Differential transcription of the MPB70 genes in two major groups of Mycobacterium bovis BCG substrains

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Substrains of Mycobacterium bovis BCG (BCG) have been divided into two major groups, high and low producers, on the basis of the amount of secretion of the MPB70 protein. The antigen is produced in high concentration by BCG Tokyo, Moreau, Russia and Sweden (high-producer substrains), whereas in BCG Pasteur, Copenhagen and Tice (low-producer substrains) it is detected at 1% (wt/wt) or less of the concentration of BCG Tokyo. To investigate why this protein is secreted differently, the MPB70 genes of BCG Tokyo and Pasteur were cloned, sequenced and compared. The MPB70 genes in two substrains showed exactly the same sequence. Even the upstream and downstream regions of the MPB70 gene were identical. MPB70 gene expression was assessed by means of Northern hybridization analysis and reverse transcriptase polymerase chain reaction. The mRNA was clearly detected in BCG Tokyo, but at a very low level in BCG Pasteur. On the basis of these results, the difference in the secretion of the MPB70 protein between BCG Tokyo and Pasteur was attributed to differential transcription efficiencies.

Keywords: BCG substrains, MPB70, RT-PCR, transcription

INTRODUCTION

The MPB70 protein was originally isolated from the Tokyo substrain of Mycobacterium bovis BCG (BCG) by Nagai et al. (1981). This protein accounted for about 10% (w/w) of the total secreted protein content in Sauton culture medium (Nagai et al., 1981). Amounts of secretion of this protein differ between BCG substrains (Miura et al., 1983). On the basis of differential secretion, BCG has been divided into two major groups. BCG Tokyo, Moreau, Russia and Sweden (high-producer substrains) secrete a lot of the MPB70 protein, whereas BCG Pasteur, Copenhagen, Glaxo and Tice (low-producer substrains) secrete little. The two groups of BCG substrains also differ in mycolic acid patterns (Minnikin et al., 1984), a DNA restriction fragment length polymorphism (Collins & De Wit, 1987), the copy number of insertion sequence IS986 (Fomukong et al., 1992), and the presence or absence of the MPB64 gene (Li et al., 1993).

Abbreviations: BCG, bacillus Calmette-Guérin; RT-PCR, reverse transcriptase polymerase chain reaction.

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The amino acid sequence of MPB70 from M. bovis BCG was reported by Patarroyo et al. (1986). Radford et al. (1988) first described the nucleotide sequence of the MPB70 gene from M. bovis AN5. It did not contain a signal region and corresponded to the sequence from position 91 to 582. The complete nucleotide sequence of the MPB70 gene from BCG Tokyo was first reported by Terasaka et al. (1989). It was from position -173 to 597. Radford et al. (1990) reported the sequence of an analogous gene from M. bovis AN5 from position -81 to 582, including a signal region. Matsumoto et al. (1995) reported the nucleotide sequence from position -157 to 726 in Mycobacterium tuberculosis. Epitope mapping of MPB70 from M. bovis was reported by Radford et al. (1990) and Pollock et al. (1994).

Recently, DNA amplification of a region of the MPB70 gene was carried out for rapid identification of M. bovis and M. tuberculosis by PCR. The PCR consistently demonstrated a positive PCR with the MPB70 primers in all BCG substrains tested, regardless of whether they were high or low producers (Li et al., 1993). However, the authors made no comparison of the sequence of the MPB70 gene, including the non-coding region, between substrains.
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**METHODS**

**Reagents.** Restriction endonucleases, modifying enzymes, vectors (pUC19), the 7-deaza sequence kit, the nick-translation kit and the ligation kit were purchased from Takara Shuzo Co.

MPB64 is another of the secreted proteins (Harboe et al., 1992; Wikar et al., 1991), and is specific to the *M. tuberculosis* complex. It was purified from the culture fluid of BCG strain Tokyo (Harboe et al., 1986). The absence of the MPB64 gene in some strains of BCG was reported by Li et al. (1993). The PCR product of the MPB64 gene was found in BCG Tokyo, Moreau, Russia and Sweden, in the virulent *M. bovis* AN5, and in *M. tuberculosis* H37Rv and H37Ra, whereas it was not found in BCG Pasteur, Copenhagen, Glaxo and Tice. The MPB70 gene was found in both the MPB64-positive and MPB64-negative groups, though the MPB64-positive groups secreted a large amount of the MPB70 protein and negative groups secreted little. The amount of the MPB70 protein thus seemed to be related to the presence of the MPB64 gene or other factors.

It was also thought that the coding or non-coding region of the MPB70 gene may differ between substrains, leading to the observed differences in MPB70 secretion. In this study, we compared the sequences of the MPB70 genes from BCG Tokyo and Pasteur. We also assessed MPB70 gene expression by Northern hybridization analysis and reverse transcriptase-PCR (RT-PCR).

**Bacterial strains and plasmids.** BCG Pasteur and Tokyo were grown in Sauton medium (Suzuki et al., 1987). *Escherichia coli* K12 strain JM109 was used as a host for plasmid pUC19; it was grown in NZYM medium [1% (w/v) NZ amine, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl, and 0.2% (w/v) MgSO4·7H2O: Sambrook et al., 1989].

**Probes.** Two kinds of DNA probes were obtained from the cloned SalI fragment containing the MPB70 gene (Terasaka et al., 1989): a 265 bp Sau3AI fragment containing the N-terminal sequence (probe A) and the other was a 625 bp SalI–SalI fragment corresponding to the C-terminal sequence (probe B) of the MPB70 gene and 149 bp beyond the coding sequence.

**DNA cloning.** Cells of BCG Tokyo and Pasteur were harvested by filtration. Genomic DNA of BCG Pasteur and Tokyo was extracted as described previously (Suzuki et al., 1987). Approximately 1 µg genomic DNA was digested with *SalI* and fractionated by 0.8% (w/v) agarose gel electrophoresis. Fractionated DNA fragments, denatured with 0.5 M NaOH, were transferred to a nylon membrane (Gene Screen Plus, NEN Research Products). Hybridizations were accomplished by using the ECL direct nucleic acid labelling and detection system as described in the manufacturer’s instructions. The DNA fragments of about 3.6 kbp which gave a clear hybridization band with the labelled probes A and B were harvested, cloned into plasmid vector pUC19, and transformed into competent *E. coli* JM109 cells. The ampicillin (50 µg ml⁻¹) resistant white colonies on the NZYM plates containing 0.1 mM IPTG and 0.004% (w/v) X-Gal were screened by the colony hybridization technique (Grunstein & Wallis, 1977). Approximately 1 µg each of probes A and B was nick-translation by using a nick translation kit and [α-³²P]dCTP (3000 Ci mmol⁻¹; 111 TBq mmol⁻¹) as described previously (Matsuo et al., 1988).

**DNA sequencing.** The sequencing strategy is presented in Fig. 2. *Sau3A1* fragments with BCG Tokyo or Pasteur DNA insert were subcloned into the BamHI site of pUC19. In the same way, *AclI* fragments were subcloned into the *AclI* site, *AclI–SalI* fragments were subcloned into the *AclI–SalI* site, *SalI*–*SalI* fragments were subcloned into the *SalI–SalI* site and *SalI–SalI* fragments were subcloned into the *SalI–SalI* site of pUC19. The nucleotide sequences of these subcloned DNA fragments were determined by the dideoxy chain-termination method using pUC plasmids (Hattori & Sakaki, 1986) and M13 primer M3 and RV (Takara Shuzo Co.). Additionally, synthetic primer p-705S (5'-GAAGGTAAAGAACACAATTG-3') was used so that the sequencing fully overlapped on both strands. The sequence data were analysed by DNASIS (Hitachi Software Service).

**Extraction of total RNAs and analysis.** Cells were disrupted with glass beads. Total RNAs were extracted according to the method of Chomczynski & Sacchi (1987) and stored at -80 °C after being dissolved in diethyl-pyrocarbonate-treated H2O [0.2% (w/v)]. Concentrations of total RNAs were determined spectrophotometrically at 260 nm and verified by judging the intensities of bands after electrophoresis in 1.2% (w/v) agarose gels.

The total RNAs (20 µg per well) were separated by electrophoresis on agarose gel containing 2.2 M formaldehyde.
(Lehrach et al., 1977), then transferred to a nylon membrane (Hybond-N+, Amersham) in 20 x SSC (3 M NaCl, 0.3 M sodium citrate). After transfer, RNAs were covalently cross-linked to the membrane by UV irradiation. Northern hybridization was carried out with probe B, using the ECL direct nucleic acid labelling and detection system.

**Synthetic oligonucleotides.** Four oligonucleotides for PCR were synthesized on the basis of the sequence of the MPB70 gene (Terasaka et al., 1989; Radford et al., 1990) and the 16S rRNA gene of BCG (Suzuki et al., 1988), using a DNA synthesizer (model 391, Applied Biosystems). Oligonucleotides p-70N (5'-TCAGGGAATTGTCGCAAGGAC-3') and p-70C (5'-CGGAGGCATTAGCAGCTGT-3') corresponded to positions 155 to 174 and 557 to 576, respectively, of the sequence of the MPB70 gene of BCG Pasteur and Tokyo (Terasaka et al., 1989). Oligonucleotides p-16N (5'-CTGAGATACGGCCCAGACTC-3') and p-16C (5'-GCCCC-GTCTAATTCTCTTGAG-3') corresponded to positions 320 to 339 and 696 to 715 of the sequence of the 16S rRNA gene of BCG (Suzuki et al., 1988).

Oligonucleotides p-70S (5'-GAAGGTAAGGAAACACACAGG-3') and p-70P (5'-GGTGGCGGCAATTGTTGTCT-3') were synthesized in the same way; they corresponded to positions 3 to 22 and 16 to 4 on the basis of the sequence of the MPB70 gene.

**Reverse transcription and amplification reaction.** Total RNAs (20 μg) were heated for 5 min at 75 °C, then total RNAs were transcribed into cDNAs in 20 μl 50 mM Tris/HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂ containing 100 pmol random hexamer, 200 U M-MLV reverse transcriptase, and 40 U RNasin by incubation for 60 min at 37 °C.

To assess the expression of the MPB70 gene, RT-PCR was performed in a total volume of 100 μl with 20 μl reverse transcriptase reaction mixture containing 2.5 U Taq polymerase, 50 mM KCl, 25 mM Tris/HCl (pH 8.3), 1.5 mM MgCl₂, 0.01% (w/v) gelatin, 100 pmol each of four primers and 25 mM of each deoxynucleotide triphosphate (dATP, dGTP, dCTP and dTTP). The thermal profile involved 30 cycles of denaturation at 94 °C for 1 min (the first cycle was for 3 min), primer annealing at 55 °C for 2 min, and extension at 72 °C (the last cycle was for 5 min).

The amplified products were analysed by electrophoresis on 12% (w/v) agarose gels containing ethidium bromide (1 ng ml⁻¹) and the products were identified by Southern hybridization by using probe B.

**Primer extension.** About 50 μg total RNA and 10 pmol oligonucleotide primer p-70P labelled with [γ-³²P]ATP at the 5' end were dissolved in 100 μl 0.4 M NaCl, 40 mM PIPES, 1 mM EDTA. The solution was incubated at 95 °C for 5 min and cooled to 42 °C. The nucleic acids were then precipitated with ethanol, washed with 80% (v/v) ethanol, dried, and redissolved in 20 μl 50 mM Tris/HCl (pH 8.3), 120 mM KCl, 8 mM MgCl₂, 2 mM dithiothreitol, 0.5 mM deoxynucleotide triphosphates with 200 U of reverse transcriptase. After incubation at 42 °C for 1 h, the reaction was stopped by ethanol precipitation; the precipitate was washed with 80% (v/v) ethanol, dried, redissolved in 7.5 μl double-distilled water, and then subjected to 8 M urea/6% (w/v) polyacrylamide gel electrophoresis.

**RESULTS**

**Southern hybridization analysis**

Chromosomal DNA from BCG Tokyo and Pasteur was digested with SalI and fractionated by 0.8% (w/v) agarose gel electrophoresis. Fragments of 3.6 kbp gave clear hybridization with probes A and B (Fig. 1). The fragments were harvested, cloned into pUC19, and used for further study.

**Nucleotide sequencing**

The DNA sequencing strategy is shown in Fig. 2, and the DNA sequence in Fig. 3. The sequence data were stored and analysed by DNASIS. The DNA sequence of the MPB70 gene of BCG Pasteur was identical to the sequence of the MPB70 gene of BCG Tokyo. The DNA sequence contained a 579 bp open reading frame beginning with ATG at position 1 and ending with a TAA stop codon at position 580. A Shine–Dalgarno (SD) sequence was found upstream of the initiation codon (Fig. 3).

**Northern hybridization analysis**

The expression of the MPB70 gene was examined by Northern hybridization analysis. Two clear hybridization

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![Probe A](image1.png) ![Probe B](image2.png)

**Fig. 2.** Restriction endonuclease map of the cloned 3.6 kbp SalI fragment, sequenced region, subcloned fragments, sequencing strategy and probes. Various restriction fragments were cut out from these plasmids and cloned into pUC19 by random cloning for sequencing. Dotted lines indicate sequenced fragments. Solid arrows indicate sequencing with M13 or M13 RV primers. The open arrow indicates the sequencing with primer p-70S.
bands were detected by probe B in BCG Tokyo but not in Pasteur (Fig. 4). The size of the mRNA of the MPB70 gene was similar to that of 16S rRNA. Under these conditions, 16S rRNA was detectable in both substrains using the 603 bp 16S rRNA PCR product as a probe (data not shown).

Amplification

Primers p-70N and p-70C amplified a 422 bp MPB70 PCR product from the coding sequence of the MPB70 gene. Primers p-16N and p-16C amplified a 603 bp 16S rRNA PCR product from the coding sequence of the 16S rRNA gene. Two pairs of the primers co-amplified a 422 bp MPB70 and a 603 bp 16S rRNA product from genomic DNA of both BCG Tokyo and Pasteur. These two products were almost the same in quantity after 30 cycles of amplification. Under these conditions, gene expression was compared by the RT-PCR method between BCG Tokyo and Pasteur (Fig. 5a). In BCG Tokyo, a 603 bp 16S rRNA PCR product and a 422 bp MPB70 PCR product were detected. In BCG Pasteur, a 603 bp rRNA PCR product was detected and the quantity of the product was almost equal to that of BCG Tokyo, but a 422 bp MPB70 PCR product was not observed. However, when an additional 30 cycles of amplification were performed, the 422 bp MPB70 PCR product was detectable. When the RNAs were treated with RNase A, PCR products were not detected. The amplified product from MPB70 gave a clear hybridization band by Southern hybridization analysis (Fig. 5b).

Primer extension

For primer extension, the primer was hybridized to MPB70 mRNA of BCG Tokyo and then extended by reverse transcriptase. Two elongation products were

Fig. 3. Nucleotide and deduced amino acid sequence of the MPB70 gene from BCG Tokyo and Pasteur. The SD sequence is underlined. Asterisks indicate the transcriptional start sites. Numbers above the alignment indicate the nucleotide sequence, whereas those at the side refer to the deduced amino acid sequence.

![Image of the nucleotide and deduced amino acid sequence]
detected, at positions -57 and -49, by the primer p-70P (Fig. 6).

**DISCUSSION**

The MPB70 genes of BCG Tokyo and Pasteur were cloned, sequenced, and compared to investigate why this protein is secreted differently in these sub-strains. Southern hybridization patterns of the two sub-strains were identical in all cases when tested with probes derived from the MPB70 gene. The MPB70 gene sequences of the two sub-strains were identical, including their promoter-like sequences. The sequence of the mature protein was perfectly identical with that determined in our previous work (Terasaka et al., 1989). Furthermore, the upstream region, including the promoter-like sequence, and the downstream region of the MPB70 gene were perfectly identical. When compared with other MPB70 sequence data (Terasaka et al., 1989; Radford et al., 1990; Matsumoto et al., 1995), the mature protein regions were perfectly identical. However, there were a few differences in the upstream or downstream regions. The DNA sequence of the *M. bovis* MPB70 gene was reported from position -81 to 582 (Radford et al., 1990). In comparison with our data, there was a single deletion at position -28 and a single insertion between positions -31 and -30 in the region upstream of the SD sequence. In the signal peptide region, there was a single deletion at position 47 and a single insertion between positions 49 and 50. The DNA sequence of the *M. tuberculosis* MPB70 gene was reported from position -157 to position 726 (Matsumoto et al., 1995). The sequence of the MPB70 gene including the non-coding region or expected regulatory region was identical to that of BCG Tokyo and Pasteur, but the amount of secretion was quite different. In *M. bovis* AN5, there were a few mutations, but these seemed not to influence MPB70 gene expression.

The nucleotide sequences of various BCG and *M. tuberculosis* genes have been reported. The 65 kDa gene sequences reported by two groups (Thole et al., 1987; Shinnick, 1987) were completely identical. The MPB/MPT64 genes (Yamaguchi et al., 1989; Oettinger & Andersen, 1994) were identical except for one silent mutation. The B component of 85-complex genes (Matsuo et al., 1988) was also identical except for a single nucleotide sequence. The A component of 85-complex genes (De Wit et al., 1990; Borremans et al., 1989) was different only in a few nucleotide changes. In all cases, these genes were highly conserved and presumed to play functionally important roles. Comparing the genes of BCG Tokyo and Pasteur in the present study, the MPB64 gene was absent in BCG Pasteur but the MPB70 genes were completely identical and the gene expression was obviously found in both BCG Tokyo and Pasteur. It was thus thought that MPB70 may play some physiologically important role.

Next, we investigated which steps concerned differential gene expression of MPB70 by Northern analysis and RT-PCR. To extract pure RNA from mycobacteria it is important to lyse the cell wall quickly, but the complex structure and impermeability of the cell walls of mycobacteria make the lysis difficult (Boddinghaus et al., 1990). We disrupted the cells mechanically using glass beads with denaturing solution for about 2 min. Then total RNAs were extracted according to the method of Chomczynski & Sacchi (1987). This procedure was simple and rapid compared to other methods for lysis mycobacterial cell walls.

Northern hybridization analysis gave a clear-cut difference between the two sub-strains. Two clear hybridization bands were observed in BCG Tokyo, but not in BCG Pasteur (Fig. 4). Since probe B contained 149 bp downstream from the stop codon of the MPB70 gene, it may have hybridized to RNA irrelevant to the MPB70 gene. The primer extension experiment with BCG Tokyo revealed two transcriptional initiation signals. These results suggested that two kinds of mRNA, different in size, were synthesized.
The RT-PCR method was so sensitive that great care had to be taken to avoid contamination of DNAs even in trace amounts (Yamaguchi et al., 1992). RNA samples were carefully examined for contamination of genomic DNAs by two means. One was that RNA samples were examined for no amplification of the 16S rRNA gene by primers without the reverse transcriptase reaction. The other was that RNA samples were treated with RNase A and then examined for no amplification by RT-PCR. The RT-PCR data indicated that MPB70 gene expression was seen in BCG Pasteur, but very weakly. This conclusion was confirmed by RT-PCR-Southern hybridization and additional amplification. The additional amplification for BCG Pasteur detected a trace amount of MPB70 gene expression, and non-specific PCR products were also detectable (Fig. 5). These products were concluded to be dimers from the size of the fragments. In the additional amplification, it was thought that the depletion of reaction components and accumulation of products may induce a non-specific reaction.

In conclusion, this study suggests that the difference in secretion of the MPB70 antigen proteins between BCG Tokyo and Pasteur is due to differential efficiencies of transcription. There was no difference in the immediate upstream region between the two substrains; regulatory protein(s) may be concerned with differential initiations of transcription. Finally, it has to be stressed that there is another possibility, of differences in mRNA stability. The biological and immunological significance of MPB70 is as yet unknown. Further study on these points is necessary.

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