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MEMBRANE-ASSOCIATED IMMUNOGENS OF MYCOBACTERIA

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(57) Claim

1. A recombinant nucleic acid encoding all or part of a membrane-associated polypeptide of a mycobacterium, wherein said polypeptide comprises a transmembrane domain and said mycobacterium is capable of inducing an immune response that is detectable with all or part of said membrane-associated polypeptide.

4. The nucleic acid of Claim 3 wherein said membrane-associated polypeptide comprises an ion-motive ATPase.

11. An isolated membrane-associated polypeptide of a mycobacterium, wherein said polypeptide comprises a transmembrane domain and said mycobacterium is capable of inducing an immune response that is detectable with all or part of said membrane-associated polypeptide.

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(54) Title: MEMBRANE-ASSOCIATED IMMUNOGENS OF MYCOBACTERIA

(57) Abstract

Nucleic acid encoding four novel immunodeterminant protein antigens of *M. bovis* BCG, which is a vaccine strain for tuberculosis, have been isolated. These genes were isolated as immunoreactive recombinant clones from a genomic library of *M. bovis* BCG DNA, constructed in pBR322 vector, and screened with sera collected from tuberculosis patients. The BCG DNA insert of one of the recombinants, pMBB51A, which expressed an antigen of Mr 90 kD, was sequenced completely and an ORF encoding 761 amino acids encoding a protein of deduced molecular weight 79 kD, was identified. This gene was identified to encode a membrane bound, ion-motive ATPase of *M. bovis* BCG. The approach described here can be used to identify immunogens of mycobacteria. In addition, the well-characterized *M. bovis* BCG antigens can be used in the prevention, diagnosis and treatment of tuberculosis. The 79 kD antigen is also useful in the design of recombinant vaccines against different pathogens. The sequence of the 79 kD membrane-associated polypeptides also are useful for the development of specific PCR amplification based diagnostic procedures for the detection of mycobacteria. Also, the promoter of the 79 kD antigen is useful for expressing homologous and/or heterologous antigens in mycobacteria.

MEMBRANE-ASSOCIATED IMMUNOGENS OF MYCOBACTERIA

Technical Field of the Invention

The invention relates to membrane-associated polypeptides of mycobacteria and, in particular, the use of such polypeptides and the nucleic acids encoding them for use as vaccines and diagnostic reagents.

Background of the Invention

The mycobacteria are a diverse collection of acid fast, gram-positive bacteria, some of which cause important human and animal diseases. In humans, the two most common mycobacteria-caused diseases are tuberculosis (TB) and leprosy, which result from infection with M. tuberculosis and M. leprae, respectively.

Tuberculosis displays all of the principal characteristics of a global epidemic disease. Currently, tuberculosis afflicts more than 35 million individuals worldwide and results in over 4 million deaths annually. In India, at any given time, almost 8 million people are reported to suffer from this disease and 500,000 deaths recorded. These figures may not cover the totality of those suffering from this disease in this country. Thus, tuberculosis appears to be a problem of major concern in India as also in many other countries of the world.

Tuberculosis is caused by M. tuberculosis, M. bovis, M. africanum and M. microti, the acid-fast, Gram positive, tubercle bacilli of the family Mycobacteriaceae. Some local pathogenic strains of M. tuberculosis have also been isolated from patients in Madras and other cities in India, which differ in some respects from M. tuberculosis H37Rv, which is a virulent strain.

In recent years, certain groups of individuals with AIDS have been found to have a markedly increased incidence of TB as well. It has now been shown that one group of mycobacteria which consists of M. avium, M. intracellulare and M. scrofulaceum, jointly known as MAIS complex, is responsible for disseminated disease in a large number of persons with AIDS (Kiehn et al., J. Clin. Microbiol., 21:168-173 (1985); Wong et al., Amer. J. Med., 78:35-40 (1985)).

Since Koch identified M. 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. However, characteristics of M. tuberculosis have hampered research to improve diagnosis and to develop more effective vaccines. In addition, the biochemical composition of the organism has made identification and purification of the cellular constituents difficult, and many of these materials once purified, lack sensitivity and specificity as diagnostic reagents. As a result, diagnostic and immunoprophylactic measures for mycobacterial diseases have changed little in the past half century. The conventional methods for the diagnosis of M. tuberculosis are troublesome and results are delayed.

Bacillus Calmette-Guerin (BCG), an avirulent strain of M. bovis (Calmette, A., Masson et Cie, Paris (1936)),

is used extensively as a vaccine against tuberculosis. Though numerous studies have found that it has protective efficacy against tuberculosis (Luelmo, F., Am. Rev. Respir. Dis., 125, 70-72 (1982)) BCG has failed to protect against tuberculosis in several trials (WHO, Tech. Rep. Ser., 651:1-15 (1980)) for reasons that are not entirely clear (Fine, P., Tubercle, 65:137-153 (1984); Fine, et al., Lancet, (ii):499-502 (1986)).

The eradication with vaccination, early diagnosis, and efficient therapy is an important objective of the drive to combat mycobacterioses. The lacunae in the present knowledge of the biology of these pathogens - their make-up, their natural history, their physiology, biochemistry and immunological reactivities, highlights the need for attempts to unravel their weaknesses, so that more efficient ways to combat this disease can be devised. To develop more effective tools for the diagnosis and prevention of these diseases, it is important to understand the immune response to infection by mycobacterial pathogens. The mycobacterial components that are important in eliciting the cellular immune response are not yet well defined. The antibody and T-cell responses to infection or inoculation with killed mycobacteria have been studied in humans and in animals. Human patients with TB or leprosy produce serum antibodies directed against mycobacterial antigens. Although antibodies may have some function in the antimycobacterial immune response, the exact function remains to be clarified since no protective role can be ascribed to these antibodies. Protection against mycobacterial diseases involves cell-mediated immunity.

Mycobacteria do not produce any directly toxic substances and consequently their pathogenicity results

from multiple factors involved in their interaction with the infected host. Intracellular parasitism probably depends on host cell trophic factors; it is conceivable that their short supply may be bacteriostatic and could play a role in the mechanism of mycobacterial dormancy.

It is generally understood that protective immunity in mycobacterial infection is mediated by specific T cells which activate macrophages into non-specific tuberculocidal activity. Evidence suggests that gamma-IFN triggers macrophages towards H_2O_2 -mediated bacterial killing, but related or other macrophage activating factor (MAF) molecules may also be involved. The causes responsible for the inadequate bactericidal function at sites of abundant T cell proliferation have not yet been explained. Dissociation between delayed-type hypersensitivity (DTH) and protective immunity led to views that T-cells of a distinct subset or specificity could be responsible for the acquired resistance to mycobacterial infection. Alternatively, interference with protection may result from corollary cellular reactions, namely by suppressor T-cells and macrophages, or from the shifting of T-cells towards helper function for B-cells.

Unlike viral and some parasite pathogens which can evade host resistance by antigenic shift, mycobacteria have a resilient cell wall structure and can suppress host immune responses by the action of their immunomodulatory cell wall constituents. Whilst the success of protective immunization towards other microbial pathogens mainly depends on quantitative parameters of immunity, it appears that mycobacterial immunomodulatory stimuli produce a regulatory dysfunction of the host immune system. This may not be possible to override simply by more resolute

immunization using vaccines of complex composition such as whole mycobacteria (e.g. BCG). Perhaps mycobacteria did not evolve potent "adjuvant" structures to boost the host immunity but rather to subvert host defenses towards ineffective cellular reactions operating to the advantage of the pathogen. Vaccination with an attenuated pathogen such as BCG could amplify further immune responses but with limited protection of the host, the potential scope for immunization with defined antigens is yet to be explored.

The purification and characterization of individual antigenic proteins are essential in understanding the fundamental mechanism of the DTH reaction on the molecular level. The possible functional role of proteins of defined structure in the pathogenesis of mycobacterial diseases as well as for diagnostic purposes remains of great interest. Numerous groups have attempted to define mycobacterial antigens by standard biochemical and immunological techniques, and common as well as species specific antigens have been reported in mycobacteria (Minden, et al., Infect. Immun., 46:519-525 (1984); Closs, et al., Scand. J. Immunol., 12:249-263 (1980); Chaparas, et al., Am. Rev. Respir. Dis., 122:533 (1980); Daniel, et al., Microbiol. Rev., 42:84-113 (1978); Stanford, et al., Tubercle, 55:143-152 (1974); Kuwabara, S., J. Biol. Chem., 250:2556-2562 (1975)).

Very little information about the mycobacterial genome is available. Initially, basic studies were conducted to estimate the genome size, G+C content and the degree of DNA homology between the various mycobacterial genomes (Grosskinsky, et al., Infect. Immun., 57, 5:1535-1541 (1989); Garcia, et al., J. Gen. Microbiol., 132:2265-2269 (1986); Imaeda, T., Int. J. Sys. Bacteriol., 35, 2:147-150 (1985); Clark-Curtiss,

et al., J. Bacteriol., 161 3:1093-1102 (1985); Baess, I. et al., B., Act. Path. Microbiol. Scand., (1978) 86:309-312; Bradley, S. G., Am. Rev. Respir. Dis., 106:122-124 (1972)). Recently, recombinant DNA techniques have been used for the cloning and expression of mycobacterial genes. Genomic DNA fragments of M. tuberculosis, M. leprae and some other mycobacterial species were used for the construction of lambda gtll phage (Young, et al., Proc. Natl. Acad. Sci., U.S.A., 82:2583-2587 (1985); Young, et al., Nature (London), 316:450-452 (1985)) or other vector-based recombinant gene libraries. These libraries were screened with murine monoclonal antibodies (Engers, et al., Infect. Immun., 48:603-605 (1985); Engers, et al., Infect. Immun., 51:718-720 (1986)) as well as polyclonal antisera and some immunodominant antigens were identified. The principal antigen among these being five 12, 14, 19, 65 & 71 kDa of M. tuberculosis (Young et al., Proc. Natl. Acad. Sci., U.S.A., 82:2583-2587 (1985); Shinnick et al., Infect. Immun., 55(7):1718-1721 (1987); Husson and Young, Proc. Natl. Sc. Acad., 84:1679-1683 (1987); and five 12, 18, 23, 36 & 65 kDa antigens of M. leprae (Young, et al., Nature (London), 316:450-452 (1985)). A few homologues of some of these antigens were also identified in some other mycobacterial species (e.g., BCG) (Yamaguchi et al., FEB 06511, 240:115-117 (1988); Yamaguchi et al., Infect. Immun., 57:283-288 (1989); Matsuo, et al., J. Bacteriol., 170, 9:3847- 3854 (1988); Radford, et al., Infect. Immun., 56, 4:921-925 (1988); Lu, et al., Infect. Immun., 55, 10:2378-2382 (1987); Minden, et al., Infect. Immun., 53, 3:560-564 (1986); Harboe, et al., Infect. Immun., 52, 1:293-302 (1986); Thole, et al., Infect. Immun., 50, 3:800-806 (1985)). These antigens, however, are either intracellular or secreted molecules.

Although M. bovis BCG has been widely used as a vaccine against tuberculosis, the determination of the membrane-associated polypeptides of mycobacterium that are capable of inducing a protective immune response is highly desirable. The use of such a membrane-associated polypeptide or the DNA encoding it provides for the generation of recombinant vaccines, e.g., mycobacterial membrane-associated immunogens expressed in, for example, a virus or bacterium such as vaccinia virus, Salmonella, etc. used as a live carrier, or the display of non-mycobacterial immunogens on the surface of a cultivable mycobacterial strain which can be used as a live recombinant vaccine.

Accordingly, it is an object herein to provide methods for identifying and isolating nucleic acids encoding a membrane-associated polypeptide of mycobacteria.

Further, it is an object herein to provide membrane-associated polypeptides of mycobacteria and the nucleic acids encoding it.

Still further, it is an object herein to provide vaccines utilizing all or part of the membrane-associated polypeptide of a mycobacterium or the DNA encoding such membrane-associated polypeptide.

Still further, it is an object to provide reagents comprising said membrane-associated polypeptide with a mycobacterium or DNA encoding it useful in diagnostic assays for mycobacterial infection.

Still further, it is an object to provide a promoter sequence comprising the promoter of said membrane associated polypeptide, which can direct gene expression in mycobacteria as well as in other microorganisms such as E. coli.

Summary of the Invention

In accordance with the foregoing objects, the invention includes compositions comprising nucleic acid encoding all or part of a membrane-associated polypeptide of a mycobacterium and the membrane-associated polypeptide encoded by said DNA. The membrane-associated polypeptide is characterized by the ability to detect an immune response to pathogenic mycobacteria or the mycobacteria from which the membrane associated polypeptide or part thereof is derived. Such mycobacteria include M. bovis, M. tuberculosis, M. leprae, M. africanum and M. microti, M. avium, M. intracellular and M. scrofulaceum and M. bovis BCG.

A particular mycobacterial membrane-associated polypeptide is a 79 kD ion-motive ATPase. Extra-cellular, intra-cellular and transmembrane domains are identified in this mycobacterial membrane-associated polypeptide based upon its DNA and deduced amino acid sequence.

The invention also includes vaccines utilizing all or part of a membrane-associated mycobacterial polypeptide or an expressible form of a nucleic acid encoding it. The invention also includes mycrobacterial promoter sequences capable of directing gene expression in mycobacteria as well as in other microorganisms such as E. coli. Such promoters are from mycobacterial genes encoding membrane-associated ATPases. A preferred promoter is that of the gene encoding the M. bovis BCG 79 kD membrane-associated polypeptide. This promoter sequence is especially useful to express genes of interest in mycobacteria.

Brief Description of the Drawings

Figure 1 illustrates the results of immunoscreening of recombinant colonies carrying M. bovis BCG DNA (panel A) and M. tuberculosis H37Rv DNA (panel B), using sera from TB patients in which the presence of M. bovis BCG antigens and M. tuberculosis H37Rv antigens capable of reacting with the antisera is indicated by a qualitative signal.

Figure 2 shows the comparison of restriction site maps of recombinant clones carrying BCG DNA identified using the immunoscreening assay described herein (panel B) with the restriction site maps of five immunodominant antigens of M. tuberculosis and M. bovis BCG genomic DNAs, respectively, (Husson and Young, Proc. Natl. Acad. Sci., U.S.A., 84:1679-1683 (1987); Shinnick et al., Infect. Immun., 55:1718-1721 (1987) (panel A)). Restriction maps in each panel have been drawn to the same scale (indicated at the top), and restriction sites are indicated above the restriction maps. The dotted line in panel A represents the non-mycobacterial DNA. Restriction enzymes: B, BamHI, E, EcoRI, G, BglII, K, KpnI, P, PvuI, X, XhoI, H,HincII, U, PvuII, Ps, PstI, Hi, HindIII. In panel A, A is SalI and S is SacI. In panel B, S is SalI.

Figure 3 illustrates the results of Western blot analysis of the sonicated supernate of recombinant clone pMBB51A which carries a BCG DNA insert identified following immunoscreening of the recombinant colonies. The top panel shows reactivity of MBB51A (lane 2) and E. coli (lane 1) with sera from TB patients. The bottom panel (part A) shows reactivity of MBB51A (lanes 1 and 2) and E. coli (lane 3) with anti-H37Rv sera raised in rabbits. Part B shows reactivity of MBB51A (lanes 1 and 2) and E. coli (lane 3) with the second

antibody alone. Arrows indicate the position of the 90 kD immunoreactive BCG protein expressed by the recombinant MBB51A, which was absent in the negative control.

Figure 4 illustrates the nucleotide sequence (Seq. ID No.: 1) of clone pMBB51A 3.25 kb insert DNA containing the M. bovis BCG immunoreactive MBB51A gene encoding an ion-motive ATPase, with a deduced molecular weight of 79 kD. The deduced amino acid sequence (Seq. ID No.: 2) is shown below the nucleotide sequence. Upstream promoter elements are underlined. Transcription termination region is indicated by inverted arrows. 5′ and 3′ flanking regions are also shown.

Figure 5 illustrates a schematic model derived for the 79 kD protein encoded by pMBB51A which represents an ion-motive ATPase of BCG. The model considers only the structural and functional features that are prominent in the other ion-motive ATPase homologs of transmembrane domains of the protein. Functionally, important amino acid residues are indicated (P), proline at position 400; (D), aspartic acid at position 443; (G), glycine at position 521; and (A), alanine at position 646. Numbers indicate amino acid residues broadly defining the limits of the transmembrane domains.

Figure 6 illustrates the results of Southern blot hybridization of BamHI digest of genomic DNAs from M. bovis BCG (lane 6), M. tuberculosis H37Rv (lane 5), M. smegmatis (lane 4) and M. vaccae (lane 3 using pMMB51A DNA insert (lane 8) as probe. Panel A shows ethidium bromide stained gel and panel B shows the results of Southern blot hybridization.

Detailed Description of the Invention

As used herein, a "membrane-associated polypeptide" of a mycobacterium is defined as any Mycobacterial membrane-associated polypeptide which is capable of detecting an immune response against the wild-type mycobacterium containing the membrane-associated polypeptide. However, based upon the observed cross-reactivity of the 79 kD membrane-associated polypeptide of an M. bovis BCG with pooled anti-sera from patients afflicted with tuberculosis and the cross-hybridization as between the DNA encoding the 79 kD membrane-associated polypeptide and the DNA of M. tuberculosis H37Rv, the membrane-associated polypeptide of the invention is not limited to that identified herein from M. bovis BCG. Rather, it encompasses not only homologs to the 79 kD ion-motive ATPase but also any and all membrane-associated polypeptides of a mycobacterium that can be used to detect an immune response by the same or a different mycobacteria in which the membrane-associated polypeptide is normally found.

As used herein, "nucleic acid" includes DNA or RNA as well as modified nucleic acid wherein a detectable label has been incorporated or wherein various modifications have been made to enhance stability, e.g., incorporation of phosphorothioate linkages in the phosphoribose backbone, etc. Such nucleic acid also includes sequences encoding the anti-sense sequence of the DNA encoding the membrane-associated polypeptide such that the now well-known anti-sense technology can be used to modulate expression of such membrane-associated polypeptides.

In some aspects of the invention, the nucleic acid sequence encoding all or part of a membrane-associated polypeptide of the mycobacterium is used as a vaccine.

When so-used the nucleic acid is generally an "expressible nucleic acid" that contains all necessary expression regulation sequences to control transcription and translation of the nucleic acid in a designated host system. In some vaccine embodiments, the DNA encodes a chimeric polypeptide containing at least one transmembrane domain of the membrane-associated polypeptide and an "immunogenic polypeptide". The transmembrane domain is used to display the immunogenic polypeptide on the surface of a particular host organism such as an attenuated live vaccine. When the membrane-associated polypeptide includes more than one transmembrane region, one or more of the transmembrane regions can be used with an immunogenic polypeptide. Thus, for example, the 79 kD ion-motive ATPase as shown in Figure 5 has at least three extracellular domains into which an immunogenic polypeptide can be engineered by well-known methods involving recombinant DNA technology. Although it is preferred that more than one transmembrane region be used to display an immunogenic polypeptide, one skilled in the art can readily vary the length of such a membrane-associated polypeptide to maximize an immunogenic response or to minimize the amount of membrane-associated polypeptide used in such applications.

As used herein, "immunogenic polypeptide" comprises all or part of any polypeptide which can potentially be utilized in a vaccine or diagnostic application. Thus, the immunogenic polypeptide can comprise heterologous immunogens, i.e., immunogens from non-mycobacterial sources, e.g., Salmonella or Shigella or from different mycobacteria from which the membrane-associated polypeptide is derived, e.g., immunogens from Mycobacterium tuberculosis fused to a membrane-associated polypeptide from M. bovis BCG. However, in

some instances homologous immunogens can be used. For example, each of the extracellular domains as set forth in Figure 5 herein can be combined and displayed by combination with one or more of the transmembrane domains from the membrane-associated polypeptide normally containing them. Alternatively, the intercellular domains can be displayed extracellularly using appropriate transmembrane regions from the same molecule.

In an alternate vaccine embodiment, all or part of the membrane-associated polypeptide of mcobacteria, rather than the DNA encoding, is used as part of a vaccine. Such proteinaceous vaccines are formulated with well-known adjuvants and administered following well-established protocols known to those skill d in the art.

In still other embodiments, the nucleic acid encoding the membrane-associated polypeptide of the invention can be used as a diagnostic for detecting infection based upon hybridization with wild-type genes contained by the infectious mycobacterium. Such detection can comprise direct hybridization of DNA extracted from an appropriate diagnostic sample or PCR amplification using the nucleotide sequence of the nucleic acid encoding the membrane-associated polypeptide of the invention to prime amplification. If PCR amplification is primed in a conserved region the presence of mycobacteria in a diagnostic sample can be determined. If primed in a non-conserved region which is species specific the diagnostic assay determined the specific mycobacterium causing an infection.

In addition, the membrane-associated polypeptide of the invention can also be used to detect the presence of antibodies in the sera of patients potentially infected

with mycobacteria. Such detection systems include radioimmunoassays and various modifications thereof which are well-know to those skilled in the art. In addition, the membrane-associated polypeptide of the invention can be used to detect the presence of a cell-mediated immune response in a biological sample. Such assay systems are also well-known to those skilled in the art and generally involve the clonal expansion of a sub-population of T cells responding to stimuli from the membrane-associated polypeptide. When so-used, the humoral and/or cell-mediated response of a patient can be determined and monitored over the course of the disease.

Recombinant clones encoding immunogenic protein antigens of M. bovis BCG have been isolated from a genomic library of M. bovis BCG DNA. In particular, DNA fragments encoding four protein antigens of M. bovis BCG have been isolated by probing a pBR322 library of M. bovis BCG DNA with sera from TB patients, absorbed on E. coli. Restriction site maps of these four recombinant clones are different from those of the five immunodominant antigens of mycobacteria (Young, et al., Proc. Natl. Acad. Sci., U.S.A., 82:2583-2587 (1987); Husson and Young, Proc. Natl. Acad. Sci., U.S.A., 84:1679-1683 (1987); Shinnick et al., Infect. Immun., 55:1718-1721 (1987)), thereby indicating that these cloned protein antigens are novel. One of the recombinant DNA clones encoded an immunoreactive protein with apparent molecular weight of 90 kD as determined by Western blot analysis. The complete nucleotide sequence of the insert DNA of this clone was determined. This clone was found to carry a mycobacterial promoter and a monocistronic ORF encoding a protein of 761 amino acids with a deduced molecular weight of 79 kD. This 79 kD protein had extensive homology with ion-motive ATPases of S. faecalis (Solioz

et al., J. Biol. chem, 262:7358-7362 (1987)), E. coli (Hesse et al., Proc. Natl. Acad. Sci., U.S.A., 81:4746-4750 (1984)) and several other organisms, and thus, represents an ion-motive ATPase or a putative K+ATPase of BCG. Using computer algorithms, this ion-motive ATPase was determined to be a membrane protein and has a homologue in M. tuberculosis H37Rv, which is pathogenic in humans, but not in M. vaccae and M. smegmatis, which are non-pathogenic. As a result, novel BCG immunogens can be available which can be useful in the prevention, diagnosis and treatment of tuberculosis and other mycobacterial infections. They can be used, for example, in the development of highly specific serological tests for screening patients for individuals producing antibodies to M. tuberculosis, or those infected with M. tuberculosis, in the development of vaccines against the disease, and in the assessment of the efficacy of the treatment of infected individuals.

Further, based on the nucleotide sequence of the pMBB51A insert DNA, appropriate oligonucleotide primers can be used for PCR amplification using as template M. bovis BCG or M. tuberculosis H37Rv DNA. Such a PCR amplification scheme can be thus useful for the detection of mycobacterial DNA in a given sample. Further, by a judicious choice of the primer design, such an amplification procedure can be adapted for taxonomic classification of mycobacterial DNAs. For example, using primers to flank a heavily conserved region such as the ATP-binding site, PCR amplification is common to all mycobacterial species, whereas using primers from non-conserved areas, amplification can be made species specific.

Example I

Isolation and Characterization of Genes Encoding Immogenic Protein Antigens of Mycobacterium bovis BCG and Mycobacterium tuberculosis H37R

A. Construction of Recombinant DNA Libraries of M. bovis BCG DNA and Mycobacterium Tuberculosis H37Rv

A recombinant DNA library of M. bovis BCG genomic DNA was constructed using pBR322 a high copy number plasmid vector (Bolivar, et al., Gene, 2:95-113 (1977)) with antibiotic markers (ampicillin and tetracycline) and several unique cloning sites. M. bovis BCG cells were harvested from a culture in late logarithmic phase of growth and high molecular weight DNA was isolated by the procedure of (Eisenach, et al., J. Mol. Biol., 179:125-142 (1986)) with slight modifications. BCG DNA was digested to completion with BamH I and shotgun cloning of these fragments into the BamH I site of pBR322 was performed. The genomic library was transformed into E. coli strain DHI and recombinants were scored on the basis of ampicillin resistance and tetracycline sensitivity. The aim of this approach was to generate restriction fragments of a broad size range so as not to restrict the library to DNA fragments of any particular size range. This cloning strategy also ensured to a large extent that any recombinants selected for expression of mycobacterial antigens should be likely to drive expression from a mycobacterial promoter rather than the Tet promoter of pBR322.

The BCG library constructed in this manner contained 2051 clones of BCG origin. In an analogous manner, a genomic library of Mycobacterium tuberculosis H37Rv DNA was constructed and 1100 clones obtained.

The BCG DNA inserts ranged in size from 0.9 to 9.5 kb. The average size of the mycobacteria DNA fragments inserted in pBR322 was estimated to be about 4 kb. Given the genome size of BCG to be 4.5 x 10^3kb (Bradley, S. G., J. Bacteriol., 113:645-651 (1973); Imaeda, et al., Int. J. Syst. Bacteriol., 32, 456-458 (1982)), about 1000 clones of this average insert size would represent comprehensively the entire genome of the microorganism.

B. Isolation of Recombinant DNA Clones Encoding BCG Mycobacterium bovis BCG and Mycobacterium tuberculosis H37Rv Protein Antigens

In order to identify recombinants expressing mycobacterial antigens, a colony immunoscreening assay (CIA) to screen recombinant colonies with appropriate antisera, was established. Sera obtained from 20 patients newly diagnosed with active pulmonary tuberculosis were pooled for use in immunoscreening. None of the patients had received treatment for tuberculosis prior to this study and their sputa were positive for acid fast bacteria in all cases. Pooled sera were absorbed on a E. coli sonicate overnight at 4°C, to eliminate antibodies cross-reactive to E. coli antigens, thereby improving signal to noise ratio during the immunoscreening.

Individual recombinant colonies were grown overnight on nitrocellulose membranes and immunoscreening was carried out as described with slight modifications. The colonies were lysed in chloroform vapor to release the cloned mycobacterial antigens, immobilized on the nitrocellulose paper. The immobilized antigens were reacted with TB sera and binding of the antibody was revealed by standard procedures using a horseradish peroxidase-protein A detection system. The signals

obtained with the recombinant clones were compared with that obtained in case of E. coli colonies harbouring pBR322 vector alone, which served as the negative control, to assess the signal to noise ratio. Further, to ascertain whether the immunoreactivity of the recombinant clones was due to anti-mycobacterial antibodies or due to a reaction with normal serum components, another CIA of the selected recombinants was performed using TB sera and normal human sera NHS which had been absorbed on E. coli in a manner analogous to that described earlier for TB sera. Only those clones reacting selectively with TB sera and not with NHS, were considered to be unambiguously suggestive of the presence of mycobacterial antigens. The use of this immunoscreening approach to identify recombinant colonies carrying mycobacterial DNA inserts capable of expressing mycobacterial antigens is described below:

Figure 1 shows the result of immunoscreening of recombinant colonies carrying M. bovis BCG DNA (panel A) or M. tuberculosis H37 Rv DNA (panel B) using sera from TB patients. The colonies were grown on nitrocellulose paper overnight, lysed to release the cloned mycobacterial antigen and allowed to react with the antibodies. The presence of mycobacterial antigen is indicated by a qualitative signal in the recombinant clones which is absent in the negative control comprising colonies harbouring pBR322 vector alone. A similar assay was repeated with normal human serum to ascertain the specificity of the cloned mycobacterial antigens. 51 recombinant colonies carrying M. bovis BCG DNA inserts and 45 recombinant colonies carrying M. tuberculosis H37Rv DNA inserts were screened by the above procedure; 14 clones of BCG origin (panel A) and 2 clones of H37Rv origin (panel B) exhibited distinct strong signals indicating the immunoreactivity of these

clones (Fig. 1). All these clones were also tested for immunoreactivity with NHS. However, with the exception of 3 clones which showed a slight reactivity to NHS, none of the clones reacted with NHS, thereby indicating that these expressed mycobacterial antigens reacted selectively with TB sera. Thus, this procedure resulted in the forthright identification of recombinant clones encoding mycobacterial antigens. This strategy can be generally applicable to mycobacterial gene banks prepared in plasmid or cosmid vectors to identify genes which are expressed in E. coli at least to the limit detectable by the immunoassay.

C. Restriction Mapping of Immunoreactive Mycobacterium bovis BCG DNA Recombinants

The insert DNAs of four of the immunoreactive BCG recombinant DNA clones isolated using the TB sera were mapped with restriction endonucleases. Figure 2, panel B, shows the genomic DNA restriction site maps deduced for the cloned BCG DNA in four recombinants, in which, A represents Sal I, B, BamH I, E, EcoR I, G, Bgl II, K, Kpn I, P, Pvu I, S, Sac I, X, Xho I. These restriction site maps were then compared with those constructed previously for the five immunodominant antigens of M. tuberculosis/M. bovis BCG (Young, et al., Proc. Natl. Acad. Sci., U.S.A., 82:2583-2587 (1985); Husson, et al., Proc. Natl. Acad. Sci., 84:1679-1683 (1987); Shinnick, et al., Infect. Immun., 55, 7:1718-1721 (1987)) (Figure 2, panel A). Since the restriction site maps shown in panels A and B have been drawn to the same scale, the differences between the two are apparent. There are no regions of similarity between the restriction site maps of immunoreactive BCG recombinant clones and those of the previously characterized immunodominant antigens of M.

tuberculosis/M. bovis BCG. Therefore, one can conclude that the cloned BCG DNA inserts in the four recombinants are novel.

Example II

Isolation and Characterization of a Gene Encoding a BCG Ion-motive ATPase

A. Identification of a Novel BCG Antigen

One of the four immunoreactive BCG clones, pMBB51A, revealed the presence of a protein of Mr 90 kD, on Western blot analysis using TB sera as well as anti-H37Rv polyclonal antiserum raised in rabbits (Figure 3). Similar Western blot analysis of pMBB51A with a pool of a few anti-mycobacterial monoclonal antibodies (TB 23, TB 71, TB 72, TB 68, TB 78; Engers et al., Infec. Immun., 48:603-605 (1985)) or with normal human sera did not reveal this immunoreactive protein of 90 kD. This confirms that pMBB51A encodes a BCG antigen which is different from those identified previously in BCG, thereby making it a novel antigen.

B. Determination of the Nucleotide Sequence of pMBB51A

In order to further characterize this novel BCG antigen, pMBB51A DNA insert was subjected to nucleotide sequencing. The BamH I-BamH I insert carried in pMBB51A was mapped for additional restriction enzyme cleavage sites. It was determined that there were at a minimum a single Pst I site and 3 Sal I sites in this sequence. Overlapping fragments derived from single and double digests of Sal I, BamH I and Sal I, BamH I and Pst I, and Pst I and Sal I, were subcloned into M13mp18 and M13mp19 vectors, in preparation for DNA sequence analysis. DNA sequencing was then carried out

using commercially available kits such as the Sequenase system and the T7 system from Pharmacia. Oligonulceotides derived from the determined sequence were synthesized and used as primers to complete the sequence of the larger inserts. Several areas of compression were encountered during the sequencing and these were resolved by using dITP in the sequencing reactions, and by changing the reaction conditions. The complete nucleotide sequence of the pMBB51A insert DNA was determined by sequencing both the strands using dGTP as well as dITP. The DNA sequence of the pMBB51A insert was determined to be 3.25 kb long with a GC content of 67.1% and is shown in Figure 4.

The determination of the DNA sequence of the 3.25 kb insert of clone pMBB51A (Figure 4) permitted the elucidation of the amino acid sequence of the 90 kD BCG antigen. In Figure 4, nucleotides are numbered from the left end of the pMBB51A insert DNA.

A search of pMBB51A insert DNA sequence for possible ORFs in all three reading frames revealed the longest ORF of 2286 bp encoding a polypeptide of 761 amino acids on one of the strands. The other strand was found to have a smaller URF of 1047 bp capable of encoding a polypeptide of 349 amino acids. The longest ORF encoding a 761 amino acid long protein corresponded to a deduced molecular weight of 79 kD which came closest to the immunoreactive BCG protein with apparent molecular weight of 90 kD, seen on the Western blot. The deduced amino acid sequence for this protein is given below the nucleotide sequence in Figure 4.

The location of this ORF on the pMBB51A insert DNA was such that there were long stretches of flanking DNA sequences, devoid of any meaningful ORFs, present on either side. This precluded the expression of this ORF

from the pBR322 Tet gene promoter and instead suggested that this ORF was being expressed from its own promoter in pMBB51A. This also suggested that E. coli may correctly utilize the M. bovis BCG transcription and translation start and stop sites in this gene.

Immediately upstream of the ORF, regulatory sequences closely matching the -35, -10 and Shine-Dalgarno sequences of E. coli, (Rosenberg, et al., Annul. Rev. Genet., 13:319-353 (1979)) were identified. The spacing between these three regulatory motifs was also very well conserved. Although the other mycobacterial promoters sequenced (Dale, et al., Molecular Biology of the Mycobacteria, chap. 8, 173-198 (1990)) show some differences from the E. coli consensus sequences in all the three regions -35, -10 and SD, the regulatory elements of pMBB51A DNA showed a maximum degree of sequence identity with E. coli in the -35 and SD sequence elements with a single mismatch in each element, and about 50% sequence identity in the Pribnow box. All the above features clearly indicated that this region is the promoter region for the mycobacterial gene contained in pMBB51A. The extent of similarity between this BCG promoter sequence and a typical E. coli promoter is remarkable and explains the functional activity of this promoter, unlike many other mycobacterial promoters, in E. coli. The translation initiation codon in this ORF was ATG at position 508 while a single translation termination codon TGA was identified at position 2790. Potential transcription termination structures capable of forming stem and loop conformations were identified in the region 3' to this ORF. The pMBB51A ORF thus represented a monocistronic gene rather than an operon. The promoter region of MBB51A gene is capable of directing gene expression in E. coli as well as in mycobacteria. This promoter sequence is useful for directing expression of

mycobacterial genes in E. coli. Further, this promoter sequence can also be used to express homologous and/or heterologous genes in a mycobacterium, thus providing a key element for the development of gene expression systems in mycobacteria.

In order to derive information about the possible biological function of the MBB51A protein, the amino acid sequence of this protein was used to search for homology against available sequences in the PIR Protein Database Release 20 (Table I) and a Genebank Nucleic Acid Database (Table II) using the Fast A suite of programmes written by (Lipman and Pearson, Proc. Natl. Acad. Sci., USA, 85:2 (1988)). The MBB51A protein sequence exhibited homology to a family of ion-motive ATPases from different organisms, ranging from bacteria to mammals. The 13 best scores from a search with ktuple 2 are shown in the upper panel of Table I and 10 best scores from a search with ktuple 1 are shown in the lower panel. In each case, MBB51A protein exhibited maximum homology (75.9% homology in a 593 amino acid overlap with 31.9% identity to a K+ transporting ATPase of S. faecalis (Solioz et al., 1987). The next best homology was observed with the B-chain of K+ transporting ATPase of E. coli (Hesse, et al., Proc. Natl. Acad. Sci., U.S.A., 81:4746-4750 (1984)) (68.8% homology in a 397 amino acid overlap with 24.2% identity). A lesser extent of homology was also seen with H+, Ca++ and Na+-ATPases from different organisms. The results of homology search thus indicated that MBB51A protein is an ion-motive ATPase of M. bovis BCG and is closely related to the other bacterial ion-motive ATPases. This is the first report of the cloning and identification of such an ATPase in mycobacteria. The BCG ion-motive ATPase showed homologies with other ion-motive ATPases with overlapping regions ranging in size from 593 amino

acids in case of S. faecalis to 82 amino acids as in case of L. donovani, (Meade, et al., Mol. Cell Biol., 7, 3937-3946 (1987)), though most of the regions of sequence identity or conservation were localized in the C-terminal half of the MBB51A protein. Further, a region of 30 amino acids in the C-terminal half of MBB51A protein was found to be shared with most of these ATPases, thereby suggesting the functional importance of this region. Detailed alignment of MBB51A protein with the K+ ATPases of S. faecalis and E. coli also indicated that several residues were conserved between the three ATPases, including the ones that are invariant in all ATPases from bacteria to man.

TABLE I

RESULTS OF HOMOLOGY SEARCH OF MBB51A AMINO ACID SEQUENCE AGAINST PIR PROTEIN DATABASE

ktuple : 2

LOCUS	SHORT DEFINITION	initn	opt
>A29576	Potassium - transporting ATPase Streptococcus	547	792
>PWECBK	Potassium - transporting ATPase, β chain - E.coli	314	270
>A25939	Proton - transporting ATPase - Neurospora	168	186
>A25823	Proton - transporting ATPase - Yeast	166	184
>PWRBFC	Calcium - transporting ATPase, fast twitch skele	152	158
>PWRBSC	Calcium - transporting ATPase, slow twitch skele	135	157
>A25344	Potassium - transporting ATPase - Rat	78	155
>RDEBHA	Mercuric reductase -Shigella flexneri plasmid	99	142
>RDPSHA	Mercuric reductase (transposon Tn501)	74	124
>RGPSHA	Mercuric resistance operon regulatory p	79	109
>A24639	Sodium/potassium-transporting ATPase, alpha	92	82
>A24414	Sodium/potassium-transporting ATPase, alpha	92	82
>B24862 Sodium/potassium-transporting ATPase, beta	83	82	

The PIR protein data base (2378611 residues in 9124 sequences) was scanned with the FASTA program. The mean of the original initial score was 27.2 with a standard deviation of 6.9. Initial scores (initn) higher than 75.6 are 6 standard deviations above the average, a level of significance that usually indicates biological relatedness. Optimization (opt) generally will improve the initial score of related proteins by introducing gaps in the sequence. Unrelated sequences usually do not have their scores improved by optimization.

ktuple : 1

>A29576	potassium-transporting ATPase - Streptococcus	744	792
>PWECBK	potassium-transporting ATPase, β chain - Eschc	386	270
>A25939	Proton -transporting ATPase - Neurospora crassa	310	186
>A25823	proton-transporting ATPase -Yeast (Saccharomy)	317	184
>B24639 Sodium/potassium-transporting ATPase, alpha (+	158	163	
>A24639	Sodium/potassium-transporting ATPase, alpha ch	175	160
>C24639	Sodium/potassium-transporting ATPase,alpha (II	192	159
>PWRBFC	Calcium-transporting ATPase, fast twitch skele	240	158
>PWSHNA	Sodium/potassium-transporting ATPase, alpha skele	214	158
>A24414	Sodium/potassium-transporting ATPase, alpha chain	214	158

TABLE II

RESULTS OF HOMOLOGY SEARCH OF MBB51A AMINO ACID SEQUENCE AGAINST GENBANK NUCLEIC ACID SEQUENCE DATABASE

ktuple : 2

LOCUS	SHORT DEFINITION		initn	opt
>STRATPK	S.faecalis K+ ATPase, complete cds.	537	800	
>ECOKDPABC	E.coli kdpABC operon coding for Kdp-ATpase	314	270	
>YSPPMA1A	S.pombe H+ ATPase, complete cds.		135	188
>NEUATPASE	N.crassa plasma membrane ATPase, complete	133	186	
>NEUATPPM	Neurospora crassa plasma membrane H+ ATPase	131	186	
>YSCPMA1	Yeast PMA1 for plasma membrane ATPase		166	184
>M17889	Figure 2. N of L.donovani ATPase and		166	170
>M12898	Rabbit fast twitch skeletal muscle Ca++ ATPas		140	158
>RABATPAC	Rabbit Ca + Mg dependent Ca++ ATPase mRNA, co		142	157
>NR1MER	Plasmid NR1 mercury resistance (mer) operon.		100	143

ktuple : 1

>STRATPK	S.faecalis K+ ATPase gene, complete cds.	744	800
>SYNCATPSB	Cyanobacterium Synechococcus 6301 DNA for AT	379	422
>ECOKDPABC	E.coli kdpABC operon coding for Kdp-ATPase p	379	270
>YSPPMA1A	S.pombe H+ ATPase gene, complete cds.	275	188
>NEUATPASE	N.crassa plasma membrane ATPase gene, comple	311	186
>NEUATPPM	Neurospora crassa plasma membrane H+ ATPase	302	186
>YSCPMA1	Yeast PMA1 gene for plasma membrane ATPase	317	184
>JO4004	Leishmania donovani. cation transporting ATP	322	170
>M17889	Figure 2. Nucleotide seguence of L.donovani		306	170
>RATATPA2	Rat Na+,K+ ATPase alpha (+) isoform catalytic	158	163	

* * *

The KdpB protein of E. coli and possibly the S. faecalis K+ ATPase are members of E1E2-ATPases which are known to form an aspartyl phosphate intermediate, with cyclic transformation of the enzyme between phosphorylated and dephosphorylated species. By analogy to other ATPases, the phosphorylated Asp residue (D) (Furst, et al., J. Biol. Chem., 260:50-52 (1985)) was

identified at position 443 in the MBB51A ATPase. This residue is the first of a pentapeptide sequence DKTGT that has been conserved in ATPases from bacteria to man, and must form an essential element of the catalytic site. Similarly, proline (P) at position 400 in MBB51A ATPase was found to be an invariant amino acid in other ATPases and is predicted to be located in a membrane spanning domain. Such membrane buried proline residues have been hypothesized to be required for the reversible conformational changes necessary for the regulation of a transport channel (Brandl, et al., Proc. Natl. Acad. Sci., U.S.A., 83:917-921 (1986)). In addition, other sequence motifs believed to be functionally important in other ion-motive ATPases were also found to be conserved in the MBB51A ATPase. These include a Gly (G) (Farley and Faller, J. Biol. Chem., 260:3899-3901 (1985)) at position 521 and Ala (A) (Ohta, et al., Proc. Natl. Acad. Sci., U.S.A., 83:2071-2075 (1986)) at position 646, and are shown in Figure 5.

Since the MBB51A ATPase was homologous to membrane associated ATPases, characterization of the membrane associated helices in MBB51A protein was performed by computer algorithms. Using a hydropathy profile (Rao, et al., Biochem. Biophys. Acta., 869:197-214 (1986)), seven transmembrane domains in the MBB51A protein were identified and are shown in Table III and Figure 5. Nearly the same transmembrane domains were also identified using the hydrophobic moment plot (Eisenberg et al., J. Mol. Biol., 179:125-142 (1984)) and are also shown in Table III and Figure 5. The average size of a transmembrane domain is around 21 residues, because 21 residues coil into an α-helix approximately the thickness of the apolar position of a lipid bilayer (32 Å). This size of a transmembrane domain is, however, flexible within the range of a few amino acids, as

determined by the functional properties of a given membrane-associated protein. The transmembrane domains identified in MBB51A protein, range in size from 20-37 residues. The first six transmembrane domains span the membrane only once, as indicated by both the hydropathy profile and the hydrophobic moment plot. The seventh transmembrane domain may traverse the membrane twice. These features along with the membrane buried proline (P) at position 400, are in accordance with the channel transport functions of ion-motive ATPases, involving a reversible change in the conformation of these proteins. Such transmembrane domains further define the intracellular and extracellular domains of this molecule. See Figure 5.

Table III

Transmembrane Domain in Fig. 5	Eisenberg Method	Rao & Argos Method
1	102 - 122	98 - 125
2	129 - 149	127 - 147
3	164 - 184	164 - 185
4	199 - 219	198 - 220
5	361 - 381	360 - 382
6	387 - 407	387 - 419
7	703 - 723	695 - 732

The hydropathy profile of MBB51A protein was nearly superimposable over that of S. faecalis K+ ATPase, even though the MBB51A ATPase has at the N-terminus, 154 extra amino acids, which were absent in S. faecalis. This clearly puts in evidence the strong evolutionary conservation of the broad domain structure between these two proteins, making it more likely for the two proteins to have a similar three dimensional structural organization.

Based on the hydropathy profile and secondary structure predictions, a schematic model of the MBB51A ATPase is presented in Figure 5. This model comprises at least seven transmembrane domains which span the membrane once are indicated along with the respective amino acid positions in Figure 5. This model further defines extracellular and intracellular domains of the MBB51A protein. Many of the residues which have been shown to be functionally important in other ion-motive ATPases and are also conserved in the MBB51A protein, are also shown. Of these, proline (P) at position 400 is membrane-buried whereas as aspartic acid(D) at 443, glycine (G) at 521 and alanine (A) at 646, face the cytoplasm.

In order to determine whether the gene encoding MBB51A ion-motive ATPase is present in other mycobacterial strains related or unrelated to BCG, like the virulent strain M. tuberculosis H37Rv and other non-tuberculous, non-pathogenic mycobacteria like M. vaccae and M. smegmatis, Southern blot hybridization with genomic DNA from the above species was performed, using as probe BCG insert DNA from pMBB51A. As shown in Figure 6, DNA hybridizable with the pMBB51A insert DNA was also present in M. tuberculosis H37Rv DNA but not in M. smegmatis and M. vaccae. This indicated that the M. tuberculosis H37Rv homologue of the pMBB51A gene has a similar genetic organization as seen in M. bovis BCG DNA, and is present on a 3.25 kb BamH I fragment.

The availability of novel Mycobacterium bovis BCG and/or Mycobacterium tuberculosis H37Rv antigens make it possible to address basic biochemical, immunological, diagnostic and therapeutic questions still unanswered about tuberculosis and Mycobacterium tuberculosis. For example, Mycobacterium tuberculosis specific antigenic determinants can be used to develop

simple and specific seroepidemiological tests to screen human populations. Such serological tests are highly specific because of the use of antigenic determinants determined by the approaches described above and known to be unique to Mycobacterium tuberculosis H37Rv. Such serological tests are useful for early diagnosis of tuberculosis, thus permitting early treatment and limiting transmission of the disease from infected individuals to others.

Resistance to tuberculosis is provided by cell mediated immunity. The antigens identified here can be further used to determine which segments of these antigens are recognized by Mycobacterium tuberculosis specific T-cells. A mixture of peptides recognized by helper T-cells provides a specific skin test antigen for use in assessing the immunological status of patients and their contacts. A mixture of such peptides is also useful in evaluating rapidly the immunological efficacy of candidate vaccines. In addition peptides recognized by Mycobacterium tuberculosis specific T-cells can be components of a vaccine against the disease.

Knowledge of the complete nucleotide sequence of pMBB51A DNA insert provides a rich source of sequence information which can be used to design appropriate primers for PCR amplification of mycobacterial genomic DNA fragments. The ion-motive ATPase of BCG has areas of heavily conserved sequences (for, e.g., the ATP binding site) which are expected to be the same for all mycobacterial species and areas of sequence divergence (for, e.g., the N-terminal region) which are different in different mycobacterial species. Based on this knowledge primers can be designed either from the conserved regions or from the diverged regions to identify whether in a given sample the target DNA is mycobacterial versus non-mycobacterial, and in case of

mycobacterial DNA, which mycobacterial species the DNA belongs.

Such amplification schemes are useful for the development of highly sensitive and specific PCR amplification based diagnostic procedures for mycobacteria. The observation that the 3.25kb pMBB51A DNA insert is present in Mycobacterium tuberculosis H37Rv and Mycobacterium bovis BCG and is absent in avirulent Mycobacterium vaccae and Mycobacterium smegmatis, which have bearing on other aspects of the biological differences between these species, manifest in terms of virulence, growth characteristics and metabolism.

Recombinant vaccines can also be constructed by incorporating the DNA encoding all or part of the membrane-associated polypeptides of the invention into an appropriate vaccine vehicle. For example, all or part of the DNA encoding the 79kD Mycobacterium bovis BCG protein or a portion of the protein can be incorporated into a vaccine vehicle capable of expressing the said DNA. Such a vaccine vehicle could be a virus for, e.g., vaccinia virus, etc., or a bacterium, e.g., mycobacteria, Salmonella, Vibrio, Bacillus, Yersinia, Bordetella, etc. to produce a vaccine capable of conferring long-lasting immunity on individuals to whom it is administered.

A special feature of the 79kD BCG ion-motive ATPase is that it is a membrane bound antigen. Therefore, it can be used to link foreign DNA sequences encoding antigenic epitopes (B-cell epitopes or T-cell epitopes) of interest, with this gene or a portion of this gene in a manner which causes the foreign epitope to be used as an immunogen. Such linkages can be engineered into extracellular or intracellular domains of MBB51A

protein, or into a combination of both types of domains. Engineering of immunogenic foreign epitopes into MBB51A DNA is accomplished by standard recombinant DNA methods known to those skilled in the art. Some of these methods involve use of unique restriction sites, in vitro mutagenesis and/or PCR-related methods. One such convenient method involves the use of a unique NdeI site at position 1090 in the MBB51A DNA where foreign DNA can be inserted. Grafting of epitopes on the cell surface induces rapid antibody response by virtue of the epitope being well-exposed on the bacterial cell, which in turn leads to direct activation of B cells. In addition, intracellular localization of an epitope induces B cell memory and a proficient T cell response. Examples of epitopes of interest known to be involved in the immune response to various pathogens include epitopes from E. coli LT toxin, foot and mouth disease virus, HIV, cholera toxin, etc.

Thus, the 79 kD antigen is useful in the design of recombinant vaccines against different pathogens. Such vaccines comprise a recombinant vaccine vehicle capable of expressing all or part of the 79 kD membrane-associated protein of mycobacteria, into which foreign epitopes have been engineered, such that the foreign epitopes are expressed on the outer surface and/or on the inner side of the cell membrane, thereby rendering the foreign epitopes immunogenic. The vaccine vehicle for this purpose may be a cultivable mycobacterium for, e.g., BCG. In these applications, the BCG ion-motive ATPase gene can be borne on a mycobacterial shuttle vector or alternately the foreign DNA encoding antigenic epitopes of the immunogenic polypeptides can be inserted into the mycobacterial genome via homologous recombination in the ion-motive ATPase gene or random integration. Such a process yields stable

recombinant mycobacterial strains capable of expressing on their surface and/or in the cytoplasm antigenic sequences of interest, which can, for example, provide protection against a variety of infectious pathogens. Targeting of recombinant antigens to the cell-wall is attractive not only because of the high immunogenicity of mycobacterial cell-walls but, in addition, because of concerns with the introduction of a live vaccine in populations with a high prevalence of HIV seropositivity. Additionally, based on the MBB51A protein, a non-living but immunogenic recombinant cell surface subunit vaccine can also be developed to provide a useful alternative to live vaccines. Alternately, other bacterial, viral or protozoan vaccine vehicles could be transformed to generate such recombinant vaccines. Examples of potential vaccine vehicles include vaccinia virus, pox-viruses, Salmonella, Yerisinia, Vibrio, Bordetella, Bacillus, etc.

Further, using such an approach, multivalent recombinant vaccines which allow simultaneous expression of multiple protective epitopes/antigens of different pathogens, could also be designed.

Equivalents

Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, many equivalents to the specific materials and components described specifically herein. Such equivalents are intended to be encompassed in the scope of the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Munshi, Anil
Kapoor, Archana

(ii) TITLE OF INVENTION: Membrane-Associated Immunogens of Mycobacteria

(iii) NUMBER OF SEQUENCES: 2

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: PCT/US/93/
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(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Trecartin, Richard F
(B) REGISTRATION NUMBER: 31,801
(C) REFERENCE/DOCKET NUMBER: FP-57004/RFT

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3250 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 508..2790

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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GGATCCCGCG GTCATCGATC GGGTCAAACA CCGCCTCGAC GGGTTCACGC TGGCGCCGCT
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GTCCACCGCC GCGGGAGGTG GTGGCCGGCA GCCACGCATC TACTACGGCA CCATCCTGAC
   120

CGGTGACCAA TACCTTCACT GCGAGCGCAC CCGCAACCGG CTGCACCACG AACTCGGCGG
   180

TATGGCCGTC GAAATGGAAG GCGGTGCGGT GGCGCAAATC TGCGCGTCCT TCGATATCCC
   240

ATGGCTGGTC ATTCGCGCGC TCTCCGATCT CGCCGGAGCC GATTCGGGGG TGGACTTCAA
   300

TCGGTTTGTC GGCGAGGTGG CGGCCAGTTC GGCCCGCGTT CTGCTGCGCT TGCTGCCGGT
   360

GTTGACGGCC TGTTGAAGAC GACTATCCGC CGGTGCGTTC ACCGCGTCAG GCGGCTTCGG
   420

TGAGGTGAGT AATTTGGTCA TTAACTTGGT CATGCCGCCG CCGATGTTGA GCGGAGGCCA
   480

CAGGTCGGCC GGAAGTGAGG AGCCACG ATG ACG GCG GCC GTG ACC GGT GAA
   531
                                 Met Thr Ala Ala Val Thr Gly Glu
                                   1               5

CAC CAC GCG AGT GTG CAG CGG ATA CAA CTC AGA ATC AGC GGG ATG TCG
   579
His His Ala Ser Val Gln Arg Ile Gln Leu Arg Ile Ser Gly Met Ser
    10                  15                  20

TGC TCT GCG TGC GCC CAC CGT GTG GAA TCG ACC CTC AAC AAG CTG CCG
   627
Cys Ser Ala Cys Ala His Arg Val Glu Ser Thr Leu Asn Lys Leu Pro
 25                  30                  35                  40

GGG GTT CGG GCA GCT GTG AAC TTC GGC ACC CGG GTG GCA ACC ATC GAC
   675
Gly Val Arg Ala Ala Val Asn Phe Gly Thr Arg Val Ala Thr Ile Asp
                45                  50                  55

ACC AGC GAG GCG GTC GAC GCT GCC GCG CTG TGC CAG GCG GTC CGC CGC
   723
Thr Ser Glu Ala Val Asp Ala Ala Ala Leu Cys Gln Ala Val Arg Arg
            60                  65                  70

GCG GGC TAT CAG GCC GAT CTG TGC ACG GAT GAC GGT CGG AGC GCG AGT
   771
Ala Gly Tyr Gln Ala Asp Leu Cys Thr Asp Asp Gly Arg Ser Ala Ser
        75                  80                  85
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GAT CCG GAC GCC GAC CAC GCT CGA CAG CTG CTG ATC CGG CTA GCG ATC
     819
Asp Pro Asp Ala Asp His Ala Arg Gln Leu Leu Ile Arg Leu Ala Ile
      90                  95                 100

GCC GCC GTG CTG TTT GTG CCC GTG GCC GAT CTG TCG GTG ATG TTT GGG
     867
Ala Ala Val Leu Phe Val Pro Val Ala Asp Leu Ser Val Met Phe Gly
105                 110                 115                 120

GTC GTG CCT GCC ACG CGC TTC ACC GGC TGG CAG TGG GTG CTA AGC GCG
     915
Val Val Pro Ala Thr Arg Phe Thr Gly Trp Gln Trp Val Leu Ser Ala
                125                 130                 135

CTG GCA CTG CCG GTC GTG ACC TGG GCG GCG TGG CCG TTT CAC CGC GTT
     963
Leu Ala Leu Pro Val Val Thr Trp Ala Ala Trp Pro Phe His Arg Val
            140                 145                 150

GCG ATG CGC AAC GCC CGC CAC CAC GCC GCC TCC ATG GAG ACG CTA ATC
    1011
Ala Met Arg Asn Ala Arg His His Ala Ala Ser Met Glu Thr Leu Ile
        155                 160                 165

TCG GTC GGT ATC ACG GCC GCC ACG ATC TGG TCG CTG TAC ACC GTC TTC
    1059
Ser Val Gly Ile Thr Ala Ala Thr Ile Trp Ser Leu Tyr Thr Val Phe
    170                 175                 180

GGC AAT CAC TCG CCC ATC GAG CGC AGC GGC ATA TGG CAG GCG CTG CTG
    1107
Gly Asn His Ser Pro Ile Glu Arg Ser Gly Ile Trp Gln Ala Leu Leu
185                 190                 195                 200

GGA AGC GAT GCT ATT TAT TTC GAG GTC GCG GCG GGT GTC ACG GTG TTC
    1155
Gly Ser Asp Ala Ile Tyr Phe Glu Val Ala Ala Gly Val Thr Val Phe
                205                 210                 215

GTG CTG GTG GGG CGG TAT TTC GAG GCG CGC GCC AAG TCG CAG GCG GGC
    1203
Val Leu Val Gly Arg Tyr Phe Glu Ala Arg Ala Lys Ser Gln Ala Gly
            220                 225                 230

AGT GCG CTG AGA GCC TTG GCG GCG CTG AGC GCC AAG GAA GTA GCC GTC
    1251
Ser Ala Leu Arg Ala Leu Ala Ala Leu Ser Ala Lys Glu Val Ala Val
        235                 240                 245

CTG CTA CCG GAT GGG TCG GAG ATG GTC ATC CCG GCC GAC GAA CTC AAA
    1299
Leu Leu Pro Asp Gly Ser Glu Met Val Ile Pro Ala Asp Glu Leu Lys
    250                 255                 260
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GAA CAG CAG CGC TTC GTG GTG CGT CCA GGG CAG ATA GTT GCC GCC GAC
   1347
Glu Gln Gln Arg Phe Val Val Arg Pro Gly Gln Ile Val Ala Ala Asp
265                 270                 275                 280

GGC CTC GCC GTC GAC GGG TCC GCT GCG GTC GAC ATG AGC GCG ATG ACC
   1395
Gly Leu Ala Val Asp Gly Ser Ala Ala Val Asp Met Ser Ala Met Thr
                285                 290                 295

GGC GAG GCC AAA CCG ACC CGG GTG CGT CCG GGG GGG CAG GTC ATC GGC
   1443
Gly Glu Ala Lys Pro Thr Arg Val Arg Pro Gly Gly Gln Val Ile Gly
            300                 305                 310

GGC ACC ACA GTG CTT GAC GGC CGG CTG ATC GTG GAG GCG GCC GCG GTG
   1491
Gly Thr Thr Val Leu Asp Gly Arg Leu Ile Val Glu Ala Ala Ala Val
        315                 320                 325

GGC GCC GAC ACC CAG TTC GCC GGA ATG GTC CGC CTC GTT GAG CAA GCG
   1539
Gly Ala Asp Thr Gln Phe Ala Gly Met Val Arg Leu Val Glu Gln Ala
    330                 335                 340

CAG GCG CAA AAG GCC GAC GCA CAG CGA CTA GCC GAC CGG ATC TCC TCG
   1587
Gln Ala Gln Lys Ala Asp Ala Gln Arg Leu Ala Asp Arg Ile Ser Ser
345                 350                 355                 360

GTG TTT GTT CCC GCT GTG TTG GTT ATC GCG GCA CTA ACC GCA GCC GGA
   1635
Val Phe Val Pro Ala Val Leu Val Ile Ala Ala Leu Thr Ala Ala Gly
                365                 370                 375

TGG CTA ATC GCC GGG GGA CAA CCC GAC CGT GCC GTC TCG GCC GCA CTC
   1683
Trp Leu Ile Ala Gly Gly Gln Pro Asp Arg Ala Val Ser Ala Ala Leu
            380                 385                 390

GCC GTG CTT GTC ATC GCC TGC CCG TGT GCC CTG GGG CTG GCG ACT CCG
   1731
Ala Val Leu Val Ile Ala Cys Pro Cys Ala Leu Gly Leu Ala Thr Pro
        395                 400                 405

ACC GCG ATG ATG GTG GCC TCT GGT CGC GGT GCC CAG CTC GGA ATA TTT
   1779
Thr Ala Met Met Val Ala Ser Gly Arg Gly Ala Gln Leu Gly Ile Phe
    410                 415                 420

CTG AAG GGC TAC AAA TCG TTG GAG GCC ACC CGC GCG GTG GAC ACC GTC
   1827
Leu Lys Gly Tyr Lys Ser Leu Glu Ala Thr Arg Ala Val Asp Thr Val
425                 430                 435                 440
```

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GTC TTC GAC AAG ACC GGC ACC CTG ACG ACG GGC CGG CTG CAG GTC AGT
   1875
Val Phe Asp Lys Thr Gly Thr Leu Thr Thr Gly Arg Leu Gln Val Ser
                445                 450                 455

GCG GTG ACC GCG GCA CCG GGC TGG GAG GCC GAC CAG GTG CTC GCC TTG
   1923
Ala Val Thr Ala Ala Pro Gly Trp Glu Ala Asp Gln Val Leu Ala Leu
            460                 465                 470

GCC GCG ACC GTG GAA GCC GCG TCC GAG CAC TCG GTG GCG CTC GCG ATC
   1971
Ala Ala Thr Val Glu Ala Ala Ser Glu His Ser Val Ala Leu Ala Ile
        475                 480                 485

GCC GCG GCA ACG ACT CGG CGA GAC GCG GTC ACC GAC TTT CGC GCC ATA
   2019
Ala Ala Ala Thr Thr Arg Arg Asp Ala Val Thr Asp Phe Arg Ala Ile
    490                 495                 500

CCC GGC CGC GGC GTC AGC GGC ACC GTG TCC GGG CGG GCG GTA CGG GTG
   2067
Pro Gly Arg Gly Val Ser Gly Thr Val Ser Gly Arg Ala Val Arg Val
505                 510                 515                 520

GGC AAA CCG TCA TGG ATC GGG TCC TCG TCG TGC CAC CCC AAC ATG CGC
   2115
Gly Lys Pro Ser Trp Ile Gly Ser Ser Ser Cys His Pro Asn Met Arg
                525                 530                 535

GCG GCC CGG CGC CAC GCC GAA TCG CTG GGT GAG ACG GCC GTA TTC GTC
   2163
Ala Ala Arg Arg His Ala Glu Ser Leu Gly Glu Thr Ala Val Phe Val
            540                 545                 550

GAG GTC GAC GGC GAA CCA TGC GGG GTC ATC GCG GTC GCC GAC GCC GTC
   2211
Glu Val Asp Gly Glu Pro Cys Gly Val Ile Ala Val Ala Asp Ala Val
        555                 560                 565

AAG GAC TCG GCG CGA GAC GCC GTG GCC GCC CTG GCC GAT CGT GGT CTG
   2259
Lys Asp Ser Ala Arg Asp Ala Val Ala Ala Leu Ala Asp Arg Gly Leu
    570                 575                 580

CGC ACC ATG CTG TTG ACC GGT GAC AAT CCC GAA TCG GCG GCG GCC GTG
   2307
Arg Thr Met Leu Leu Thr Gly Asp Asn Pro Glu Ser Ala Ala Ala Val
585                 590                 595                 600

GCT ACT CGC GTC GGC ATC GAC GAG GTG ATC GCC GAC ATC CTG CCG GAA
   2355
Ala Thr Arg Val Gly Ile Asp Glu Val Ile Ala Asp Ile Leu Pro Glu
                605                 610                 615
```

```
GGC AAG GTC GAT GTC ATC GAG CAG CTA CGC GAC CGC GGA CAT GTC GTC
   2403
Gly Lys Val Asp Val Ile Glu Gln Leu Arg Asp Arg Gly His Val Val
            620                 625                 630

GCC ATG GTC GGT GAC GGC ATC AAC GAC GGA CCC GCA CTG GCC CGT GCC
   2451
Ala Met Val Gly Asp Gly Ile Asn Asp Gly Pro Ala Leu Ala Arg Ala
        635                 640                 645

GAT CTA GGC ATG GCC ATC GGG CGC GGC ACG GAC GTC GCG ATC GGT GCC
   2499
Asp Leu Gly Met Ala Ile Gly Arg Gly Thr Asp Val Ala Ile Gly Ala
    650                 655                 660

GCC GAC ATC ATC TTG GTC CGC GAC CAC CTC GAC GTT GTA CCC CTT GCG
   2547
Ala Asp Ile Ile Leu Val Arg Asp His Leu Asp Val Val Pro Leu Ala
665                 670                 675                 680

CTT GAC CTG GCA AGG GCC ACG ATG CGC ACC GTC AAA CTC AAC ATG GTC
   2595
Leu Asp Leu Ala Arg Ala Thr Met Arg Thr Val Lys Leu Asn Met Val
                685                 690                 695

TGG GCA TTC GGA TAC AAC ATC GCC GCG ATT CCC GTC GCC GCT GCC GGA
   2643
Trp Ala Phe Gly Tyr Asn Ile Ala Ala Ile Pro Val Ala Ala Ala Gly
            700                 705                 710

CTG CTC AAC CCC CTG GTG GCC GGT GCG GCC ATG GCG TTC TCA TCG TTC
   2691
Leu Leu Asn Pro Leu Val Ala Gly Ala Ala Met Ala Phe Ser Ser Phe
        715                 720                 725

TTC GTG GTC TCA AAC AGC TTG CGG TTG CGC AAA TTT GGG CGA TAC CCG
   2739
Phe Val Val Ser Asn Ser Leu Arg Leu Arg Lys Phe Gly Arg Tyr Pro
    730                 735                 740

CTA GGC TGC GGA ACC GTC GGT GGG CCA CAA ATG ACC GCG CCG TCG TCC
   2787
Leu Gly Cys Gly Thr Val Gly Gly Pro Gln Met Thr Ala Pro Ser Ser
745                 750                 755                 760

GCG TGATGCGTTG TCGGGCAACA CGATATCGGG CTCAGCGGCG ACCGCATCCG
   2840
Ala

GTCTCGGCCG AGGACCAGAG GCGCTTCGCC ACACCATGAT TGCCAGGACC GCGCCGATCA
  2900

CCACCGGCAG ATGAGTCAAA ATCCGCGTGG TGCTGACCGC GCCGGACAGC GCATCCACAA
  2960
```

TCACATAGCC GGTCAGTATG GCGACGAACG CCGTCAGAAC ACCGGCCAGG CCGGCGGCGG
3020

CGCTCGGCCA TAGCGCCGCG CCCACCATGA TCACACCGAG CGCAATCGAC CACGACGTGA
3080

CTCGTTGAGC AAGTGGGTGC CGGCACCCGT CGGGTGCTGA TGGGTCAGGC CGACGTCTAG
3140

GCCAAACCCC TGCACGGTGC CCAGGGCGAT CTGCGCGATG CCCACGCACA GCAACGCCCA
3200

ACGTCGCCAG GTCATCGGTG AATGTTGCCG CCGCGGCGCC CGGCGGATCC
3250

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 761 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Thr Ala Ala Val Thr Gly Glu His His Ala Ser Val Gln Arg Ile
1 5 10 15

Gln Leu Arg Ile Ser Gly Met Ser Cys Ser Ala Cys Ala His Arg Val
20 25 30

Glu Ser Thr Leu Asn Lys Leu Pro Gly Val Arg Ala Ala Val Asn Phe
35 40 45

Gly Thr Arg Val Ala Thr Ile Asp Thr Ser Glu Ala Val Asp Ala Ala
50 55 60

Ala Leu Cys Gln Ala Val Arg Arg Ala Gly Tyr Gln Ala Asp Leu Cys
65 70 75 80

Thr Asp Asp Gly Arg Ser Ala Ser Asp Pro Asp Ala Asp His Ala Arg
85 90 95

Gln Leu Leu Ile Arg Leu Ala Ile Ala Ala Val Leu Phe Val Pro Val
100 105 110

Ala Asp Leu Ser Val Met Phe Gly Val Val Pro Ala Thr Arg Phe Thr
115 120 125

Gly Trp Gln Trp Val Leu Ser Ala Leu Ala Leu Pro Val Val Thr Trp
130 135 140

Ala Ala Trp Pro Phe His Arg Val Ala Met Arg Asn Ala Arg His His
145 150 155 160

Ala Ala Ser Met Glu Thr Leu Ile Ser Val Gly Ile Thr Ala Ala Thr
165 170 175

Ile Trp Ser Leu Tyr Thr Val Phe Gly Asn His Ser Pro Ile Glu Arg
180 185 190

Ser Gly Ile Trp Gln Ala Leu Leu Gly Ser Asp Ala Ile Tyr Phe Glu
195 200 205

Val Ala Ala Gly Val Thr Val Phe Val Leu Val Gly Arg Tyr Phe Glu
210 215 220

Ala Arg Ala Lys Ser Gln Ala Gly Ser Ala Leu Arg Ala Leu Ala Ala
225 230 235 240

Leu Ser Ala Lys Glu Val Ala Val Leu Leu Pro Asp Gly Ser Glu Met
245 250 255

Val Ile Pro Ala Asp Glu Leu Lys Glu Gln Gln Arg Phe Val Val Arg
260 265 270

Pro Gly Gln Ile Val Ala Ala Asp Gly Leu Ala Val Asp Gly Ser Ala
275 280 285

Ala Val Asp Met Ser Ala Met Thr Gly Glu Ala Lys Pro Thr Arg Val
290 295 300

Arg Pro Gly Gly Gln Val Ile Gly Gly Thr Thr Val Leu Asp Gly Arg
305 310 315 320

Leu Ile Val Glu Ala Ala Ala Val Gly Ala Asp Thr Gln Phe Ala Gly
325 330 335

Met Val Arg Leu Val Glu Gln Ala Gln Ala Gln Lys Ala Asp Ala Gln
340 345 350

Arg Leu Ala Asp Arg Ile Ser Ser Val Phe Val Pro Ala Val Leu Val
355 360 365

Ile Ala Ala Leu Thr Ala Ala Gly Trp Leu Ile Ala Gly Gly Gln Pro
370 375 380

Asp Arg Ala Val Ser Ala Ala Leu Ala Val Leu Val Ile Ala Cys Pro
385 390 395 400

Cys Ala Leu Gly Leu Ala Thr Pro Thr Ala Met Met Val Ala Ser Gly
405 410 415

Arg Gly Ala Gln Leu Gly Ile Phe Leu Lys Gly Tyr Lys Ser Leu Glu
420 425 430

Ala Thr Arg Ala Val Asp Thr Val Val Phe Asp Lys Thr Gly Thr Leu
435 440 445

Thr Thr Gly Arg Leu Gln Val Ser Ala Val Thr Ala Ala Pro Gly Trp
450 455 460

Glu Ala Asp Gln Val Leu Ala Leu Ala Ala Thr Val Glu Ala Ala Ser
465 470 475 480

Glu His Ser Val Ala Leu Ala Ile Ala Ala Ala Thr Thr Arg Arg Asp
485 490 495

Ala Val Thr Asp Phe Arg Ala Ile Pro Gly Arg Gly Val Ser Gly Thr
500 505 510

Val Ser Gly Arg Ala Val Arg Val Gly Lys Pro Ser Trp Ile Gly Ser
515 520 525

Ser Ser Cys His Pro Asn Met Arg Ala Ala Arg Arg His Ala Glu Ser
530 535 540

Leu Gly Glu Thr Ala Val Phe Val Glu Val Asp Gly Glu Pro Cys Gly
545 550 555 560

Val Ile Ala Val Ala Asp Ala Val Lys Asp Ser Ala Arg Asp Ala Val
565 570 575

Ala Ala Leu Ala Asp Arg Gly Leu Arg Thr Met Leu Leu Thr Gly Asp
580 585 590

Asn Pro Glu Ser Ala Ala Ala Val Ala Thr Arg Val Gly Ile Asp Glu
595 600 605

Val Ile Ala Asp Ile Leu Pro Glu Gly Lys Val Asp Val Ile Glu Gln
610 615 620

Leu Arg Asp Arg Gly His Val Val Ala Met Val Gly Asp Gly Ile Asn
625 630 635 640

Asp Gly Pro Ala Leu Ala Arg Ala Asp Leu Gly Met Ala Ile Gly Arg
645 650 655

Gly Thr Asp Val Ala Ile Gly Ala Ala Asp Ile Ile Leu Val Arg Asp
660 665 670

His Leu Asp Val Val Pro Leu Ala Leu Asp Leu Ala Arg Ala Thr Met
675 680 685

Arg Thr Val Lys Leu Asn Met Val Trp Ala Phe Gly Tyr Asn Ile Ala
690 695 700

Ala Ile Pro Val Ala Ala Ala Gly Leu Leu Asn Pro Leu Val Ala Gly
705 710 715 720

Ala Ala Met Ala Phe Ser Ser Phe Phe Val Val Ser Asn Ser Leu Arg
725 730 735

Leu Arg Lys Phe Gly Arg Tyr Pro Leu Gly Cys Gly Thr Val Gly Gly
740 745 750

Pro Gln Met Thr Ala Pro Ser Ser Ala
755 760

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A recombinant nucleic acid encoding all or part of a membrane-associated polypeptide of a mycobacterium, wherein said polypeptide comprises a transmembrane domain and said mycobacterium is capable of inducing an immune response that is detectable with all or part of said membrane-associated polypeptide.

2. The nucleic acid of Claim 1 wherein said mycobacterium is selected from the group consisting of M. bovis, M. tuberculosis, M. leprae, M. africanum, and M. microti, M. avium, M. intracellular and M. scrofulaceum.

3. The nucleic acid of Claim 1 wherein said mycobacterium is M. bovis BCG.

4. The nucleic acid of Claim 3 wherein said membrane-associated polypeptide comprises an ion-motive ATPase.

5. The nucleic acid of Claim 4 wherein said ATPase has a deduced molecular weight of about 79kD.

6. The nucleic acid of Claim 1 wherein said membrane-associated polypeptide is encoded by a DNA sequence capable of hybridizing with nucleic acid containing all or part of the DNA SEQUENCE ID NO: 1.

7. The nucleic acid of Claim 6 wherein said nucleic acid encodes at least an extracellular domain of said membrane-associated polypeptide.

8. The nucleic acid of Claim 6 wherein said nucleic acid encodes at least an intracellular domain of said membrane-associated polypeptide.

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9. The nucleic acid of Claim 6 wherein said nucleic acid encodes at least one transmembrane domain of said membrane-associated polypeptide.

10. The nucleic acid of Claim 9 wherein said nucleic acid encodes a chimeric polypeptide comprising said at least one transmembrane domain and an immunogenic polypeptide.

11. An isolated membrane-associated polypeptide of a mycobacterium, wherein said polypeptide comprises a transmembrane domain and said mycobacterium is capable of inducing an immune response that is detectable with all or part of said membrane-associated polypeptide.

12. The membrane-associated polypeptide of Claim 11 wherein said mycobacterium is selected from the group consisting of M. bovis, M. tuberculosis, M. leprae, M. africanum, and M. microti, M. arium, M. intracellular and M. scrofulaceum.

13. The membrane-associated polypeptide of Claim 11 wherein said mycobacterium is M. bovis BCG.

14. The membrane-associated polypeptide of Claim 13 wherein said membrane-associated polypeptide comprises an ion-motive ATPase.

15. The membrane-associated polypeptide of Claim 14 wherein said ATPase has a deduced molecular weight of about 79kD.

16. The membrane-associated polypeptide of Claim 11 wherein said membrane-associated polypeptide is encoded by a nucleic acid capable of hybridizing with a nucleic acid encoding all or part of DNA SEQUENCE ID NO:1.

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17. The membrane-associated polypeptide of Claim 16 wherein said polypeptide comprises at least an extracellular domain of said membrane-associated polypeptide.

18. The membrane-associated polypeptide of Claim 16 wherein said polypeptide comprises at least an intracellular domain of said membrane-associated polypeptide.

19. The membrane-associated polypeptide of Claim 16 wherein said polypeptide comprises at least one transmembrane domain of said membrane-associated polypeptide.

20. The membrane-associated polypeptide of Claim 19 wherein said polypeptide comprises a chimeric polypeptide comprising said at least one transmembrane domain and an immunogenic polypeptide.

21. A vaccine comprising all or part of a membrane-associated polypeptide of a mycobacterium or expressible nucleic acid encoding all or part of said polypeptide, in a recombinant vaccine vehicle capable of expressing said DNA, wherein the vaccine vehicle comprises a virus or a bacterium.

22. The vaccine of Claim 21 wherein said membrane-associated polypeptide is an ion-motive ATPase of a mycobacterium.

23. An isolated nucleic acid comprising a promoter sequence from an ion-motive ATPase of a mycobacterium.

24. An isolated nucleic acid comprising a sequence which encodes an *M. bovis* BCG protein having a sequence shown in SEQ. ID NO. 2.

25. An isolated nucleic acid comprising a sequence which encodes a protein of a mycobacterium other than an *M. bovis* BCG protein wherein said protein

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is a homolog of the BCG protein having the sequence shown in SEQ ID NO. 2;

is an immunogenic membrane-associated protein of said mycobacterium, and

is encoded by DNA which will hybridize with a DNA probe derived from the sequence presented in SEQ ID NO. 1.

Dated this 7th day of May, 1997.

Archana Ka[poor
AND
Anil Munshi
By their Patent Attorneys
Davies Collison Cave

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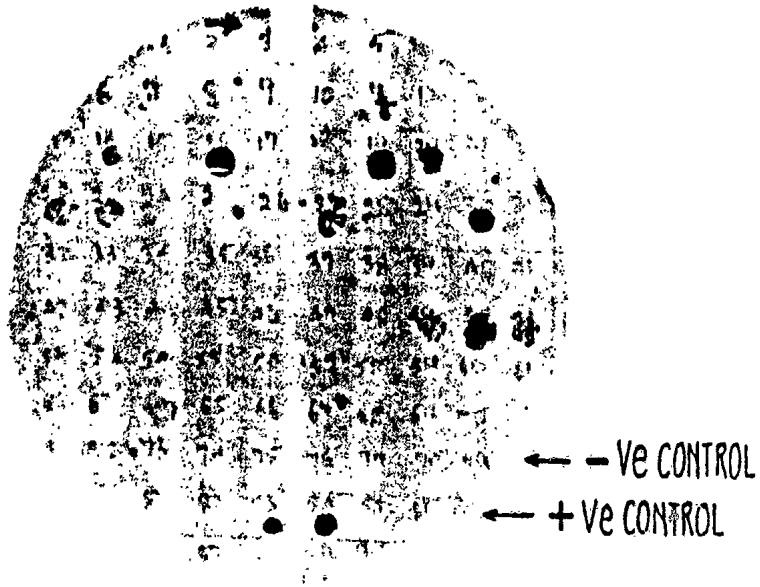


Fig. 1A

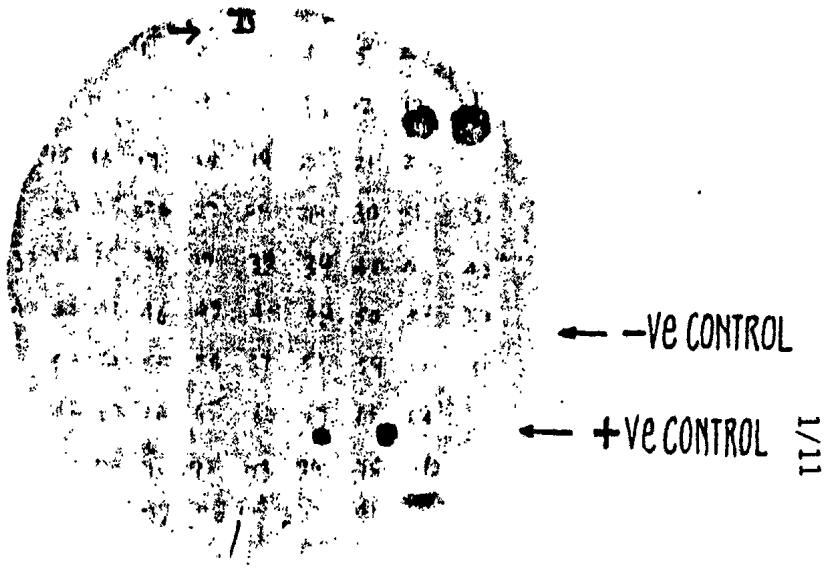
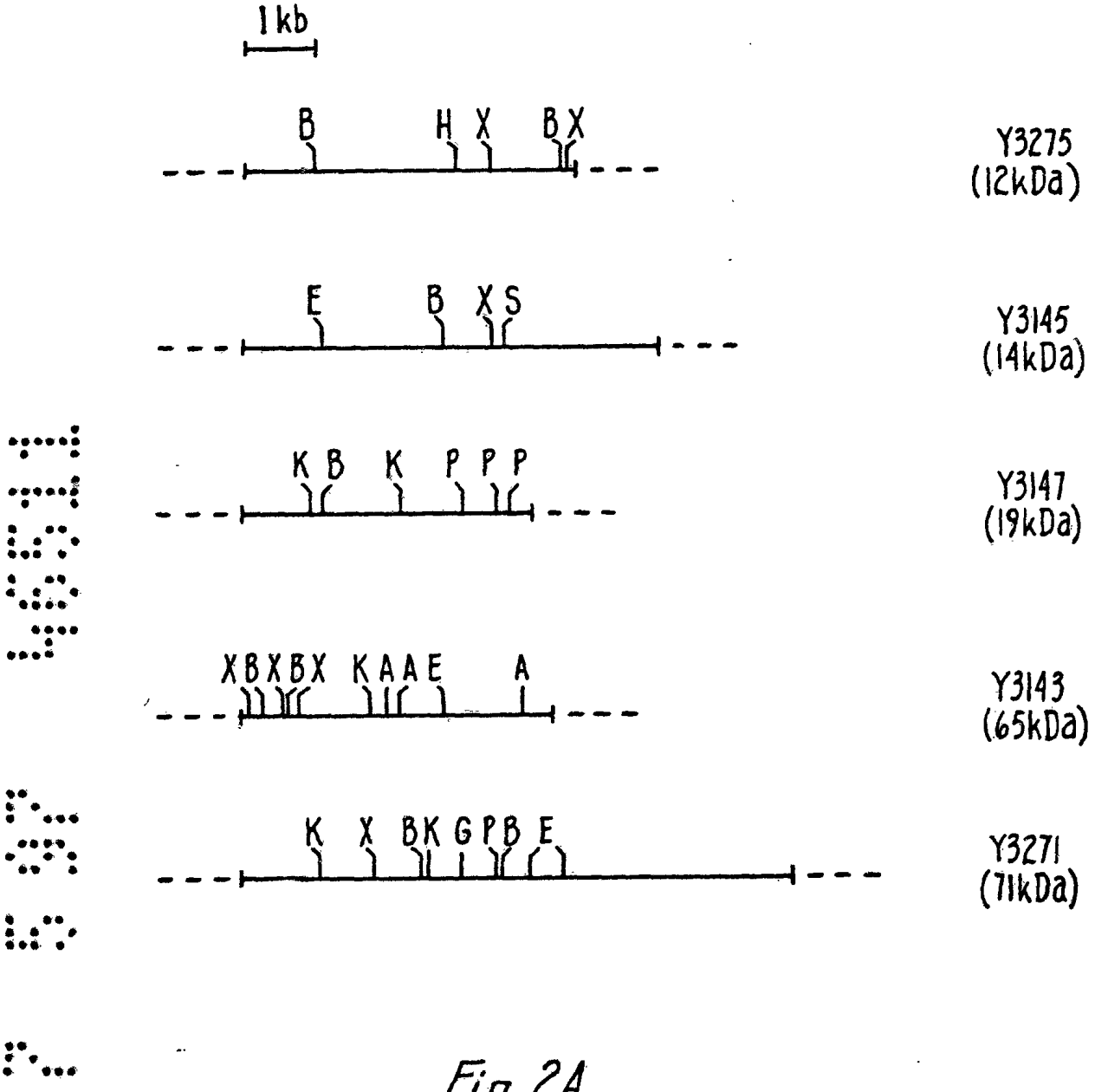


Fig. 1B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Y3143
(65kDa)
K X B K G P B E
Y3271
(71kDa)

Fig. 2A

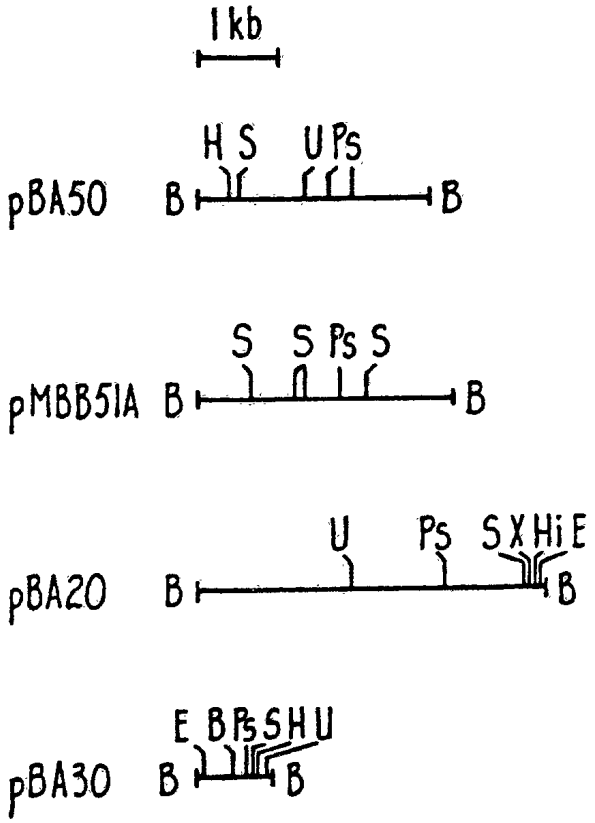
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Fig. 2B

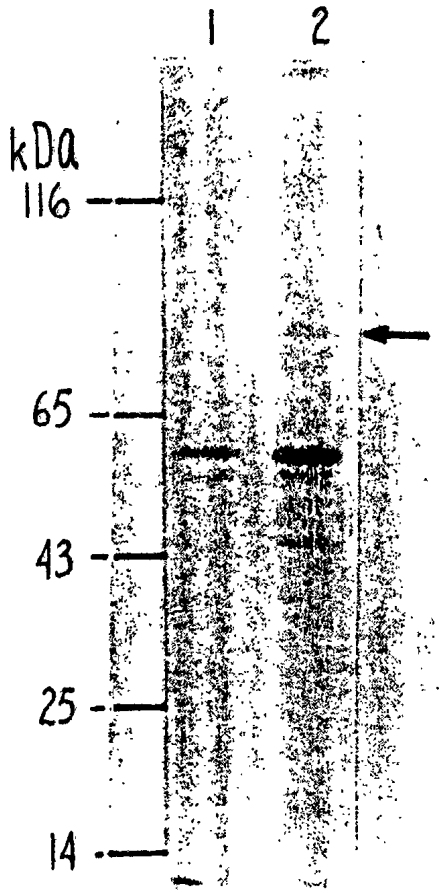

Fig. 3A

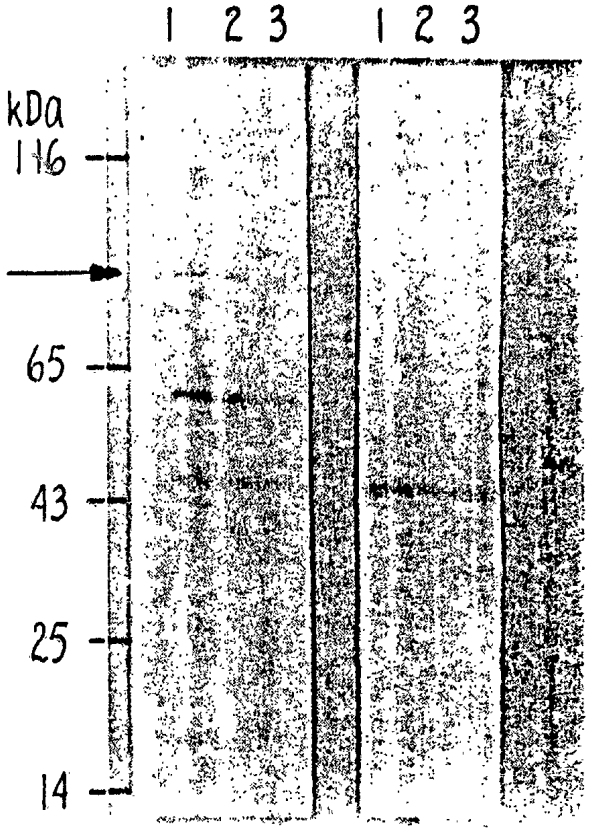

Fig. 3B Fig. 3C

7 5 97 46511

Fig. 4A

```
                                                                                  80
GGATCCCGCGGTCATCGATCGGGTCAAACACCGCCTCGACGGGTTCACGCTGGCGCCGCTGTCCACCGCCGCGGGAGGTG
GTGGCCGGCAGCCACGCATCTACTACGGCACCATCCTGACCGGTGACCAATACCTTCACTGCGAGCGCACCCGCAACCGG
CTGCACCACGAACTCGGCGGTATGGCCGTCGAAATCGAAGGCGGTGCGGTGGCGCAAATCTGCGCGTCCTTCGATATCCC
ATGGCTGGTCATTCGCGCGCTCTCCGATCTCGCCGGAGCCGATTCGGGGGTGGACTTCAATCGGTTTGTCGGCGAGGTGG

                                                -35                  -10        480
CGGCCAGTTCGGCCCGCGTTCTGCTGCGCTTGCTGCCGGTGTTGACGGCCTGTTGAAGACGACTATCCGCCGGTGCGTTC

ACCGCGTCAGGCGGCTTCGGTGAGGTGAGTAATTTGGTCATTAACTTGGTCATGCCGCCGCCGATGTTGAGCGGAGGCCA

                        S/D      508
CAGGTCGGCCGGAAGTGAGGAGCCACG ATG ACG GCG GCC GTG ACC GGT GAA CAC CAC GCG AGT GTG
                             MET Thr Ala Ala Val Thr Gly Glu His His Ala Ser Val
                               1

    550                                                                  600
CAG CGG ATA CAA CTC AGA ATC AGC GGG ATG TCG TGC TCT GCG TGC GCC CAC CGT GTG GAA
Gln Arg Ile Gln Leu Arg Ile Ser Gly MET Ser Cys Ser Ala Cys Ala His Arg Val Glu
                          20
                                                          650
TCG ACC CTC AAC AAG CTG CCG GGG GTT CGG GCA GCT GTG AAC TTC GGC ACC CGG GTG GCA
Ser Thr Leu Asn Lys Leu Pro Gly Val Arg Ala Ala Val Asn Phe Gly Thr Arg Val Ala
                          40
                                                  700
ACC ATC GAC ACC AGC GAG GCG GTC GAC GCT GCC GCG CTG TGC CAG GCG GTC CGC CGC GCG
Thr Ile Asp Thr Ser Glu Ala Val Asp Ala Ala Ala Leu Cys Gln Ala Val Arg Arg Ala
                          60
                                  750
GGC TAT CAG GCC GAT CTG TGC ACG GAT GAC GGT CGG AGC GCG AGT GAT CCG GAC GCC GAC
Gly Tyr Gln Ala Asp Leu Cys Thr Asp Asp Gly Arg Ser Ala Ser Asp Pro Asp Ala Asp
                          80
                 800
CAC GCT CGA CAG CTG CTG ATC CGG CTA GCG ATC GCC GCC GTG CTG TTT GTG CCC GTG GCC
His Ala Arg Gln Leu Leu Ile Arg Leu Ala Ile Ala Ala Val Leu Phe Val Pro Val Ala
                         100
```

Fig. 4B

```
 850                                                                    900
GAT CTG TCG GTG ATG TTT GGG GTC GTG CCT GCC ACG CGC TTC ACC GGC TGG CAG TGG GTG
Asp Leu Ser Val MET Phe Gly Val Val Pro Ala Thr Arg Phe Thr Gly Trp Gln Trp Val
                        120
                                                        950
CTA AGC GCG CTG GCA CTG CCG GTC GTG ACC TGG GCG GCG TGG CCG TTT CAC CGC GTT GCG
Leu Ser Ala Leu Ala Leu Pro Val Val Thr Trp Ala Ala Trp Pro Phe His Arg Val Ala
                        140
                                        1000
ATG CGC AAC GCC CGC CAC CAC GCC GCC TCC ATG GAG ACG CTA ATC TCG GTC GGT ATC ACG
MET Arg Asn Ala Arg His His Ala Ala Ser MET Glu Thr Leu Ile Ser Val Gly Ile Thr
                        160
                           1050
GCC GCC ACG ATC TGG TCG CTG TAC ACC GTC TTC GGC AAT CAC TCG CCC ATC GAG CGC AGC
Ala Ala Thr Ile Trp Ser Leu Tyr Thr Val Phe Gly Asn His Ser Pro Ile Glu Arg Ser
                1100    180
GGC ATA TGG CAG GCG CTG CTG GGA AGC GAT GCT ATT TAT TTC GAG GTC GCG GCG GGT GTC
Gly Ile Trp Gln Ala Leu Leu Gly Ser Asp Ala Ile Tyr Phe Glu Val Ala Ala Gly Val
                        200
 1150                                                                  1200
ACG GTG TTC GTG CTG GTG GGG CGG TAT TTC GAG GCG CGC GCC AAG TCG CAG GCG GGC AGT
Thr Val Phe Val Leu Val Gly Arg Tyr Phe Glu Ala Arg Ala Lys Ser Gln Ala Gly Ser
                        220
                                                        1250
GCG CTG AGA GCC TTG GCG GCG CTG AGC GCC AAG GAA GTA GCC GTC CTG CTA CCG GAT GGG
Ala Leu Arg Ala Leu Ala Ala Leu Ser Ala Lys Glu Val Ala Val Leu Leu Pro Asp Gly
                        240
                                        1300
TCG GAG ATG GTC ATC CCG GCC GAC GAA CTC AAA GAA CAG CAG CGC TTC GTG GTG CGT CCA
Ser Glu MET Val Ile Pro Ala Asp Glu Leu Lys Glu Gln Gln Arg Phe Val Val Arg Pro
                        260
                           1350
GGG CAG ATA GTT GCC GCC GAC GGC CTC GCC GTC GAC GGG TCC GCT GCG GTC GAC ATG AGC
Gly Gln Ile Val Ala Ala Asp Gly Leu Ala Val Asp Gly Ser Ala Ala Val Asp MET Ser
                        280
```

1400

GCG ATG ACC GGC GAG GCC AAA CCG ACC CGG GTG CGT CCG GGG GGG CAG GTC ATC GGC GGC
Ala MET Thr Gly Glu Ala Lys Pro Thr Arg Val Arg Pro Gly Gly Gln Val Ile Gly Gly
300

1450 1500

ACC ACA GTG CTT GAC GGC CGG CTG ATC GTG GAG GCG GCC GCG GTG GGC GCC GAC ACC CAG
Thr Thr Val Leu Asp Gly Arg Leu Ile Val Glu Ala Ala Ala Val Gly Ala Asp Thr Gln
320

1550

TTC GCC GGA ATG GTC CGC CTC GTT GAG CAA GCG CAG GCG CAA AAG GCC GAC GCA CAG CGA
Phe Ala Gly MET Val Arg Leu Val Glu Gln Ala Gln Ala Gln Lys Ala Asp Ala Gln Arg
340

1600

CTA GCC GAC CGG ATC TCC TCG GTG TTT GTT CCC GCT GTG TTG GTT ATC GCG GCA CTA ACC
Leu Ala Asp Arg Ile Ser Ser Val Phe Val Pro Ala Val Leu Val Ile Ala Ala Leu Thr
360

1650

GCA GCC GGA TGG CTA ATC GCC GGG GGA CAA CCC GAC CGT GCC GTC TCG GCC GCA CTC GCC
Ala Ala Gly Trp Leu Ile Ala Gly Gly Gln Pro Asp Arg Ala Val Ser Ala Ala Leu Ala
380

1700

GTG CTT GTC ATC GCC TGC CCG TGT GCC CTG GGG CTG GCG ACT CCG ACC GCG ATG ATG GTG
Val Leu Val Ile Ala Cys Pro Cys Ala Leu Gly Leu Ala Thr Pro Thr Ala MET MET Val
400

1750 1800

GCC TCT GGT CGC GGT GCC CAG CTC GGA ATA TTT CTG AAG GGC TAC AAA TCG TTG GAG GCC
Ala Ser Gly Arg Gly Ala Gln Leu Gly Ile Phe Leu Lys Gly Tyr Lys Ser Leu Glu Ala
420

1850

ACC CGC GCG GTG GAC ACC GTC GTC TTC GAC AAG ACC GGC ACC CTG ACG ACG GGC CGG CTG
Thr Arg Ala Val Asp Thr Val Val Phe Asp Lys Thr Gly Thr Leu Thr Thr Gly Arg Leu
440

1900

CAG GTC AGT GCG GTG ACC GCG GCA CCG GGC TGG GAG GCC GAC CAG GTG CTC GCC TTG GCC
Gln Val Ser Ala Val Thr Ala Ala Pro Gly Trp Glu Ala Asp Gln Val Leu Ala Leu Ala
460

Fig.4C

```
                                1950
GCG ACC GTG GAA GCC GCG TCC GAG CAC TCG GTG GCG CTC GCG ATC GCC GCG GCA ACG ACT
Ala Thr Val Glu Ala Ala Ser Glu His Ser Val Ala Leu Ala Ile Ala Ala Ala Thr Thr
                        480
                2000
CGG CGA GAC GCG GTC ACC GAC TTT CGC GCC ATA CCC GGC CGC GGC GTC AGC GGC ACC GTG
Arg Arg Asp Ala Val Thr Asp Phe Arg Ala Ile Pro Gly Arg Gly Val Ser Gly Thr Val
                        500
 2050                                                                2100
TCC GGG CGG GCG GTA CGG GTG GGC AAA CCG TCA TGG ATC GGG TCC TCG TCG TGC CAC CCC
Ser Gly Arg Ala Val Arg Val Gly Lys Pro Ser Trp Ile Gly Ser Ser Ser Cys His Pro
                        520
                                                        2150
AAC ATG CGC GCG GCC CGG CGC CAC GCC GAA TCG CTG GGT GAG ACG GCC GTA TTC GTC GAG
Asn MET Arg Ala Ala Arg Arg His Ala Glu Ser Leu Gly Glu Thr Ala Val Phe Val Glu
                        540
                                            2200
GTC GAC GGC GAA CCA TGC GGG GTC ATC GCG GTC GCC GAC GCC GTC AAG GAC TCG GCG CGA
Val Asp Gly Glu Pro Cys Gly Val Ile Ala Val Ala Asp Ala Val Lys Asp Ser Ala Arg
                        560
                                2250
GAC GCC GTG GCC GCC CTG GCC GAT CGT GGT CTG CGC ACC ATG CTG TTG ACC GGT GAC AAT
Asp Ala Val Ala Ala Leu Ala Asp Arg Gly Leu Arg Thr MET Leu Leu Thr Gly Asp Asn
                        580
                2300
CCC GAA TCG GCG GCG GCC GTG GCT ACT CGC GTC GGC ATC GAC GAG GTG ATC GCC GAC ATC
Pro Glu Ser Ala Ala Ala Val Ala Thr Arg Val Gly Ile Asp Glu Val Ile Ala Asp Ile
                        600
 2350                                                                2400
CTG CCG GAA GGC AAG GTC GAT GTC ATC GAG CAG CTA CGC GAC CGC GGA CAT GTC GTC GCC
Leu Pro Glu Gly Lys Val Asp Val Ile Glu Gln Leu Arg Asp Arg Gly His Val Val Ala
                        620
                                                        2450
ATG GTC GGT GAC GGC ATC AAC GAC GGA CCC GCA CTG GCC CGT GCC GAT CTA GGC ATG GCC
MET Val Gly Asp Gly Ile Asn Asp Gly Pro Ala Leu Ala Arg Ala Asp Leu Gly MET Ala
                        640
```

Fig. 4D

7 5 97 46511

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                                                  2500
ATC GGG CGC GGC ACG GAC GTC GCG ATC GGT GCC GCC GAC ATC ATC TTG GTC CGC GAC CAC
Ile Gly Arg Gly Thr Asp Val Ala Ile Gly Ala Ala Asp Ile Ile Leu Val Arg Asp His
                        660
                            2[illegible]0
CTC GAC GTT GTA CCC CTT GCG CTT GAC CTG GCA AGG GCC ACG ATG CGC ACC GTC AAA CTC
Leu Asp Val Val Pro Leu Ala Leu Asp Leu Ala Arg Ala Thr MET Arg Thr Val Lys Leu
                        680
                2600
AAC ATG GTC TGG GCA TTC GGA TAC AAC ATC GCC GCG ATT CCC GTC GCC GCT GCC GGA CTG
Asn MET Val Trp Ala Phe Gly Tyr Asn Ile Ala Ala Ile Pro Val Ala Ala Ala Gly Leu
                        700
 2650                                                                  2700
CTC AAC CCC CTG GTG GCC GGT GCG GCC ATG GCG TTC TCA TCG TTC TTC GTG GTC TCA AAC
Leu Asn Pro Leu Val Ala Gly Ala Ala MET Ala Phe Ser Ser Phe Phe Val Val Ser Asn
                        720
                                                          2750
AGC TTG CGG TTG CGC AAA TTT GGG CGA TAC CCG CTA GGC TGC GGA ACC GTC GGT GGG CCA
Ser Leu Arg Leu Arg Lys Phe Gly Arg Tyr Pro Leu Gly Cys Gly Thr Val Gly Gly Pro
                        740
                                                                  ------------->
CAA ATG ACC GCG CCG TCG TCC GCG TGA TGCGTTGTCGGGCAACACGATATCGGGCTCAGCGGCGACCGCA
Gln MET Thr Ala Pro Ser Ser Ala TER
                            761
<------------
TCCGGTCTCGGCCGAGGACCAGAGGCGCTTCGCCACACCATGATTGCCAGGACCGCGCCGATCACCACCGGCAGATGAGT
CAAAATCCGCGTGGTGCTGACCGCGCCGGACAGCGCATCCACAATCACATAGCCGGTCAGTATGGCGACGAACGCCGTCA
GAACACCGGCCAGGCCGGCGGCGGCGCTCGGCCATAGCGCCGCGCCCACCATGATCACACCGAGCGCAATCGACCACGAC
GTGACTCGTTGAGCAAGTGGGTGCCGGCACCCGTCGGGTGCTGATGGGTCAGGCCGACGTCTAGGCCAAACCCCTGCACG
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CGCCCGGCGGATCC
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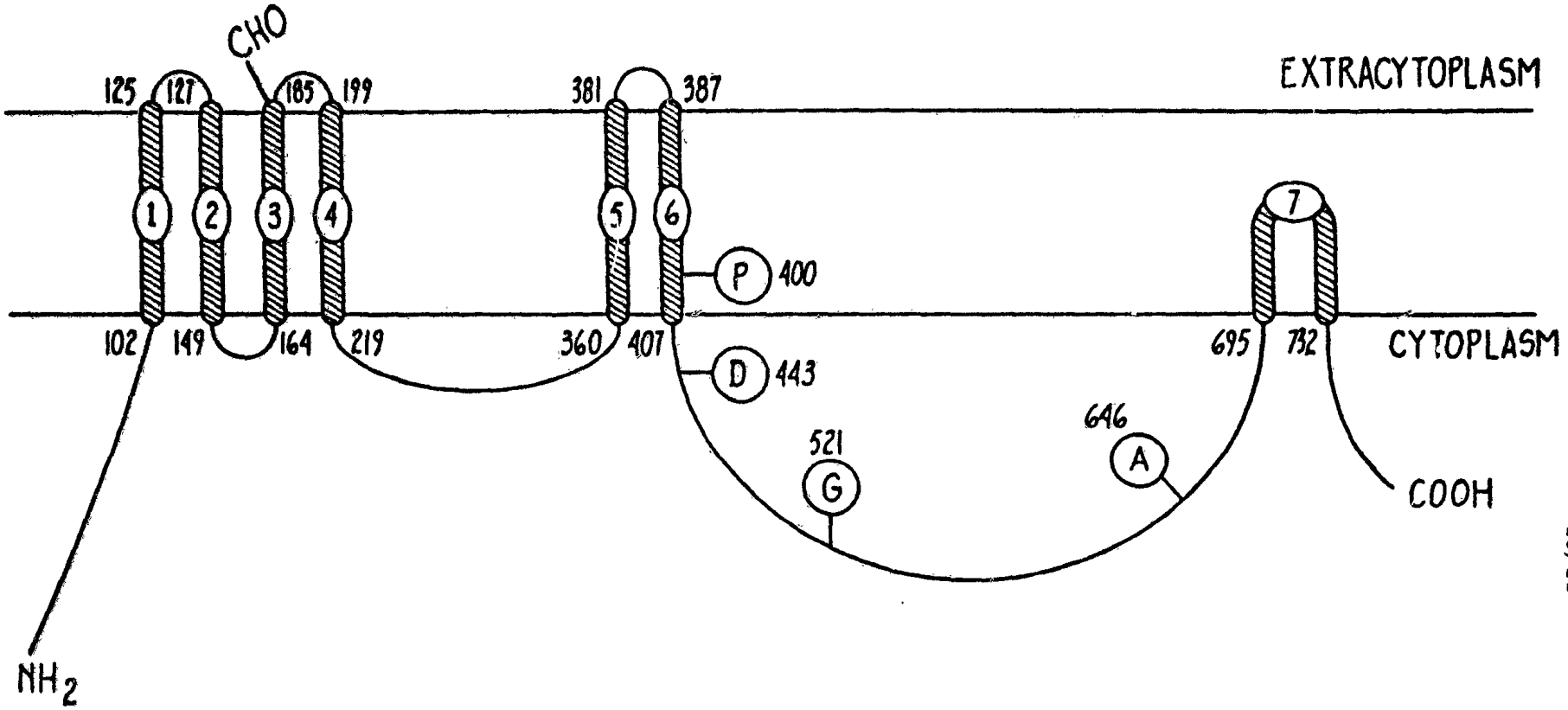
Fig.4E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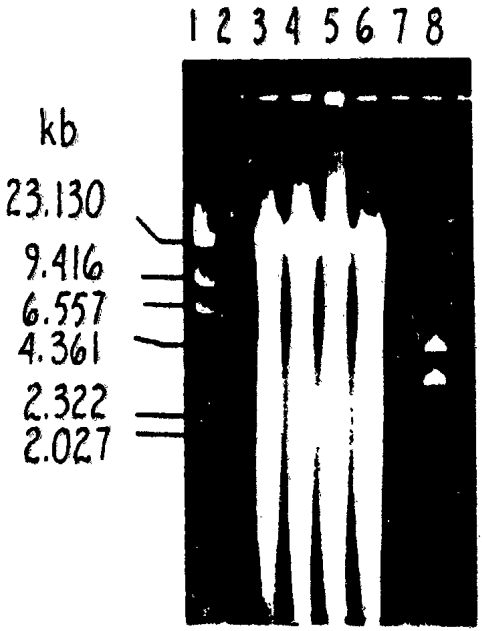
127
185
199
381
387
EXTRACYTOPLASM
1
2
3
4
5
6
7
P
400
102
149
164
219
360
407
695
732
CYTOPLASM
D
443
646
521
A
G
COOH
NH2

Fig. 5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kb
23.130
9.416
6.557
4.361
2.322
2.027

Fig. 6A

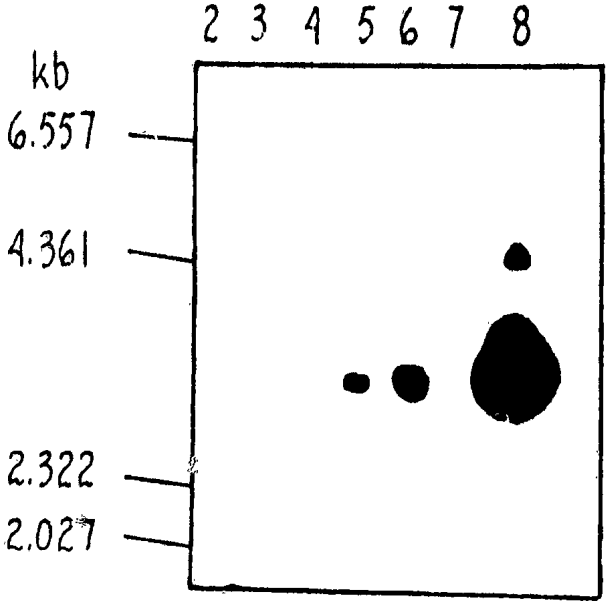
2 3 4 5 6 7 8
kb
6.557
4.361
2.322
2.027

Fig. 6B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/06080

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C07K 15/04, 13/00; C12N 9/14, 15/31, 15/55; A61K 39/04
US CL :530/350; 435/195; 424/92; 536/23.7, 24.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/350; 435/195; 424/92; 536/23.7, 24.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	Proceedings of the National Academy of Sciences of the USA, Volume 82, issued May 1985, R. A. Young et al., "Dissection of *Mycobacterium tuberculosis* antigens using recombinant DNA", pages 2583-2587, especially page 2583.	1-3, 11, 12, 23 --- 4-10, 13-22
X --- Y	Journal of Clinical Microbiology, Volume 28, Number 3, issued March 1990, H. S. Rumschlag et al., "Immunologic Characterization of a 35-Kilodalton Recombinant Antigen of *Mycobacterium tuberculosis*", pages 591-595, especially the abstract.	1, 2, 11, 12 --- 3, 13, 21

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
03 SEPTEMBER 1993

Date of mailing of the international search report
24 SEP 1993

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/06080

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Nature, Volume 351, issued 06 June 1991, C. K. Stover et al., "New use of BCG for recombinant vaccines", pages 456-460, especially pages 456-457.	20
Y	Infection and Immunity, Volume 50, Number 3, issued December 1985, J. E. R. Thole et al., "Cloning of *Mycobacterium bovis* BCG DNA and Expression of Antigens in *Escherichia coli*", pages 800-806, especially pages 800-801.	1-22
A	Scandanavian Journal of Immunology, Volume 12, issued 1980, O. Closs et al., "The Antigens of *Mycobacterium bovis*, Strain BCG, Studied by Crossed Immunoelectrophoresis: a Reference System", pages 249-263.	1-22
A	Infection and Immunity, Volume 55, Number 6, issued June 1987, D. B. Young et al., "Screening of a Recombinant Mycobacterial DNA Library with Polyclonal Antiserum and Molecular Weight Analysis of Expressed Antigens", pages 1421-1425.	1-22
A	Molecular Microbiology, Volume 6, Number 2, issued 1992, "Mycobacterial protein antigens: a compilation", pages 133-145.	1-22

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/06080

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

Keyword databases:	Medline, AIDSline, Pascal, Biosis, NTIS, Derwent Biotech. Abs., WPI, USPTO-APS
Search terms:	Mycobact?, tuberculos?, lepr?; antigen, vaccine; clon?, recombinant?, cDNA; ATPase, transport?, antiport?
Sequence databases:	GenBank, EMBL, GeneSeq, SwissProt, PIR