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Expression of the *Mycobacterium bovis P36* gene in *Mycobacterium smegmatis* and the baculovirus/insect cell system

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Abstract

In the present study we evaluated different systems for the expression of mycobacterial antigen P36 secreted by *Mycobacterium bovis*. P36 was detected by Western blot using a specific antiserum. The *P36* gene was initially expressed in *E. coli*, under the control of the T7 promoter, but severe proteolysis prevented its purification. We then tried to express P36 in *M. smegmatis* and insect cells. For *M. smegmatis,* we used three different plasmid vectors differing in copy number and in the presence of a promoter for expression of heterologous proteins. P36 was detected in the cell extract and culture supernatant in both expression systems and was recognized by sera from *M. bovis*-infected cattle. To compare the expression level and compartmentalization, the MPB70 antigen was also expressed. The highest production was reached in insect cell supernatants. In conclusion, *M. smegmatis* and especially the baculovirus expression system are good choices for the production of proteins from pathogenic mycobacteria for the development of mycobacterial vaccines and diagnostic reagents.

Key words
- *Mycobacterium tuberculosis*
- *Mycobacterium smegmatis*
- Insect cells
- Baculovirus
- Secreted proteins
- P36
- MPB70
- Antigen

Introduction

The purification and characterization of individual mycobacterial antigens are essential for the understanding of the pathogenic mechanisms of mycobacteria and the immune response against them. This may also contribute to the knowledge of the virulence of other intracellular bacteria in which cellular immunity is also involved. The extremely long duplication time and the virulence of mycobacteria have prevented for years the identification of antigens and virulence factors. The application of the tools of molecular biology has produced important progress in the knowledge of *Mycobacterium tuberculosis* and *M. leprae* antigens and of the immune response against them (1,2). Expression of mycobacterial antigens and virulence factors in a nonpathogenic, fast growing organism such as *Escherichia coli* offers potential advantages over their purification from pathogenic mycobacteria such as safer working conditions and more rapid results. In addition, expression can be enhanced by placing the gene under the control of a strong promoter or by employing useful protein domains or peptides. However, some diagnostic assays based on *E. coli*-cloned mycobacterial antigens were reported to be less

sensitive than tests in which the antigen is directly purified from a mycobacterium (3). Post-translational modifications such as glycosylation, which has been reported in mycobacteria (4,5) but not in *E. coli*, have been proposed as an explanation for this difference (3). In this respect, *M. smegmatis* (6,7) and the baculovirus/insect cell system (8) seem to be more appropriate hosts for the expression of mycobacterial antigens for several reasons: the transcriptional and translational machinery of *M. smegmatis* and that of the slow growing mycobacteria may be similar because both bacteria belong to the same genus. Moreover, protein from other mycobacteria expressed in *M. smegmatis* may suffer proper modification and folding (6). On the other hand, the baculovirus system may reach a high level of expression, up to 50% of the total proteins in infected cultures (8), and may induce various modifications. Finally, a reduced reaction of antibodies or T cells with host antigens may be obtained. Human or cattle sera frequently have high titers of antibodies against *E. coli* proteins.

In this study we investigated the P36 antigen, a 36-34-kDa protein that was identified and characterized from *M. bovis* by our group (9) and from *M. tuberculosis* by Berthet et al. (10). One of our aims was to purify this protein to study the immune response against P36 and to use it in diagnostic assays. When we expressed P36 in *E. coli* the protein was produced but highly degraded. In this study we describe the cloning and expression of the *P36* gene in *M. smegmatis* and in the baculovirus/insect cell system. Because of the importance of the MPB70 antigen for the diagnosis of bovine tuberculosis, its gene was also cloned in *M. smegmatis*.

Material and Methods

Bacteria, viruses, insect cells and media

The *Escherichia coli* strains used in this work were *E. coli* DH5α and *E. coli* BL21(DE3), obtained from Life Technologies (Gaithersbourg, MD). They were grown in LB media supplemented with ampicillin (100 µg/ml) when required. *M. smegmatis* mc^2155 was used as the mycobacterial host for gene expression (11). *M. bovis* AN5 was used as the source of native P36 and MPB70. Mycobacteria were cultured in MADC-TW media (11) and *M. smegmatis* was also cultured in 3% tryptic soy broth (Difco, Detroit, MI). AcMNPV was used as wild type baculovirus. Sf9 insect cells were grown in serum-free Sf 900 II medium (Life Technologies) at 27°C in 25-cm^2 flasks. All manipulations involving *M. bovis* were conducted under a P3 containment.

Cloning procedures

The *P36* gene was inserted into the pYUB18, pMV261 and pYUB178 shuttle vectors described below. In the following constructions the *P36* gene was obtained from pMBA123, a plasmid containing the *P36* gene and regulatory sequences inserted into pBluescript KSII (9). The *P36* gene and regulatory sequences were released with *Xho*I (NEB, Beverly, MA) from pMBA123, filled-in with the Klenow fragment of DNA polymerase I and purified from agarose gels with Gene Clean (Bio 101, La Jolla, CA). The fragment was ligated to the pYUB18 (11) vector digested with *Bam*HI and filled-in with the Klenow fragment, giving rise to pMBA60, or to the *Eco*RV-digested pYUB178 vector (12), giving rise to pMBA62.

Plasmid pMBA123 was digested with the enzymes *Xho*I and *Rsa*I, yielding a 1.0-kb fragment that contains the *P36* gene. The fragment was purified from the agarose gel as described above and cloned in the pMV261 vector (12) previously digested with *Sal*I and *Pvu*II. The resulting plasmid was called pMBA61.

The *MPB70* gene was cloned in pMV261 as follows: a 700-bp band was amplified

from the *M. bovis* genome using primers D 5'CAGCAAGGGGCTACAGGTTT3' and R 5'CTAATGCCTCCGGCGTAATC3', based on the MBP70 sequence (13). The amplification conditions were: 94°C for 2 min, followed by 30 cycles of: 94°C for 30 s, 55°C for 2 min and 72°C for 2 min. The amplification product was detected in agarose gels and purified by the Wizard PCR (Promega, Madison, WI). The fragment ends were phosphorylated with polynucleotide kinase (NEB) and ligated to the vector pMV261 digested with *Eco*RV, to produce the plasmid called pMBA77.

The *P36* gene was cloned in the baculovirus vector pVL1393 (14) as follows: the *P36* gene was amplified from pMBA123 using the synthetic oligonucleotides: bac pst 5'AACTGCAGATTATGCCGAACCGACGCCGACGC3' and bac xba 5'GCTCTAGATTAGGCGACCGGCACGGTGATTG3' containing the *Pst*I and *Xba*I cleavage site, respectively. The resulting PCR product of the expected molecular weight was purified by agarose gel electrophoresis, digested with the mentioned enzymes and inserted into the corresponding sites of pVL1393 under the control of the polyhedrin protein promoter, resulting in pMBA90.

pMBA125 has the same insert as pMBA123, but in the opposite direction so that expression will be under the direction of the T7 promoter.

The correct orientation and integrity of inserts from plasmid constructions were confirmed by restriction analysis, PCR amplification and sequence analysis. Plasmids were purified with Wizard mini preps kit (Promega). The general characteristics of the plasmid constructs obtained are shown in Table 1.

Table 1 - Main features of plasmid constructions.

[1]The insert orientation is opposite to that of pMBA123.

Plasmid	Vector	Cloning site	Insert size	Promoter	Gene
pMBA123	pBluescript KS	*Xho*I	1.3 kb	lac Z-P36	*P36*
pMBA125	pBluescript KS	*Xho*I	1.3 kb[1]	T7-P36	*P36*
pMBA60	pYUB18	*Bam*HI	1.3 kb	P36	*P36*
pMBA61	pMV261	*Sal*I-*Pvu*II	1.0 kb	Hsp60	*P36*
pMBA62	pYUB178	*Eco*RV	1.3 kb	P36	*P36*
pMBA77	pMV261	*Eco*RV	0.75 kb	Hsp60	*MPB70*
pMBA90	pVL1393	*Pst*I-*Xba*I	0.95 kb	Polyhedrin	*P36*

Transformation of *Mycobacterium smegmatis*

Competent cells of *M. smegmatis* strain mc^{2}155 were prepared and transformed as described by Jacobs et al. (11).

Preparation of cell fractions

E. coli cultures were induced for 2 h by the addition of 10 mM isopropyl-b-thiogalactopyranoside (IPTG). For *E. coli* BL21, 100 µg/ml rifampicin was added at the time of IPTG induction. Cells were harvested by centrifugation and resuspended in loading buffer for PAGE (2% sodium dodecyl sulfate (SDS), 0.125 M Tris HCl, pH 6.8, 1% 2-mercaptoethanol, 0.02% bromophenol blue, and 10% glycerol). Cell extracts were obtained by boiling for 5 min. Cell extracts from *M. smegmatis* or insect cells were obtained also by centrifugation and boiling in loading buffer for PAGE. Proteins from culture supernatants were precipitated by the addition of up to 10% trichloroacetic acid and resuspended in 1/100 of the original volume in loading buffer for PAGE.

Western and Southern blots

Western and Southern blots were performed as previously described (15,16).

Transfection of insect cells

Transfection of insect cells to produce recombinant baculoviruses was performed as described by O'Reilly (17). Sf9 cells (17)

were infected at a multiplicity of infection (m.o.i.) = 5.

Results

Expression of *P36* gene in *E. coli*

In a previous study (9) we reported the expression of the *P36* gene in *E. coli,* but severe degradation prevented P36 purification from *E. coli* cell extracts (strain DH5α). In order to obtain a high P36 expression level and stability in *E. coli*, plasmid pMBA125 was introduced into the *E. coli* strain BL21(DE3). This plasmid (pMBA125) carries the *P36* gene downstream of the T7 promoter. The *E. coli* strains bear the gene of T7 RNA polymerase from phage T7 under the control of an IPTG-inducible promoter. In addition, *E. coli* BL21(DE3) lacks the Lon (18) and OmpT (19) proteases, which especially degrade recombinant products. Western blot assays using rabbit anti-P36 sera (Figure 1) demonstrated higher P36 expression levels and a more stringent degree of IPTG regulation in *E. coli* BL21 (DE3) than in *E. coli* DH5α. However, the degradation increased with the production level, because only a 29-kDa degradation product is seen in *E. coli* DH5α, while several bands are demonstrated in *E. coli* BL21(DE3).

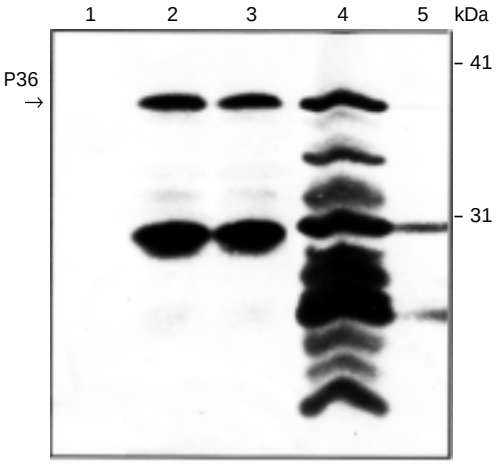

Figure 1 - Detection of P36 expressed in extracts of *E. coli.* Western blot: anti-P36 was used as first antibody. *Lanes*: 1, *E. coli* DH5α; 2 and 3, *E. coli* DH5α (pMBA123); 4 and 5, *E. coli* BL21(DE3) (pMBA125). *Lanes*: 2, 4: Cell extracts from IPTG-induced bacteria; 3, 5: cell extracts from noninduced bacteria.

Cloning of *P36* and *MPB70* genes in *M. smegmatis*

To compare the expression level in terms of the vector used, the *P36* gene and its regulatory sequence were cloned in three different vectors, pYUB18, pYUB178 and pMV261. The main characteristics of the three vectors are as follows: pYUB18 is a low copy number cosmid (11), pYUB178 lacks a replication origin for mycobacteria, and is inserted into the genome of bacilli at a mycobacteriophage integration site (12). Both plasmids lack a promoter for the expression of cloned genes. pMV261 is a moderate copy number plasmid and contains the inducible promoter of the BCG *hsp60* gene (12). Following the insertion of the *P36* gene into pMV261, pYUB178, or pYUB18, the recombinant plasmids pMBA61, pMBA62, pMBA60 were obtained, respectively. The *MPB70* gene was cloned in *M. smegmatis* to compare its expression level and antibody reactivity to that of *P36*. The cloning of the *MPB70* gene into the pMV261 vector originated plasmid pMBA77 (see Table 1). *M. smegmatis* was transformed with these plasmids and their presence was confirmed by the preparation of *M. smegmatis* plasmid DNA, followed by *E. coli* transformation and restriction analysis or PCR amplification. A Southern blot was performed to demonstrate the presence of pMBA62 in *M. smegmatis* (data not shown).

P36 expression in *M. smegmatis*

Expression of the *P36* gene in extract and culture supernatants from different recombinant *M. smegmatis* strains was demonstrated by Western blots using specific anti-P36 rabbit sera (Figure 2). The highest production of P36 was observed with *M. smegmatis* (pMBA61). The recombinant protein was found both in the supernatant and in the cell extract. Addition of H_2O_2 as an inductor of the hsp60 promoter had no effect on P36

production (data not shown). Lower levels of P36 expression were observed in *M. smegmatis* (pMBA60) and *M. smegmatis* (pMBA62) (Figure 2). No P36 was observed in the culture supernatant of *M. smegmatis* (pMBA62).

P36 proteolysis was reduced with respect to recombinant P36 expressed in *E. coli*. A 33-kDa protein reacting weakly with anti-P36 rabbit sera in cell extracts of nonrecombinant *M. smegmatis* was sometimes observed, indicating the existence of a weak crossreactive protein. Although the stronger P36 production took place in *M. smegmatis* (pMBA61), no recombinant protein band was observed in Coomassie blue-stained gels (data not shown).

In order to precisely assess the molecular sizes of the supernatant and of the intracellular forms of P36 protein produced by recombinant *M. smegmatis,* we submitted a small amount of each sample to SDS-PAGE to detect sharper bands. In the Western blot we observed that the intracellular form is heavier than the supernatant form (data not shown). The *M. bovis* AN5 supernatant showed two bands corresponding to both *M. smegmatis* forms.

The immune recognition of the recombinant P36 was studied using a few serum samples from cattle with macroscopic tuberculosis lesions which had been previously assayed for reactivity against the *E. coli* recombinant P36 (9). The sera reacted against the P36 antigen produced by *M. smegmatis* (Figure 3A). Two sera recognized no other protein in the supernatant while the other sera recognized a 45-kDa band. Sera from healthy cattle did not react with supernatants or cell extract.

MPB70 expression in *M. smegmatis*

The *MPB70* gene was cloned in pMV261 vector. MPB70 protein was detected as a single band in culture supernatant using a monoclonal antibody. A very low level of MPB70 protein was observed in cell extract (Figure 4). A panel of cattle sera reacting against *E. coli* recombinant MBP70 (Bigi F, unpublished results) was assayed for the immune recognition of *M. smegmatis*-produced MPB70. The sera reacted against the

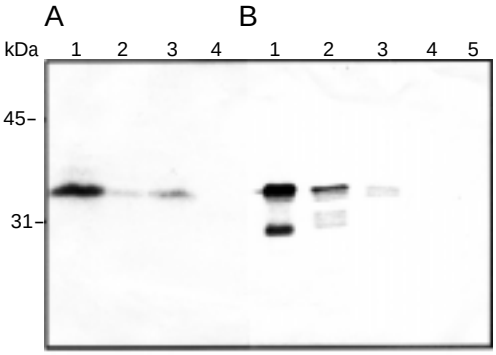

Figure 2 - *A*, Detection of P36 expressed in *M. smegmatis.* Anti-P36 was used as first antibody. Western blot of cell extracts: *Lanes*: 1, *M. smegmatis* (pMBA61); 2, *M. smegmatis* (pMBA62); 3, *M. smegmatis* (pMBA60); 4, *M. smegmatis. B*, Western blot of culture supernatants: *Lanes*: 1, *M. bovis* AN5; 2, *M. smegmatis* (pMBA61); 3, *M. smegmatis* (pMBA60); 4, *M. smegmatis* (pMBA62); 5, *M. smegmatis*. One milliliter of culture was used for preparation of extract and supernatant.

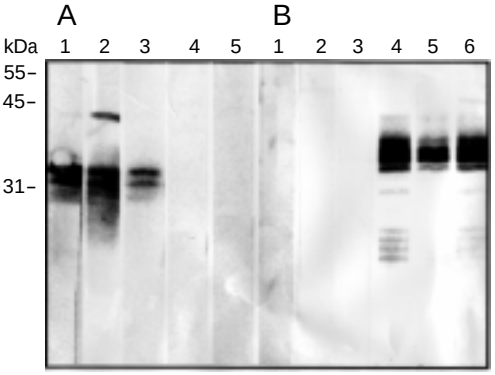

Figure 3 - *A,* Recognition of *M. smegmatis*-expressed P36 by cattle sera. Western blot was performed using the culture supernatant from *M. smegmatis* (pMBA61). Sera from infected (lanes 1-3) or healthy (lanes 4 and 5) cattle were used. *B*, Recognition of P36 expressed in insect cells (3 days post-infection) by cattle sera. Western blot was performed using the culture supernatant from insect cells infected with recombinant baculoviruses. Sera from healthy (lanes 1-3) or infected (lanes 4-6) cattle were used.

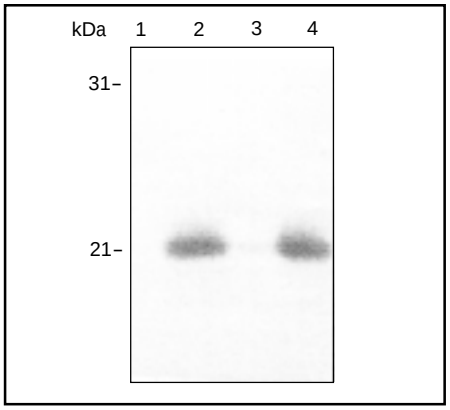

Figure 4 - Detection of MPB70 expressed in *M. smegmatis.* Western blot: anti-MPB70 was used as first antibody. *Lanes*: 1, *M. smegmatis* culture supernatant; 2, *M. bovis* AN5 culture supernatant; 3, *M. smegmatis* (pMBA77) cell extract; 4, *M. smegmatis* (pMBA77) culture supernatant. One milliliter of culture was used for preparation of extract and supernatant.

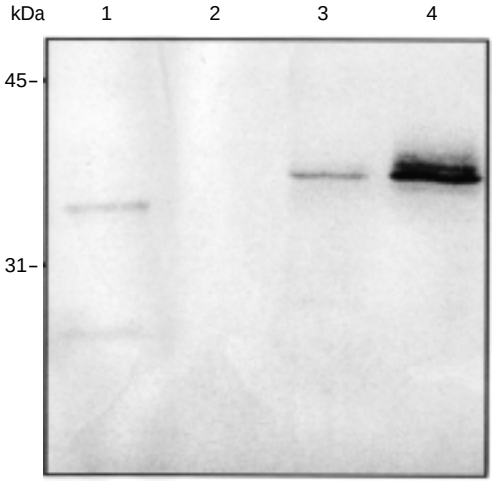

Figure 5 - Detection of P36 expressed in insect cells. Western blot: anti-P36 was used as first antibody. *Lanes*: 1, *M. bovis* AN5 culture supernatant; 2, cell extract from insect cells infected with nonrecombinant baculoviruses; 3, cell extract from insect cells infected with recombinant baculoviruses; 4, culture supernatant from insect cells infected with recombinant baculoviruses (50 µl non-TCA concentrated supernatant). Except for lane 4, 1 ml of culture was used for preparation of extract and supernatant.

recombinant MPB70 antigen (data not shown). No other *M. smegmatis* protein was recognized. Sera from healthy cattle did not react with supernatants.

Expression of *P36* gene in insect cells

Insect cells were infected with recombinant baculovirus carrying the *P36* gene. In this construction the start codon of the *P36* gene is ATG (instead of GTG) to optimize expression in this eukaryotic system. The presence of the *P36* gene was demonstrated in infected Sf9 insect cells by dot blot of cell DNA using the *P36* gene as a probe. Sf9 cells were infected at a multiplicity of infection of 5. Culture supernatants and cell extracts were obtained at 1.5, 3 and 4 days post-infection (dpi). The expression of P36 in extract and culture supernatant was demonstrated by Western blot using specific anti-P36 sera. The recombinant protein was found both in the supernatant and in the cell extract (Figure 5), with a molecular mass slightly higher than the P36 produced by *M. bovis*. In the supernatant, P36 appeared first at 1.5 dpi, reached its maximum expression level at 3 dpi and was not found at 4 dpi, probably due to degradation (data not shown). A main P36 band with slightly smaller bands was seen, indicating that degradation is highly reduced in insect cells. Several proteins migrated in the region of P36, making it difficult to detect a P36-specific band in Coomassie blue-stained gels (data not shown). In cell extracts, P36 appeared at 1.5 dpi, reaching also its maximum expression level at 3 dpi and slightly decreasing at 4 dpi (data not shown). Anti-P36 serum recognized no protein in the cell extract or culture supernatant from nonrecombinant baculovirus-infected insect cells. P36 production was higher in insect cells than in *M. bovis,* because when only 5 µl of nonconcentrated insect cell supernatant was submitted to SDS-PAGE, the protein was clearly detected by Western blot. On the contrary, no P36 band was detected when 5 µl of nonconcentrated *M. bovis* was loaded.

The same panel of sera from infected cattle as used in section 3.3 recognized P36 secreted by insect cells (Figure 3B). These cattle sera recognized no other protein in the insect cell culture supernatant. Sera from healthy cattle did not react with the supernatants.

Comparison of relative P36 production in *M. smegmatis* and baculovirus-infected insect cells

Since P36 is very poorly stained by Coomassie blue or silver nitrate (Bigi F, unpublished observations, and Berthet FX, personal communication) we decided to determine the relative P36 production level in the different expression systems by Western blot. To compare the relative production level of P36 protein in *M. smegmatis*, baculovirus-infected insect cells, *E. coli* and *M. bovis*, we determined the minimal number of cells producing detectable P36 by Western blot (Table 2). Since the sera and the antigen used in this comparison are the same, this method permits an approximate analysis of production level, even though the absolute amount of protein (i.e., as determined in Coomassie blue- stained gels) is not known. The highest

relative P36 production was reached in insect cells. The accumulation in the supernatant was 20 times higher than in cell extracts. *E. coli* showed an intermediate level of expression, while the supernatant and cell extracts of *M. smegmatis* had the same amount of P36 protein, similar to that of the producing organism, *M. bovis*. The production per cell in the insect cell supernatant was 4600 times higher than in the *M. smegmatis* supernatant. The protein concentration of each fraction is also given in Table 2. The comparison was based on the number of cells and not on protein concentration because of the highly different protein concentration of cell extracts and culture supernatants.

Table 2 - Amount of cells that produce detectable P36 in Western blots.

[1]Five-day culture of *M. smegmatis* (pMBA61); [2]Three-day post-infection culture; [3]Three-week culture.

Source of P36	Culture volume (µl)	Cell density (cell/ml)	Number of cells	Protein (µg/ml)
M. smegmatis supernatant[1]	300	1.0×10^8	0.3×10^8	10
M. smegmatis extract[1]	300	1.0×10^8	0.3×10^8	200
Insect cell supernatant[2]	5	1.3×10^6	6.5×10^3	50
Insect cell extract[2]	100	1.3×10^6	1.3×10^5	220
E. coli cell extract	50	2.0×10^9	1.0×10^8	500
M. bovis AN5 supernatant[3]	300	4.0×10^8	1.3×10^8	40

Discussion

The objective of this study was to compare different systems for the expression of mycobacterial antigen genes. For these expression assays we chose the P36 antigen, a secreted protein identified and cloned in our laboratory (9). This protein contains several amino acidic PGLTS repeats, which is the main antigenic region of the protein (Bigi F, unpublished observations).

The gene encoding P36 was introduced in *E. coli* BL21 under the control of the strong promoter T7. P36 production was higher compared to the *E. coli* strain (DH5α) where the *P36* gene is under the direction of *lacZ* promoter. The degree of gene regulation by IPTG addition was more stringent in *E. coli* BL21 than in *E. coli* DH5α, a result possibly explained by the fact that in BL21 it is the RNA polymerase gene, not the recombinant gene, which is under the control of P*lac* (20). Although *E. coli* BL21 is a protease-deficient strain, the extent of proteolysis was still high, preventing the purification of the antigen and indicating that Lon and OmpT proteases are not responsible for the degradation.

To improve P36 protein production, stability and presumably post-translational modifications, the gene encoding P36 was introduced into *M. smegmatis* and in the baculovirus/insect cell system. At the same time, we transformed *M. smegmatis* with the gene encoding antigen MBP70 (21,22). We used MPB70 protein to compare its expression level, compartmentalization and antibody reactivity with that of P36. Plasmid constructs containing the genes coding for the two antigens were introduced into *M. smegmatis*. These constructs correspond to different vectors (integrative or replicative, with and without a strong promoter). The highest P36 production was obtained with a replicative plasmid containing the *hsp60* promoter (pMBA61). No induction of *P36* gene expression was achieved by adding H_2O_2 to the culture medium, as reported by others (12). A lower production was obtained with *M. smegmatis* transformed with a replicative promoter-less plasmid (pMBA60) and with the integrative promoter-less plasmid (pMBA62). This lower P36 production was observed using both promoter-less vectors, demonstrating that the P36 promoter is active in *M. smegmatis*. Recombinant MBP70 antigen was produced by *M. smegmatis* (pMBA77). No production was obtained when the *MPB70* gene was cloned in pYUB18 (data not shown), suggesting that the higher the gene copy number, the stronger the production.

P36 protein was found both in the culture

supernatant and cell extract of *M. smegmatis* (pMBA61). Since in *M. bovis* P36 is a secreted protein, the secretion process seems to be more efficient in *M. bovis* than in *M. smegmatis*. The supernatant and extract forms were detected as bands of slightly different sizes, with the extract form being heavier. This result suggests that the cell extract form may be the processing precursor of the secreted form. However, it is unclear why bands of both sizes are observed in the extracellular fluid of *M. bovis*. A higher secretion rate was observed with MBP70 produced by *M. smegmatis* mainly in the culture supernatant.

P36 stability was higher both in *M.* smegmatis cell extract and culture supernatant than in *E. coli*, suggesting that *M. smegmatis* has fewer proteases or that P36 acquires a folding that allows it to resist proteolysis.

The *P36* gene was also expressed in the baculovirus/insect cell system under the control of the polyhedrin promoter. Again, the protein could be identified both in the culture supernatant and in the cell extract. A higher production and secretion level was reached because recombinant P36 protein was easily detected in Western blots in nonconcentrated supernatants. It has been shown that baculovirus-infected insect cells may secrete recombinant proteins (23). The fact that the protein was not observed in the supernatant culture at 4 dpi may indicate that strong proteolysis arises late in the infection, probably because cell lysis released proteases. Recombinant P36 antigen had a higher molecular mass than that produced in *M. bovis*, perhaps due to post-translational protein modifications occurring in insect cells. To our knowledge, there is only one previous case of mycobacterial protein expression in the baculovirus system. This was the expression of the *M. tuberculosis* chaperonin 10 protein by Atkins et al. (24). Compared to P36, higher expression of chaperonin 10 was achieved; however, chaperonin 10 is a nonsecreted protein and is already abundant in mycobacteria. A sample of few sera from infected cattle recognized the recombinant P36 produced by *M. smegmatis* or insect cells. As previously reported (25), cattle sera recognized no protein cell extract or supernatants from insect cells infected by nonrecombinant baculoviruses. Recombinant MPB70 produced by *M. smegmatis* was also recognized by cattle sera. These results establish the basis for a diagnostic assay.

A semiquantitative analysis of the relative production level of recombinant P36 showed that the highest production was obtained in the baculovirus/insect cell supernatant. In this system there seems to be a good rate of exportation because the extracellular/cellular ratio was higher than in *M. smegmatis.*

A high yield of mycobacterial proteins in systems such as those described here could be the first step to study the immune recognition or the biological function of these antigens. In an applied approach, the recombinant antigens could be useful for diagnosis or prevention studies.

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