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Expression of Proteins of *Mycobacterium tuberculosis* in *Escherichia coli* and Potential of Recombinant Genes and Proteins for Development of Diagnostic Reagents

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Recombinant plasmids containing DNA from *Mycobacterium tuberculosis* were transformed into *Escherichia coli*, and three colonies were selected by their reactivity with polyclonal antisera to *M. tuberculosis*. The three recombinant vectors contained DNA inserts of different sizes flanking a common 4.7-kilobase (kb) sequence. Each recombinant produced 35- and 53-kilodalton proteins (35K and 53K proteins, respectively) which were absent in the control *E. coli*. In Western blotting experiments, both proteins bound several antisera to *M. tuberculosis* but not antisera to other commonly isolated mycobacteria. Rabbits immunized with the recombinant 35K protein produced antisera which bound to both the 35K and 53K protein bands, a single 35K protein band present in a culture filtrate of *M. tuberculosis*, and single protein bands with differing molecular weights in whole-cell homogenates from other *Mycobacterium* spp. An additional recombinant vector containing a 2.2-kb subclone of the 4.7-kb sequence was constructed and, when used as a probe, demonstrated homology with various fragments of chromosomal digests of selected mycobacteria. Reactivity of this probe to *Mycobacterium bovis* and *M. bovis* BCG was indistinguishable from reactivity to *M. tuberculosis*. Immunoglobulin G reactivity to the 35K antigen was detected in antisera from 8 of 20 persons with active tuberculosis, 4 of 18 persons with leprosy, and none of 14 healthy controls. In contrast, reactivity to various proteins in *M. tuberculosis* culture filtrate was present in 18 of 20 patients with tuberculosis, 16 to 18 patients with leprosy, and 5 of 14 controls. The production of *M. tuberculosis* proteins by *E. coli* circumvents many difficulties encountered in the growth and manipulation of *M. tuberculosis* and may facilitate the development of better diagnostic and immunizing reagents.

Tuberculosis continues to present diagnostic problems for physicians and control problems for public health officials. The disease most commonly affects the lungs, but it may involve almost any tissue and produce diverse, difficult-to-diagnose clinical syndromes. Since Koch identified *Mycobacterium tuberculosis* as the causative agent of tuberculosis in 1882, many scientific studies and public health efforts have been directed at diagnosis, treatment, and control of this disease. In the developed world, these efforts have been largely successful, yet transmission of tuberculosis continues, with over 20,000 new cases being diagnosed annually in the United States alone (2). In the United States, recent reports indicate that tuberculosis may soon be on the increase, with new cases associated with human immunodeficiency virus infections and other factors (3). In the developing world, tuberculosis is an even more important cause of morbidity and mortality in both children and adults, and there is little evidence that disease rates are declining (10).

Control of tuberculosis in developed and developing countries involves different problems, many of which pertain to health care resources and delivery. However, the characteristics of *M. tuberculosis* have hampered research to improve diagnosis and to develop more effective vaccines. The organism is potentially infectious to laboratorians, and several weeks are usually required to isolate the organism from clinical specimens. In addition, the biochemical composition of the organism has made identification and purification of cellular constituents difficult, and many of these materials, once purified, lack sensitivity or specificity as diagnostic reagents (4). To circumvent these difficulties, researchers have begun to develop monoclonal antibodies and to clone mycobacterial DNA into other microorganisms, including *Escherichia coli* (5, 17). Such immunologic and genetic systems allow identification of gene products from *M. tuberculosis* which will provide better diagnostic tests and more effective vaccines. In this paper, we describe the construction and characterization of recombinant plasmids in *E. coli* that express potentially useful antigenic proteins of *M. tuberculosis*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and antisera. *M. tuberculosis* $H_{37}Ra$ (derived from Trudeau Mycobacterial Culture Collection [presently housed at the American Type Culture Collection, Rockville, Md.] no. 201) was the source of DNA for this study. *E. coli* JM107 was the host for the recombinant plasmids, which were constructed with pUC13 (J. Messing, St. Paul, Minn.) (16). *E. coli* JM109(pUC8) was used as a control nonrecombinant. Heat-killed cells of serologic reference strains of *Mycobacterium kansasii*, *Mycobacterium avium* serotypes 4 and 8, and *Mycobacterium scrofulaceum* serotype 42 and the homologous antisera prepared in rabbits were produced in our laboratories. Other *Mycobacterium* spp. that were the source of DNA for Southern blots included *M. bovis* TMC 401, *M. bovis* BCG Rosenthal, *M. smegmatis* TMC 1525, *M. gordonae* TMC 1324, and *M. avium* serotypes 1B, 4B, and 9B. Four antisera to *M. tuberculosis*, three made in rabbits ($H_{37}Rv$ and M-322t, prepared at Centers for Disease Control, Atlanta, Ga.; and

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4589, T. Daniel, Cleveland, Ohio) and one made in a burro (Burro 254 against strain Erdman TMC 107; S. Chaparas, Bethesda, Md.), were used for either detection or characterization of recombinants. Human antisera were obtained from the collection established by the Steering Committee on the Immunology of Leprosy, United Nations Development Program/World Bank/World Health Organization, and from healthy volunteers at the Centers for Disease Control.

Construction of recombinant plasmids. Chromosomal DNA was isolated from *M. tuberculosis* $H_{37}Ra$ by established methods (1, 7). This DNA and cesium chloride-ethidium bromide-purified vector pUC13 DNA were separately digested with restriction enzyme *Bam*HI or *Eco*RI (New England BioLabs, Inc., Beverly, Mass.). pUC13 DNA was dephosphorylated and mixed with chromosomal DNA from *M. tuberculosis* $H_{37}Ra$. The DNA was then ligated with T4 DNA ligase according to the recommendations of the manufacturer (New England BioLabs), and *E. coli* JM107 was transformed with the ligation mixture (9). Recombinant transformants were selected on plates containing 200 μg of carbenicillin per ml, 0.4 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside per ml, and 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG).

Screening and characterization of recombinant *E. coli*. Approximately 5,000 colonies (2,500 each from *Bam*HI and *Eco*RI digests) were screened by an adaptation of a colony antigen detection technique (13). In brief, recombinant colonies were blotted onto nitrocellulose paper (BA85, 0.45-μm pore size; Schleicher & Schuell, Inc., Keene, N.H.) which was then soaked in 1% bovine serum albumin for 1 h to block nonspecific binding. The blots were incubated with polyclonal rabbit antiserum raised against *M. tuberculosis* M-322t. Antibody binding was detected by subsequent incubation with ^{125}I-labeled protein A and autoradiography. Three recombinants which bound antisera were designated TB-2, TB-5, and TB-6. The molecular nature of this antiserum binding was further demonstrated by Western blot analysis (6). Whole-cell protein preparations of recombinant *E. coli* grown overnight at 37°C in LB medium (14) were electrophoretically separated on 0.75 or 1.5 mM sodium dodecyl sulfate-12.5% polyacrylamide gel. These proteins were electrophoretically transferred to nitrocellulose sheets at 7 V/cm for 3 h in 25 mM Tris buffer (pH 8.6) containing 192 mM glycine and 20% (vol/vol) methanol. Nonspecific binding was blocked with 1% bovine serum albumin or 0.03% Tween 20. Blots were incubated at 25°C for 90 min with a 1/1,000 dilution of burro antiserum to *M. tuberculosis* and then with ^{125}I-labeled protein A, and then they were autoradiographed. Antibody binding was also detected with horseradish peroxidase-conjugated protein A (Bio-Rad Laboratories, Richmond, Calif.) as described by the manufacturer. In experiments with human antisera, casein was substituted for bovine serum albumin as described by Kenna et al. (11). Human sera were reacted overnight at 4°C at dilutions of 1/50. Production of recombinant proteins was compared in the presence and absence of IPTG.

Specificity of recombinant proteins. Rabbit antisera prepared against heat-killed *M. kansasii*, *M. scrofulaceum*, and *M. avium* were mixed 2:1 with washed *E. coli* to absorb anti-*E. coli* antibodies. These sera were tested to determine the dilutions which optimized the mycobacterial activity and minimized the cross-reactive *E. coli* activity. The antisera against *M. kansasii*, *M. scrofulaceum*, and *M. avium* were subsequently used at dilutions of 1/1,500, 1/5,000, and 1/1,000, respectively.

Production of polyclonal rabbit antisera to recombinant proteins. The 35- and 53-kilodalton proteins (35K and 53K proteins, respectively) were purified by the technique of Parekh et al. (15). Briefly, proteins were electrophoresed in a preparative polyacrylamide gel and transferred to nitrocellulose paper. The nitrocellulose paper strips containing appropriate bands were excised, and proteins were eluted with acetonitrile. The proteins were suspended in deionized H_2O and mixed with an equal volume of Freund incomplete adjuvant, and 50 to 100 μg was injected subcutaneously into 30 sites on the back of a 6-week-old New Zealand White rabbit. The rabbit was given subcutaneous booster doses of 50 to 100 μg of protein at 2 and 4 weeks, and serum was obtained at 6 and 8 weeks. This antiserum was used in Western blotting experiments with sonic extracts of *M. leprae* (biopsy OPD102457, taken in Bombay, India) and cell homogenates of *Mycobacterium fortuitum* TMC 1529, *Mycobacterium chelonae* TMC 1542, *M. kansasii* TMC 1204, *M. gordonae* TMC 1324, *M. avium* P55 serotype 4B, and *M. avium* 23435 serotype 8A, which were prepared as previously described (8).

Restriction mapping and subcloning of recombinant *E. coli*. Plasmid DNA from the recombinants was purified by isopycnic cesium chloride-ethidium bromide sedimentation (12). The location of restriction sties was determined by using single and double digests of restriction enzymes *Bam*HI, *Hin*dIII, *Pst*I, *Nsi*I, *Sal*I, *Eco*RI, *Sph*I, and *Sst*I (New England BioLabs). A 2.2-kilobase subclone of the *M. tuberculosis* DNA insert in TB-2 was generated by the *Sph*I digest of TB-2 DNA and referred to as TB-11 [strain JM107(pLWM2110)].

Hybridization of recombinant vector with selected mycobacteria. Purified plasmid DNA from TB-11 was labeled with ^{32}P with a nick translation kit (New England Nuclear Corp., Boston, Mass.), according to the instructions of the manufacturer. Chromosomal DNA from *M. tuberculosis* $H_{37}Ra$, *M. gordonae*, *M. bovis*, *M. smegmatis*, *M. bovis* BCG, and *M. avium* serotypes 1B, 4B, and 9B was restricted with *Bam*HI and electrophoresed for 16 h (8 mA) in a 0.8% agarose gel in Tris-acetate buffer (0.04 M Tris-acetate, 0.001 M EDTA; pH 8.0). The DNA was transferred to nitrocellulose paper, hybridized under stringent (T_m, −10°C) and less stringent (T_m, −20°C) conditions with ^{32}P-labeled TB-11 probe, and autoradiographed for 24 h at −70°C (12). Unrestricted chromosomal DNA was also fixed to nitrocellulose paper with a slot-blotting apparatus (Schleicher & Schuell) and hybridized with TB-11 DNA that had been labeled with biotin according to the instructions of the manufacturer (BluGene; Bethesda Research Laboratories, Inc., Gaithersburg, Md.).

RESULTS

Three colonies, two from *Bam*HI and one from *Eco*RI digests, bound rabbit antiserum M-322t. These colonies, identified as TB-2 [JM107(pLWM2020)], TB-5 [JM107(pLWM2050)], and TB-6 [JM107(pLWM2060)], also bound two additional rabbit sera, 4589 and $H_{37}Rv$, and one burro serum, all raised against *M. tuberculosis*. The restriction digests of pLWM2020, pLWM2050, and pLWM2060 demonstrated overlapping inserts of the same sequences which were 4.7, 4.9, and 12.2 kilobases respectively. The results of the restriction digestions of two recombinants, pLWM2020 and pLWM2050, are shown in Fig. 1.

Western blot analysis with the various sera against *M. tuberculosis* demonstrated that two proteins, 35K and 53K, were present in all three recombinants but were absent in the

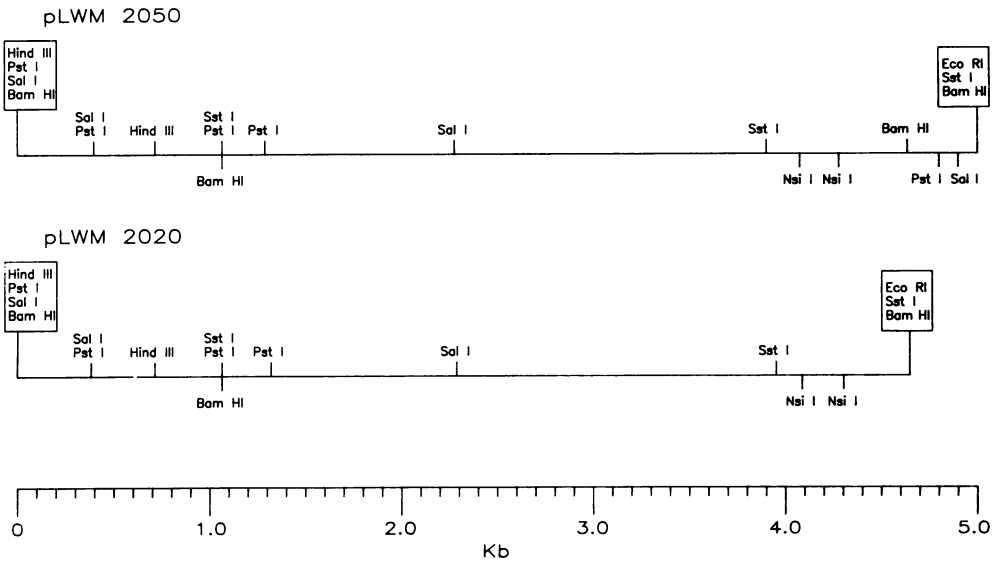

FIG. 1. Restriction maps of pLWM2020 and pLWM2050. Restriction sites present in the vector are shown within boxes. Both recombinants include an identical 4.7-kilobase (Kb) sequence. Recombinant pLWM2050 has an additional sequence which may or may not represent a naturally occurring contiguous DNA sequence.

control *E. coli* JM109(pUC8), without inserts, and in *E. coli* with inserts of other DNA sequences from *M. tuberculosis* (Fig. 2). The two recombinant proteins were produced in the absence of IPTG, but addition of this *lac* inducer increased production. Whole-cell preparations of recombinant *E. coli* TB-2, TB-5, TB-6, and TB-11 were tested with antisera to mycobacteria other than *M. tuberculosis*. These antisera did not bind to the 35K or 53K protein, although each antiserum possessed some residual anti-*E. coli* activity (Fig. 3).

Rabbit antisera raised against the 35K protein reacted with both the 35K and 53K proteins and with a single 35K band in *M. tuberculosis* culture filtrates (Fig. 4). Human sera from several groups of patients were reacted with proteins from *M. tuberculosis* culture filtrate and with nonrecombinant and recombinant *E. coli* (Fig. 4; Table 1). Reactivity to the 35K protein was detected in 8 of 20 persons infected with *M. tuberculosis*, 4 of 18 persons infected with *M. leprae* (6 and

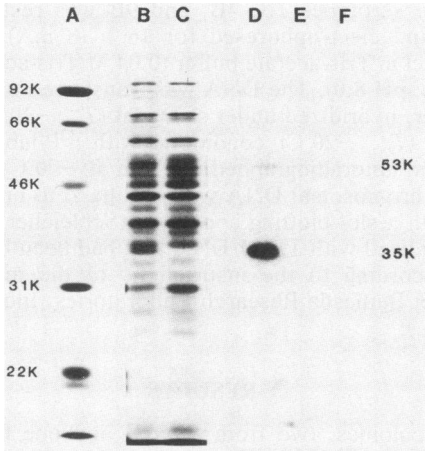

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel of whole-cell protein preparations of recombinant and control *E. coli* stained with Coomassie blue and autoradiographed. Two additional protein bands, 35K and 53K, are present in sodium dodecyl sulfate-polyacrylamide gel of TB-2 (lane B) and absent in that of control *E. coli* JM109(pUC8) without *M. tuberculosis* DNA (lane C). Lane A, molecular weight standards; lanes D, E, and F, autoradiographs of whole-cell proteins which were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose paper, and reacted with rabbit anti-*M. tuberculosis* sera and ^{125}I-labeled protein A (6). The 35K and 53K proteins are present in TB-2 (lane D) but absent in a control *E. coli* which has a different insert of DNA from *M. tuberculosis* (lane E) and in an *E. coli* which lacks any insert from *M. tuberculosis* (lane F).

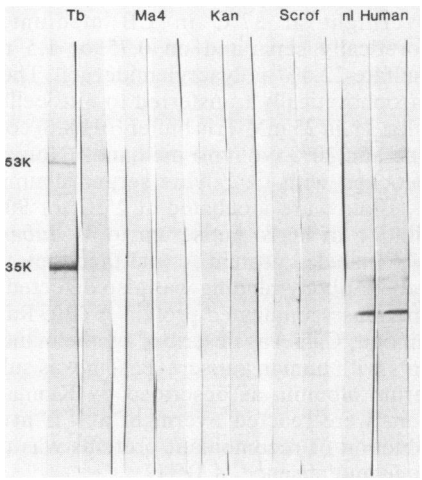

FIG. 3. Specificity of the 35K and 53K proteins to antisera directed against *M. tuberculosis* and other clinically important mycobacteria. Western blots of whole-cell proteins from TB-2 (left strip of nitrocellulose paper in each pair) and *E. coli* JM109(pUC8) were reacted with five antisera (*M. tuberculosis* [lanes Tb], *M. avium* serotype 4 [lanes Ma4], *M. kansasii* [lanes Kan], *M. scrofulaceum* [lanes Scrof], and person uninfected with *M. tuberculosis* [lanes nl Human]) and a horseradish-peroxidase antibody detection system (Bio-Rad) with protein A. Although each serum binds to some *E. coli* proteins, only antiserum to *M. tuberculosis* recognizes the 35K and 53K proteins.

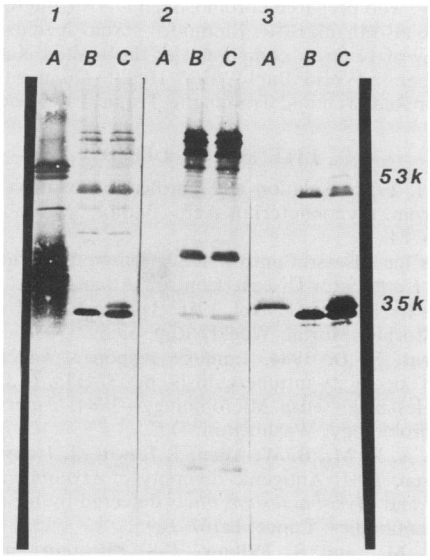

FIG. 4. Western blots of *M. tuberculosis* culture filtrate (lanes A) and whole-cell proteins of control *E. coli* JM109(pUC8) (lanes B) and the recombinant TB-2 (lanes C). Blots were incubated with serum from a patient with tuberculosis (section 1) and a healthy individual (section 2). Section 3 was incubated with rabbit serum raised against the 35K antigen from TB-2. Sera from the patient with tuberculosis and the rabbit bound to the 35K protein in the recombinant *E. coli*. The rabbit antiserum also recognized only a single 35K protein in the *M. tuberculosis* culture filtrate.

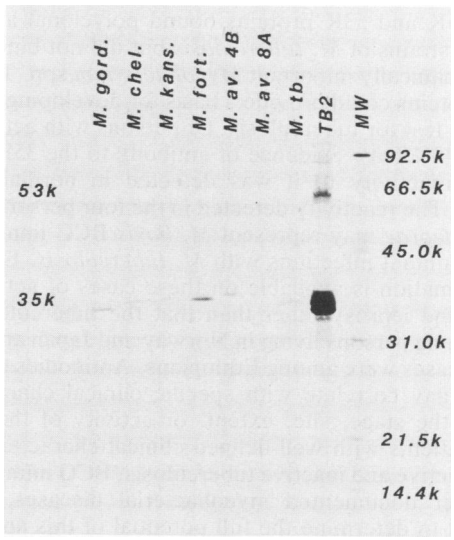

FIG. 5. Western blot of culture filtrates from *M. gordonae*, *M. chelonae*, *M. kansasii*, *M. fortuitum*, *M. avium* serotype 4B, *M. avium* serotype 8A, *M. tuberculosis* $H_{37}Ra$, and whole-cell proteins from the recombinant TB-2. The blot was incubated with rabbit serum raised against the 35K antigen from TB-2. MW, Molecular size standards; k, kilodaltons. Single proteins of different molecular weights were detected in the various mycobacterial culture filtrates.

12 persons with lepromatous and tuberculoid leprosy, respectively), and none of 14 well persons. In contrast, reactivity to proteins present in *M. tuberculosis* culture filtrate was frequent in sera from persons infected with *M. tuberculosis* and *M. leprae* but was also present in 5 of 14 well persons. In this control group, only one person had a history of a positive tuberculin skin test. No single pattern of reactivity to *M. tuberculosis* culture filtrate could discriminate patients with active tuberculosis from patients with leprosy or from controls.

Since the TB-11 subclone was the smallest fragment which still produced the 35K and 53K proteins, this plasmid was used as a probe to examine selected mycobacteria. For unrestricted chromosomal DNA blotted to nitrocellulose paper, the greatest binding of TB-11 was to *M. tuberculosis*, *M. bovis*, and *M. bovis* BCG. Much less binding to *M. avium*, *M. gordonae*, *M. leprae*, and *M. smegmatis* was detected. In Southern blotting experiments under stringent conditions, TB-11 bound only to *M. tuberculosis*, *M. bovis*, BCG, and *M. gordonae*. The two BCG isolates, *M. bovis*, and *M. tuberculosis* $H_{37}Ra$ had two chromosomal fragments and the *M. gordonae* had a single fragment that shared homology with TB-11. The small *M. bovis*, BCG, and *M. tuberculosis* fragments comigrated with the *M. tuberculosis* DNA insert in TB-11. Under less stringent conditions, homology with *M. avium* but not with *M. smegmatis* was detected (data not shown). Culture filtrates or cell homogenates from other *Mycobacterium* species, with the exception of cell sonic extracts of *M. leprae*, bound the sera from the rabbit immunized with the 35K protein. Although a single protein was present in positive mycobacterial strains, these proteins had slightly different molecular weights (Fig. 5).

TABLE 1. Occurrence of antibodies against recombinant 35K protein and *M. tuberculosis* culture filtrate in selected patient populations

Patient group (no. of patients)	% of patients with antibodies to:	
	Recombinant 35K protein	*M. tuberculosis* culture filtrate
Active tuberculosis (20)	40	90
Leprosy (18)	22	88
Healthy control (14)	0	30

DISCUSSION

Our research is directed towards the development of better tests and reagents for the diagnosis of tuberculosis. We have, therefore, tried to maximize the likelihood of identifying antigenic proteins relevant to naturally occurring infections by choosing to screen recombinant *E. coli* with polyclonal antiserum that was obtained after the injection of live tubercle bacilli. Recombinant organisms can serve as a source of DNA probes or proteins which will be the basis for diagnostic tests or for production of monoclonal antibodies.

We have succeeded in expressing *M. tuberculosis* proteins in *E. coli* by conventional recombinant DNA techniques with pUC13 as the vector. Synthesis of these proteins in the absence of IPTG (*lac* inducer) suggests that *M. tuberculosis* promoters can function in *E. coli*. The three recombinant *E. coli* strains produced the same two 35K and 53K proteins. The reactivity of both proteins to rabbit antiserum produced against the 35K protein suggests that they are polymers, subunits, or degradation products of the same protein. This explanation is also supported by the reactivity of rabbit antiserum prepared to the 35K protein with only a 35K protein in the culture filtrate from *M. tuberculosis*. The 35K protein is a minor component among the culture filtrate antigens. The scarcity may be why is has not been identified in previous attempts to develop useful serologic tests for tuberculosis.

The 35K and 53K proteins bound polyclonal antisera to different strains of *M. tuberculosis* but did not bind antisera to other clinically important *Mycobacterium* spp. Therefore, these proteins could provide a basis for developing a specific serologic test for tuberculosis. Of persons with active tuberculosis, 40% have evidence of antibody to the 35K protein, and no reactivity to it was detected in normal, healthy controls. The reactivity detected in the four persons infected with *M. leprae* may represent *M. bovis* BCG immunization or concomitant infections with *M. tuberculosis*. Little clinical information is available on these cases of active tuberculosis and leprosy other than that the tuberculosis cases were among persons living in Norway and Japan and that the leprosy cases were among Ethiopians. Antibodies to the 35K protein may correlate with specific clinical characteristics such as the stage, site, extent, or activity of the disease. Many patients with well-defined clinical characteristics, including active and inactive tuberculosis, BCG immunization, and other documented mycobacterial diseases, must be screened to determine the full potential of this antigen as a serologic reagent.

DNA homology between the TB-11 probe and closely related organisms such as *M. bovis* BCG is not unexpected. Homology with other mycobacteria under less stringent hybridization conditions and the presence of similar-molecular-weight proteins in culture filtrates from other mycobacteria indicate that related epitopes are present in various mycobacteria. The present clone includes a rather large DNA sequence of 2.2 kilobases. Nevertheless, it is potentially useful as a probe to recognize *M. tuberculosis*. It is likely that oligonucleotide probes generated after sequencing of this protein gene will be useful in the detection of *M. tuberculosis*. The binding of rabbit polyclonal antiserum to similar-molecular-weight proteins in a variety of mycobacteria may represent a cross-reactivity which could limit the usefulness of this protein or antibody to this protein as diagnostic reagents. On the other hand, it is also likely that antigenic dissection of this protein with monoclonal antibodies will identify sequences with various degrees of specificity that would have additional diagnostic potential.

Further studies are necessary to determine if these proteins are specific for cellular immune reactions or if they would be useful as immunizing agents directed at preventing primary infection with tuberculosis. Since the diagnostic potential of skin testing with tuberculin-purified protein derivative, the accepted skin test preparation, is limited by cross-reactions due to infections with mycobacteria other than *M. tuberculosis*, a more specific skin test reagent would have important public health applications. Similarly, a more effective and acceptable vaccine would be of even greater importance. This approach of cloning *M. tuberculosis* proteins may provide the basis for diagnostic tests and vaccines which will make control and eventual eradication of tuberculosis possible.

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