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Evidence for Absence of the MPB64 Gene in Some Substrains of *Mycobacterium bovis* BCG

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Substrains of *Mycobacterium bovis* BCG have been divided in two major groups, high producers and low producers of the secreted proteins MPB64 and MPB70. Of these, *Mycobacterium tuberculosis* secretes only the analog MPT64 during growth on Sauton medium. It has been confirmed that high-producer and low-producer substrains of BCG as well as *M. tuberculosis* contain the gene for the MPB/MPT70 protein. By contrast, polymerase chain reaction and hybridization experiments are reported here which indicate that the MPB64 gene is absent in the BCG substrains Copenhagen, Pasteur, Glaxo, and Tice, in which previous methods did not permit distinction between secretion of small amounts or absence of the protein in culture fluids.

The MPB64 protein was purified from the culture fluid after growth of *Mycobacterium bovis* BCG substrain Tokyo on Sauton medium (8). It belongs to the group of proteins actively secreted by mycobacteria (9, 20), and the gene, originally cloned and sequenced by Yamaguchi et al. (23), contains a typical signal sequence. Different substrains of BCG vary markedly in production and secretion of this protein, and apparently high-producing and low-producing substrains were identified by radioimmunoassay (RIA) inhibition tests for MPB64 in culture fluids with polyclonal rabbit anti-MPB64 (8). This distinction corresponds fully to the division of BCG substrains into high and low producers of MPB70 (7, 16), another actively secreted protein whose cloned gene (19) also contains a typical signal sequence.

In *Mycobacterium tuberculosis*, there is a striking contrast concerning the corresponding proteins. In RIA inhibition tests based on the use of labelled MPB70 and monospecific, polyclonal rabbit anti-MPB70, this protein was barely detectable in *M. tuberculosis* culture fluid, while an MPB64 analog was readily detected by RIA inhibition tests as well as two-dimensional electrophoresis (8).

Based on the striking species specificity of MPB64 and its occurrence in these two major pathogens, *M. tuberculosis* and virulent strains of *M. bovis*, we chose primers covering the positions 459 to 699 of the MPB64 gene in our work to establish a polymerase chain reaction (PCR) technique for the diagnosis of *M. tuberculosis* in clinical samples. This region has previously been used by Manjunath et al. (13) for this purpose. The reaction has provided positive signals in all the *M. tuberculosis* isolates tested so far.

As a part of these investigations, different substrains of BCG were also tested, with the surprising observation that this region of the gene could only be amplified in some of the substrains. Other primers covering a larger part of the gene were then tested with similar results. We report these studies and hybridization experiments, all indicating that the MPB64 gene is absent in some substrains of BCG.

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MATERIALS AND METHODS

Bacterial strains. The BCG substrains tested were the same as in our previous studies (7, 15). BCG Tokyo substrain 172 and BCG Copenhagen substrain 1331 were the reference high- and low-producer strains of MPB64 and MPB70, respectively. In addition, the following substrains were tested: BCG Glaxo substrain 1077, BCG Moreau, BCG Pasteur substrain 1173P2, BCG Sweden, BCG Tice, and BCG Russia. BCG Copenhagen was obtained from the State Serum Institute, Copenhagen, Denmark, and all other strains were from National Institute of Health, Tokyo, Japan. The bacteria were grown on Ogawa slants and obtained for PCR studies from the slant surface, while separate samples were transferred for further cultivation in Sauton liquid medium.

M. tuberculosis H37Rv (ATCC 27294), H37Ra (collection of the National Institute of Public Health, Oslo, Norway), and two routine isolates of *M. tuberculosis* (defined by growth characteristics, colony appearance, positive niacin test, and presence of cord factor) from two patients with pulmonary tuberculosis were grown on Loewenstein-Jensen medium for PCR studies.

For comparative assay of MPT64 content in *M. tuberculosis* H37Rv and H37Ra, culture fluids were provided by Peter Andersen, State Serum Institute, Copenhagen, Denmark, after growth of their reference strains for 6 weeks on Sauton medium.

M. bovis AN5, MNC no. 433, was obtained from the State Serum Institute, Copenhagen, and kindly grown by Finn Saxegaard at The Veterinary Institute, Oslo, for PCR studies and characterization of protein content in culture fluid.

Staphylococcus aureus used for a control in the hybridization test was a routine isolate (defined by growth characteristics, colony appearance, and coagulase and DNase positivity).

Terminology. Purification of MPB70 was originally described by Nagai et al. (16). The term MPB was then introduced for the designation of a protein purified from *M. bovis* BCG, with a number denoting the relative mobility in 7.7% polyacrylamide gels at a running pH of 9.5 by the method of Davis (4a). Purification of MPB64 was reported by

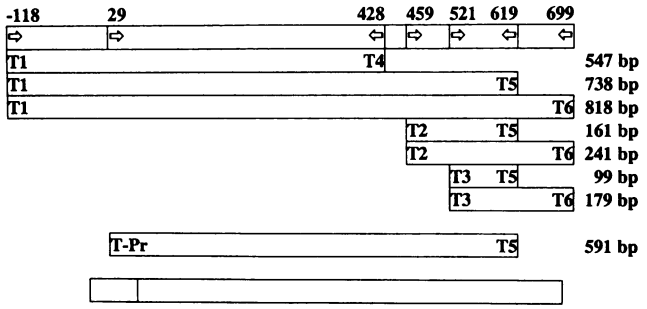

FIG. 1. Positions of oligonucleotides used for amplification of the MPB64 gene. Numbers on top indicate their 5′ ends according to the numbering of Yamaguchi et al. (23). The arrows indicate the direction of the primers. The length of each PCR product and the length of the probe are shown to the right. The coding region for the protein including the N-terminal signal peptide is indicated below.

Harboe et al. (8). The term MPT64 is used to denote the corresponding protein in *M. tuberculosis*.

ELISA. The enzyme-linked immunosorbent assay (ELISA) was set up based on our standard techniques for studies of mycobacterial antigens (21). Briefly, Immunoplate II (lot 0984; NUNC, Copenhagen, Denmark) 96-well plates were coated with 100 μl of BCG culture fluid providing 1 μg of total protein per well. Blocking was done with phosphate-buffered saline (pH 7.4) containing 5 mg of bovine serum albumin per ml. The monoclonal antibody L24.b4, previously shown to react with MPB/MPT64 (22) purified by protein A affinity chromatography, was added as a second layer, applying 100 μl diluted to 1:100 per well. Washing was done with phosphate-buffered saline with 0.1% Tween 20 four times between each step. Detection was by sheep anti-mouse immunoglobulin $F(ab')_2$ fragments conjugated with horseradish peroxidase (Amersham International plc., Amersham, United Kingdom) diluted 1:2,000 with ABTS [2,2′-azino-di-(3-ethylbenzthiazolium sulfate)] as the substrate. All reaction mixtures were set up in triplicate, the median value being used for recording and calculations. The optical density values are recorded as the difference in optical density between antigen-coated neighboring wells processed with or without monoclonal antibody, all other steps and reagents being equal. The latter control wells consistently gave values below 0.040.

PCR. Colonies were scraped from Ogawa and Loewenstein-Jensen cultures, washed, and suspended in distilled water. The suspensions were boiled for 10 min and cleared by centrifugation at 12,000 × *g* in an Eppendorf centrifuge for 30 s. The supernatants were analyzed by PCR.

The seven possible combinations of six primers from the MPB64 protein-coding gene (Fig. 1; Table 1) were used for amplification. Amplification with primers from the 16S rRNA gene region (1) was used for a quality control of the samples. The strains were also tested with primers corresponding to the MPB70 protein gene (19).

The standard amplifications were done in 100 μl of 50 mM KCl–10 mM Tris-HCl (pH 8.3)–1.5 mM $MgCl_2$ (PCR buffer), 60 μM each deoxynucleotide triphosphate (Pharmacia Fine Chemicals, Uppsala, Sweden), 0.25 μM each primer, and 1 U of Ampli-Taq DNA polymerase (Perkin Elmer-Cetus, Norwalk, Conn.) in a TempCycler (Coy Laboratory Products Inc., Ann Arbor, Mich.). The amplifications were performed on 50 μl of sample solution, and 35 cycles were run (1 min at 95°C, 1 min at 55°C, and 1 min and 40 s at 72°C [including ramp times], with a final elongation step of 5 min at 72°C). Low-stringency amplification was performed for two cycles with an annealing temperature of 35°C followed by 33 cycles as described above (10).

TABLE 1. Sequences of the PCR primers

Primer (reference)	Sequence (5′-3′)	Position
For the MPB64 gene (23):		
Sense		
T1	CAGGCATCGTCGTCAGCAGC	−118 to −99
T2	TCCGCTGCCAGTCGTCTTCC	459 to 478
T3	TATCGATAGCGCCGAATGCC	521 to 540
T-Pr	CGGCTGTCGTTTTGCTCTGT	29 to 48
Antisense		
T4	GTGATTGGCTTGCGATAGGC	428 to 409
T5	GCAACTCCCCCGGGTTGAAG	619 to 600
T6	GTCCTCGCGAGTCTAGGCCA	699 to 680
For the MPB70 gene (4, 19):		
Sense	GAACAATCCGGAGTTGACAA	195 to 214
Antisense	AGCACGCTGTCAATCATGTA	566 to 547
For 16S rRNA gene (1):		
Sense	GGTGGTTTGTCGCGTTGTTC	584 to 603
Antisense	TGCACACAGGCCACAAGGGA	1046 to 1027

The PCR product (15 μl) was fractionated on agarose (NuSieve 3:1 agarose; FMC Bioproducts, Rockland, Maine), electrophoresed, and visualized with ethidium bromide (Sigma Chemical Co., St. Louis, Mo.). DNA bands with the expected size indicated positive reactions. Negative controls containing all PCR reagents except DNA were run in parallel with the samples. Recommended precautions were taken to avoid DNA contamination (11).

Isolation of DNA for hybridization. Colonies from Ogawa slants were harvested and washed in 50 mM Tris-HCl–5 mM EDTA (pH 8.0) (TE). The pellet was resuspended in TE containing 4 mg of lysozyme (Sigma) per ml and incubated for 1.5 h at 37°C with shaking. Bacterial membranes were disrupted by adding sodium dodecyl sulfate (SDS) and proteinase K (Sigma) to final concentrations of 1% and 500 μg/ml, respectively, and increasing the temperature to 60°C. After 1 h, the DNA was extracted three times with an equal volume of phenol, phenol-chloroform (1:1), and chloroform and precipitated with isopropanol–3 M sodium acetate (13:1). The DNA pellets were washed with 70% ethanol, dried, redissolved in TE, and stored at −20°C. The amount of DNA was measured by the agarose plate method (17).

Production of labelled probe. The labelled MPB64 probe (nucleotides 29 to 619) was produced by PCR, 35 cycles with 0.25 μM T-Pr primer and 0.25 μM T5 primer (Table 1) in a final volume of 75 μl containing PCR buffer, 30 ng of DNA from BCG substrain Tokyo, and 40 μM each dATP, dGTP, and dTTP, 10 μM dCTP, 60 μCi of [α-^{32}P]dCTP (3,000 Ci/mM) (Amersham), and 1.5 U of Ampli-Taq DNA polymerase. The probe was purified with CHROMA SPIN-100 columns (Clontech Laboratories, Inc., Palo Alto, Calif.). The purified PCR product (5 μl) was electrophoresed on 1% SeaKem agarose gel (FMC Bioproducts), and the appropriate segment was cut out of the gel and assayed for radioactivity. A part of the 16S rRNA gene (nucleotides 584 to 1046, Table 1) was similarly labelled.

Dot-blot hybridization. A 2-μg sample of each mycobacte-

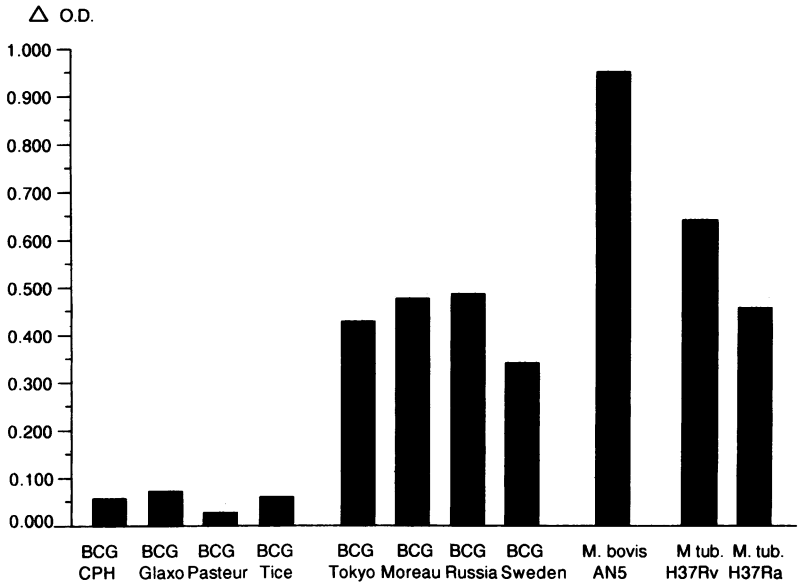

FIG. 2. ELISA for MPB64 in culture fluids of substrains of *M. bovis* BCG and virulent *M. bovis* AN5 and for MPT64 in two strains of *M. tuberculosis*. Δ O.D., change in optical density.

rial DNA was applied to a 0.45-μm-pore-size nitrocellulose membrane (Bio-Rad Laboratories, Richmond, Calif.) and air dried. The DNA on the membrane was denatured in 1.5 M NaOH and neutralized with 0.5 M Tris-HCl (pH 7.4), and the membrane was baked at 78°C for 2 h. Prehybridization was done for 4 h at 42°C in 3× SSC (17) (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–50% deionized formamide–0.1% bovine serum albumin–100 μg of poly(A) per ml–100 μg of sheared, denatured calf thymus DNA per ml. After 10 μl (2×10^9 cpm) of the probe was added to the same solution, the hybridization was performed for 4 h at 50°C for the MPB64 probe and 42°C for the 16S rRNA probe. The membrane was washed twice for 30 min in 2× SSC–0.1% SDS, twice for 30 min in 0.1× SSC–0.1% SDS at room temperature, and twice for 1 h in 0.1× SSC–0.5% SDS at 70°C for the MPB64 probe and 55°C for the 16S rRNA probe. Finally, Kodak X-Omat film was exposed to the membrane at −70°C for 15 h after the membrane was rinsed in 2× SSC at room temperature for 5 min and sandwiched between intensifying screens.

RESULTS

ELISA. The results of the ELISA for demonstration of MPB64 and MPT64 in culture fluids are shown in Fig. 2. The reference high-producer and low-producer substrains, BCG Copenhagen and BCG Tokyo, respectively, show markedly different optical density values in the assay, and the other BCG substrains fall into two similar markedly different categories. These results were identical with the previous findings with two-dimensional electrophoresis used to detect the spot at the typical location for MPB64 and with RIA inhibition tests based on labelled MPB64 and polyclonal rabbit anti-MPB64 (8). The bacterial samples providing these culture fluids were those directly applied in the PCR tests. The virulent strain *M. bovis* AN5 gave the highest optical density value, while *M. tuberculosis* H37Rv and the attenuated strain H37Ra showed an MPT64 content of similar magnitude to that of the high-producer BCG substrains tested.

PCR. Table 2 shows that the substrains Tokyo, Sweden, Moreau, and Russia were all positive, whereas the substrains Copenhagen, Glaxo, Pasteur, and Tice were repeatedly negative on PCR with all seven primer combinations. *M. bovis* AN5 and the *M. tuberculosis* H37Rv and H37Ra strains were also positive. All BCG strains were positive with the MPB70 primers and with the 16S rRNA primers, and so also were the *M. bovis* AN5 and *M. tuberculosis* H37Rv and H37Ra strains. The low-stringency amplifications gave results with respect to specific bands that were identical to those obtained by the standard method.

Hybridization. Results of hybridization with the MPB64 gene probe are presented in Fig. 3A. The MPB64 gene probe was washed away from the DNA of the BCG substrains Copenhagen, Glaxo, Pasteur, and Tice, but not from the Tokyo, Moreau, Russia, and Sweden substrains nor from *M. bovis* AN5 or *M. tuberculosis* H37Rv. When the hybrids were washed at 60°C instead of 70°C, the MPB64 gene probe persisted on all the mycobacterial DNA dots (data not shown).

Results of hybridization with the 16S rRNA gene probe are presented in Fig. 3B. When the hybrids were washed at 55°C, the 16S rRNA gene probe persisted on all the mycobacterial DNA dots. When washed at higher temperatures, the probe disappeared from all the mycobacterial DNA dots simultaneously (data not shown).

The MPB64 gene probe and the 16S rRNA gene probe did not hybridize with the dots containing equal amounts of DNA from *S. aureus* used as negative controls under the conditions described in Materials and Methods.

DISCUSSION

As noted above, the present ELISA gave results similar to those of the previous RIA inhibition tests based on polyclonal, monospecific rabbit anti-MPB64 (8). The BCG sub-

TABLE 2. Comparison of PCR results on BCG substrains, *M. bovis*, and *M. tuberculosis*

Strain	PCR result with the following primer[a]:								
	MPB64							MPB70	16S rRNA
	T1–T4	T1–T5	T1–T6	T2–T5	T2–T6	T3–T5	T3–T6		
BCG Copenhagen	−	−	−	−	−	−	−	+	+
BCG Glaxo	−	−	−	−	−	−	−	+	+
BCG Pasteur	−	−	−	−	−	−	−	+	+
BCG Tice	−	−	−	−	−	−	−	+	+
BCG Tokyo	+	+	+	+	+	+	+	+	+
BCG Moreau	+	+	+	+	+	+	+	+	+
BCG Russia	+	+	+	+	+	+	+	+	+
BCG Sweden	+	+	+	+	+	+	+	+	+
M. bovis AN5	+	+	+	+	+	+	+	+	+
M. tuberculosis H37Rv	+	+	+	+	+	+	+	+	+
M. tuberculosis H37Ra	+	+	+	+	+	+	+	+	+

[a] For identification of primers, see Fig. 1 and Table 1.

strains giving low values still gave an absorption value distinctly above background. Lack of production of MPB64 protein could not be demonstrated by this technique because of the specificity of the MPB64-reactive monoclonal antibody which cross-reacts with the proteins of the actively secreted antigen 85 complex (22), and monoclonal antibodies to MPB64 without significant cross-reactivity with the 85 complex have to our knowledge not yet been described.

Antigenic differences among BCG substrains were initially demonstrated by gel precipitation techniques, while only limited information was provided on the constituents showing distinct differences (12, 18). Gradually, this led to definition of two main groups of BCG substrains characterized by secretion of high or low amounts of the defined proteins MPB70 (7, 15) and MPB64 (8). Characterization of two separate groups of BCG substrains has also been obtained by mycolic acid patterns (14), a restriction fragment length polymorphism at the DNA level (2), and insertion sequence analyses in which most BCG substrains have a single copy of the insertion sequence IS*986* at the same chromosomal site, while other substrains have an additional copy at a different, common location (6). No data are available to explain how these observations are connected, providing the identical allocation of the BCG substrains tested in two distinct groups.

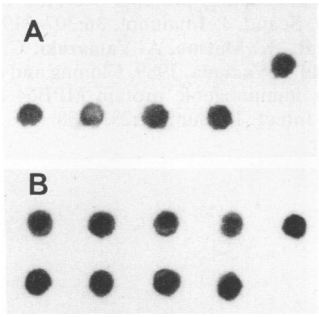

FIG. 3. Hybridization with the MPB64 probe (A) and with the 16S rRNA probe (B) produced by PCR amplification of DNA from *M. bovis* BCG substrain Tokyo. DNA was extracted from the different bacteria and applied to the membrane in the following order from left to right: top row, BCG substrains Copenhagen, Pasteur, Glaxo, Tice, and Moreau; lower row, BCG substrains Russia, Sweden, Tokyo, *M. tuberculosis* H37Rv, and *S. aureus*.

While the production pattern of the MPB64 and MPB70 proteins is identical in the substrains of BCG tested, the conditions are entirely different in *M. tuberculosis*. The analog MPT64 was readily detectable in *M. tuberculosis* by two-dimensional electrophoresis and RIA inhibition tests (8) and in the present ELISA illustrated in Fig. 2 regarding H37Rv as well as H37Ra. By contrast, similar assays did not disclose any sign of significant production of an MPB70 analog in *M. tuberculosis* (7, 8).

Cousins et al. (3, 4) have used DNA amplification of a region of the MPB70 gene by PCR for rapid identification of *M. bovis* and *M. tuberculosis*. The amplification reaction produced a single 372-bp product which was readily detected by agarose gel electrophoresis in all 14 *M. tuberculosis* strains tested. Thus, there is a striking difference at the protein and gene levels in *M. tuberculosis*. While the gene is readily detected, *M. tuberculosis* is a low producer of a protein analogous to MPB70. The PCR consistently demonstrated a positive signal in all their strains of virulent *M. bovis* and in two strains of *M. bovis* BCG; one of the latter was indicated to be from a human isolate, while the other was not further defined. In the present study, we obtained a positive PCR with the MPB70 primers in all the BCG substrains tested, including four high-producer and four low-producer substrains as defined at the protein secretion level. This agrees with the notion that the different protein patterns observed are probably due to differences in regulation of MPB70 protein production.

Amplification trials on the MPB64 gene gave contrasting results. Here they complied completely with previous groupings of BCG substrains inasmuch as the Tokyo, Sweden, Moreau, and Russia substrains, the virulent *M. bovis* AN5 strain, and the *M. tuberculosis* H37Rv and H37Ra strains contained the MPB64 gene, whereas the Copenhagen, Glaxo, Pasteur, and Tice substrains seemed to lack the gene. With all the different combinations of primers used in the PCR, no product was observed for the negative substrain group. Even an annealing temperature of 35°C for the first two cycles did not result in any visible PCR product. Base agreement at the 3′ end of the primers used is critical for the amplification reaction to give a product. It seems unlikely that point mutations should occur at all the six chosen primer ends.

The hybridization results confirmed the PCR results and are strong evidence that the MPB64 gene is missing in the substrains Copenhagen, Glaxo, Pasteur, and Tice, while it is

present in the substrains Tokyo, Sweden, Moreau, and Russia. Because of the very high stringency of the hybridization conditions, it is likely that there was virtually complete correspondence between probe and gene for the MPB64-positive group. The relatively high stringency required for the negative group could be due to the presence of similar nucleotide sequences elsewhere in the mycobacterial genome, in accordance with gene duplications with modifications as a mechanism in the evolution of the genome.

BCG vaccination trials have shown a striking variation with regard to protection against tuberculosis in different areas of the world. Explanations for this variation have recently been discussed by Fine (5), who concludes: "It now seems more reasonable to accept that several mechanisms may be involved, and that masking by atypical mycobacterial infections, variation in BCG strains and geographic variations in pathogenesis have all played a role in generating the observed variation in BCG's efficacy." Demonstration of variation in genetic constitution of different substrains of BCG is relevant in relation to this discussion and is shown here with regard to an immunogenic secreted protein.

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