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Identification of *Mycobacterium leprae* Antigens from a Cosmid Library: Characterization of a 15-Kilodalton Antigen That Is Recognized by Both the Humoral and Cellular Immune Systems in Leprosy Patients

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Screening of the *Mycobacterium leprae* cosmid library with pooled sera from lepromatous leprosy (LL) patients by a colony immunoblot technique resulted in the identification of about 100 colonies that produced immunologically reactive proteins. Twenty-four of these clones were purified, analyzed, and found to comprise two groups according to the reactivity of the recombinant proteins with LL sera and to the DNA restriction patterns of the recombinant plasmids and cosmids. Proteins specified by clones from group I reacted strongly with LL patients' sera on a Western blot (immunoblot), demonstrating a 15-kDa protein band designated A15. The A15 antigen also reacted with pooled sera from patients with tuberculoid leprosy from the United States and Brazil. Clones from group II did not show any reactive protein band on a Western blot, when reacted with patients' sera. DNAs from cosmids of group II all contain a 10-kb *Pst*I fragment that hybridized to the unique repetitive *M. leprae* DNA. Sequence analysis of a 1.2-kb fragment containing the entire coding sequence of A15 revealed three open reading frames (ORFs), only one of which (ORF II) contains sufficient genetic information to encode for A15. Part of the A15 gene was found to exist also in a group of λgt11::*M. leprae* clones previously isolated in our laboratory by immunological screening with LL patients' sera. One of the λgt11 clones (L8) expresses a β-galactosidase fusion protein with 89 amino acids from the C terminus of A15. An important result was that the fusion protein was clearly recognized by T cells from leprosy patients. Interestingly, *Mycobacterium tuberculosis*-stimulated T cells from *M. leprae* nonresponder (LL as well as borderline tuberculoid) patients were able to respond to the isolated recombinant *M. leprae* antigen, indicating that nonresponsiveness to *M. leprae* antigens can be reversible. The sequence of the *M. leprae* DNA fused to the β-galactosidase gene of λgt11 clone L8 was identical to that of a λgt11::*M. leprae* clone isolated recently that expresses an immunologically reactive fusion protein (S. Laal, Y. D. Sharma, H. K. Prasad, A. Murtaza, S. Singh, S. Tangri, R. Misra, and I. Nath, Proc. Natl. Acad. Sci. USA 88:1054–1058, 1991). Besides the complete sequence of the A15 gene, sequencing data of two flanking ORFs are presented. Downstream from ORF II (A15), ORF III has a high degree of similarity to the genes for tomato ATP-dependent proteases that are members of a larger class of highly conserved proteases ubiquitous among prokaryotes and eukaryotes. ORF I, located upstream from ORF II, shares a high degree of similarity to the carboxy terminus of the *Escherichia coli lysU* gene, which codes for the stress- and heat-shock-inducible lysyl-tRNA synthetase. Sequences homologous to the A15 gene were detected in chromosomal DNA from *Mycobacterium avium*, *Mycobacterium bovis* BCG, and *M. tuberculosis*.

Studies of *Mycobacterium leprae* antigens are important for understanding the host immune response against the bacterium as well as for elucidating possible virulence factors. Screening of the λgt11::*M. leprae* expression library with monoclonal antibodies (MAbs) raised against the bacterium resulted in the identification of a relatively limited number of reactive clones (29), most of which belong to the conserved family of the heat shock proteins (8, 14, 26, 28). Concerned that the MAbs did not detect the entire complement of *M. leprae* antigens, Sathish et al. screened the λgt11::*M. leprae* library with pooled sera from lepromatous leprosy (LL) and tuberculoid leprosy (TT) patients (22). Using extensively adsorbed sera, Sathish et al. identified 19 antigenic determinants that reacted with antibodies in LL patients' sera and 5 that reacted with antibodies in TT patients' sera (22). Two other groups have also screened the λgt11::*M. leprae* library with LL patients' sera, but each of those groups was able to identify only a single antigen (3, 10).

Among the clones identified by Sathish et al., three specified nonfusion proteins that were expressed either from their own promoters or from the *lac* promoter of the vector but that used their own translational signals (22). The 65-kDa *M. leprae* protein and some other mycobacterial antigens (14, 27) were also reported to be expressed possibly from their own transcription-translation signals. The relatively low (for mycobacteria) G+C content (56%) of *M. leprae* (6) might favor expression of *M. leprae* genes in *Escherichia coli*, which has a similar G+C content. We therefore decided to screen an *M. leprae* cosmid library with pooled LL patients' sera in order to identify the mycobacterial antigens that are capable of being expressed from their own transcription-translation signals. Besides identifying new, potentially immunologically important *M. leprae* antigens, such clones

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TABLE 1. Description of MAbs used in this study

Pool	Size (kDa) of *M. leprae* protein(s) recognized	No. of MAbs
I	12	3
	16	2
	18	5
	26	3
	32–34	1
	34	1
	35	1
	36	1
	Low molecular size[a]	4
II	55–65	1
	65	8
	High molecular size[a]	4

[a] The low and high molecular sizes have not been characterized (22).

might be further analyzed to study regulation of this subclass of antigen-coding genes.

Since each cosmid clone contains about 45 kb of mycobacterial DNA, it is theoretically possible to screen all of the *M. leprae* genome by using approximately 1,000 clones, assuming that all *M. leprae* sequences are equally represented in the cosmid library. However, only those sequences that lie in phase with the *bla* promoter of the vector (pHC79) and the sequences whose promoters are recognized by the *E. coli* transcription-translation machinery will be expressed.

We report here the isolation and characterization of two groups of cosmid clones that specify proteins reactive with LL patients' sera and DNA sequence analysis of a gene from one group that encodes a 15-kDa protein antigen (A15) that stimulates proliferation of T cells from leprosy patients.

MATERIALS AND METHODS

Immunological screening of recombinant libraries. *E. coli* strain LE392 (15) was infected with the pHC79::*M. leprae* cosmid library (ALX-1) as described by Clark-Curtiss et al. (6). Bacterial transductants containing recombinant cosmids were plated on L agar plates (11) supplemented with 10 μg of tetracycline per ml to a density of 800 colonies per plate. DNA from the pYA626::*M. leprae* plasmid library, ALX-3 (6), was used to transform *E. coli* LE392. Recombinant clones were selected in the same way as the cosmid clones.

The colonies were screened by a colony immunoblot assay. After the colonies were transferred to nitrocellulose membranes (Schleicher & Schuell Inc., Keene, N.H.), they were lysed by chloroform vapors for 30 min at 37°C. The proteins were blocked and reacted with LL patients' sera at a dilution of 1:1,000, as described previously (22); the sera were kindly provided by R. H. Gelber (The Kuzell Institute, San Francisco, Calif.), T. P. Gillis (Hansen's Disease Research Center, Carville, La.), and T. H. Rea (University of Southern California Medical Center, Los Angeles, Calif.).

Immunological characterization of recombinant proteins. Three pools of sera from leprosy patients were used in this study. The LL and TT sera originating from patients in the United States were described before (22), and a third pool of TT sera from Brazilian patients was kindly provided by Elizeu Carvalho (Universidade do Estado do Rio de Janeiro, Brazil). This serum pool was extensively adsorbed as described previously (22). The two TT sera pools were used at a dilution of 1:500. The MAbs that were used (diluted 1:1,000) are listed in Table 1. The MAbs described before (22) were obtained from the World Health Organization Immunology of Leprosy clone bank or were generous gifts from A. Basten (University of Sydney, New South Wales, Australia) and A. H. J. Kolk (The Royal Tropical Institute, Amsterdam, The Netherlands). Immunoreactive proteins were detected by using anti-human or anti-mouse immunoglobulins conjugated to alkaline phosphatase, as recommended by the manufacturer (Sigma Chemical Co., St. Louis, Mo.).

Protein extracts from clones identified by colony immunoblot were separated by electrophoresis on sodium dodecyl sulfate (SDS)-polyacrylamide gels (7.5, 10, 12, and 15%) under reducing conditions. Proteins were electroblotted onto nitrocellulose membranes (pore size, 0.2 μm) and reacted with LL patients' sera as described above. For enzyme-linked immunosorbent assay (ELISA), selected cosmid clones were grown overnight at 37°C. One milliliter of cells (5×10^8/ml), killed with phosphate-buffered saline (PBS) containing 0.6% Formalin, was washed three times with PBS and resuspended in the initial volume in bicarbonate buffer (pH 9.6). Aliquots of 100 μl were applied in duplicate to 96-well ELISA plates. The cells were adsorbed to the plates overnight at 4°C, and unattached cells were washed away with PBS. Plates were blocked with 1% bovine serum albumin (BSA) for 1 h; the cells were reacted with the individual MAbs (listed in Table 1) for 2 h at room temperature (RT), washed twice with PBS containing 0.1% BSA, and reacted with sheep anti-mouse immunoglobulin–alkaline phosphatase conjugate (Sigma) for 1 h at RT. After the secondary antibodies were washed off, diethanolamine (pH 9.8) containing 1 mg of *p*-nitrophenyl phosphate per ml (Sigma) was added. One and a half hours later, the plates were read at 405 nm on an automated microplate reader (Bio-Tek Instruments, Burlington, Vt.).

T-cell proliferation experiments. T-cell lines were raised by stimulating 10^6 peripheral blood mononuclear cells per ml from in vivo-primed leprosy patients with 1 μg of a *Mycobacterium tuberculosis* sonicate, provided by P. Klatser (Royal Tropical Institute, Amsterdam, The Netherlands). Five days after the addition of antigen, 20% T-cell growth factor (Biotest, Frankfurt, Germany) was added. T cells were frozen at day 11 or 12 as described previously (18). T cells (10^4) and 5×10^4 irradiated (20 Gy) autologous or human leukocyte antigen-DR-matched peripheral blood mononuclear cells as antigen-presenting cells were cultured in Iscove's modified Dulbecco's medium (IMDM) containing 10% pooled human sera and an optimal antigen concentration in 96-well flat-bottomed microtiter plates (Greiner, Frickenhausen, Germany); phytohemagglutinin (2 μg/ml; Wellcome Diagnostics, Dartford, United Kingdom) and plain IMDM were used as controls. The cultures were set up in triplicate and incubated at 37°C in a fully humidified atmosphere containing 5% CO_2 for 72 h. Eighteen hours before termination, 1 μCi of [^{3}H]thymidine (specific activity, 5.0 μCi/mmol; Radiochemical Centre, Amersham, United Kingdom) was added. The samples were harvested on glass fiber filters with a semiautomatic sample harvester. [^{3}H]thymidine incorporation was assessed by counting with a liquid scintillation counter.

DNA manipulations. Standard DNA manipulation techniques were done according to Maniatis et al. (13). The 1.2-kb *Pst*I fragment common to group I clones (see Results) was subcloned in pBluescript/SK$^-$ (Stratagene, La Jolla, Calif.) in both orientations. Both plasmids were used to create nested deletions by using the Erase-A-Base kit

(Promega Biotec, Madison, Wis.). The *M. leprae* DNA inserts from *M. leprae*::λgt11 clones were amplified by polymerase chain reaction, digested with *Eco*RI, and subcloned into the *Eco*RI site of the expression vector, pEX2 (25). *E. coli* POP2136 (25) was used as a host for pEX2 and derivatives. Amplification was as follows. One isolated plaque was picked with a toothpick, resuspended in 5 μl of distilled water, and boiled for 5 min. The phage suspension (1 μl) was added to 49 μl of a solution of 50 mM KCl, 2 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 2 mg of BSA per ml, 0.25 mM deoxynucleoside triphosphate (dNTP), and 20 pmol of primers (GTAATGGTAGCGACCGGCGC and GGCGACG ACTCCTGGAGCCCG). *Taq* polymerase (0.5 U; Perkin-Elmer Cetus, Norwalk, Conn.) was added, and the reaction was performed in a polymerase chain reaction processor (Perkin-Elmer Cetus) using 35 cycles (90 s at 95°C, 2 min at 60°C, and 3 min at 72°C).

For Southern blot DNA hybridizations, probes were labeled with digoxigenin-dUTP, hybridized at 65°C, and detected by using the nonradioactive Genius kit (Boehringer Mannheim, Indianapolis, Ind.), according to the protocol provided (1), or the probes were labeled with [^{32}P]dCTP and the hybridizations were conducted as described previously (6). Blots were washed with 2× SSC (1× SSC = 0.15 M NaCl plus 0.015 sodium citrate [pH 8.0]) for 30 min at RT and for 30 min at 65°C when the nonradioactively labeled probe was used. With the ^{32}P-labeled probe, hybridizations were carried out at 65°C, and the blots were washed at RT with 2× SSC, at 65°C with 2× SSC–1% SDS, and at RT with 0.1× SSC (6).

Production of recombinant fusion proteins. Expression of *cro-lacZ* hybrid genes from pEX2 derivatives (pIHB1001 and pIHB1003) was induced as described previously (30). Induced cells were suspended in 100 mM Tris-HCl (pH 8.0) containing 10 mM EDTA to an optical density of 2.0 at 600 nm, freeze-thawed once, and sonicated on ice (three bursts of 30 s each with a 1-min rest) with a Branson sonifier operating at 80 W to give complete lysis. For T-cell proliferation assays, lysates containing the fusion proteins were centrifuged for 10 min at 12,000 × *g*, and the insoluble pellet, enriched for the fused proteins, was resuspended in IMDM and used as antigen.

Nucleotide sequencing and data analysis. Nucleotide sequencing was performed on double-stranded DNA templates by the dideoxy-chain termination method (21) with either the Sequenase version 2.0 kit (United States Biochemical Corporation, Cleveland, Ohio) or the T7 sequencing kit (Pharmacia LKB, Uppsala, Sweden). For sequencing of pEX2 derivatives, a synthetic oligonucleotide primer that is complementary to the DNA sequence 12 to 26 nucleotides upstream of the *Eco*RI site of pEX2 (25) was used. Computer analysis of the DNA sequences was performed with the Genetic Computer Group Sequence Analysis software package (7). GenBank data bank was searched with FASTA AND TFASTA programs (20).

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper have been submitted to GenBank (accession no. M67510).

RESULTS

Immunological characterization of reactive proteins. Initially, screening of the pYA626::*M. leprae* plasmid library and the pHC79::*M. leprae* cosmid library with pooled LL patients' sera resulted in the identification of about 100 reactive cosmid clones and two reactive plasmid clones (Fig. 1). Cell extracts from 22 isolated cosmids and the two plasmid clones were used to detect the corresponding reactive proteins by SDS-polyacrylamide gel electrophoresis and Western blotting with the same pooled patients' sera. Surprisingly, specific protein bands were detected in only a portion of the clones, which are designated as group I. Some of them are presented in Fig. 2 (lanes 3, 4, and 5). All reactive bands appeared to be about 15 kDa in size. One of the clones derived from the plasmid library (pYA1114) also specified an immunologically reactive protein with a similar molecular size (lane 1).

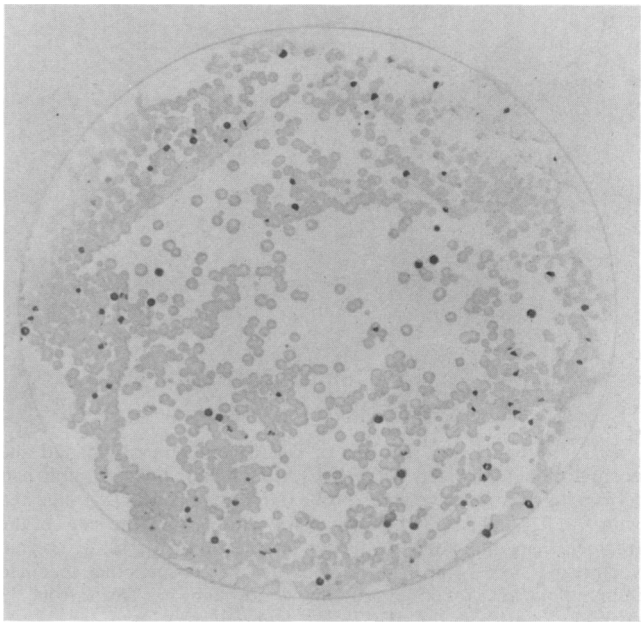

FIG. 1. Screening of the *M. leprae* cosmid library with pooled sera from LL leprosy patients by colony blot immunoassay.

The absence of any detectable reactive protein bands on SDS-polyacrylamide gels ranging from 7.5 to 15% in 18 of the 22 isolated cosmids (for example, Fig. 2, lanes 6 through 8) was investigated further. One possibility is that the recombinant protein(s) is recognized only in the native form as detected in the colony immunoassay and not in the denatured form as present on a Western blot. However, running the protein extracts on nondenaturing gels did not change the results. It is also possible that the proteins reactive on colony immunoblot were expressed only when

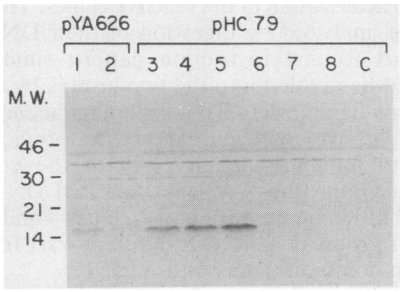

FIG. 2. Western blot of two clones from the plasmid (pYA626) library (lanes 1 and 2) and six clones from the cosmid (pHC79) library (lanes 3 through 8) reacted with sera from LL patients. Lane C, extract from a clone containing the cosmid vector pHC79.

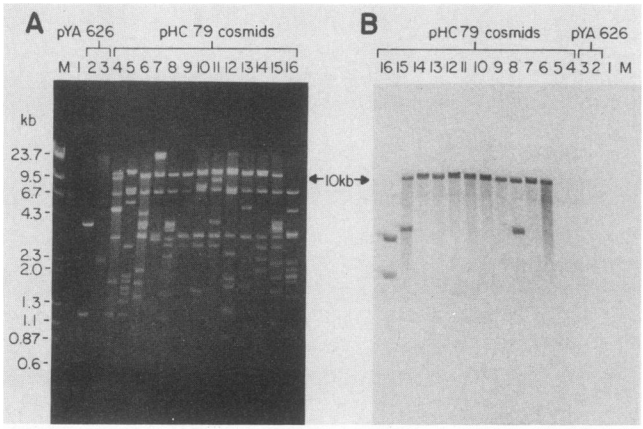

FIG. 3. Restriction patterns of DNA from several plasmid and cosmid clones digested with *Pst*I (A) and their hybridization patterns with the repetitive DNA probe (B). Lanes: M, λ DNA digested with *Hin*dIII and φX174 DNA digested with *Hae*III (the sizes of the fragments are indicated to the left of the photograph); 1, cosmid vector (pHC79); 2 and 3, DNA from the two plasmids that specified immunologically reactive proteins; 4 through 16, DNA from 13 cosmid clones from the pHC79::*M. leprae* library.

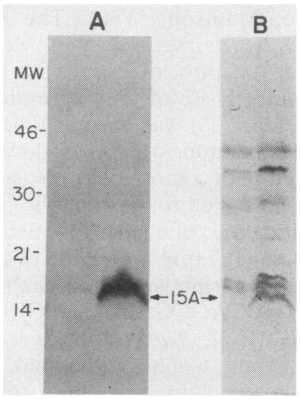

FIG. 4. Reactivity of extracts from pYA1164 expressing the A15 antigen (right lane in each panel) and extract from the vector, pBluescript/SK$^-$ (left lane in each panel) with pooled sera from LL patients (A) and TT leprosy patients from Brazil (B). Molecular weight markers (in thousands) are indicated on the left.

cells were grown in agar plates and not in liquid medium or that the reactive proteins were secreted from the cells. We tested these possibilities by running SDS-polyacrylamide gels with plate-derived bacteria and with protein concentrate from the supernatant fraction of liquid cultures of the reactive clones. No specific reactive bands were observed on Western blots.

To determine whether the immunoreactive proteins were recognized by MAbs against *M. leprae* proteins, we reacted the 24 clones (22 cosmids and two plasmids) with two pools of MAbs by the colony immunoblot technique. One pool consisted of MAbs reactive with mycobacterial proteins of 50 kDa and larger, and the other pool consisted of MAbs reactive with proteins of less than 40 kDa (Table 1). The five clones (four cosmids and one plasmid) that specified the 15-kDa immunoreactive band (group I) did not react with either of the pools. The other 18 cosmid clones (group II) reacted with both pools. Three of the cosmid clones from group II were analyzed by an ELISA, in which proteins specified by each of these three clones reacted with six MAbs. These MAbs recognize proteins of 16, 18, 26, 32 to 34, 50 to 65, and 65 kDa from *M. leprae*. However, when the individual MAbs were used to probe Western blots containing protein extracts from the three cosmids, no specific reactive bands were observed.

DNA characterization of the reactive clones. The 24 isolated clones were analyzed by digestion of their DNAs with the enzyme *Pst*I to test restriction pattern similarities. The DNAs could be divided into the two groups by their restriction patterns (Fig. 3A). The grouping according to the restriction patterns matched exactly the previous grouping according to immunoreactivity. All of the cosmid clone members of group I have at least two *Pst*I DNA fragments (1.7 and 1.2 kb) in common (Fig. 3A, lanes 4 and 5), whereas members of group II have two different *Pst*I fragments (10 and 3 kb) in common (lanes 6 through 15).

The redundancy of the number of the clones having similar restriction patterns in groups I and II led us to test whether those clones contained the repetitive *M. leprae* DNA sequences (5). Southern hybridization of the *Pst*I digests of the immunoreactive clones with a DNA probe derived from plasmid pYA1065 (5) demonstrated that members of group II all contain a 10-kb *Pst*I DNA fragment that hybridized with the *M. leprae* repetitive DNA and that some contain additional fragments which also shared homology with the repetitive DNA (Fig. 3B).

Since a 1.2-kb *Pst*I fragment was common to the plasmid clone, pYA1114 (Fig. 3A, lane 2), and to group I cosmids, and since the plasmid clone also specified an immunologically reactive protein of 15 kDa (Fig. 2, lane 1), it was likely that this fragment harbored the gene encoding the 15-kDa protein. To test this possibility, the 1.2-kb *Pst*I fragment isolated from pYA1114 was hybridized to *Pst*I digests of cosmids belonging to group I. The probe recognized a common 1.2-kb fragment shared by all the cosmids tested (data not shown). The 1.2-kb *Pst*I fragment from pYA1114 was subcloned in both orientations into pBluescript/SK$^-$, resulting in plasmids pYA1164 and pYA1165. Induced with IPTG (isopropyl-β-D-thiogalactopyranoside), plasmid pYA1164 expressed a 15-kDa protein that was reactive with LL patients' sera (Fig. 4A, right lane). Plasmid pYA1165 (opposite orientation) also expressed a 15-kDa immunoreactive protein band (though weakly), suggesting that it contains mycobacterial promoter-like sequences that are recognized by *E. coli*.

Reactivity with TT leprosy patients' sera. The recombinant mycobacterial antigen A15, expressed in pYA1164, reacted positively on a Western blot with the sera obtained from TT leprosy patients in the United States and in Brazil. Both sera reacted specifically with a protein band around 15 kDa; the reactivity of the protein specified by pYA1164 with the sera from the Brazilian TT patients is shown in Fig. 4B, right lane.

Recognition by T cells from leprosy patients. Plasmid pIHB1001, which efficiently expressed a fusion protein containing almost 80% of the deduced A15 protein, was used to see whether A15 could be recognized by *M. tuberculosis*-stimulated T cells derived from leprosy patients. *M. tuberculosis* rather than *M. leprae* was used to generate the T-cell lines in order to be able to compare responses in both borderline tuberculoid (BT) and borderline lepromatous (BL) patients. The latter patients are nonresponders to *M. leprae*, and it is difficult to grow *M. leprae*-reactive T cells from such individuals. Autologous peripheral blood mono-

TABLE 2. T-cell proliferative responses

Antigen source[a]	Stimulation index[b] of [^{3}H]thymidine in T-cell lines from patient				
	LL	BT-1	TT-1	TT-2	BT-2
M. leprae	3	2	49	113	11
Purified protein derivative[c]	59	9	110	588	26
E. coli (pIHB1001)[d]	31	13	45	51	1
E. coli (pEX2)	8	1	2	2	1
Medium	1	1	1	1	1

[a] Concentrations (μg/ml) of the antigens tested were 1.0 for *M. leprae*, 5.0 for the purified protein derivative, and 0.1 to 10 for pIHB1001 and pEX2.

[b] The data are expressed as stimulation indices determined by the amount of [^{3}H]thymidine incorporated into T cells in the presence of the indicated antigen/the amount of [^{3}H]thymidine incorporated into T cells in the absence of antigen.

[c] From *M. tuberculosis*.

[d] pIHB1001 expressed the C-terminal 89 amino acids of A15 as a fusion with β-galactosidase.

nuclear cell-derived antigen-presenting cells were used to present the *M. leprae* sonicate, the purified protein derivative from *M. tuberculosis*, the *E. coli* lysate of induced pIHB1001, and the *E. coli* lysate of induced pEX2 to the T-cell lines (Table 2). *M. tuberculosis*-generated T-cell lines from BT as well as LL patients appeared to respond to the *E. coli* fraction containing the fusion protein expressed by pIHB1001 (Table 2 and unpublished data). As a control, one T-cell line is shown that does not respond to the antigen, showing that the A15 antigen is not nonspecifically mitogenic. The antigen therefore contains an epitope(s) that is important for human T-cell response. Moreover, T-cell lines from *M. leprae* nonresponder (LL as well as BT) patients were able to respond to the isolated recombinant antigen, indicating that T-cell nonresponsiveness to whole *M. leprae* can be reversed by stimulation with this particular antigen. Finally, the observation that these *M. tuberculosis*-generated T cells are able to respond to the *M. leprae* fusion protein suggests the presence of a cross-reactive antigen in *M. tuberculosis*.

Nucleotide sequence. The entire 1.2-kb *PstI* fragment was sequenced and revealed three open reading frames (ORFs), only one of which (ORF II) corresponds in size to the 15-kDa protein (Fig. 5).

Sequence analysis of ORF II revealed putative −35 and −10 promoter regions as well as a ribosomal binding site near the 5′ end of the coding sequence. A stem structure located downstream from ORF II might be a rho-independent terminator (Fig. 6). The G+C content of ORF II is 56%, similar to the estimated value of the *M. leprae* genome (6).

The sequence preceding ORF II has been termed ORF I and specifies 57 amino acids. The nucleotide sequence of ORF I (Fig. 6) is similar to a portion (carboxy terminus) of the *E. coli lysU* gene (4, 12), which encodes for lysyl-tRNA synthetase (50.9% identity). The third ORF (ORF III) (Fig. 5 and 6) specifies 97 amino acids that are similar to sequences of the variable regions of the tomato ATP-dependent proteases CD4a and CD4b (63.9% identity), which are members of a class of highly conserved genes (9).

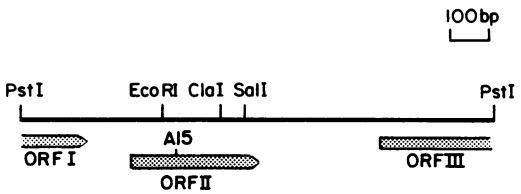

FIG. 5. Partial restriction map of the 1.2-kb fragment that contains the gene coding for A15 and the three suggested ORFs.

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CTGCAGCCGCCGAGGAAGATGAGGCGATGGCACTTGATGAGGAATTTCTGGCGGCACTGG   60
  A  A  A  E  E  D  E  A  M  A  L  D  E  E  F  L  A  A  L  E

AGTACGCGATGCCGCCATGCACTGGAACTGGAATGGGTATCGACCGGTTGTTGATGTCTT  120
  Y  A  M  P  P  C  T  G  T  G  M  G  I  D  R  L  L  M  S  L

TGACTGGACTGTCAATTAGGGAGACTGTTTTGTTCCCGATTGTTCGGCCGCACTCCAACT  180
  T  G  L  S  I  R  E  T  V  L  F  P  I  V  R  P  H  S  N  *

GAACTGTTCGTGTTGTGTCGAGATTGAGTATGCGGTGTTCTTATGGCAGATTAGTATCTG  240

GGGAGGTTGATCCAAACTGCTCAGGAAGAAACGTGAGGGTAAGATAATGGCGAAGAAAGT  300
                                      M  A  K  K  V

GACCGTCACCTTGGTCGATGATTTCGATGGTGCGGGCGCCGCCGATGAAACGGTCGAATT  360
  T  V  T  L  V  D  D  F  D  G  A  G  A  A  D  E  T  V  E  F

CGGGCTTGACGGGGTGACCTACGAGATCGACCTTACGAACAAGAATGCCGCGAAACTGCG  420
  G  L  D  G  V  T  Y  E  I  D  L  T  N  K  N  A  A  K  L  R

TGGCGATCTGAGGCAATGGGTGTCCGCCGGACGGCGCGTCGGCGGTCGGCGGCGAGGGCG  480
  G  D  L  R  Q  W  V  S  A  G  R  R  V  G  G  R  R  R  G  R

TTCCAATTCTGGACGCGGCCGTGGGGCGATCGATCGCGAACAGAGCGCGGCGATCCGGGA  540
  S  N  S  G  R  G  R  G  A  I  D  R  E  Q  S  A  A  I  R  E

ATGGGCTCGTCGGAACGGACATAATGTGTCGACTCGTGGTCGTATTCCGGCCGACGTCAT  600
  W  A  R  R  N  G  H  N  V  S  T  R  G  R  I  P  A  D  V  I

TGACGCATTCCACGCGGCGACTTAAAATAAAAAGTTCTGTACTGACGCCCGGGCTCTAGG  660
  D  A  F  H  A  A  T  *

ACCCGGGCGTCAGTACTTTAAAAAAATATGTTGCTTTCGTTGCTGGCGAACTGATCGCCA  720

GCAACTGGCGGAAGCGATTCGGCGGTTTCTTCGTTTCTGGGGTTAAGAACCTGGTGTCGC  780

AGGACTACGAAGCCTCGGATACTCCAAGCCACTGGAAACGGCAATATTGCCGACCATTAC  840

AGTGGACGGCAGGTACACGGTTCGGGTCCCATGGGCTGGAACGGGTCGTATGGATGGTGT  900

AGGGAGAGCAGGTAACCGCCGATGTTCGAAAGATTTACCGATCGTGCCCGCAGGGTGGTC  960
                  M  F  E  R  F  T  D  R  A  R  R  V  V

GTCCTGGCACAGGAAGAGGCCCGGATGCTCAACCATAACTACATCGGCACCGAGCACATT 1020
  V  L  A  Q  E  E  A  R  M  L  N  H  N  Y  I  G  T  E  H  I

TTGCTGGGTCTTATCCCTGAAGGCGAAGGTGTCGCGGCGAAATCGTTGGATTCGTTGGGG 1080
  L  L  G  L  I  P  E  G  E  G  V  A  A  K  S  L  D  S  L  G

ATTTCACTTGAAGCCGTTCGCAGTCAGGTCGAAGATATTATCGGCCAGGGTCAGCAGGCG 1140
  I  S  L  E  A  V  R  S  Q  V  E  D  I  I  G  Q  G  Q  Q  A

CCGTCGGGGCATATCCCGTTTACGCCTCGTGCCAAGAAGGTTCTTGAGCTGAGCTTGCGT 1200
  P  S  G  H  I  P  F  T  P  R  A  K  K  V  L  E  L  S  L  R

GAGGCGCTGCAG 1212
  E  A  L  Q
```

FIG. 6. The nucleotide sequence and the deduced amino acids of the three ORFs found in the DNA coding for A15. A putative ribosome binding site and promoter −10 and −35 regions are underlined in front of ORF II. The two arrows downstream from ORF II indicate the position of a stem structure that is a putative rho-independent terminator.

Relationship of the DNA coding for A15 with previously identified λgt11::*M. leprae* clones and homology with other mycobacterial DNAs. To investigate the relationship between the gene coding for A15 and previously isolated λgt11::*M. leprae* clones (22), the 1.2-kb DNA fragment from pYA1164 was hybridized with DNAs from those clones. The probe hybridized to three clones (L8, L21, and L33) that had been grouped into one hybridization group (22). This indicated that these clones contain part or all of the *M. leprae* gene coding for A15. The 2.3- and 1.8-kb *Eco*RI DNA inserts from clones L8 and L21 were subcloned into the expression vector pEX2, resulting in plasmids pIHB1001 and pIHB1003, respectively. Induction of these plasmids resulted in the expression of Cro–β-galactosidase fusion proteins of 124 and 145 kDa. Sequence analysis of the *M. leprae* DNA immediately fused to the *cro-lacZ* gene in pIHB1001 revealed that this sequence is identical to nucleotides 356 through 714 of ORF II that codes for A15. Thus, the

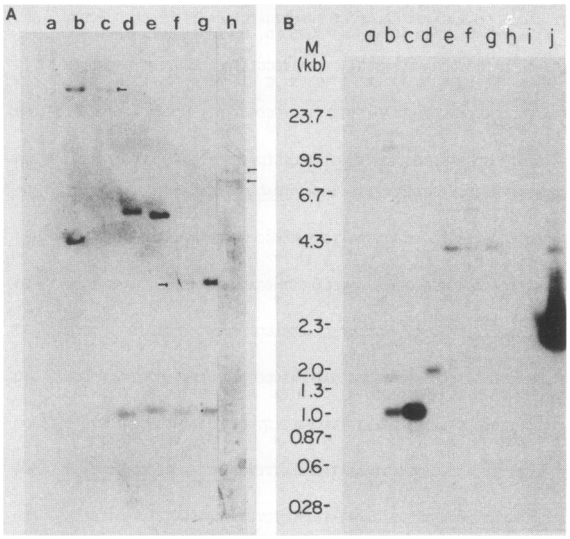

FIG. 7. (A) Hybridization pattern of the *M. leprae* 1.2-kb *Pst*I fragment with *Eco*RI digests of DNA from various mycobacteria. Lanes: a, *E. coli*; b, *M. fortuitum*; c, *M. phlei*; d, *M. scrofulaceum*; e, *M. avium*; f, *M. bovis*; g, *M. tuberculosis*; h, *M. leprae*. Arrows indicate the position of weak hybridization bands that resulted from the presence of smaller amounts of DNA for *M. bovis*, *M. phlei*, and *M. leprae*. (B) Hybridization pattern of the A15 gene fragment with *Pst*I-digested chromosomal DNA. Lanes: a, molecular size markers (sizes are indicated to the left of the photograph); b and c, *M. leprae* from two different isolates; d, *M. avium*; e, *M. bovis* BCG; f, *M. tuberculosis* H37Rv; g, *M. tuberculosis* H37Ra; h, *E. coli*; i, *M. avium* TMC 724 (partial digestion); j, plasmid subclone containing the A15 gene fragment.

C-terminal 89 amino acids of A15 are expressed as a fusion protein in pIHB1001.

The sequence of the *M. leprae* DNA in pIHB1003 (data not shown) is not homologous to ORF II and probably expresses another gene (the beginning of the *lysU* homolog?) positioned upstream from the 1.2-kb *Pst*I fragment that was sequenced here.

A nucleotide data base search revealed that the sequence of nucleotides 356 through 714, which is part of ORF II from the *Eco*RI site (Fig. 5 and 6), is identical to a sequence published recently by Laal et al. (10) that encoded a β-galactosidase fusion protein that was immunoreactive with sera from LL patients. The authors suggested that the β-galactosidase fusion protein was capable of eliciting a T-cell proliferative response in cells derived from leprosy patients and healthy contacts in a fashion similar to the response induced by the native *M. leprae* bacterium (10).

To test whether the identified gene(s) shares homology with other mycobacterial DNAs, the 1.2-kb *Pst*I fragment was hybridized with various mycobacterial DNAs digested with *Eco*RI (Fig. 7A). The results suggested the existence of homologous sequences in slowly growing as well as fast-growing mycobacteria. It is interesting to note that the mycobacterial DNAs demonstrated two hybridization bands, suggesting that the *Eco*RI site is conserved in the mycobacteria tested. When the coding sequence for just the 15-kDa antigen was used as a probe, hybridization was observed between the probe DNA and chromosomal DNA fragments from *M. leprae* and several slowly growing mycobacteria (Fig. 7B) but not with fast-growing mycobacteria such as *Mycobacterium fortuitum*, *M. phlei*, and *M. vaccae* (data not shown).

DISCUSSION

A number of mycobacterial antigens were identified from λgt11 libraries, some of which are expressed as nonfusion proteins (22, 27), suggesting that they might be expressed from their own regulatory sequences. Since no specific promoter sequences were reported in the above cases, it is not known whether these antigens are expressed from their own transcription-translation sequences or from the vector's *lac* promoter in conjunction with the mycobacterial ribosome binding sites. Other studies have suggested that some mycobacterial gene promoters might be recognized by *E. coli* (23, 24).

The results presented here demonstrate that a cosmid library could serve directly for the isolation of genes specifying some antigens recognized by leprosy patients' sera. This approach could also be applied to the identification of genes specifying immunologically reactive antigens from other pathogens. It is obvious that this approach is limited to the isolation of a specific subclass of reactive proteins: those whose transcription-translation sequences are recognized by *E. coli*. Nevertheless, one might efficiently screen the genome of any bacterium by using one to three agar plates and, thus, save several DNA manipulations to characterize the intact gene within its original DNA context.

Although we have identified and partially characterized only 22 reactive cosmid clones, it is striking that all of them fell into only two categories, namely, groups I and II.

The nature of the antigens expressed by the cosmids belonging to group II remains unclear. It is worthwhile to note here that there have been other investigators who have also found that clones that produced immunologically reactive proteins in the plaque immunoassay did not produce immunologically reactive proteins detected by Western blot analysis (22, 27). Six MAbs that recognized mycobacterial antigens of 16, 18, 26, 32 to 34, 50 to 65, and 65 kDa reacted positively with proteins specified by each of the three different cosmids of group II that were tested. Since these are cosmid clones that each contain approximately 45 kb of *M. leprae* DNA, it is conceivable that each cosmid could express several protein antigens.

A possible explanation for the inability to detect an immunologically reactive protein band on a Western blot assay is that the antigen(s) is of very small molecular mass (<10 kDa), which makes it impossible to be detected by this technique. Further studies are under way to define and characterize the antigenic determinant(s) expressed by group II clones.

It is unclear why, in this study, we did not detect the same three nonfusion antigens that were isolated previously in our laboratory (22) by screening the λgt11::*M. leprae* library with the same sera, especially since it has been found that at least one of them is expressed from its own transcription-translation signals (2). A possible explanation is that, since we initially selected only strongly immunoreactive clones from the cosmid library, we did not find the same three nonfusion λgt11 clones that had been shown to react weakly with the LL patients' sera (22).

Group I clones expressed the A15 antigen that has the human B-cell epitope(s) recognized at the polyclonal level in both LL and TT patients' sera. We also showed that the A15 antigen contains important epitope(s) for T cells from both TT and LL patients. Since the patients studied here differed in their human leukocyte antigen phenotypes, the epitope(s) can be recognized in the context of different human leukocyte antigen molecules. Whether one or several T-cell

epitopes are involved requires further epitope mapping with synthetic peptides. In addition, since *M. tuberculosis*-stimulated T cells were able to respond to the *M. leprae* fusion protein, an homologous antigen seems to be expressed by *M. tuberculosis* as well. Importantly, it was noted that *M. tuberculosis*-stimulated T cells from *M. leprae* nonresponder (LL as well as BT) patients were able to respond to the isolated recombinant *M. leprae* antigen, indicating that nonresponsiveness to *M. leprae* antigens could be reversible. We have previously reported similar results in a substantial proportion of lepromatous nonresponder patients. Latent T-cell activity to *M. leprae* was revealed in these patients upon stimulation of their peripheral T cells with isolated fractions of the bacillus (17) or with the recombinant hsp65 (19). These antigens may be important candidates for the immunotherapy and immunoprophylaxis of lepromatous leprosy.

Shortly before submission of this paper, Laal et al. (10) reported the isolation of an *M. leprae*::λgt11 clone, termed LSR2, that expressed a β-galactosidase fusion protein of about 10 kDa that reacted with LL patients' sera. The fused protein elicited a T-cell proliferative response in cells derived from leprosy patients, which was similar to the response elicited with the *M. leprae* bacterium. Their published sequence is identical to the fused *M. leprae* sequence found in the λgt11 clone (L8) identified previously in our laboratory (22). The DNA fragment from L8 was cloned into pIHB1001 (this work) and expressed about 80% of the putative amino acids of A15 fused to Cro–β-galactosidase, essentially the same as the LSR2 clone identified by Laal et al. (10). Our T-cell proliferation results demonstrated that this antigen is recognized by *M. tuberculosis*-selected T cells from both LL and TT patients, a result which is in contrast to the finding of Laal et al. that unselected peripheral T cells from LL patients do not respond (10). The difference between their results and ours could be because we first selected for T-cell activity against mycobacterial antigens by stimulation with *M. tuberculosis* and therefore were better able to detect T-cell recognition of the A15 antigen in patients who were nonresponsive to the whole repertoire of *M. leprae* antigens.

The searching of sequence data banks has not identified any similar sequence in other organisms; thus, it was not possible to postulate the identity of this protein. Sequence analysis did not reveal any major hydrophobic domain near the N-terminal part of the deduced protein that indicated the existence of a signal sequence; thus, it is not likely that this protein is secreted or is part of the mycobacterial cell wall.

There are putative ribosomal binding sites just before the start codon of ORF II and the −10 and −35 regions further upstream (Fig. 6). Further studies should be done to evaluate their uses in *M. leprae* and in *E. coli*. In any case, our results suggest indirectly that a mycobacterial promoter-like sequence is recognized, which allows *E. coli* to express A15 both in the cosmid clones and in pYA1165, when ORF II is in the opposite direction to the *lac* promoter of the plasmid vector.

Sequence analysis of the entire 1.2-kb *Pst*I fragment revealed a stem and loop structure downstream of ORF II that might serve as a rho-independent terminator of ORF II.

The GenBank data search did show similarities between the deduced amino acids of ORF I and the *E. coli* gene *lysU*, which encodes for one of the two lysyl-tRNA synthetases in this bacterium (4, 12). It is interesting to note that *lysU* is regulated by σ32 and therefore has been assigned to the heat shock regulon (16).

The sequence of ORF III (downstream from ORF II) revealed a high degree of similarity to the N-terminal sequences of the tomato ATP-dependent proteases CD4a and CD4b (9). These proteins have been shown to be members of a recently identified family of highly conserved ATP-dependent proteases (including *clpA* and *clpB* of *E. coli*) that are ubiquitous among prokaryotes and eukaryotes (9). It is not clear why the mycobacterial-derived amino acids are more similar to the eukaryotic counterpart.

Hybridization of the 1.2-kb *Pst*I DNA fragment to various mycobacterial chromosomal DNA digests demonstrated the existence of similar sequences within their genomes; this result is not surprising, since the 1.2-kb fragment contains ORF I and ORF III, as well as ORF II. When hybridization was carried out with a probe that consisted only of ORF II sequences, the probe hybridized only to chromosomal DNA fragments from *M. leprae* and several slowly growing mycobacteria (*Mycobacterium avium*, *M. bovis* BCG, and *M. tuberculosis*). This result, together with experiments with *M. tuberculosis*-generated T cells from leprosy patients, lends further support to the suggestion of the presence of a cross-reactive antigen in *M. tuberculosis* and perhaps in other slowly growing mycobacteria.

In conclusion, A15 might be regarded as one of the major *M. leprae* antigens recognized by the human immune system, since it has a B-cell epitope(s) that is recognized by antibodies from patients from different geographical regions (this work) (10) and since it also contains at least one T-cell epitope that is recognized by human T cells from leprosy patients (this work) (10) as well as from healthy contacts (10).

Our results, as well as those of Laal et al. (10), demonstrated that the approach of searching for *M. leprae* antigens that might be involved in eliciting a cellular protective response in humans by probing for immunoreactive antigens that are recognized by B cells has proven to be justified.

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