

Nucleotide Sequence Analysis and Seroreactivities of the 65K Heat Shock Protein from *Mycobacterium paratuberculosis*

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Mycobacterium paratuberculosis is the causative agent of Johne's disease, a chronic enteritis in ruminants. It has also been implicated as a possible cause of Crohn's disease, an inflammatory bowel disease of unknown etiology. The mycobacterial 65K heat shock proteins (hsp-65K) are among the most extensively studied mycobacterial proteins, and their immunogenic characteristics have been suggested to be the basis for autoimmunization in chronic inflammatory diseases. In this context, we isolated and sequenced the hsp-65K-encoding gene from our *M. paratuberculosis* PTB65K genomic library. A high degree of identity was found between the open reading frame (ORF) of the PTB65K gene and those of *Mycobacterium tuberculosis* (89.6%), *Mycobacterium leprae* (86.6%), and *Mycobacterium avium* 18 (98.8%). The amino acid sequence alignment of the PTB65K protein with the hsp-65K homologs revealed that the *M. tuberculosis* and *M. leprae* proteins each differed by 36 amino acid residues and that the *M. avium* 18 protein differed by 8 residues. We also investigated the humoral immune responses of animals with Johne's disease and patients with Crohn's disease against the recombinant PTB65K antigen. Immunoblot analysis showed that sera from only 3 of 10 clinically ill and 5 of 25 subclinically ill cows reacted with PTB65K. In addition, sera from two of two sheep and one of two goats with clinical symptoms of Johne's disease also reacted with PTB65K; 0 samples from 10 normal cows reacted. In humans, sera from 7 of 13 patients with Crohn's disease, 3 of 4 with tuberculosis, 5 of 6 with leprosy, 5 of 12 with non-inflammatory bowel disease, and 0 of 4 with ulcerative colitis reacted with the recombinant PTB65K antigen. These results indicate that this PTB65K heat shock protein is uninformative when used for serodiagnosis of Johne's disease in animals. However, in humans, the high intensity of antibody reactions of some sera from Crohn's disease patients compared with that from noninflammatory bowel disease patients showed a positive correlation with mycobacterial diseases.

Mycobacterium paratuberculosis is the etiologic agent of paratuberculosis or Johne's disease, an incurable chronic intestinal disease in ruminants (6). Paratuberculosis occurs worldwide and is economically important in the cattle industry. Eradication programs for this disease have been limited by the lack of accurate, reliable, and rapid diagnostic tests to identify and eliminate infected animals (6, 8). Although primarily considered the cause of a ruminant disease, *M. paratuberculosis* has also been isolated and/or identified from primates suffering from chronic ileitis (33) and patients with Crohn's disease, an inflammatory bowel disease of unknown etiology but with histopathologic features similar to those of Johne's disease in animals (4, 11, 23, 29, 31, 36, 44, 53).

An association of some immunodominant antigens of *M. paratuberculosis* and autoimmunity has been suggested for the pathogenesis of both Johne's disease and Crohn's disease (29, 30, 42). Hence, recently there has been an increased interest in the potential role of mycobacteria, especially *M. paratuberculosis*, in the pathogenesis of Crohn's disease. The 65K protein is a member of GroEL family of heat shock proteins. They are among the most extensively studied immunogenic mycobacterial proteins. These highly conserved mycobacterial hsp-65K proteins have been shown to be major stimulants of cellular

and humoral immunity against mycobacterial antigens (45, 50, 51). In addition, they have been implicated in the generation of autoimmune responses (20), in the pathogenesis of adjuvant-induced arthritis in animal models (26, 52), and in a general role in inflammatory responses (41). However, because of the extremely slow growth of *M. paratuberculosis* and the difficulties in isolating it from patients and even from infected animals (4), its role in the pathogenesis of these diseases remains undefined.

To overcome these limitations and to facilitate the identification and isolation of a specific *M. paratuberculosis* immunodominant antigen(s) or epitope(s), we previously constructed an *M. paratuberculosis* genomic library in an expression vector (14, 15). Using hyperimmune rabbit anti-*M. paratuberculosis* CBA-1 and IT-13 monoclonal antibodies (which recognize members of the 65K family of several mycobacterial species) and a PCR-generated DNA fragment of this molecule in *M. paratuberculosis* as a probe, we previously isolated and characterized a recombinant clone that expressed the *M. paratuberculosis* 65K (PTB65K) heat shock protein antigen. In the present study, the nucleotide sequence of the gene encoding this molecule was determined and compared with those of other mycobacterial 65K heat shock protein-encoding genes. The deduced amino acid sequences of the products were also compared. In addition, we have investigated and evaluated the reactivities of the *M. paratuberculosis* recombinant PTB65K protein against the humoral immune responses of animals with Johne's disease and of humans with Crohn's disease.

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MATERIALS AND METHODS

Bacterial strains and plasmids. *M. paratuberculosis* Linda (ATCC 43015), an isolate from a patient with Crohn's disease (provided by P. Brennan, Department of Microbiology, Colorado State University, Fort Collins), was grown in 7H9 broth (pH 5.9) supplemented with 100 ml of Middlebrook OADC enrichment (Difco Laboratories, Detroit, Mich.) per liter, 0.05% Tween 80, and 2 mg of mycobactin J (Allied Monitor, Fayette, Mo.) per liter as described previously (23). *Escherichia coli* INV alpha F' (a derivative of strain DH1 [Invitrogen Corp., San Diego, Calif.]) was used as a host for transforming the pcDNAII phagemid vector (from Invitrogen); it and its recombinant variants were grown in LB medium containing 200 µg of ampicillin per ml, as suggested by the manufacturer. Stock cultures of *M. paratuberculosis* and *E. coli* were stored at -70°C in their respective growth media supplemented with 25% glycerol.

DNA manipulations and mapping. We previously constructed a genomic library of *M. paratuberculosis* in an expression vector (pcDNAII) and screened it with antibodies and DNA probes (14, 15). We also identified and purified four recombinant clones containing 2.9-kb *Bam*HI inserts that hybridized to a 383-bp DNA probe which was specific for the mycobacterial 65K antigen-encoding genes. The antigenic identity of their expressed products was confirmed by immunoblots with IT-13 and CBA-1 monoclonal antibodies (15). One clone, designated pMptb 20, was selected for nucleotide sequencing and protein analysis.

Plasmid DNA was prepared by the alkaline lysis method (43). Restriction endonuclease digests were performed as recommended by the manufacturer (GIBCO BRL Life Technologies, Inc., Grand Island, N.Y.). The DNA insert mapping was accomplished by restriction pattern analyses of plasmid digests by agarose gel electrophoresis following standard procedures (43).

Subcloning and DNA sequencing. Plasmid DNA from the immunoreactive clone pMptb 20 was digested, and the *M. paratuberculosis* *Bam*HI 2.9-kb restriction fragment was electroeluted by agarose gel electrophoresis on a DEAE-cellulose membrane (NA-45; Schleicher & Schull, Keene, N.H.) as described previously (15). After cleavage with the appropriate endonucleases, restriction fragments of interest were electroeluted and subcloned into the multiple cloning site of the pcDNAII phagemid vector; 10 subclones were generated (Fig. 1A). These recombinant phagemids were transformed into INV alpha F' *E. coli*, and each transformant was tested for expression of mycobacterial antigen by sodium dodecyl sulfate (SDS) immunoblotting. For sequencing, each transformant was grown and rescued as plasmid DNA or as single-stranded phage. Initially, with the M13 phage universal or reverse primer, recombinant double-stranded plasmids or single-stranded phage were sequenced by the Nucleic Acids Core in the Institute for Molecular Genetics at Baylor College of Medicine by automated fluorescent DNA sequencing technology (46). Custom oligonucleotide primers (Genosys Biotechnologies, Inc., The Woodlands, Tex.) were also designed for regions of known sequence to produce overlapping fragments.

DNA and amino acid sequence analysis. Computer analysis was performed at the facilities of the Molecular Biology Computational Resource, Baylor College of Medicine. DNA sequence analysis was performed with the Genetics Computer Group (GCG) package, version 8 software (University of Wisconsin, Madison). DNA multiple alignment of four mycobacterial 65K-encoding genes was prepared by the use of the Treealign method of Jotun Hein (24). The nucleic acid and predicted amino acid sequences for PTB65K were compared with those in the EMBL and GenBank databases, including the Protein Identification Resource (22) database, with the use of the BLAST programs (1) provided via the worldwide web (2) (National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, Md.).

Antibodies and serum samples. Rabbit hyperimmune anti-*M. paratuberculosis* serum was produced in our laboratory by repeated subcutaneous immunization with *M. paratuberculosis* sonicate as described before (14). Specific monoclonal antibodies (IT-13, CBA-1, and ML-30) for mycobacterial 65K molecules, provided by the United Nations Development Program/World Bank/World Health Organization Special Program for Research and Training in Tropical Diseases, A. Bengaard Andersen, Mycobacterial Department, Staten Serum Institut, Copenhagen, Denmark, and J. Ivanyi, Tuberculosis and Related Infections Unit, Hammersmith Hospital, London, respectively, were also used (15).

A total of 51 animal serum samples, obtained from the National Animal Disease Center Mycobacteriosis Unit, Ames, Iowa; from M. Collins, University of Wisconsin School of Veterinary Medicine, Madison; and from Allied Monitor, Inc., Fayette, Mo., were used. Included among these samples were sera from two sheep, two goats, and 10 cows with clinical and 25 cows with subclinical naturally acquired Johne's disease, 2 cows inoculated with *M. paratuberculosis*, and 10 normal cows without detectable *M. paratuberculosis* infection.

Sera from 40 patients, including 13 with Crohn's disease, 5 with ulcerative colitis, 12 with non-inflammatory bowel disease (non-IBD) (including gastric and duodenal ulcers), 4 with tuberculosis (obtained from patients attending the VA Hospital), and 6 with disease classified as lepromatous leprosy who were untreated (obtained from P. Brennan, Colorado State University), were also tested.

To avoid any cross-reactivity with *E. coli* proteins, including *E. coli* hsp-65k, all sera were adsorbed as described previously for the rabbit antiserum (14, 15). Briefly, each serum sample was adsorbed by a mixture of nonrecombinant *E. coli* INV alpha F (Invitrogen Corp.) sonicate suspended in 2 ml of phosphate-buffered saline (PBS; 0.01 M Na₂HPO₄ · 7H₂O-NaH₂PO₄ · H₂O, 0.15 M NaCl

[pH 7.0]) and a boiled pellet. Each sonicate was prepared from 100 ml of an overnight LB broth culture containing 200 µg of ampicillin per ml. The sera were adsorbed at a dilution of 1:30 (vol/vol, serum/sonicate-pellet mixture) for 16 h at 4°C. The adsorption of each sample was tested and verified by its lack of reactivity with a blot strip containing fractionated nonrecombinant *E. coli* proteins. The adsorbed serum was then stored at -70°C in aliquots until use.

Immunoblotting. Immunoblotting procedures were performed as described previously (15). The protein content of *M. paratuberculosis* sonicates was estimated by the Coomassie blue dye-binding assay with respect to an albumin standard according to the instructions of the manufacturer (Bio-Rad Laboratories, Richmond, Calif.). For recombinant and nonrecombinant *E. coli* lysates, bacterial cells from 1 ml of culture (optical density at 600 nm of 2.0) were harvested by centrifugation and suspended in 50 µl of sterile deionized water per bacterial pellet. Bacterial proteins were solubilized by boiling for 5 min in an equal volume of gel sample buffer (Tris-Cl [pH 6.8], 2.5% SDS, 100 mM dithiothreitol [DTT; Sigma Chemical Co.], 10% [vol/vol] glycerol, 0.002% [wt/vol] bromophenol blue) and fractionated by SDS-12% PAGE. Protein blotting was performed with a Milliblot-SDE Transfer System (Millipore Intertech, Bedford, Mass.) onto Immobilon-P transfer membranes (polyvinylidene difluoride; Millipore Intertech) as described by the manufacturer.

Immunoblot strips containing fractionated proteins from clone pMptb 20, which contained the MTB65K-encoding gene and its expressed product, were tested against each individual adsorbed serum or monoclonal antibody. Filters were washed in TNT buffer (10 mM Tris [pH 7.5], 150 mM NaCl, 0.05% [vol/vol] Tween 20) and in blocking buffer (TNT containing 5% nonfat milk). Filters were then incubated with adsorbed serum (diluted in blocking buffer; 1:500 for rabbit antiserum, 1:30 for animal sera, and 1:50 for patient sera or 1:100 and 1:1,000 for monoclonal antibodies with ascites fluid and supernatant, respectively) overnight at 4°C. Color was visualized with appropriate anti-immunoglobulin G (IgG)-peroxidase conjugates and 4-chloro-1-naphthol substrate. Color development was complete in 15 to 20 min. The filters were then washed in distilled water and dried.

Nucleotide sequence accession number. The nucleotide sequence of the 65K-hsp of *M. paratuberculosis* Linda has been submitted to the EMBL data library under accession number U15989.

RESULTS

Cloning of *M. paratuberculosis* 65K-encoding gene. In a previous report, the creation of an *M. paratuberculosis* expression library and the isolation of recombinant clone pMptb 20 expressing the PTB65K heat shock protein were described (15). To evaluate and confirm the specificity and the molecular weight of the expressed product, SDS-immunoblot strips containing *M. paratuberculosis* sonicate and recombinant (pMptb 20) and nonrecombinant *E. coli* lysates were incubated with the adsorbed hyperimmune rabbit antisera and the specific monoclonal antibodies CBA-1, IT-13, and ML-30. As we have shown previously (15), the reactivities were specific to PTB65K, confirming the identity of the expressed product as *M. paratuberculosis* 65K antigen. These results demonstrate that the PTB65K-encoding gene is located within a 2.9-kb *Bam*HI insert fragment. Restriction enzyme analysis of the recombinant phagemid carrying the PTB65K gene and subsequent subcloning of selected restriction fragments and their expression analyses on SDS immunoblots of whole-cell lysate proteins indicate that subclone 6 (pMptb 20/S6) expressed a truncated peptide of approximately 25 kDa (Fig. 1A and B, lane 6). This subclone, containing an 828-bp fragment starting from base 1, was ligated into the *Pst*I and *Kpn*I sites of the pcDNAII vector. The nucleotides encoding the amino acids included 690 bp, which included 677 bp from the initiation codon and 13 bp in the cloning site of the vector (where the stop codon was formed) extending from the *Kpn*I towards the *Hind*III site. The 690 bp encode 230 amino acids, which is comparable to the theoretical molecular weight of 25,300 (Fig. 1A and B). This also indicates that the open reading frame (ORF) was located within this fragment and that the gene encoding PTB65K is transcribed in the direction indicated by the arrow in Fig. 1A. However, the monoclonal antibodies CBA-1 and ML-30 (IT-13 was not tested because of its depletion at the source) did not react to subclone 6. This may indicate that their determinants were not located on this subclone. In addition, the lack of expression of subclone

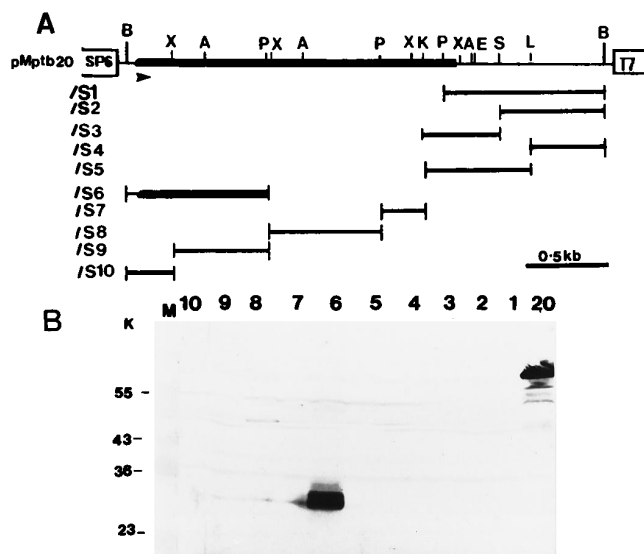


FIG. 1. Physical map of pMptb 20 and subclones used for sequencing and expression analysis. (A) Heavy bars represent the expressed PTB65K-encoding gene and subclone 6 (pMptb 20/S6). Thin bars represent subclones created in the same vector, and the arrow indicates the direction of transcription. The open brackets labeled SP6 and T7 denote promoter sequences on the pCDNAII vector. S1 to S10 on the left are pMptb subclones. Restriction sites: A, *Sac*I; B, *Bam*HI; E, *Eco*RI; K, *Kpn*I; L, *Sal*I; P, *Pst*I; S, *Sph*I; X, *Xba*I. (B) Immunoblots of expressed products of the subclones. Rabbit anti-*M. paratuberculosis* serum was used as the primary antibody. Lane numbers are subclone numbers. Lane 20, lysate from the whole pMptb 20 clone; lane M, molecular weight markers. The numbers to the left are molecular weights (in thousands).

9 (pMPTb 20/S9 [Fig. 1A and B, lane 9]), which starts from nucleotide 226, located 58 bases upstream of the first ATG codon, indicates that the promoter of this PTB65K-encoding gene is located upstream of GTG (at position 151), the initiation codon for this peptide. It is also possible that a potential second promoter (between GTG and ATG) is interrupted and not complete in this subclone.

DNA sequence analysis of the 65K-encoding gene. The nucleotide sequence of 2,643 bases extending from the Sp6 towards the T7 site of clone pMptb 20 is shown in Fig. 2. The DNA had a G+C content of 67.67%, similar to that calculated for the mycobacterial genome. Analysis of the sequence revealed an ORF of 1,749 bases, starting with the GTG codon at positions 151 to 153 and terminating with the TGA stop codon at positions 1897 to 1899. This is in the same reading frame that corresponds to the size of the expressed protein. The ORF and its deduced amino acid sequence are shown in Fig. 2. Analysis of the ORF with the GCG package predicted that it encodes a peptide consisting of a 582-amino-acid translation product with a theoretical relative molecular weight of 61,175 and an isoelectric point of 4.91. The G+C content in the ORF is 67.5%. The first AUG in the ORF occurs at position 274, and a polypeptide starting at this position would have 541 amino acids, with a molecular weight of 56,858. However, the expressed antigen migrated at about 65,000 by SDS-PAGE (15) and on immunoblots (Fig. 1B, lane 20). Codon usage analysis revealed a strong preference (91%) for G or C in the third base position. This codon composition and bias are consistent with the published sequences of other mycobacterial structural genes (44, 49). Scanning of the putative promoter sequence upstream of the ORF revealed a potential Shine-Dalgarno ribosome-binding site, GGCAC (bases -18 to -13), and possible -10 TAAGAAT (bases -31 to -25) and

-35 TTGCAC (bases -54 to -49) sequences with 17-bp spacing, which is the optimal distance for promoters in prokaryotes (Fig. 2). The potential formation of a hairpin-loop structure for transcriptional termination is apparent: nucleotide -10 upstream of the putative initiation codon and 8 bases downstream of the putative translation stop codon, as indicated by the arrows in Fig. 2.

PTB65K-encoding gene and its homology to other mycobacterial hsp-65K genes. Comparative computer analyses of the nucleotide and predicted amino acid sequences and those found in the GenBank database demonstrated that the PTB65K-encoding gene from *M. paratuberculosis* Linda is the homolog to the 65K-encoding genes of the *Mycobacterium* species *M. tuberculosis* (45), *M. bovis* (50), *M. leprae* (34), and *M. avium* 18 (9). *M. avium* 18 was previously known as *M. paratuberculosis* 18, which was a bovine strain that was mycobactin independent (5, 25, 35, 54). It has already been shown that the DNA sequences of the 65K-encoding genes of *M. tuberculosis* and *M. bovis* are identical. A high degree of identity was found between the ORF of the PTB65K-encoding gene and the *M. tuberculosis* (89.6%), *M. leprae* (86.6%), and *M. avium* 18 (98.8%) genes, with the greatest identity being within the area of alignment.

The deduced amino acid sequence of PTB65K showed a high degree of similarity to that of the GroEL hsp-65K family of chaperonins, especially to the sequenced 65K-encoding genes of other mycobacteria. The amino acid sequence of PTB65K was aligned with the corresponding published sequences for homologous hsp-65K proteins from other pathogenic mycobacteria in the GenBank database, and the following relationships were revealed: 93% identity with the *M. tuberculosis* protein, 89% identity with the *M. leprae* protein, and 98% identity with the *M. avium* 18 protein. Figure 3 shows the extent of sequence identity between these mycobacterial polypeptides. In addition, PTB65K also showed 46% identity in a 582-amino-acid alignment within the complete sequence of the human mitochondrial hsp60 protein (27).

Conservation of genetic elements is also apparent. The putative ribosome-binding regions of both the *M. leprae* and *M. paratuberculosis* genes were identical except for the -10 sequence, which differed at only one position (six of seven bases). However, the promoter sequence of the *M. paratuberculosis* gene was similar to that of the *M. leprae* gene but not identical to those of any of the other mycobacterial promoters that have been sequenced. Inverted sequences similar to those found upstream and downstream of PTB65K have also been identified for several mycobacterial structural genes (38, 40, 51).

Serological reactivity of the *M. paratuberculosis* 65K protein.

Initially, to determine whether the humoral immune response could recognize *M. paratuberculosis* antigens, SDS immunoblot strips containing *M. paratuberculosis* sonicate were incubated with adsorbed sera from animals with Johne's disease and patients with Crohn's disease as well as controls. Although reactivities were observed with all the sera tested, including the controls (Fig. 4 and 5, lanes L), no specific pattern for a specific disease could be established. This was not surprising because of the cross-reactivities among pathogenic and environmental mycobacteria and other unrelated bacterial species. To investigate if any of these serum samples recognized the PTB65K antigen, copies of SDS immunoblot strips containing the recombinant *E. coli* (pMptb 20) lysates were incubated with each adsorbed serum. One copy of these immunoblot strips was always tested for reactivity with the adsorbed rabbit antiserum as a positive control.

When animal sera were tested against the PTB65K antigen, the results were uninformative serologically. Weak reactivities

1 GGATCCTGGAGATCCATCTGGTGGACCCGCGGCGGTTTCGGCGGCTTCTACGAGCAGCTGCTCGACCTGCCCTAGTTGCGACCCGACCTGTTCTTG
-35
101 CACTCGGCATGGGCGAGTGTCTAAGAACTGGCACTCGCGACCACTGAGTGTAGGTGCGGACGGTGAAGCTCGGCTCTGCTCGCCGACGGCCAGC
-10 SD V L G R D G E A R L C R R P T A A

201 AGCCTGGTTCGTCGCGGGCACTGCACCCGGCCAGGACGTGTCATCCCCAAATCCGGAGGAATCACTTCGCAATGGCCAAGACAATTGCGTACGACGAA
A W S S V A G T A P G Q D V S S P I R R N H F A M A K T I A Y D E

301 GAGGCCCGTCGCGGCTCGAGCGGGGCTCAACGCCCTCGCCGACGCGGTAAAGGTACGTTGGGCCCCAAGGTCGCAACGTCGTCCTAGAGAAGAAT
E A R R G L E R G L N A L A D A V K V T L G P K G R N V V L E K K

401 GGGGTTCCCCACGATCACCAACGATGGTGTGTCCATCGCCAAGGAGATCGAGTGGAGGACCCGTACGAAAAGATCGCGCCGAGCTGGTCAAGGAAT
W G S P T I T N D G V S I A K E I E L E D P Y E K I G A E L V K E V

501 CGCCAAGAAGACCGACGCTCGCCGTTGACGGCACGACGCGCCACGGTGTCTCGCCAGGCGTGGTCCGCGAGGGCTGCGCAACGTCGCGGCCGGC
A K K T D D V A G D G T T T A T V L A Q A L V R E G L R N V A A G

601 GCCAACCCGCTGGGTCTCAAGCGCGGCATCGAGAAGGCCGTCGAGAAGGTACCGAGACCCCTGCTCAAGTCGGCCAAGGAGTTCGAGACCAAGGACCA
A N P L G L K R G I E K A V E K V T E T L L K S A K E V E T K D Q

701 TCCTGCCACCGCGCCATCTCCGCGGGCGACAGTTCGATCGCGACCTGATCGCCGAGGCGATGGACAAGGTGGCAACGAGGGCGTCATCACCGTCGA
I A A T A A I S A G D Q S I G D L I A E A M D K V G N E G V I T V E

801 GGAGTCCAACACCTTCGGCTGCAGCTCGAGCTACCGAGGGTATGCGGTTTCGACAAGGGTTACATCTCGGGCTACTTCGTCACGGACGCCGAGCGTCAG
E S N T F G L Q L E L T E G M R F D K G Y I S G Y F V T D A E R Q

901 GAAGCGTCCGAGGACCCGTTCATCTGCTGGTCAGTCCAAGGTCTCGACCGTCAAGGACCTCTGCGCTGCTGGAGAAGGTATCCAGGCCGGCA
E A V L E D P F I L L V S S K V S T V K D L L P L L E K V I Q A G

1001 AGCCGCTGTTGATCATCGCCGAGGACGTGAGGGCGAGGCCCTGTCCACCTGGTTCGTCACAAGATCCGCGGCACCTTCAAGTCGGTGGCCGTCGAAGGC
K P L L I I A E D V E G E A L S T L V V N K I R G T F K S V A V K A

1101 GCCCGGCTTCGCGCACCGCCGAAGGGATGCTTCAGGACATGGCCATCCTCACCGGCGCCAGGTTCATCAGCGAAGAGGTGGCCCTGTCGCTGGAGAGC
P G F G D R R K A M L Q D M A I L T G G Q V I S E E V G L S L E S

1201 GCCGACATCTGCTGCTCGGTAAGGCCCGCAAGGTGCTGTCACCAAGGACGAGACCCATCGTCGAGGGCGCGGTGACTCCGACGCCATCGCCGGCC
A D I S L L G K A R K V V V T K D E T T I V E G A G D S D A I A G

1301 GGGTGGCCAGATCCGCACCGAGATCGAAGACGCGACTCCGACTACGACCGGAGAAGTGCAGGAGCGGCTGGCCAAGTGGCCGGCGGCTGGCGGT
R V A Q I R T E I E N S D S D Y D R E K L Q E R L A K L A G G V A V

1401 GATCAAGGCCGCGCGCCGACCGAGGTGAGCTCAAGGACGCGAAGCACCATCGAGGACCGGTCCGCAACGCCAAGGCCGCGTGGAGGAGGATC
I K A G A A T E V E L K E R K H R I E D A V R N A K A A V E E G I

1501 GTCGCGGCGGTGGCGTGGCCCTGCTGCACGCGATCCCGGCTCTGGACGAGCTGAAGCTCGAGGGCGAAGAGGCGACCGGCCCAACATCGTCCGGTGG
V A G G G V A L L H A I P A L D E L K L E G E E A T G A N I V R V

1601 CCCTCGAGCGTCCGCTGAAGCAGATCGCCTTCAACGGTGGCCCTGGAGCCCGGCGTGGTGGCCGAGAAGTCCGCAACTCGCCCGCGGTACCGCCCTCAA
A L E R P L K Q I A F N G G L E P G V V A E K V R N S P A G T G L N

1701 CGCCGCCACCGGTAAGTACGAGGACCTGCTCAAAGCCGGCATTACCGAACCGGTTAAGGTACCCGCTCGGCGTGCAGAACCGCGCTCCATCTCGGGG
A A T G K Y E D L L K A G I T E P V K V T R S A L Q N A A S I S G

1801 CTGTTCTGACCACCGAAGCGGTGCTCGCCGACAAACCGGAAAAGACGGCCCTCCCGGGCGACCCGACCGCGGCATGGGCGCATGGACTTCTGAG
L F L T T E A V A D K P E K T A P P A G D P T G G M G G M D F *

1901 TCCGTTACGGAAAAGCCCGGTCCTGAAATNTCGAGTGGCCGGGANTTTTCGTCGCCGCGTGGGCGGAGTTTACCGCNCAGCGTGGGGCTACGT
2001 CCGTCCACACCCGAAATCGTGCACAGCCCCAAGCTGCCTGACCCGCCGCGGTGTCGACGGCGATTATGCCGATGCCCCGTTCTGGGCACTGA
2101 CGCCTGCACCGCGCGCGTACCCGACGTCACCTTGCAGACCGGTATCGCAAGGTGTTCCGCGACGTCACATCGCGAAGGACCGGAGCTGACGCCG
2201 GCCGCAAAAGCGCGCGCGGTGGTGTGCGCGGTGCGACACTGGCCGTTTGTCCGACGCGGATCCATGGACCAAGTGGCTCGATGCGGCCGNGCC
2301 CGCCGAAATCGTGGCGGATCGGCACGGTCAGCGCGCATCCTGTCGCGAGTANAACGCTGGCCGATGACGAGGCGGACAGCGTGTCCGGAATGCGG
2401 GTAACGACCGCCCGGACGGCGTTCGANATCGGTTGCGGCTGCCCGCCGCAAGGGTGCCTGCTCGACGCGTTACTCAACGCGACGGGTATCAA
2501 GCCCGCCGACGTCGTCGCTGTCGCGGACCGNACCGGGCGCCCGGCAATTCGCGGCTGCGCGCTCGTGGAGTTGCCGACGGCGGTGCGGAGTNTC
2601 CGAAGAAAACAAGGTTNCGGGTGTCTTNGTNCGGCCGACTTC

FIG. 2. Nucleotide sequence of clone pMpb 20. The nucleotide sequence of the *M. paratuberculosis* 65K protein (PTB65K) ORF and its predicted 582-amino-acid sequence of 61,175 Da is indicated. A putative Shine-Dalgarno (SD) ribosome-binding site, possible promoter sequence (-10 and -35), and initiation codons are underlined. *, putative stop codon. Arrows indicate inverted sequences.

M. paratuberculosis	VLGRDGE-----ARLCRRPTAAAWSSVAGTAPGQDVSSP IRRNHFAMAKTIA YDEEARRGLERGINALADAVKVTLLGPKGRNVVLEKKWGSPTITNDGV	94
M. avium 18A.....	53
M. tuberculosisA.....	53
M. leprae	MP.....TQPASCG..SR.LHP.SV.NGGCRH.VTLA.FL.....S.....	100
M. paratuberculosis	STIAKEIELEDPEYEKIGAEVLKVEAKKTDDVAGDGTTTATVLAQALVREGLRNVAAGANPLGLKRGIEKAVEKVTETLLKSAKEVETKDIQAATAAISAGD	194
M. avium 18G.....E.....	153
M. tuberculosisG.....E.....	153
M. lepraeK.....D.....E.....	200
M. paratuberculosis	QSIGDLIAEAMDKVNEGVI TVEESNTFGLQLEL TEGMRFDKGYISGYFVTD AERQEA VLEDPFILLVSSKVSTVKDLLP LLEKVIQAGKPLLI IAE DVE	294
M. avium 18P.....Y.....G.....	253
M. tuberculosisP.....Y.....G.....	253
M. lepraeE.Y.....S.....	300
M. paratuberculosis	GEALSTLVNKRIRGTFKSVAVKAPGFGDRRKAMLQDMAILTGGQVISEEVGLSLESADTSLLGKARKVVVTKDETTIVEGAGSDAIAGRVAQIRTEIEN	394
M. avium 18I.....	353
M. tuberculosisT.N.L.....T.....Q.....	353
M. lepraeA.....T.NT.L.....M.....T.....	400
M. paratuberculosis	SDSYDREKLQERLAKLAGGVAVIKAGAATEVELKERKHRIEDAVRNAKAAVEEGIVAGGGVALLHAI PALDELKLEGE EATGANIVRVALERPLKQIAF	494
M. avium 18P.....	453
M. tuberculosisT.Q.A.T.....D.....K.....A.....	453
M. lepraeT.Q.A.....K.....T.D.....K.....A.....	500
M. paratuberculosis	NGGLEPGVVAEKVRNSPACTGLNAATGKYEDLLKAGITEPVKVTRSALQNAASIAGLFLITEAVVADKPEKTAPPAGDPTGGMGMDP	582
M. avium 18E.....AD.....A.A.....	541
M. tuberculosisS.....L.....H.....Q.....V.....A.VAD.....EKASVP-GG.D.....	540
M. lepraeS.M.....LSV.H.....E.....VAD.....A.S.....	588

FIG. 3. Comparison of amino acid sequences of four mycobacterial 65K antigens. Identical residues are represented by dots, and gaps that had to be introduced to maximize sequence alignment are indicated by dashes. The strains were *M. paratuberculosis* Linda and *M. avium* 18, which was recently reclassified (formerly *M. paratuberculosis* 18) (see text for details).

were observed with only 3 of 10 (30%) clinical and 5 of 25 (20%) subclinical samples from cows. In addition, sera from neither of the two passively immunized cows nor any of the 10 normal cows reacted to the PTB65K antigen. To determine if sera from other naturally infected animal species with Johne's disease could recognize this recombinant clone, sera from two sheep and two goats with paratuberculosis were tested. Sera from both sheep and only one goat with clinical symptoms reacted weakly with this antigen. Figure 4 shows some of these reactivities. These results indicate that the humoral immune responses of different species to this antigen differ.

When sera from Crohn's disease, ulcerative colitis, and non-IBD patients were tested, 7 of 13 (54%) from Crohn's disease and 5 of 12 (42%) from non-IBD patients reacted to PTB65K antigen; 0 of the 5 ulcerative colitis patients' sera reacted to this antigen. Although some sera from non-IBD patients rec-

ognized this antigen, the reactivities of Crohn's disease patients' sera were of higher intensity, as observed visually, indicating the potential association with mycobacterial infection in at least this subset of patients. To study this possibility, we compared their humoral reactivities with those of sera from patients with other mycobacterial diseases. As shown in Fig. 5, sera from 75% of tuberculosis and 83% of leprosy patients reacted positively to PTB65K protein, with intensities similar to those of sera from Crohn's disease patients. This was not

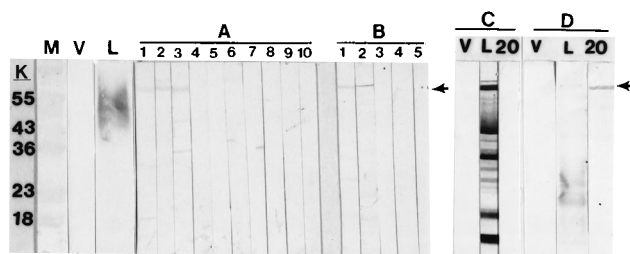


FIG. 4. Immunoblot analysis of reactivities of PTB65K with sera from infected animals with paratuberculosis (Johne's disease). Reactivities with sera from cows with clinical (A) and subclinical (B) paratuberculosis and a goat (C) and sheep (D) with clinical paratuberculosis are shown. The blot strips with lysates of *E. coli* containing nonrecombinant vector in lane V, sonicates of *M. paratuberculosis* Linda in lane L, and clone pMptb 20, which contains the PTB65K-encoding gene, in lane 20 were incubated with the indicated adsorbed animal serum. Lane M, molecular weight markers; numbers to the left show molecular weights (in thousands). Arrows indicate the expected position of the PTB65K protein.

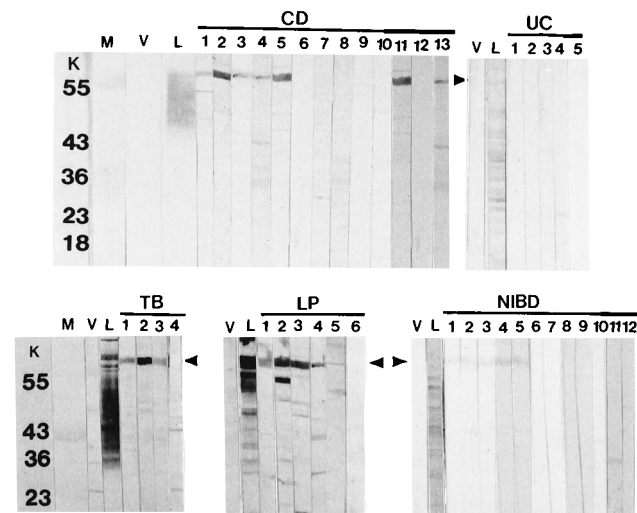


FIG. 5. Immunoblot analysis of reactivities of PTB65K with sera from patients with Crohn's disease (CD), ulcerative colitis (UC), tuberculosis (TB), leprosy (LP), and non-IBD (NIBD). Lanes V and L are blot strips, as indicated in the legend to Fig. 4, that were incubated with the indicated adsorbed patient sera. Lane M, molecular weight markers; numbers to the left show molecular weights (in thousands). Arrows indicate the expected position of the PTB65K protein.

surprising because of PTB65K's high homology to other mycobacterial hsp-65K proteins (Fig. 3). These results indicate the potential involvement of mycobacteria in the pathogenesis of Crohn's disease.

DISCUSSION

M. paratuberculosis is an obligate intracellular parasite. As a consequence of the difficulties associated with its isolation, i.e., its extremely slow growth rate and mycobactin dependency, immunological, diagnostic, and therapeutic studies have been limited. This intracellular pathogen causes paratuberculosis (Johne's disease) and recently has been implicated as a possible cause of Crohn's disease. Information on the antigenic determinants or epitopes that are recognized by antibodies and/or T cells from animals with Johne's disease and individuals with Crohn's disease is lacking. Furthermore, no clinical or laboratory markers to identify animals with subclinical disease or individuals who are afflicted with Crohn's disease are currently available. The availability of well-characterized *M. paratuberculosis* antigens should make it possible to address some problems in paratuberculosis and, perhaps, Crohn's disease that have been difficult to investigate.

The 65K antigen seems to play a dominant role in the immune response to pathogenic mycobacteria (18, 28, 39). Its possible implication in the etiology of autoimmune arthritis (52), its cross-reactivity with human lactoferrin (an iron-binding glycoprotein that is found in plasma and on mucosal surfaces) (21), and its general role in inflammatory responses (41) merit a detailed analysis at the epitope level. In this context, we have sequenced and characterized a recombinant *M. paratuberculosis* 65K-hsp (PTB65K) that we previously isolated from our expression library (15).

Sequence analysis of our PTB65K-encoding gene revealed the expected high G+C content (67.5%) that is usually found in mycobacterial genomes and in the other mycobacterial 65K-hsp-encoding gene sequences (9, 34, 45, 50). The DNA sequence identity between *M. paratuberculosis* and *M. tuberculosis*, *M. leprae*, and *M. avium* was 89.6%, 86.6%, and 98.8% (19 mismatches), respectively, showing the closest relatedness to the last. Similarly, the upstream region of our PTB65K-encoding gene contained the possibility of two putative initiation codons at nucleotide 151 (GTG, Val) and at 274 (ATG, Met), as was reported for the other 65K-hsp-encoding genes. The theoretical molecular mass and the two potential initiation codons are similar to those described for the hsp-65K of *M. leprae*. Because the molecular weights of the expressed antigens of PTB65K and subclone 6 (pMptb 20/S6) on immunoblots were about 65,000 and 25,000, respectively, as shown in Fig. 1B, lanes 20 and 6, the putative initiation codon for our PTB65K gene was the GTG at nucleotide 151, as described for *M. leprae* (34), and not at the first ATG, as described previously for *M. tuberculosis* or *M. avium* 18 (9, 45). If the initiation codon was at nucleotide 274 (ATG), then the approximate molecular weights of PTB65K and the subclone 6 protein would be 56,858 and 20,790 and not 61,175 and 25,300, respectively, which closely correspond to the immunoblot analysis results. However, deletion-insertion sequences between the two initiation codons were noticed among the four nucleotide sequences: 4, 6, and 30 nucleotides from *M. paratuberculosis* and *M. avium* and 24 nucleotides from *M. leprae* compared with the *M. tuberculosis* sequence (data not shown). The consensus sequence of the promoter is identical to that of *M. leprae* with the exception of the -10 nucleotides, with one base mismatch (data not shown).

The 65K antigen is a highly conserved molecule which had a

high degree of sequence homology in bacteria and humans (21, 27, 37). This was apparent when the amino acid sequence of PTB65K revealed 93%, 89%, and 98% identity with the amino acid sequences of the 65K-hsps of *M. tuberculosis*, *M. leprae*, and *M. avium* 18, respectively. The four regions of 10 or more amino acid residues that were described to be fully identical between the human mitochondrial hsp60 and the *M. leprae* and *M. tuberculosis* proteins are also conserved in PTB65K and the *M. avium* 18 protein (37). These regions are located at amino acid residues 125 to 136, 285 to 304, 313 to 322, and 444 to 455 (Fig. 3). However, the PTB65K of *M. paratuberculosis* differed from that of *M. avium* 18 by 8 amino acid residues and from those of *M. tuberculosis* and *M. leprae* by 36 residues within the aligned regions. The most variable segments among the four mycobacterial species tested within the aligned regions were clustered at the C termini and located at amino acid residues 455 to 472, 510 to 533, and 566 to 576. The homology between the *M. leprae* and *M. paratuberculosis* proteins at the N termini was only 45.6%, with 6 amino acid residues deleted from the *M. paratuberculosis* protein. These differences in amino acid residues at the C and/or N terminus of PTB65K may contribute to the creation of a specific epitope(s) or motifs for *M. paratuberculosis*. We have shown previously that one amino acid substitution within a protein may change the antigenic structure of that molecule (16, 17).

The humoral immune response of animals with Johne's disease and humans with Crohn's disease against the mycobacterial 65K-hsp has been assessed previously (7, 10, 30, 32, 47). Most of these authors used a complex mixture of antigens in an enzyme or immunoblot assay. Such tests for the serodiagnosis of paratuberculosis or Crohn's disease were found to be limited and uninformative because of low sensitivity and specificity. To assess the humoral immune response in animals with subclinical and clinical Johne's disease as well as in patients with Crohn's disease and negative controls, we tested 51 animal and 40 patient serum samples against our recombinant PTB65K antigen. Our immunoblot results showed variability in the immune response to this antigen. Similar to the results of others, there was no specific pattern of reactivity that corresponded with a specific stage of the disease, nor was there any correlation between infected or uninfected animals and this antigen (Fig. 4).

Recently, it has been suggested that serum IgA and not IgG in Crohn's disease and ulcerative colitis patients may interact significantly with antigens from *M. paratuberculosis* (48). On the contrary, none of our 10 Crohn's disease patient serum IgA samples reacted to PTB65K antigen, although one of two sera from ulcerative colitis patients did produce a faint band (data not shown). We have no explanation for this controversial result, especially when recombinant hsp-65 from *M. bovis* BCG was used in their studies. Perhaps the isolation of gut IgA might be more informative than serum IgA, as suggested recently (6). However, our results were somewhat more informative when human, not animal, IgG was evaluated against PTB65K. The antibody responsiveness to PTB65K varied among individuals (Fig. 5). The 42% of non-IBD patients (gastric or duodenal ulcer patients) who were positive for PTB65K antigen were likely infected with *Helicobacter pylori*, which we recently showed to cross-react with the epitope of the 65K-specific monoclonal antibody ML-30 (19). A comparable observation was reported by Thole et al. (50), who found that about 30% of their control group reacted to the recombinant hsp-65K of *M. bovis* BCG. However, when the intensity of the reactivities of our positive samples was analyzed, 54% of the Crohn's disease patients had higher titers of IgG antibodies to PTB65K than the positive non-IBD individuals. The same cor-

relation was recently made by Elsaghier et al. (12), who found that 52% of Crohn's disease patients had significantly elevated titers to the recombinant hsp-65K relative to the controls. Furthermore, the same high intensity was observed when we compared the reactivities of Crohn's disease sera to the reactivities of tuberculosis and leprosy patients' sera. Immunologic cross-reactivity was not unexpected because of the extensive amino acid sequence identity between PTB65K and the other mycobacterial hsp-65K proteins. Together, these observations suggest that those individuals who have enhanced immune responsiveness to mycobacterial hsp-65K may have a mycobacterial infection. The heightened response of some Crohn's disease patients to mycobacterial hsp-65K may indicate a potential association with mycobacterial infection. The same conclusion was recently drawn when Crohn's disease patient sera were tested against other *M. paratuberculosis* antigens (13). However, more data are needed to confirm this potential association and to determine whether these anti-PTB65K antibody activities can be correlated with disease pattern (active or not active) or disease severity.

In conclusion, PTB65K may not be useful for serodiagnosis, but its reactivity may potentially be informative about the infecting agent and/or the stage of disease in some Crohn's disease patients. Furthermore, it is possible that a specific epitope(s) found on this clone, in conjunction with other epitopes found on other antigens of *M. paratuberculosis*, could form the basis for development of a practical serological test for paratuberculosis and, perhaps, Crohn's disease. Its application in the development of a vaccine for these maladies is also of interest. The availability of PTB65K antigen and its encoding gene would facilitate some of these studies.

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