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Cloning and B-Cell-Epitope Mapping of MPT64 from *Mycobacterium tuberculosis* H37Rv

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The gene of the immunogenic protein MPT64 found in culture filtrates of *Mycobacterium tuberculosis* H37Rv was cloned and sequenced. A comparison showed *mpt64* and the gene encoding MPB64 from *Mycobacterium bovis* BCG Tokyo to be identical except for one silent mutation. The regions encoding the promoter and the signal peptide were also well conserved for the two sequences. Southern blot experiments on genomic mycobacterial DNA showed the presence of *mpt64* in the *M. tuberculosis* substrains H37Rv, H37Ra, and Erdman and in the *M. bovis* BCG substrains Tokyo, Moreau, and Russian, whereas the *M. bovis* BCG substrains Glaxo, Pasteur, Canadian, Tice, and Danish 1331 and *Mycobacterium leprae* lack the gene. Southern blot analyses revealed differences in the restriction enzyme patterns within the *M. tuberculosis* substrains as well as within the *M. bovis* BCG substrains, indicating either different chromosomal localization of *mpt64* or that mutations have occurred at different locations on the chromosomes. N-terminal and C-terminal deletion mutants were constructed for the mapping of B-cell epitopes on MPT64 with five monoclonal antibodies, C24b1, C24b2, C24b3, L24b4, and L24b5. Western blot (immunoblot) analysis revealed that the murine antibodies bind to one linear and three conformational epitopes.

Tuberculosis, caused by the slowly growing bacterium *Mycobacterium tuberculosis*, is still a world health problem. Some 8 million new cases occur each year, and the deaths of 2 to 3 million people annually may be ascribed to tuberculosis (20). The disease is predominantly raging in developing countries, but during recent years the incidence has been increasing even in industrialized parts of the world, like in certain urban regions of the United States (6).

Biochemical, immunological, and molecular biological characterization of *M. tuberculosis* has led to the identification of several antigens which may be useful in the development of improved diagnostic methods and/or vaccines (22). The proteins actively secreted by *M. tuberculosis* have for several reasons attracted special attention. These proteins have, e.g., been suggested as major immune targets during the early phase of an infection (4). *M. tuberculosis* has been shown to secrete more than 33 different proteins (3). One of the predominant proteins is MPB64, a 24-kDa protein initially isolated from culture filtrates of *Mycobacterium bovis* BCG Tokyo (10). Recently, we isolated five monoclonal antibodies (MAbs) recognizing four different epitopes on the *M. tuberculosis* version of the protein (designated MPT64) (2). The protein is produced and secreted by the virulent strains of the tuberculosis complex (*M. tuberculosis*, *Mycobacterium africanum*, and *M. bovis*, including *M. bovis* BCG) (2). However, recently it was demonstrated that not all substrains of *M. bovis* BCG carry the gene (12).

In this study, the cloning and structure analyses of *mpt64*, including the precise mapping of four B-cell epitopes defined by mono- and polyclonal antibodies, are reported. Furthermore, evidence for the different chromosomal localizations of *mpt64* within the species belonging to the tuberculosis complex is presented.

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MATERIALS AND METHODS

Bacterial strains. The *Escherichia coli* strains used in this study were INVaF′ [*endA1 recA1 hsdR17* (r_K^- m_K^+) *supE44* λ^- *thi-1 gyrA relA1* ρ*80 lacZ*Δ*M15*Δ(*lacZYA-argF*) *deoR*$^+$ F′] (InVitrogen, San Diego, Calif.) and XL1-Blue (8).

The pMAL-*p* expression vector (New England Biolabs, Beverly, Mass.) was used for the expression studies.

The mycobacterial strains used in this study are listed in Table 1. The mycobacterial genomic DNA was prepared as described by Andersen et al. (1). The *Mycobacterium leprae* Armadillo-derived chromosomal DNA was obtained from J. M. Colston, Mill Hill, London, United Kingdom.

DNA technology. Standard procedures were used for the preparation and handling of DNA, as described by Maniatis et al. (13).

Synthesis and design of probes. Oligonucleotide primers were synthesized automatically on a DNA synthesizer (Applied Biosystems ABI-391, PCR model), deblocked, and purified by ethanol precipitation.

Four oligonucleotides were synthesized on the basis of the nucleotide sequence from MPB64 described by Yamaguchi et al. (21) in expectation of some sequence homology between the MPB64 and MPT64 genes. Five oligonucleotides were synthesized on the basis of the nucleotide sequence from *mpt64* determined in this study (Table 2). The oligonucleotides were engineered to include an *Eco*RI restriction enzyme site at the 5′ end and at the 3′ end, allowing a later subcloning.

DNA cloning. The *mpt64* gene was cloned from *M. tuberculosis* H37Rv chromosomal DNA by PCR technology as described by Innis et al. (11).

In brief, the standard amplifications were carried out in a thermal reactor (Hybaid, Teddington, United Kingdom) by incubation of 100 ng of chromosomal *M. tuberculosis* H37Rv DNA brought to a final volume of 37 μl with Milli Q water (Millipore Corp., Bedford, Mass.) at 70°C for 5 min and then cooled on wet ice for 10 min. A 13-μl volume of PCR master mix was added. The PCR master mix contained 192 mM KCl, 38.5 mM Tris-HCl (pH 8.3), 5.8 mM $MgCl_2$, 0.77 mM each deoxynucleoside triphosphate, and 3.8 μM each oligonucle-

TABLE 1. Mycobacterial strains used in this study

Strain	Source
M. tuberculosis	
H37Rv	ATCC[a] 27294
Erdman	A. Lazlo, Ottawa, Canada
H37Ra	ATCC 25177
M. bovis BCG	
Danish 1331	BCG laborabory, SSI[b]
Tokyo	WHO[c]
Moreau	Our collection, SSI[d]
Russian	Our collection, SSI
Glaxo	Our collection, SSI
Pasteur	Our collection, SSI
Canadian	Our collection, SSI
Tice	Our collection, SSI
M. leprae armadillo derived	J. M. Colston

[a] American Type Culture Collection, Rockville, Md.
[b] Statens Seruminstitut, Copenhagen, Denmark.
[c] W. H. O. International Laboratory for Biological Standards, Statens Seruminstitut, Copenhagen, Denmark.
[d] Our collection, Mycobacteria Department, Statens Seruminstitut, Copenhagen, Denmark.

otide primer. The reaction mixture was overlaid with 100 μl of mineral oil. Denaturation of the DNA was carried out at 94°C for 5 min. The reaction mixture was brought to the annealing temperature, 60°C, and 1.5 U of AmpliTaq DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.) was added to the master mix. The amplifications were performed for 30 cycles at 72°C for 3 min, 94°C for 1 min 20 s, and 60°C for 2 min. At the end of the cycles, the primer extension step was carried out for 7 min. Ten microliters of the PCR product was fractionated by 1.5% (wt/vol) agarose gel electrophoresis and visualized with ethidium bromide. Negative controls containing all the PCR reagents except template DNA were run in parallel with the samples.

The PCR product was cloned in the pCR1000 vector as described for the TA cloning system (InVitrogen).

DNA sequencing. The nucleotide sequence of the cloned 628-bp *M. tuberculosis* H37Rv PCR fragment pTO01, containing the structural gene of MPT64, and the nucleotide sequence of the cloned 508-bp PCR fragment pTO03, containing the promoter region and the signal peptide sequence, were determined by the dideoxy chain termination method with a Sequenase DNA sequencing kit version 1.0 (U.S. Biochemical Corp., Cleveland, Ohio) according to the instructions provided. Both strands of the DNA were sequenced.

Southern blotting. Four micrograms of mycobacterial genomic DNA was digested with *Eco*RI, electrophoresed in a 0.8% agarose gel, and transferred onto GeneScreen Plus membranes (NEN Research Products, Boston, Mass.). The 628-bp *Eco*RI *mpt64* fragment from pTO01 was nick translated with a kit from Boehringer Mannheim, Mannheim, Germany, and used as a probe. Hybridization was performed at 65°C in an aqueous solution containing 1% sodium dodecyl sulfate (SDS), 1 M NaCl, 10% dextran sulfate, 100 μg of denatured salmon sperm DNA per ml, and [α-^{32}P]dCTP nick-translated *mpt64* probe according to the instructions provided. Washing of the membrane was performed as described by the manufacturer.

Subcloning of *mpt64*. An *Eco*RI site was engineered immediately 5′ of the first codon of *mpt64* so that only the coding region of the gene encoding MPT64 would be expressed, and an *Eco*RI site was incorporated right after the stop codon at the 3′ end.

DNA of the recombinant plasmid pTO01 was cleaved at the *Eco*RI sites. The 628-bp fragment was purified from an agarose gel and subcloned into the *Eco*RI site of the pMAL-*p* expression vector (New England Biolabs). Vector DNA containing the gene fusion was used to transform the *E. coli* XL1-Blue by the standard procedures for DNA manipulation.

The endpoints of the gene fusion were determined by the dideoxy chain termination method as described in the DNA sequencing section. Both strands of the DNA were sequenced.

Construction of *mpt64* deletion mutants. DNA of the recombinant plasmid pTO01 was cleaved in *mpt64* at the *Cla*I, the *Stu*I, or the *Sma*I site (Fig. 1). The DNA was treated with the Klenow fragment of DNA polymerase I (Bethesda Research Laboratories, Gaithersburg, Md.) to make the ends blunt. Subsequently, the DNA was digested with *Eco*RI, and the 327-bp *Eco*RI-*Stu*I, the 459-bp *Eco*RI-*Cla*I, and the 542-bp *Eco*RI-*Sma*I fragments were purified from a 2% (wt/vol) agarose gel.

The pMAL-*p* vector was cleaved at the unique *Sal*I site and made blunt with the Klenow fragment of DNA polymerase I. The DNA was afterwards digested at the unique *Eco*RI site, and the large *Eco*RI-*Sal*I fragment was purified from a 0.8% (wt/vol) agarose gel.

TABLE 2. Sequences of the *mpt64* oligonucleotides[a]

Orientation and oligonucleotide	Sequence (5′→3′)	Position (nt)
Sense		
MPT64-1	GAA TTC GCG CCC AAG ACC TAC TGC	207–225
MPT64-4	GAT GCG AAT TCG AAA ATT ACA TCG CCC	337–352
MPT64-5	GAT GCG AAT TCA AGG TCT ACC AGA ACG	479–496
MPT64-6	GAT GCG AAT TCC AGG CCT ATC GCA AGC	543–559
MPT64-7	GAT GCG AAT TCA GCA AGC AGA CCG GAC	637–652
MPT64-8	GAT GCG AAT TCG ACC CGG TGA ATT ATC	685–700
MPT64-9	CTC GAA TTC TGC TAG CTT GAG	1–14
Antisense		
MPT64-2	GAA TTC TAG GCC AGC ATC GAG TCG	826–807
MPT64-3	GAA TTC CGG CGT TCT GGT AGA CC	500–483

[a] MPT64-1, MPT64-2, MPT64-3, and MPT64-9 were constructed from the MPB64 nucleotide sequence (21). The other oligonucleotide constructions were based on the nucleotide sequence obtained from *mpt64* reported in this work. Nucleotides (nt) underlined are not contained in the nucleotide sequence of MPB/T64. The positions correspond to the nucleotide sequence shown in Fig. 1.

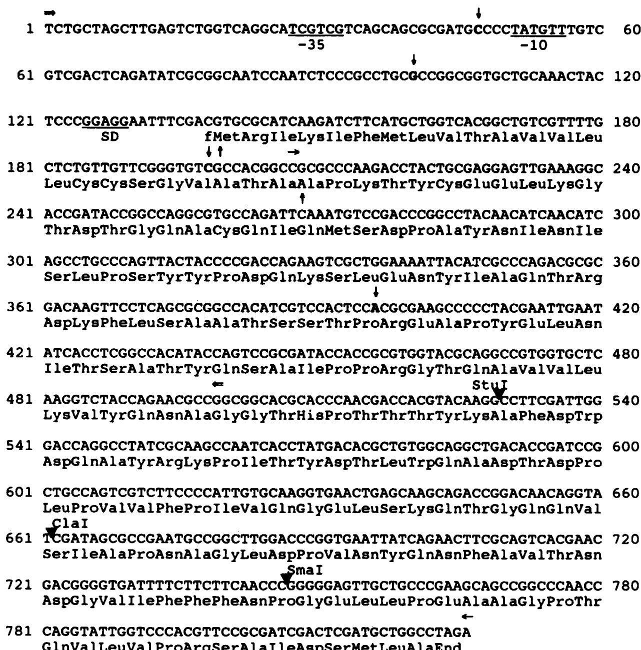

FIG. 1. Nucleotide sequence of *mpt64* and the deduced amino acid sequence of the gene product. The potential ribosome binding site is underlined and indicated by SD. The putative *mpt64* Pribnow boxes (−35 and −10 sequences) are underlined and marked. The translation initiation codon GTG is shown by the first arrow (↑) at position 139, and the mature amino terminus is indicated by the second arrow (↑) at position 209. The stop codon is indicated by End. The start and end positions of plasmid pTO01 are indicated by arrows (→ and ←) at positions 207 and 826, respectively, and the start and end positions of plasmid pTO03 are indicated by arrows (⇒ and ⇐) at positions 1 and 499, respectively. The differences from the nucleotide sequence of MPB64 (21) are indicated by arrows (↓) at positions 47, 100, 198, and 453.

In addition, one C-terminal deletion mutant was engineered by PCR using the primers MPT64-1 and MPT64-3 (Table 2). The 299-bp *Eco*RI-digested fragment was subcloned in pMAL-*p*.

To create deletion mutants from the N-terminal part of the gene as well, five oligonucleotides, MPT64-4, MPT64-5, MPT64-6, MPT64-7, and MPT64-8 (Table 2), all containing *Eco*RI sites were produced to create an in-frame fusion with *malE* of the pMAL-*p* vector by PCR, as described in the DNA cloning section. The *Eco*RI-digested PCR fragments were subcloned in the *Eco*RI site of the pMAL-*p* expression vector.

Ligations of various possible constructions were performed. The ligated DNA was transformed into *E. coli* XL1-Blue and plated on Luria-Bertani agar with ampicillin, tetracycline, and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). White colonies were picked randomly, and plasmid DNA was cleaved with *Bam*HI and *Hin*dIII and analyzed by agarose gel electrophoresis to determine the size of the mycobacterial insert.

Deletion mutants containing DNAs of the right sizes were sequenced by the dideoxy chain termination method as described in the DNA sequencing section to confirm the in-frame fusion to *malE* in pMAL-*p*. Both strands of the DNA were sequenced in all the constructions.

Crude protein extracts of *E. coli* expressing complete or truncated MPT64. Single colonies of *E. coli* carrying the recombinant pMAL-*p* plasmids were inoculated into Luria-Bertani broth containing 50 μg of ampicillin and 12.5 μg of tetracycline per ml and grown at 37°C to 2×10^8 cells per ml. Isopropyl-β-D-thiogalactopyranoside (IPTG) was then added to a final concentration of 0.3 mM, and growth was continued for a further 2 h. The bacteria were harvested and suspended in sample buffer (62 mM Tris-HCl [pH 6.8], 2% SDS, 0.7 M β-mercaptoethanol) (one-fifth of the original volume) and boiled for 5 min.

PAGE and immunoblotting. Samples of crude *E. coli* protein extracts (15 μl) were separated by SDS–10% polyacrylamide gel electrophoresis (PAGE) before being stained with Coomassie brilliant blue R250 or transferred onto nitrocellulose sheets by electroblotting.

Protein standards of known molecular mass were obtained from Bio-Rad, Richmond, Calif. Nitrocellulose sheets were soaked in phosphate-buffered saline (PBS), pH 7.6, containing 0.5% Tween 20 as a blocking agent. PBS, pH 7.6, containing 0.05% Tween 20, was used for dilution of antibodies and for washing. The nitrocellulose-bound samples were probed with MAbs or a polyclonal purified rabbit serum against *M. tuberculosis* H37Rv absorbed against *E. coli* XL1-Blue(pMAL-*p*) as described earlier (2). The detecting antibodies were horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin (P260; DAKO A/S, Glostrup, Denmark) or horseradish peroxidase-conjugated swine anti-rabbit immunoglobulin G (P217; DAKO A/S). A color reaction was obtained by using 3.3′,5.5′-tetramethylbenzidine as the substrate.

Computer program to predict B-cell epitopes. Potential B-cell epitopes were predicted by a computer program for protein structure prediction entitled Surfaceplot (Synthetic Peptides Incorporated, Edmonton, Alberta, Canada). The surface profile is based on hydrophilicity, accessibility, and flexibility parameters (14).

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper have been deposited in the EMBL data library under accession number X75361.

RESULTS

Cloning of *mpt64*. The gene, the signal sequence, and the promoter region of MPT64 were cloned by PCR technology as two fragments of 628 and 508 bp in pCR1000, designated pTO01 and pTO03.

DNA sequence. The nucleotide sequences of pTO01 and pTO03 and the deduced amino acid sequence are shown in Fig. 1. The DNA sequence contained an open reading frame starting with a GTG codon at positions 139 to 141 and ending with a termination codon (TAG) at positions 823 to 825. The nucleotide sequence of the first 23 codons was expected to encode the signal sequence. On the basis of the known N-terminal amino acid sequence (Ala-Pro-Lys-Thr-Tyr-X-Glu) of the MAb C24b1-purified MPT64 (2) and the features of the signal peptide, it is presumed that the signal peptidase recognition sequence (Ala-X-Ala) (19) is located in front of the N-terminal region of the mature protein at position 208. Therefore, a structural gene encoding MPT64, *mpt64*, derived from *M. tuberculosis* H37Rv was found at positions 208 to 822 of the sequence shown in Fig. 1. The nucleotide sequence of *mpt64* differed by only with few nucleotides from the nucleotide sequence of MPB64 described by Yamaguchi et al. (21) (Fig. 1). In *mpt64* at position 453 a substitution of an adenine for a guanine was found. From the deduced amino acid sequence, this change occurs at the third position of the codon as a silent mutation. In the signal sequence at position 198, a cytosine is substituted for a guanine, also as a silent mutation.

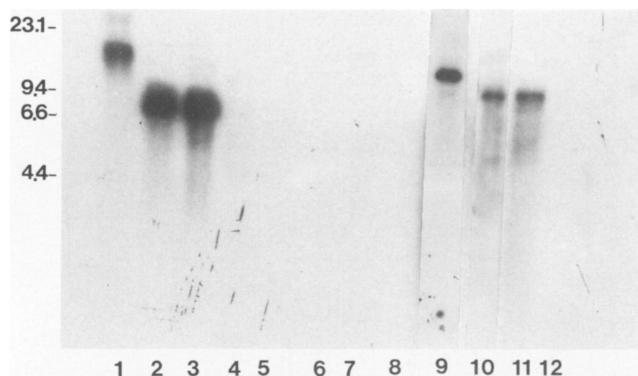

FIG. 2. Southern hybridization pattern with nick-translated *mpt64* probe to *Eco*RI-digested chromosomal DNA from various mycobacterial species. Lanes: 1, *M. bovis* BCG Tokyo; 2, *M. bovis* BCG Moreau; 3, *M. bovis* BCG Russian; 4, *M. bovis* BCG Glaxo; 5, *M. bovis* BCG Pasteur; 6, *M. bovis* BCG Canadian; 7, *M. bovis* BCG Tice; 8, *M. bovis* BCG Danish 1331; 9, *M. tuberculosis* H37Rv; 10, *M. tuberculosis* H37Ra; 11, *M. tuberculosis* Erdman; 12, *M. leprae*. The numbers at the left indicate the size of standard DNA fragments in kilobase pairs. Lanes 9 and 10 are from a separate Southern blot experiment.

In the nonstructural region of the promoter and the Shine-Dalgarno sequence, two differences occurred, one addition at position 47 of a cytosine and one deletion of a guanine at position 100. The promoter-like sequences and the Shine-Dalgarno sequence were identical with those of the MPB64 gene of *M. bovis* BCG Tokyo (21). Thus, it is concluded that *mpt64* consists of 618 bp and that the deduced amino acid sequence contains 205 residues with a molecular weight of 24,433 and that MPT64 is homologous to MPB64.

Presence of *mpt64* in different mycobacterial species. In order to determine the distribution of *mpt64* within species belonging to the tuberculosis complex and in *M. leprae*, the 628-bp *Eco*RI *mpt64* fragment from pTO01 was used as a probe in a Southern blot experiment. The result is shown in Fig. 2. The probe hybridized to *Eco*RI fragments of approximately 14 kb in *M. tuberculosis* H37Rv, of approximately 11 kb in the *M. tuberculosis* substrains H37Ra and Erdman, of approximately 20 kb in *M. bovis* BCG Tokyo, and to a fragment of between 6.6 and 9.4 kb in the *M. bovis* BCG substrains Moreau and Russian, but the probe did not hybridize to any *Eco*RI fragment from the *M. bovis* BCG substrains Glaxo, Pasteur, Canadian, Tice, and Danish 1331 or *M. leprae.*

B-cell epitope mapping on MPT64 fusion proteins with MAbs. Because of our interest in the immunological potential of MPT64, we wanted to localize the domains to which the anti-MPT64 MAbs bind. A series of deletion mutants produced from pTO01 were constructed. The deletion mutants were expressed in the *E. coli* pMAL-*p* expression vector, which is designed to allow the fusion protein to be exported to the periplasm. The recombinant proteins are fused to the maltose-binding protein, encoded by *malE*, and the expression is regulated by the strong IPTG-inducible P_{tac} promoter. By manipulating the gene encoding the fusion protein rather than the nonfused protein, it was possible to establish that the truncated molecules were actually being produced by the use of polyclonal antibodies raised against the maltose-binding protein. In total, nine plasmids, carrying four N-terminal and five C-terminal deletions, were constructed (Fig. 3).

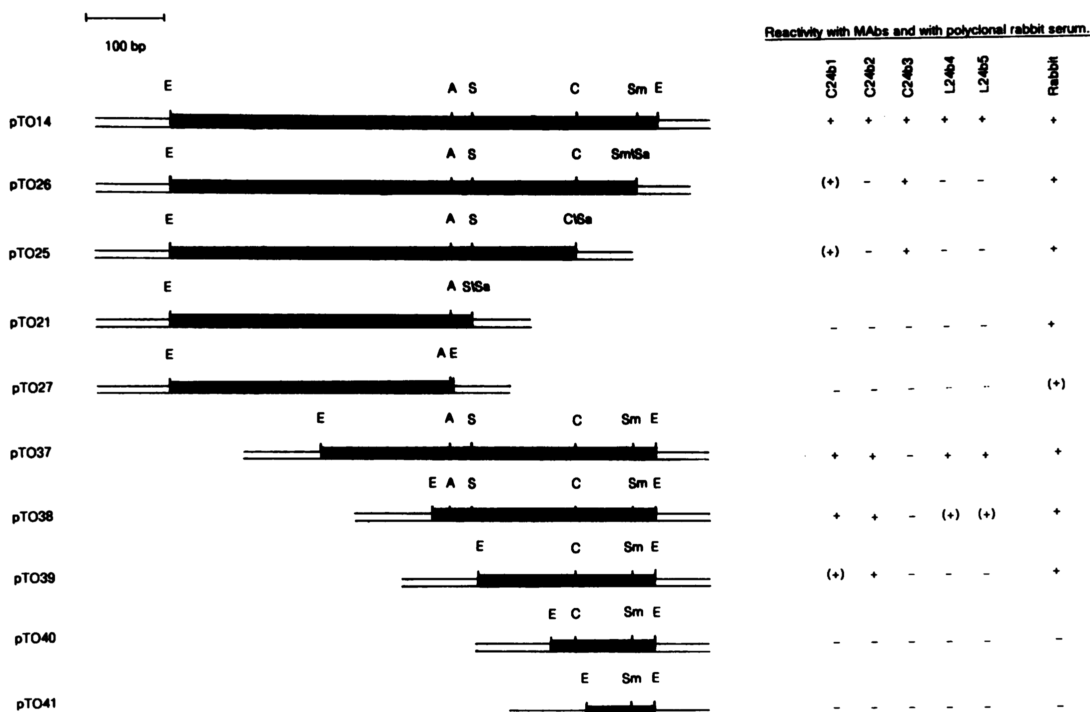

FIG. 3. Physical map of recombinant plasmids expressing various regions of *mpt64* and reactivity of MAbs and absorbed polyclonal rabbit serum to fusion proteins expressed by the plasmids. On the left part of the figure, the open bars represent vector DNA, the solid bar represents *mpt64*, and the transcription of the gene is from left to right. At the right, are listed the reactivities of MAbs and absorbed polyclonal serum to crude protein extracts from induced *E. coli* XL1-Blue cells harboring various plasmids, established by Western blot (immunoblot) analysis. +, strong; (+), weak; and −, no reactivity. The restriction sites are *Acc*I (A), *Cla*I (C), *Eco*RI (E), *Stu*I (S), *Sal*I (Sa), and *Sma*I (Sm).

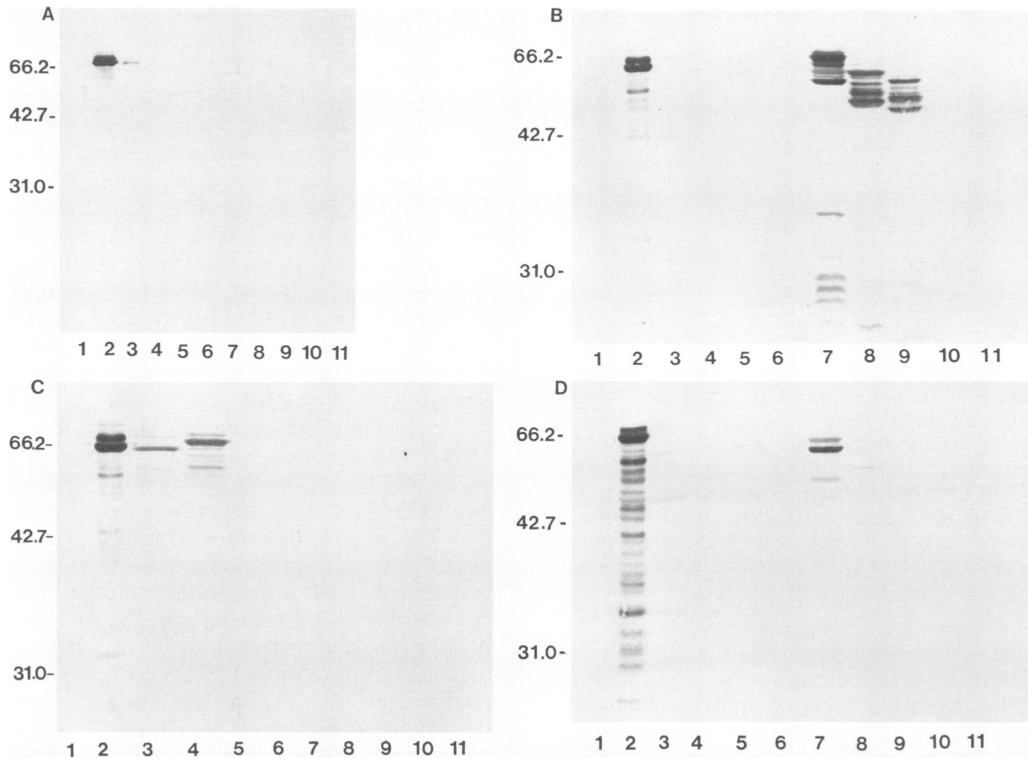

FIG. 4. Immunoblotting analysis of MAbs, using crude protein extract from induced *E. coli* XL1-Blue cells harboring various plasmids (see Materials and Methods) separated on 10% polyacrylamide gel. Lanes: 1, pMAL-*p* (negative control); 2, pTO14; 3, pTO26; 4, pTO25; 5, pTO21; 6, pTO27; 7, pTO37; 8, pTO38; 9, pTO39; 10, pTO40; 11, pTO41. Molecular mass (in kilodaltons) is indicated at the left. The gels were incubated with MAbs C24b1 (A), C24b2 (B), C24b3 (C), and L24b5 (D).

Crude protein extracts of *E. coli* XL1-Blue transformed with these plasmids were subjected to SDS-PAGE. The reactivities of a panel of anti-MPT64 MAbs towards the fusion proteins were analyzed in immunoblotting experiments. The results are summarized in Fig. 3, and four examples are shown in Fig. 4A to D. Some of the recombinant fusion proteins are partly proteolytically degraded because crude *E. coli* protein extracts were used (for an example, see Fig. 4D, lane 2). Crude protein extracts of *E. coli* XL1-Blue harboring the pMAL-*p* plasmid were used as a negative control (Fig. 4A to D, lanes 1). No specific bands were seen. The immunoblotting experiments with the recombinant fusion proteins indicated that the MAbs bind to four regions. From the binding pattern, we drew the following conclusions. MAb C24b1 recognizes an epitope, which could be linear, located within the 31-residue amino acid sequence between Gln-113 and Leu-143 (Fig. 5). MAb C24b2 binds to an epitope encoded by the plasmids pTO37, pTO38, and pTO39, which express N-terminally truncated versions of the protein. The ability to bind the MAb is abolished when more than 112 of the N-terminal residues are removed. The gene product of pTO40 expressing residues from 143 onwards did not bind C24b2. Beyond being dependent on the sequence flanked by Gln-113 and Leu-143, the binding of the MAb is also dependent on the presence of the C-terminal part of the protein, as demonstrated by the fact that C24b2 is unable to bind the gene product of pTO26, which lacks the ultimate 25 amino acids. Therefore, the C24b2-defined epitope is of the conformational type and comprises the two sequences Gln-113

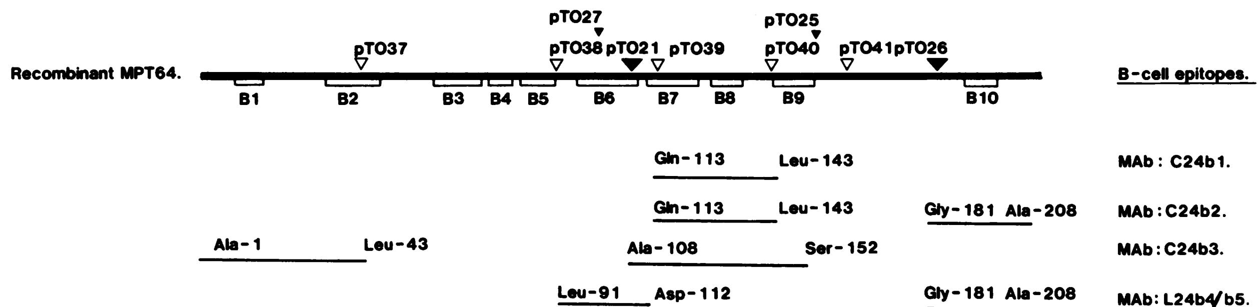

FIG. 5. Physical map of recombinant MPT64 (solid bar). The B-cell epitopes predicted by the Surfaceplot program are numbered B1 to B10 (open bars). The B-cell epitopes deduced from Western blot analysis with MAbs C24b1, C24b2, C24b3, and L24b4/b5 are shown as solid lines. The start (open triangle) and end (closed triangle) positions of the different deletion mutants are shown.

to Leu-143 and Gly-181 to Ala-208 (Fig. 5). MAb C24b3 also seems to recognize a structural epitope composed of sequences from two distinct regions of the molecule. The binding is dependent on the expression of the sequence Ala-108 to Ser-152. Furthermore, the deletion of as few as 43 N-terminal amino acids (pTO37) is detrimental for the binding of the MAb with the protein. It is concluded that the C24b3-defined epitope is composed of two structural domains found in the sequences Ala-1 to Leu-43 and Ala-108 to Ser-152 (Fig. 5). MAbs L24b4 and L24b5 both react with an epitope between Leu-91 and Asp-112 coded by the plasmids pTO37 and pTO38 but not by the plasmids pTO39, pTO40, and pTO41. None of the C-terminal deletion mutants are detected by these MAbs, indicating the dependence on the correct expression of the entire C-terminal region. Deletion of the 25 C-terminal residues in pTO26 abolish binding of the MAbs. Therefore, we conclude that the L24b4/b5 epitope is of the conformational type composed by the amino acid sequences Leu-91 to Asp-112 and Gly-181 to Ala-208 (Fig. 5).

Comparison with predicted B-cell epitopes. The amino acid sequence of MPT64 was analyzed by a computer program for protein structure prediction of potential B-cell epitopes. Ten of the predicted epitopes are shown in Fig. 5. The constructed C- and N-terminally truncated versions of MPT64 covered all the predicted B-cell epitopes. In this study, four different B-cell epitopes were found with the MAbs. Of these, the linear C24b1 epitope probably corresponds to one of the two predicted epitopes B7 and B8 shown in Fig. 5.

B-cell epitope mapping of MPT64 fusion proteins by polyclonal rabbit antibodies against *M. tuberculosis* H37Rv. In order to map B-cell epitopes on MPT64 other than those detected by the MAbs, a polyclonal rabbit serum raised against *M. tuberculosis* H37Rv absorbed against an *E. coli* extract was used. The results are summarized in Fig. 3. The polyclonal rabbit immunoglobulins recognized the same structural regions as those defined by the murine MAbs. In addition, the polyclonal immunoglobulins identified an epitope between Gly-99 and Lys-107, as shown by the binding to the gene product of pTO21 and pTO27. This epitope may correspond to the predicted epitope B6 (Fig. 5). None of the predicted epitopes in the amino-terminal part of recombinant MPT64 gave any reaction.

DISCUSSION

In this study, PCR technology was used for cloning *mpt64* from *M. tuberculosis* H37Rv. The deduced amino acid sequence of MPT64 revealed a protein of 205 amino acids with a molecular weight of 22,433. In comparison of *mpt64* with the nucleotide sequence of MPB64 from *M. bovis* BCG Tokyo (21), the two were shown to be identical except for one single silent mutation.

Several clones containing the PCR-cloned *mpt64* were sequenced, and all of the clones had the same nucleotide composition as shown in Fig. 1. Therefore, it is concluded that the differences between the nucleotide sequences of MPT64 and MPB64 found in this study are true differences and not differences obtained by misreading of chromosomal DNA by the AmpliTaq DNA polymerase as described earlier (15).

Identical nucleotide sequences of *M. tuberculosis* and *M. bovis* BCG genes have been reported earlier. DNA sequence analysis of the 65-kDa genes from *M. tuberculosis* and from *M. bovis* BCG have shown that not only the coding sequences of the genes (16, 18) but also the sequences upstream of the 65-kDa gene (17) were completely identical. In parallel, the genes of the 85A components cloned from *M. tuberculosis* (7) and *M. bovis* BCG (9) have been shown to be identical except for a silent single nucleotide change.

The distributions of *mpt64* in *M. tuberculosis* substrains and *M. bovis* BCG vaccine substrains have been discussed lately (2, 12). In this study, we wished to draw definitive conclusions concerning the distribution of *mpt64* within species belonging to the tuberculosis complex. Southern blot experiments showed the presence of *mpt64* in the three *M. tuberculosis* isolates and in the *M. bovis* BCG Tokyo, Moreau, and Russian substrains, whereas the Glaxo, Pasteur, Canadian, Tice, and Danish 1331 substrains lack the gene. The results confirm the observations by Li et al. (12). A point of interest is how and when the *M. bovis* BCG vaccine substrains lost the gene encoding MPB64. All the substrains originate in one way or the other from the same ancestor, *M. bovis* BCG Pasteur. Furthermore, the hybridization studies showed differences in the restriction enzyme patterns within species belonging to the tuberculosis complex. The differences seen in this study are a result of either different localization of *mpt64* on the chromosome or single chromosomal mutations of *Eco*RI sites or a product of both possibilities. The function of the gene product is as yet unknown, but it is apparently a dispensable one.

On the basis of the MAb reactivity patterns with the fusion proteins, B-cell epitopes were mapped to four regions. The results confirm the characterization of the MAbs described earlier (2). As a consequence of the murine MAb reactivity patterns, three of the four identified epitopes are concluded to be of the conformational type. For example, the C24b3 epitope depends on two structural domains found in the sequences Ala-1 to Leu-43 and Ala-108 to Ser-152. It may be that deletions in one end of the molecule in some instances influence the structure or accessibility of the other end of the protein. The epitopes mapped by the murine MAbs were recognized also in the rabbit model, as polyclonal rabbit anti-H37Rv immunoglobulins apparently identified the same structural regions. However, further experiments (e.g., blocking experiments using MAbs) may clarify this point definitively. B-cell epitopes can be predicted by computer programs, e.g., the Surfaceplot computer program. However, these programs only predict the linear B-cell epitopes. In this work, only one putative linear epitope which reacted with one MAb was found. The linear epitopes of MPT64 are apparently less dominant than the structural epitopes. In vitro-synthesized peptides have been widely applied for the mapping of B-cell epitopes. Clearly, this is a powerful and informative approach. However, by the synthetic peptide method it is not possible to detect epitopes of a composed nature or so-called conformational epitopes. We realize that even peptides of only 15 to 20 residues have a secondary structure (5), which may influence antibody binding, but for screening purposes the synthesis of peptides of more than 30 to 40 amino acids is still problematic. By genetic manipulation of genes, it is possible, in a simple and inexpensive way, to localize and map structurally separated but immunologically important domains of a protein.

MPT64 and MPB64 have been characterized as powerful skin test immunogens which can distinguish guinea pigs immunized with mycobacteria belonging to the tuberculosis complex from guinea pigs immunized with other mycobacteria (3, 10, 10a). We are in the process of mapping the T-cell epitope(s) of MPT64 with the recombinant proteins constructed in this study.

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