmol/μl). Mineral oil (100 μl) was added, the mixture was heated to 94°C for 6 min, and then 0.5 μl of *Taq* polymerase (5 U/μl) was added. Samples were cycled 30 times through a sequence of 94°C for 1 min, 40°C for 1.5 min, and 72°C for 1.5 min and then subjected to a final 5 min of extension at 72°C.

RESULTS

Detection of 18-kDa antigen-related sequences in other mycobacteria. In view of the T cell-specific cross-reactivity between the *M. leprae* 18-kDa protein and antigenic determinants from *M. tuberculosis*, we initially attempted to discover 18-kDa protein-like proteins and protein coding sequences in preparations from *M. tuberculosis*. We were unable to detect antibody-binding material in Western blots (immunoblots) of electrophoretically fractionated *M. tuberculosis* proteins by using the 18-kDa antigen-specific monoclonal antibody L5 or polyclonal antibodies raised against purified recombinant 18-kDa protein or against synthetic peptides derived from the 18-kDa protein amino acid sequence (data not shown). DNA hybridization with a radioactively labelled probe derived from the *M. leprae* 18-kDa antigen gene sequence did not reveal any similar sequences in preparations of *M. tuberculosis* DNA, at either a low or a high hybridization stringency (31). However, at a low stringency, the 18-kDa antigen gene probe hybridized to DNA sequences from *M. avium* and *M. intracellulare* (Fig. 1), a result confirming other recent results (20).

Isolation and characterization of duplicated 18-kDa antigen genes in *M. avium* and *M. intracellulare*. Plasmid libraries were constructed from *Eco*RI- and *Pst*I-digested DNAs from *M. avium* and *M. intracellulare* and screened by low-stringency hybridization with a radioactively labelled *M. leprae* 18-kDa antigen gene probe. DNA fragments that

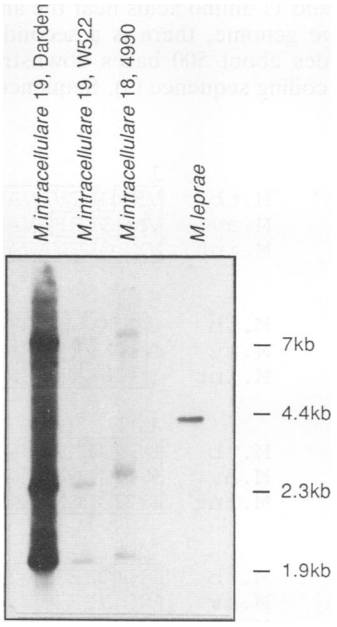

FIG. 3. Comparison of three *M. intracellulare* isolates by Southern blot analysis. DNAs from three separate *M. intracellulare* isolates were digested with *Pst*I, fractionated by electrophoresis on a 1% agarose gel, Southern blotted, and probed at a low stringency with the *M. leprae* 18-kDa antigen gene probe.

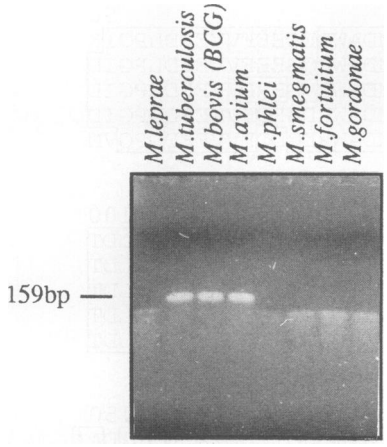

FIG. 4. Detection of 19-kDa antigen gene sequences in mycobacterial DNAs by the PCR. DNAs from various mycobacteria were subjected to PCR amplification as described in Materials and Methods by use of oligonucleotide primers derived from the *M. tuberculosis* 19-kDa antigen gene sequence.

hybridized were subcloned into M13 vectors and sequenced, and the sequences were compared with the *M. leprae* probe sequence. Two 18-kDa antigen gene-like sequences each were isolated from the *M. avium* and *M. intracellulare* genomes. As shown in Fig. 2, when these were translated into amino acid sequences, they were found to be very similar to the *M. leprae* 18-kDa protein sequence, showing between 70 and 80% amino acid identity and 84 and 88% amino acid similarity. One of the 18-kDa protein-like sequences from *M. avium* and one from *M. intracellulare* (MintC73 and MavC124, respectively) were almost the same length as the *M. leprae* 18-kDa protein sequence, while the other two (MintC541 and MavC83) were truncated, lacking, respectively, 9 and 11 amino acids near the amino terminus. In the *M. leprae* genome, there is a second open reading frame that resides about 500 bases downstream from the 18-kDa protein coding sequence (5). Sequences very similar to this open reading frame were present about 500 bases downstream from the "full-length" *M. avium* and *M. intracellulare* 18-kDa protein coding sequence homologs (MintC73 and MavC124) but not downstream from the truncated ones (MintC541 and MavC83) (data not shown).

Duplicate 18-kDa antigen genes are unlikely to be due to intraspecies polymorphisms. Although the DNA preparations used for library construction were from single isolates of *M. avium* and *M. intracellulare*, they were not clonally derived, so it was possible that the duplicate gene sequences were a reflection of polymorphisms within a heterogeneous population of mycobacteria rather than an indication of truly multiple gene copies within each species. For testing this possibility, DNA preparations from three different isolates of *M. intracellulare* were digested with *Pst*I, fractionated by electrophoresis, blotted, and probed with the *M. leprae* 18-kDa antigen gene probe at a low stringency. As shown in Fig. 3, the same three bands, at 1.9, 2.3, and 7kb, were present in all three DNA preparations, a result giving no indication of intraspecies polymorphisms but rather supporting the view that duplicate 18-kDa antigen genes exist within the genome of *M. intracellulare*.

Relationship between *M. tuberculosis* and *M. avium* and *M. intracellulare*. Ashbridge et al. (1) have cloned and sequenced the gene for the 19-kDa antigen from *M. tuberculosis* and shown that, although this antigen is highly antigenic and of a similar size, it is not related to the *M. leprae* 18-kDa antigen. Using oligonucleotide primers derived from the *M. tuberculosis* 19-kDa antigen coding region, we used PCR to test for the presence of similar genes in other mycobacterial DNA preparations. As shown in Fig. 4, 19-kDa antigen gene-like genes were apparent in DNAs from *M. bovis* (subsequently found to be identical to the *M. tuberculosis* 19-kDa antigen gene) and *M. avium* but not from the other mycobacteria tested, including *M. leprae*. Using the radioactively labelled *M. tuberculosis* 19-kDa antigen gene as a probe, we isolated hybridizing clones from the *M. avium* and *M. intracellulare* plasmid libraries described above. As shown in Fig. 5, single 19-kDa antigen gene-like genes were found in both the *M. avium* and *M. intracellulare* genomes, and their deduced amino acid sequences displayed a striking

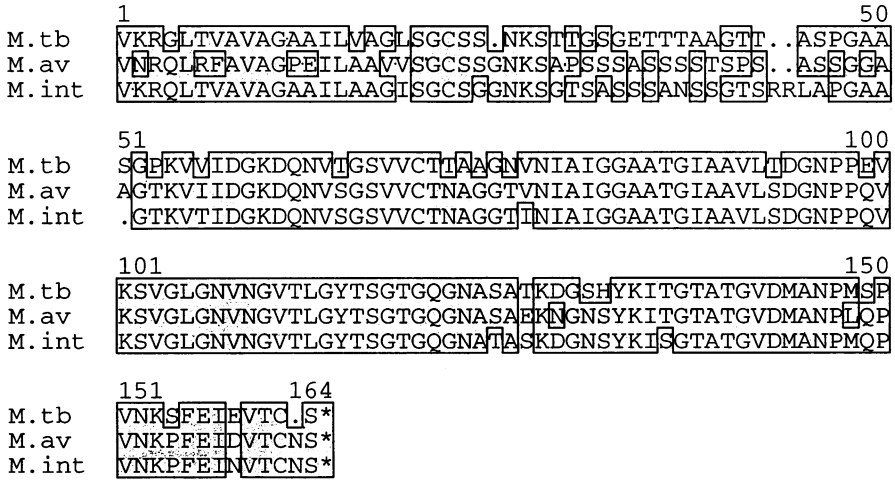

FIG. 5. Comparison of 19-kDa proteins from *M. tuberculosis* (M.tb), *M. avium* (M.av), and *M. intracellulare* (M.int). Shown are deduced amino acid sequences of genes coding for 19-kDa protein-like proteins isolated from *M. avium* and *M. intracellulare* plasmid libraries with an *M. tuberculosis* 19-kDa antigen gene probe. Predicted molecular weights for the proteins are as follows: *M. tuberculosis*, 15,114; *M. avium*, 15,472; and *M. intracellulare*, 15,471. Percentages of amino acid similarity (identity) are shown in Fig. 6.

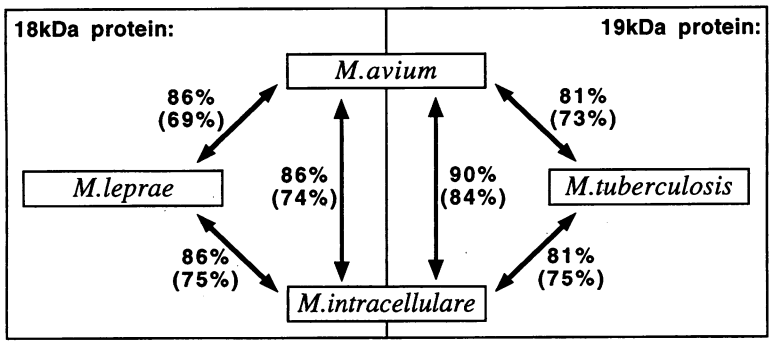

FIG. 6. Relatedness among the 18-kDa and 19-kDa proteins of mycobacteria. Percentages of amino acid similarity (identity) among the *M. leprae*, *M. avium*, *M. intracellulare*, and *M. tuberculosis* 18-kDa and 19-kDa protein genes are given.

degree of identity with their *M. tuberculosis*-derived counterpart (Fig. 6).

Attempts to identify an 18-kDa antigen gene-like gene in *M. tuberculosis* DNA. Because of the high degree of similarity among the *M. leprae*, *M. avium*, and *M. intracellulare* 18-kDa antigen genes and among the *M. tuberculosis*, *M. avium*, and *M. intracellulare* 19-kDa antigen genes (Fig. 6), we attempted to identify an 18-kDa antigen gene-like sequence in the *M. tuberculosis* genome by two methods. First, we prepared radioactively labelled probes containing the *M. leprae* 18-kDa antigen gene or a mixture of the five 18-kDa antigen gene sequences (one from *M. leprae*, two from *M. avium*, and two from *M. intracellulare*) and used them to probe at a low stringency Southern blots and plasmid libraries made from *M. tuberculosis* DNA. Using this approach, we were unable to detect any *M. tuberculosis* 18-kDa antigen gene-like sequences. The second approach was to synthesize degenerate oligonucleotide primers based on highly conserved regions of the *M. leprae*, *M. avium*, and *M. intracellulare* 18-kDa antigen gene sequences (Fig. 7) and to attempt to amplify 18-kDa antigen gene-like bands from *M. tuberculosis* DNA by PCR. This method was also unsuccessful.

DISCUSSION

Although comparative DNA hybridization studies of genomic DNA indicate that *M. leprae* is only remotely genetically related to other species of mycobacteria (13), many of the immunodominant antigens of *M. leprae* are very similar to those of other mycobacteria and to families of highly conserved stress proteins. One exception was the 18-kDa antigen which, until the discovery of cross-reactivity with *M. habana* (*M. simiae* serovar 1) and more recently of low-stringency hybridization homologies with various other mycobacteria (20), was thought to be antigenically unique to *M. leprae*. When *M. habana* was subjected to heat shock but not other forms of environmental stress, expression of the protein was significantly increased, a result suggesting that, like the other immunodominant mycobacterial antigens, the 18-kDa antigen is also a stress protein in mycobacteria. Moreover, protection of mice against infection with *M. leprae* could be achieved by immunizing them with *M. habana* (16).

The observations that T cells primed with the *M. leprae* 18-kDa antigen respond to challenge with *M. tuberculosis* bacteria (9, 14) and that *M. tuberculosis*-immunized T cells respond to *M. leprae* 18-kDa antigen determinants (9, 25) suggest that *M. tuberculosis* has either an 18-kDa antigen homolog or cross-reactive epitopes on unrelated molecules. Oligonucleotide primers derived from the 18-kDa antigen gene sequence have been used in PCR assays specifically to detect the presence of *M. leprae* DNA from as few as 100 mycobacteria in infected tissue (31). In view of the potential use of 18-kDa antigen gene sequences as tools for the early detection of *M. leprae* infection and the significance of the 18-kDa antigen as a T cell immunogen, it is important to discover whether this molecule is present in other mycobacteria and if so, how it is distributed. We have found that *M. avium* and *M. intracellulare* each contain two 18-kDa antigen genes that are very similar to the 18-kDa antigen gene of *M. leprae*. Eukaryotes commonly contain multiple copies of genes encoding heat shock proteins (18), and recently some mycobacterial heat shock proteins and other antigens were found to have duplicate (8) or multiple (29) copies. Given that *M. avium* and *M. intracellulare* are genetically only 5 to

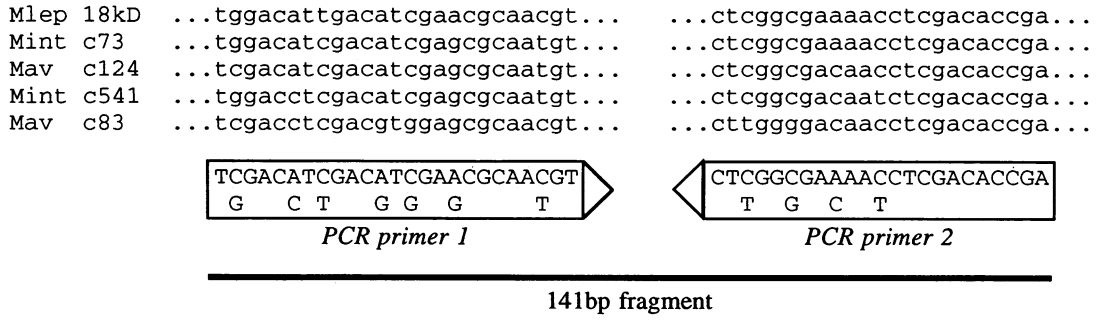

FIG. 7. Degenerate primers used in attempts to detect an 18-kDa antigen gene-like gene in *M. tuberculosis* DNA.

10% similar to *M. leprae*, as determined by DNA hybridization analysis (13), but are about 25% similar to *M. tuberculosis* (3) and that the 19-kDa antigens of *M. avium*, *M. intracellulare*, and *M. tuberculosis* are all very closely related (Fig. 6), it is surprising that we have been unable as yet to detect an 18-kDa antigen homolog in *M. tuberculosis* DNA. Other strongly immunogeneic antigens of mycobacteria are also highly conserved in *M. leprae* and *M. tuberculosis* (27, 28, 32). Cellular immunology studies from a number of laboratories (discussed above) have indicated antigenic cross-reactivity between *M. tuberculosis* and the 18-kDa protein of *M. leprae*. However, until an 18-kDa antigen homolog is discovered in *M. tuberculosis*, this phenomenon may be the result of a fortuitously similar epitope on an unrelated antigen from *M. tuberculosis*. Finally, if *M. tuberculosis* does not have an 18-kDa antigen homolog, then it is pertinent to ask what protein(s) in this organism carries out the function of such a conserved, widely distributed molecule and whether that protein(s) is also immunologically important. Recently, a 19-kDa protein purified from enriched membrane fractions of *M. tuberculosis* was sequenced and found to show a low degree of homology to the *M. leprae* 18-kDa antigen (27% identity in a 79-amino-acid overlap containing a highly conserved stretch of 11 residues) (17). It will be interesting to determine whether this protein cross-reacts antigenically with the *M. leprae* 18-kDa antigen.

ACKNOWLEDGMENTS

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