Molecular characterisation of a novel lipoglycoprotein from *Mycobacterium tuberculosis* and *Mycobacterium bovis*.

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Abstract

In Great Britain a recent independent scientific review into bovine tuberculosis concluded that the best prospect for control of TB in the national herd is to develop a vaccine. The secreted antigens of the *Mycobacterium tuberculosis* complex have received much interest as possible vaccine candidates due to their ability to confer protection against tuberculosis in small animal models. The recent discovery that some of these mycobacterial antigens are glycosylated, a modification ubiquitous in eukaryotic proteins, may provide some novel insights into the properties of these antigens.

The antigen MPB70 is the major secreted protein of *Mycobacterium bovis*, the causative agent of tuberculosis in cattle. It has previously been reported that this antigen, encoded for by the gene *mpb70*, is present in at least two forms, a 22 kDa unglycosylated form and a 25 kDa glycosylated form. A clone was isolated from an *M. tuberculosis* H37Rv mycobacterial shuttle cosmid library which expressed both the 22 and 25 kDa antigens.

Genetic analysis of this cosmid revealed that the two antigens were encoded by separate genes. The gene encoding the 25 kDa antigen (MPT83), subsequently designated *mpt83*, is situated 2.4 kb upstream of *mpt70* and transcribed in the same direction. Using a mycobacterial expression system and alkaline phosphate (PhoA) fusions, it was shown that MPT83 but not MPT70 is glycosylated by *Mycobacterium smegmatis*. Moreover, neither fusion protein was glycosylated in *E. coli*, demonstrating that glycosylation of MPT83 was specific to the mycobacterial species. The use of site directed mutagenesis in conjunction with the PhoA reporter system identified both O and N-linked glycosylation sites within MPT83.

Preliminary investigations into the role of glycosylation in the alteration of immune recognition showed a possible influence of glycosylation on a T cell epitope of MPT83.

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To me, the quote, 'To travel hopefully is better than to arrive, and the true success is to labour' (El Dorado - Robert Louis Stevenson, 1850-1894) is a perfect description of my time spent during this venture. I have learnt so much during the course of my research, from so many different people, that it is impossible to recall them all here.

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List of abbreviations

A	adenine
aa	amino acid
AIDS	acquired immune deficiency syndrome
ATP	adenosine triphosphate
BCG	bacille-Calmette-Guérin
bp	base pairs
BSA	bovine serum albumin
C	cytosine
ConA	concanavalin A
CFP	culture filtrate protein
cpm	counts per minute
CTL	cytotoxic T cell
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DTH	delayed type hypersensitivity
ELISA	enzyme-linked immunosorbent assay
EtBr	ethidium bromide
G	guanine
h	hour(s)
HEL	hen egg lysozyme
HIV	human immunodeficiency virus
His-tag	histidine tag
IgG	immunoglobulin G
IPTG	isopropyl-β-thiogalactopyranoside
kb	kilobase pairs
kDa	kilodalton
LB	Luria-Bertani
LN	lymph nodes
M	molar
M.	*Mycobacterium*
MAb	monoclonal antibody
MCS	multiple cloning site
µg	microgram
µl	microlitre
µM	micromolar
mg	milligram
ml	millilitre
mM	millimolar
MPB	mycobacterial protein of *M. bovis*
MPT	mycobacterial protein of *M. tuberculosis*
ng	nanogram
nM	nanomolar

ORF	open reading frame
PHLS	Public Health Laboratory Service
PPD	purified protein derivative
RBS	ribosome binding site
RNA	ribonucleic acid
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
STPK	serine-threonine protein kinase
UV	ultra violet
v/v	volume/volume
WHO	World Health Organisation
w/v	weight per volume

Published work associated with this thesis

Hewinson, R.G., Michell, S.Ll., Russell, W.P., McAdam, R.A. and Jacobs Jr, W.R.: Molecular characterisation of MPT83: a seroreactive antigen of *Mycobacterium tuberculosis* with homology to MPT70. **Scandinavian Journal of Immunology** 43 (1996) 490-499.

Part 1

Identification of the of the gene encoding MPT83

Chapter 1

Mycobacteria and the secreted antigens of the *Mycobacterium tuberculosis* complex

1.1 Introduction

Tuberculosis (TB) remains the leading cause of death in the world from a single infectious disease (Bloom and Murray, 1992). It has been estimated that in 1992 there were 8 million new cases of TB and 2.7 million deaths (Snider Jr. *et al.*, 1994). The global health emergency is highlighted by the prediction that by the year 2005, the annual incidence of new cases of TB will rise to 10 million, with the number of deaths reaching 3.5 million (Mizrahi, 1997). Consequently the need for basic research into treatment, diagnosis and prevention is of utmost importance.

Records of tuberculosis date back to the time of Hippocrates (c. 460 - 377 B. C.) when it was known as 'phthisis', derived from the Greek for wasting away (Castiglioni, 1933). It was not until 1722 that the nature of tuberculosis was speculated to be infectious rather than constitutional (Doetsch, 1978). The first credible demonstration that TB was contagious was made by Villemin in 1865. Villemin demonstrated that rabbits inoculated with pus and fluid from bovine and human TB lesions developed TB, establishing the belief in a specific agent as the cause for tuberculosis (Bloom and Murray, 1992).

In 1882 Robert Koch presented a seminal piece of work that changed the understanding of infectious diseases and tuberculosis. He reported on the observation and culture (the first report of bacterial culture on solid media) of the bacillus for TB, which he described as 'tuberkelbacillen' (tubercle bacilli) (Koch, 1932). Koch's criteria for proof that the tubercle bacilli was the etiological agent of TB have been generalised into Koch's Postulates: ". . . it was necessary to isolate the bacilli from the body; to grow them in pure culture . . .; and, by administering the isolated bacilli to animals to reproduce the same morbid condition . . ."

Soon after Koch's isolation of the tubercle bacillus, it was shown that definite differences existed between strains of tubercle bacillus isolated from human and bovine origin, and that both were capable of causing TB in humans (Smith, 1898). In 1901 a Royal Commission on

tuberculosis was established in Great Britain to determine "whether the disease in animals and man is one and the same thing, whether animals and man can be reciprocally infected with it, under what conditions, if at all, the transmission of the disease from animals to man takes place and what are the circumstances favourable or unfavourable to such transmission." The final conclusion reached by the Royal Commission in 1911 was that tuberculous cows were a hazard to health and that measures should be implemented to improve the situation.

The first voluntary national programme for the eradication of the disease in cattle, by tuberculin testing (section 1.5) and slaughter of positive reactors, was introduced in 1935, and made compulsory in 1950. In adition, the introduction of wide-scale pasteurisation of milk in the 1930's dramatically reduced the transmission from cattle to man. In the 1930's, prior to pasteurisation of milk, 6% (estimated to be about 2,000 per year) of human deaths due to TB were caused by *Mycobacterium bovis* (Hardie and Watson, 1992). By 1960 all areas in Great Britain were declared attested, and the incidence of disease had declined to what were regarded as negligible levels (< 0.5% of herds). Continuation of the test and slaughter policy led to a further decrease in the incidence of disease to a relatively static level.

However, since 1988 there has been a steady increase in the proportion of total herds with positive reactors to the tuberculin test (Krebs, 1997). Although the risk to humans in Great Britain of *M. bovis* infection is negligible, in 1995 only 32 (1%) of the 3,200 tuberculosis isolates cultured in Great Britain were caused by *M. bovis* (PHLS data), control of bovine TB is essential to ensure that such a risk is controlled. Bovine TB still has severe implications on animal heath and welfare as it causes reduced productivity and premature death, thus having severe economic implications for affected herds. Such factors have highlighted the need for research into the development of a vaccine for the control of TB in the British herd and for a diagnostic test that will allow differentiation between vaccinated and infected cattle (Krebs, 1997).

1.2 The genus *Mycobacterium*

The prefix *myco* is derived from the Greek 'μυκεσ' meaning fungus. The generic name *Mycobacterium* was first attributed in 1896 in the German edition of 'Atlas of Bacteriology', due to the mold-like growth of the tubercle bacillus on the surface of a liquid culture (Lehmann and Neumann, 1896). Subsequently the tubercle bacilli was classified as *Mycobacterium tuberculosis*. Few mycobacteria are pathogenic, with most having an innocuous relationship with humans and animals.

The mycobacteria are rod-like unicellular organisms which, although they behave anomalously in the Gram test, are classified as Gram positive prokaryotes. As for other Gram positive bacteria, the cell envelope of mycobacteria contains peptidoglycan. Whereas the peptidoglycan of other bacteria usually has proteins and polysaccharides attached to it, the mycobacterial polymer is predominantly lipid-linked to the mycolic arabinogalactan complex (Lederer *et al.*, 1975). The high lipid content of the cell wall is a major characteristic of mycobacteria (Brennan, 1989).

Along with the genera *Nocardia*, mycobacteria are described as 'acid fast'. That is they are resistant to decolouration by weak mineral acids after staining with arylmethane dyes. Although not unique to mycobacteria, acid fastness is commonly used to distinguish mycobacteria using the Ziehl Neelsen stain (Bishop and Neumann, 1970), the distinguishable long chains of mycobacterial mycolic acids correlating with their high degree of acid fastness (Minnikin and Goodfellow, 1980).

Mycobacteria are aerobic (though a few, including *M. bovis*, grow better at lower oxygen tensions), non-sporing, non-motile and uncapsulated, although some species appear slimy due to surface lipids called mycocides (mycocerosic acids and glycosyl phthiocerol dimycocerosates). Many of the mycobacteria are pigmented, either in the dark (scotochromogens) or on exposure to light

(photochromogens). The pigments are a class of polyterpene lipids called carotenoids. Pigmented forms are common among the airborne mycobacteria in which the pigments protect against the rays of the visible and near-UV light ranges.

The nutritional requirements of mycobacteria are relatively simple, and the organisms favour a slightly acidic environment for growth. Nutritional requirements include oxygen, nitrogen, carbon, sodium, phosphorous, sulphur, potassium, iron and magnesium (Grange, 1988). All these nutrients are present in Löwenstein-Jensen medium with glycerol and asparagine supplying carbon and nitrogen respectively. The tubercle bacilli *M. bovis* grows better with pyruvate as the carbon source.

1.2.1 The species of mycobacteria

Presently, the critical features for including a species in the genus *Mycobacterium* are: (i) acid-alcohol fastness; (ii) C_{60} - C_{90} mycolic acids which are cleaved to C_{22} - C_{26} fatty acid methyl esters by pyrolysis; and (iii) between 61-71% G+C content of DNA (Lévy-Frébault and Portaels, 1992). Shinnick and Good (1994) compiled a table of 71 reported mycobacterial species, which can be divided into two main groups on the basis of growth.

Slow growing mycobacteria

Slow-growing mycobacteria require more than 7 days to form visible colonies on solid media and can be divided into four groups: (i) The *M. tuberculosis* complex (MTb complex); (ii) slow growing non-chromogens; (iii) slow growing scotochromogens; and (iv) the slow growing photochromogens (Runyon, 1959). The MTb complex contains four pathogenic species; *M. tuberculosis*, with two variants, classical and Asian; *M. africanum* (African I and African II); *M. bovis* and *M. microti*. Collins *et al.*, (1992) proposed a simple classification for members of the MTb complex on the basis of four simple tests shown in Table 1.

Although not virulent, the many strains of *M. bovis* BCG are also included within this group.

The other three groups of slow growing mycobacteria contain the mainly opportunist pathogens for man, including the *M. avium* group. This group is composed of *M. avium* (the avian tubercle bacillus), *M. intracellulare, M. lepraemurium and M. paratuberculosis. M. avium* and *M. intracellulare* are variants of the same species and are commonly referred to as the *M. avium-intracellulare* complex (MAI complex). These species are the most pathogenic of the opportunist mycobacteria.

There is one species of pathogenic mycobacteria that does not fall into any of the above groupings, *M. leprae.* The causative agent of the chronic disease leprosy is still to be cultured *in vitro.*

Table 1.1 Subdivision of the *Mycobacterium tuberculosis* complex.
(Collins *et al.*, 1982)

Species and variant	TCH* susceptibility	Pyrazinamide susceptibility	Nitratase	Oxygen preference
M. tuberculosis				
Classical	Resistant	Sensitive	Positive	Aerobic
Asian	Sensitive	Sensitive	Positive	Aerobic
M. africanum				
African I	Sensitive	Sensitive	Negative	micro-aerophilic
African II	Sensitive	Sensitive	Positive	micro-aerophilic
M. bovis				
bovine	Sensitive	Resistant	Negative	micro-aerophilic

*thiophen-2-carboxylic acid hydrazide

Rapid growing mycobacteria

Of the species that make up this group only *M. chelonei* and *M. fortuitum* cause human disease, the others are considered to be free living saprophytes. Amongst these saprophytes *M. phlei*, *M. smegmatis* and *M. thermoresistibile* are thermophilic.

Initial reports estimated the mycobacteria to have genomes with molecular weights ranging between $3x10^9$ and $5.5x10^9$ Da (Baess and Mansa, 1978). In general the pathogenic strains have smaller genomes than the saprophytic mycobacteria. Studies using pulsed-field gel electrophoresis (PFGE) in conjunction with rare-cutting restriction enzymes (*Dra* I (TTTAAA), *Asn* I (ATTAAT)) generated DNA restriction fragments, the sum of which was estimated to be about 4 Mbp (Varnerot *et al.*, 1992; Cole and Smith, 1994). Subsequently the genome of *M. tuberculosis* H37Rv has been sequenced and has been shown to contain 4,411,529 base pairs (bp) (Cole *et al.*, 1998).

1.3 Basic biology of *M. tuberculosis* infection

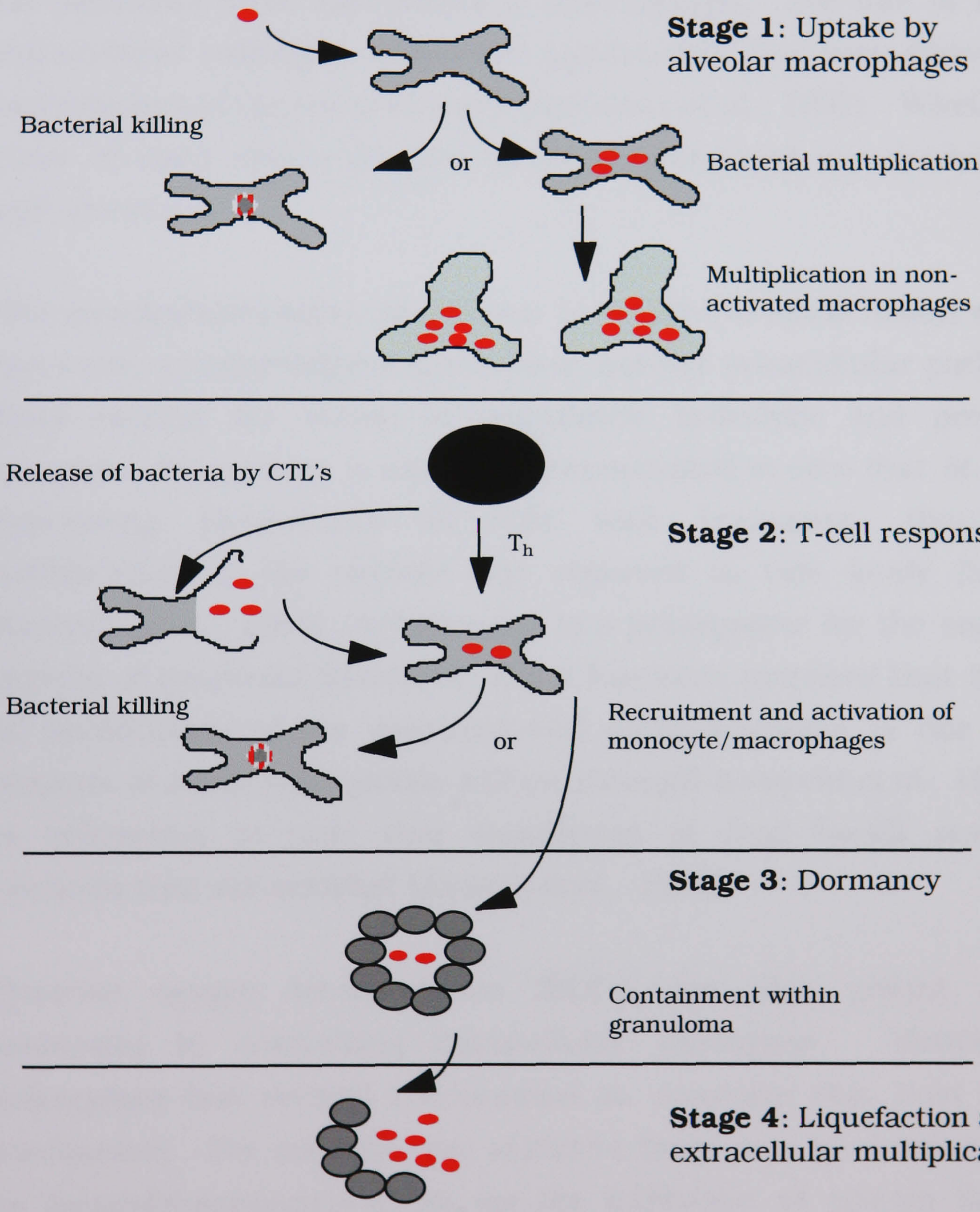
Mycobacterium tuberculosis and *M. bovis* are facultative intracellular pathogens having the ability to enter and replicate within cells. Intracellular living provides an environment guarded from extracellular host defences but also requires potent evasion mechanisms from the hosts intracellular degradative machinery.

1.3.1 Phagocytosis of *M. tuberculosis* by alveolar macrophages

After inhalation of organism-containing aerosol particles or dust-associated particles, the bacillus is phagocytosed by alveolar macrophages (Fig. 1.1, stage 1). The phagocytosed mycobacteria encounter a wide range of microbicidal actions within the macrophage which act to eliminate the foreign pathogen. The precise details of how macrophages mediate killing or inhibit growth of intracellular pathogens are unknown. However, the mechanisms by which *M.*

tuberculosis evades some of the better characterised anti-microbial functions of macrophages has been the subject of much research and is extensively reviewed elsewhere (Chan and Kaufmann, 1994).

Fig. 1.1 Schematic representation of *M. tuberculosis* infection. (Young and Duncan, 1995).



The mechanism of entry is unknown although both mannose and complement receptors have been implicated (Schlesinger, 1998) (Kang

and Schlesinger, 1998). Pathogenic mycobacteria have been shown to associate with the complement protein C2a to form a C3 convertase. This convertase generates C3b which is deposited on the mycobacteria. following this opsonisation the mycobacteria are taken up via the macrophage receptor CR1 (Schorey *et al.*, 1997). Also, the binding of terminal mannosyl units of lipoarabinomannan from some virulent *M. tuberculosis* to mannose receptors has led to the proposal that mannose capped lipoarabinomannan (ManLAM) may serve as a ligand for adherence of *M. tuberculosis* to macrophages. The fate of another intracellular pathogen, *Salmonella typhimurium*, has been shown to be independent of the route of entry (Rathman *et al.*, 1997). Whether the route of entry effects the fate of the phagocytosed mycobacterium is still unclear.

The mycobacteria-laden phagosome is thought to evade fusion with the lysosome, a major defence mechanism against intracellular pathogens, thus evading the action of degradative hydrolytic and proteolytic enzymes. In contrast, it has been demonstrated *in vitro* that *M. avium*-containing phagosomes do fuse with lysosomes, though no acidification of the vacuole was observed in this study (Sturgill-Koszycki *et al.*, 1994). Acidification is a prerequisite for the enzymatic activity of lysosomal hydrolase, and it has been proposed that the lack of acidification of the mycobacterial phago-lysosome is due to the absence of a vesicular proton-ATPase (Sturgill-Koszycki *et al.*, 1994). It is interesting to note that engulfment of dead bacilli results in vacuoles that are acidified (Russell *et al.*, 1997).

Reactive oxygen intermediates (ROI's) are also potent effector molecules in controlling intracellular pathogens. However, *M. tuberculosis* has evolved mechanisms to overcome this host defence mechanism. For example, the oxidative burst may be down-regulated by lipoarabinomannan (LAM) via the inhibition of protein kinase C (Chan *et al.*, 1991), an enzyme important in the activation of the oxidative burst. Also, the secreted protein superoxide dismutase (SOD) may provide additional protection against ROI's (Zhang *et al.*, 1991).

As has been observed for other intracellular pathogens, mycobacteria up-regulate and repress expression of a number of proteins in response to the intracellular environment (Aranda *et al.*, 1992; Abshire and Neidhardt, 1993, and Lee and Horwitz, 1995). Identification of these proteins will aid in understanding the intracellular survival mechanisms of *M. tuberculosis* and in the development of future vaccines. Recently gene 'knockout' experiments have shown that the 16 kDa α-crystallin protein of *M. tuberculosis* is required for prolonged growth inside macrophages although the mutant strain grows normally in culture (Yuan *et al.*, 1998). In addition, *M. tuberculosis* has also been shown to specifically regulate the expression of certain macrophage genes (Ragno *et al.*, 1998), although whether this phenomenon serves to aid or impair survival of the bacterium is unclear.

If the tubercle bacillus is successfully killed by the initial alveolar macrophages, then the infection process is arrested and pathogenesis is not apparent. However, if the bacilli resist the microbicidal mechanisms they multiply until the macrophage bursts releasing the daughter bacilli (Fig. 1.1). The released bacteria are engulfed by monocytes, which have been attracted to the site of infection by the release of chemokines from the alveolar macrophage at the initial site of infection (Zhang *et al.*, 1995). These monocytes which have been recruited from the bloodstream are as yet unactivated and allow logarithmic growth of the tubercle bacilli intracellularly (Fig. 1.1, stage 1).

1.3.2 T-cell response to *M. tuberculosis* infected macrophages

T lymphocytes activated in the draining lymph nodes are eventually attracted to the site of infection. T cells recognise mycobacterial peptides presented on the cell surface of the macrophages (reviewed in chapter 6). In response to the recognition of presented peptides $CD4^+$ T helper cells (T_h cells) release a range of effector molecules called cytokines including interferon gamma (IFN-γ) (Fig. 1.1, stage 2).

The cytolytic T cells (CTL's) generally recognise protein antigens from the cytosol of the macrophage whereas T_h cells generally recognise protein antigens presented from the phagolysosomal compartment. If the macrophage is unable to kill the mycobacteria, secreted antigens, possibly translocated to the cytoplasm, become a source of antigens for T cell recognition. Recognition of these antigens by CTL's may result in lysis of the inadequate macrophage releasing the mycobacteria to disseminate or be taken up by other activated macrophages. This dynamic interplay between lysis and activation of macrophages within a productive granuloma is, in part, dependent on the antigens presented, the outcome of which may be detrimental or beneficial to the host.

1.3.3 Granuloma encapsulation

The productive granuloma may become encapsulated by a fibrotic wall. Encapsulation further contributes to containment of the mycobacteria as the necrotic centre has a reduced partial oxygen pressure, an unfavorable growth condition for *M. tuberculosis*. At this stage the mycobacteria probably enter a stage of dormancy (Fig. 1.1, stage 3). However, *M. bovis* is microaerophilic (Grange, 1988) raising the question whether *M. bovis* ever enters a stage of dormancy. *Escherichia coli* have been shown to adapt to such stressful conditions by entering a stationary phase which involves the synthesis of a set of stress proteins under the transcriptional control of the alternative sigma factor *rpoS* (Hengge-Aronis, 1993). The identification of a stationary-phase stress response factor in *M. tuberculosis*, *sigF*, suggests that such a mechanism may also be utilised in mycobacterial persistence (DeMaio *et al.*, 1996). Moreover, the product of this gene has been reported to be strongly induced *in vitro* in response to oxygen depletion (Gomez *et al.*, 1997). Development of a drug against the gene product of *sigF* may prevent entry into dormancy, thus reducing the lengthy regimes of chemotherapy currently used against TB infection.

The bacilli may remain dormant but viable within the granuloma for the life of the host. However, liquefaction of the granuloma to form a

cavity with a caseous foci may occur, generating an excellent growth medium for the bacilli. Subsequent extracellular multiplication of the bacilli causes destruction of the local tissues resulting in the release of bacilli to other parts of the lung and to the environment (Fig. 1.1, stage 4). This 'reactivation' occurs when the host's resistance is lowered such as in immunocompromised hosts with human immunodeficiency virus (HIV) or in old age. Patients latently infected with *M. tuberculosis* have a 0.2% annual risk of reactivation, compared with a 5 - 10% annual risk associated with co-infection with HIV and *M. tuberculosis* (Dolin *et al.*, 1994).

1.4 Vaccination against tuberculosis

In 1908 two French researchers, Albert Calmette and Camille Guérin began attempts to attenuate a virulent strain of *M. bovis* by serial passage. After repeated passaging of this strain on a potato-glycerin medium supplemented with ox bile they noted a change in colony morphology. Over the next decade they reported the protective effects of this variant strain in a range of mammals and in 1921 it was first administered to a child. This stain was termed BCG (Bacillus of Calmette and Guérin) and in 1928 it was recommended for the prevention of TB by the League of Nations. However, a meta-analysis on the efficacy of BCG concluded that on average it was only 50% protective in preventing TB (Colditz *et al.*, 1994). Subsequent studies have shown efficacies of between 0 and 80% against *M. tuberculosis* infection in man and *M. bovis* infection in cattle (Fine *et al.*, 1994; (O'Reilly and Daborn, 1995 and Buddle *et al.*, 1995). Reasons for this variable efficacy have been discussed elsewhere (Bloom and Fine, 1994).

In 1955 Bloch and Segal studied the effect of live and dead strains of mycobacteria on the survival time of mice after challenge with virulent *M. tuberculosis*. They found that live BCG was more effective than phenol-killed BCG in protection (Bloch and Segal, 1955). These findings were supported by the work of Youmans (1979) with the

avirulent *M. tuberculosis* strain H37Ra. Comparison of the respective dose response curves for killed and live *M. tuberculosis* H37Ra showed that living cells were significantly more effective in protection against *virulent M. tuberculosis* (Youmans, 1979). From these studies it can be postulated that, in addition to the need for *in vivo* multiplication of bacilli for protection, there may also be a role for actively secreted antigens and proteins expressed only *in vivo*, in protective immunity against TB. In support of this hypothesis immunisation with extracellular proteins of *M. tuberculosis* demonstrates a degree of protection against TB in both guinea pig and mouse models of infection (Pal and Horwitz, 1992; Andersen, 1994).

1.5 Diagnosis of tuberculosis

The standard diagnostic test for tuberculosis in humans and cattle is the intradermal tuberculin test. This test measures a delayed-type hypersensitivity (DTH) response of the host to injected mycobacterial antigens. The injected mycobacterial antigen is termed 'tuberculin purified protein derivative' (PPD). PPD consists of proteins released into the growth medium from stationary cultures of *M. tuberculosis*, that are precipitated with trichloroacetic acid (TCA) after autoclaving, filtration and concentration of the culture.

Due to the conservation of many proteins between different bacteria it is not surprising that PPD is not a species-specific reagent but is widely cross-reacting. Consequently, the tuberculin skin test fails to distinguish between infection and BCG-vaccination in humans and cattle. Tuberculin skin testing is also compromised by exposure to environmental mycobacteria. However, the tuberculin test has been a useful tool in the identification of humans infected with *M. tuberculosis* in countries which do not BCG vaccinate, and of cattle infected with *M. bovis*.

The identification of *M. bovis* infected cattle by the skin test coupled with the removal of such individuals has greatly reduced the incidence of *M. bovis* infection in cattle. Nevertheless, confirmation of diagnosis

of TB routinely requires laboratory culture of the organism which can take as long as 3 to 4 weeks.

Given the potential role of secreted antigens in protective immunity and in TB diagnosis, much research has focused on the identification and characterisation of secreted antigens of the MTb complex.

1.6 Protein secretion

Protein secretion describes the translocation of a protein across the bilaminar lipid membrane surrounding the cytoplasm. The process is directed by a secretion signal peptide that has characteristic physio-chemical properties (Creighton, 1993):-

(i) A net positively charged region at the amino-terminus of 2 -5 residues after the initiating methionine (*n* region).

(ii) A stretch of 7-15 primarily hydrophobic residues (*h* region).

(iii) Approximately five neutral residues defining the end of the signal peptide (*c* region).

The mechanism of protein secretion in *E. coli* has been studied in detail (Bassford *et al.*, 1991). The Sec dependent mechanism is believed to involve the sequential transfer of the uncleaved protein across the membrane by the proteins Sec A/B, Sec E/F and SecY. Subsequent cleavage of the leader peptide is performed by the protein leader peptidase I (or by leader peptidase II for lipoprotein signal peptides, described in Chapter 3) to release the mature processed protein. Alternatively, a mechanism involving a protein complex termed the signal recognition complex (SRP) maybe used to translocate the protein prior to cleavage (Bassford *et al.*, 1991). The identification of homologous genes within the TB genome to genes encoding the Sec proteins of *E. coli* suggest that mycobacteria may use similar mechanisms for protein secretion. This is supported by the observation that *E. coli* is able to secrete mycobacterial proteins containing typical signal peptides (Hewinson and Russell, 1993).

The identification of secreted proteins of *M. tuberculosis* that do not possess a classical signal peptide (including superoxide dismutase (Zhang *et al.*, 1991), ESAT-6 (Sørensen *et al.*, 1995), and glutamine synthetase (Harth and Horwitz, 1997)) suggest that signal peptide independent mechanisms may also exist in mycobacteria. Signal peptide independent mechanisms previously described include; ABC (ATP-binding cassette) protein secretion machines (Wandersmann, 1998) and the type III secretion systems of various bacteria (Lee, 1997).

1.7 Secreted antigens of the MTb complex

Due to the heating process required to kill the virulent bacteria, and the acidic precipitation processes used in the production of PPD, many of its protein constituents are denatured and degraded (Nagai *et al.*, 1974). As preparations of BCG had been shown to be closely cross-reactive with *M. tuberculosis* in delayed skin-reactions (Chaparas and Hedrick, 1973), Nagai *et al.* (1981) sought to identify the active components of tuberculin PPD by purifying culture filtrate proteins from avirulent *M. bovis* BCG (Tokyo strain), thereby avoiding the heat sterilisation process. During this study a protein designated MPB70 (70 referring to the relative mobility in 7.7% polyacrylamide gels and MPB referring to its origin (mycobacterial protein from BCG)) was isolated which accounted for more than 10% of the proteins in the culture filtrate. Nagai *et al.* concluded that, "MPB70 is probably not an autolyzed protein, but one secreted into the culture medium as a natural product when the cells are growing actively" (Nagai *et al.*, 1981).

The definition of actively secreted proteins has been the subject of many studies. Abou-Zeid *et al.* (1987) confirmed the secreted nature of MPB70 and also identified MPB64 as secreted antigens by [^{35}S]-methionine labelling of multiplying mycobacteria in short term cultures (Abou-Zeid *et al.*, 1987). More recently Nagai *et al.* (1991) purified and obtained N-terminal sequence for 12 antigens from the culture filtrate of *M. tuberculosis*.

Using a quantitative comparison of the amounts of these proteins in culture filtrates and sonicates Wiker *et al.* (1991) devised a localisation index (LI) to define the secretion efficiencies of mycobacterial proteins.

Identification of proteins of *M. tuberculosis* present in the culture filtrate at different time-points led Andersen *et al.* (1991) to suggest that these mycobacterial proteins may be divided into three major classes:-

"i, **Excreted proteins** are produced in large quantities by *M. tuberculosis* during the first few days of culture. They accumulate in large quantities in the medium but are present only in trace amounts in the intact bacilli.

ii, **Secreted proteins** of the outer cell wall are gradually released during growth of the bacilli. The concentration of these antigens increases steadily during the culture period.

iii, **Cytoplasmic antigens**; released from dead bacteria appear suddenly in high concentrations during the late logarithmic growth phase."

The first two of these groups may be interpreted as to represent antigens actively secreted into the culture filtrate and antigens gradually released into the medium from the cell wall, such as lipoproteins, respectively. In a recent study, Sonnenberg and Belisle (1997) observed 205 protein spots following two-dimensional analysis of culture filtrate proteins (CFP's) from a 14 day culture of *M. tuberculosis* (Sonnenberg and Belisle, 1997). Characterisation of these proteins by N-terminal amino acid sequencing and/or antibody reactivity revealed that only 32 could be identified. These results suggest that there are many more components of the culture filtrate to be investigated. Table 1.2 lists the major excreted and secreted antigens of the MTb complex identified to date and the location of their genes in the completed genome sequence.

Table 1.2 Proteins identified in the culture medium of bacilli from the MTb complex.¶

MPT/B	Other designation(s)	MW (10^3) (by SDS-PAGE)	Signal Peptide	ORF No.†
12		12.5	Yes	Rv2367c
32	apa	45/47	Yes	Rv1860
35	Catalase	80-84	No	Rv1908c
44	Ag 85A, (P32)	31	Yes	Rv3804c
45	Ag 85C	31.5	Yes	Rv0129c
46	Thioredoxin	14	No	Rv1470
51		27	Yes	Rv3803c
57	GroES (BCGa)	31	No	Rv2878c
58	SOD	25	No	Rv3418c
59	Ag 85B,(α antigen)	30	Yes	Rv3846
63		18	Yes	Rv1886c
64		26	Yes	Rv1980c
70		22	Yes	Rv2875
80	MPB70 Homologue	22	Yes	
83		26	Yes	Rv2873
	19 kDa antigen	19	Yes	Rv3763
	38 kDa antigen (Antigen 78)	38	Yes	Rv0934
	Glutamine synthetase	56	No	Rv2220
	DnaK	70	No	Rv0350
	L-alanine dehydrogenase	40	No	Rv2780
	ESAT-6	6	No	Rv3875

¶ Compiled from (Nagai *et al.*, 1991; Fifis *et al.*, 1991; Andersen *et al.*, 1991, and Cole *et al.*, 1998).

†ORF Number as described in Cole *et al.*, (1998).

1.8 MPB70

As mentioned above MPB70 was found to be the major component of the culture filtrate of *M. bovis* BCG Tokyo. Following the isolation of MPB70 (Nagai *et al.*, 1981), the authors demonstrated that in skin tests, MPB70 was a BCG-specific antigen, eliciting a DTH reaction

only in guinea pigs sensitised with dried *M. bovis* BCG Tokyo in oil, but not in guinea pigs sensitised with similarly prepared cells of *M. tuberculosis*, *M. kansasii*, *M. intracellulare* or *M. phlei*. However, no such DTH reaction was observed if guinea pigs were sensitised with heat-killed *M. bovis* BCG Tokyo (Miura *et al.*, 1983). Miura *et al.* (1983) further demonstrated that a DTH response to MPB70 was dependent on the strain of *M. bovis* BCG used in sensitisation of guinea pigs, observing no skin reactions to MPB70 in guinea pigs sensitised with viable *M. bovis* BCG Pasteur or Copenhagen.

Harboe and Nagai (1984) demonstrated, by use of radioimmunoassay (RIA), that substrains of *M. bovis* BCG could be divided into two groups based on their production of MPB70. The groups were termed high or low producers of MPB70, and the levels of MPB70 observed in these groups correlated with previous results of cell mediated responses to MPB70 by guinea pigs immunised with different substrains of BCG (Miura *et al.*, 1983). Strains such as *M. bovis* BCG Tokyo, Moreau and Russia, along with the virulent *M. bovis* Ravenel were found to be high producers of MPB70, whereas *M. bovis* BCG Pasteur, Glaxo and Copenhagen along with *M. tuberculosis* were classified as low producers, expressing less than 100 times the amount of MPB70 compared to that produced by *M. bovis* BCG Tokyo. The T cell immunogenicity of MPB70 has also been reported by Pollock *et al.* (1994) who identified three T cell epitopes of this antigen that were recognised by lymph nodes from cattle experimentally infected with *M. bovis*.

In addition to the cell mediated immunogenicity of MPB70 described previously (Nagai *et al.*, 1981; and Miura *et al.*, 1983), MPB70 was also found to induce strong humoral immune responses in rabbits (Harboe and Nagai, 1984). This response also exhibited species specificity, with anti-MPB70 antibodies rapidly formed in large amounts after immunisation with BCG Tokyo and only in small amounts after immunisation with BCG Copenhagen. The above properties of MPB70 made this antigen a potential candidate for the development of a diagnostic test for *M. bovis* infection.

In a study to investigate the feasibility of assaying for anti-MPB70 antibodies in cattle as an indication of *M. bovis* infection Harboe *et al.* (1990) demonstrated a rapid increase in anti-MPB70 antibodies in cattle infected with *M. bovis* following skin testing with PPD produced from *M. bovis*. No such increase was observed in uninfected cattle. Furthermore anti-MPB70 antibodies reacted with the MPB70 protein in the bovine PPD preparations, demonstrating that MPB70 is present in tuberculin in a form which can directly induce antibody formation in an anamnestic manner in infected animals.

However, a more recent study evaluating the use of MPB70 in an anamnestic ELISA for the detection of tuberculous cattle showed that only 9 out of 29 *M. bovis* culture positive, skin test negative cattle were identified as tuberculous using such an assay (Yearsley *et al.*, 1998). This poor performance is probably due to the short time scale, 12 to 14 days, between the skin-testing and the ELISA as Harboe *et al.* (1990) clearly demonstrated that the anamnestic response to MPB70 following skin-testing was negligible at this time point. Nevertheless such a serological assay may be of use in conjunction with the skin test in the control of bovine tuberculosis, as the former can detect cattle with generalised tuberculosis which are anergic in response to the skin test (Yearsley *et al.*, 1998).

The species specificity of MPB70 was attributed to *M. bovis* due to the failure of guinea pigs sensitised with *M. tuberculosis* to elicit a DTH response to MPB70 upon skin testing (Nagai *et al.*, 1981). However, the identification of MPB70 specific T cells from humans infected with *M. tuberculosis* (Roche *et al.*, 1994; Mustafa *et al.*, 1998) and antibodies against MPB70 from mice infected with *M. tuberculosis* (Hewinson *et al.*, 1996) raises the possibility that although *M. tuberculosis* produces only low amounts of MPT70 when grown *in vitro* (Harboe and Nagai, 1984), the protein may be upregulated during the course of *M. tuberculosis* infection.

1.8.1 *mpb70*; the gene encoding MPB70

The sequence of the gene encoding MPB70 was first described for *M. bovis* BCG Tokyo (Terasaka *et al.*, 1989), and was subsequently described for the pathogenic *M. bovis* strain AN5 which is used for bovine tuberculin production (Radford *et al.*, 1990).

Although, *in vitro*, *M. tuberculosis* expresses approximately one thousandth of the amount of MPT70 expressed by BCG Tokyo (Harboe and Nagai, 1984), its gene, *mpt70*, shows no differences either within the coding sequence or in the upstream or downstream regions of the gene compared to *mpb70* from the high producing strain *M. bovis* BCG Tokyo (Matsumoto *et al.*, 1995). It has been postulated that the differences in levels of expression of MPT70 from *M. tuberculosis* and MPB70 from *M. bovis* BCG Tokyo are due to different efficiencies in transcription (Matsuo *et al.*, 1995). Although slight differences have been reported between the nucleotide sequences of *mpt70* and *mpb70* from *M. bovis* AN5, the mature proteins encoded by these genes are identical (Fig. 2.4) and it is likely that the *M. bovis* AN5 sequence may be incorrect as *mpb70* from both *M. bovis* BCG Tokyo and Pasteur are identical to that of *mpt70*.

The *mpb70* gene encodes for a protein with a predicted molecular weight of 16.3 kDa, although on SDS-PAGE MPB70 appears to have a relative molecular weight of 22 kDa (Fifis *et al.*, 1989). The protein contains a classical secretion signal peptide of 30 amino acids. The protein is processed and secreted by *E. coli*, and the signal peptide of MPB70 has been used to obtain secretion of the *M. tuberculosis* 19 kDa antigen by *E. coli* (Hewinson and Russell, 1993).

1.8.2 Heterogeneity of MPB70

The first evidence for heterogeneity of MPB70 was presented by Nagai *et al.* (1981) during their purification of MPB70 from BCG Tokyo. In this study they identified a protein, called MPB70a, that had the same

mobility in SDS-PAGE as MPB70 though was more acidic as determined by ion-exchange chromatography. This protein was later classified as MPB80 by Miura *et al.*, (1983). Harboe *et al.*, (1986) demonstrated that MPB70 and MPB80 were identical for their first 30 amino acids and suggested that the difference in charge of the two proteins may be attributable to modifications of some amino acid side-chains.

Fifis *et al.* (1991) sought to identify further species specific antigens from the culture filtrate of *M. bovis* for use in improved serological assays for bovine TB by using specific monoclonal antibodies (Wood *et al.*, 1988). During this study they identified two antigens that were recognised by the MAb SB10, which also recognised MPB70. Both these MPB70 cross-reactive antigens of 25 kDa and 23 kDa were shown to have carbohydrate associated with them and it was postulated that these two antigens were different glycosylated versions of MPB70 (Fifis *et al.*, 1991). The identification of this 25 kDa MPB70 cross-reactive protein supported previous observations of Hasløv *et al.*, (1987) who observed a similar phenomenon in BCG Tokyo with a polyclonal antibody raised against MPB70. Sensitisation of guinea pigs with live BCG Tokyo induced T cell responses to the The 23 kDa antigen with the development of DTH responses (Wiker *et al.*, 1995)

In Great Britain the Badger (*Meles meles)* has been identified as a wildlife reservoir of *Mycobacterium bovis* infection and is considered a potential source of bovine tuberculosis (Muirhead *et al.*, 1974). Investigations into the epidemiology of *M. bovis* infection in badgers necessitated the development of a diagnostic test that would allow rapid diagnosis in the field. Preliminary research into the development of a serological assay for the detection of *M. bovis* in badgers demonstrated that the 25 kDa MPB70 cross-reactive antigen is recognised, in Western blotting, by sera from *M. bovis* infected badgers but not by uninfected badgers (Goodger *et al.*, 1991).

Fifis *et al.*, (1992) also demonstrated that the 25 kDa MPB70 cross-reactive antigen, when used in an ELISA, gave stronger reactions, and

was recognised by more sera from infected cattle than MPB70. However, this 25 kDa antigen was far more cross-reactive with sera from cattle infected with *M. avium*, *M. paratuberculosis* and *Nocardia asteroides* than MPB70 an observation that may be explained by the presence of cross-reactive carbohydrate epitopes on this antigen (Krambovitis, 1987). In addition to the serodominance of this 25 kDa antigen over MPB70 during *M. bovis* infection in cattle, it is also the first antigen to be recognised by IgG antibodies following experimental *M. bovis* infection in badgers (Goodger *et al.*, 1991) and cattle (O'Loan *et al.*, 1994).

1.9 Objective of this study

Given the serodominance of the 25 kDa antigen of *M. bovis* described above, it is an interesting candidate for both a sub-unit vaccine, and as a diagnostic reagent, for bovine tuberculosis. The aim of this thesis was therefore the molecular characterisation of this 25 kDa antigen.

Chapter 2

Identification and expression of the gene for the 25 kDa mycobacterial antigen MPT83.

2.1 Introduction

The search for a specific serological test for *M. bovis* infection in cattle has focused attention on the characterisation of species specific antigens. Using a *Mycobacterium bovis* specific antibody, SB10 (Wood *et al.*, 1988), Fifis *et al.* (1989) identified and purified three antigens from *M. bovis* AN5 culture filtrate. Two of these antigens were identified as the previously described cross reacting antigens MPB70 and MPB80 (Harboe *et al.*, 1986). Further investigations by Fifis *et al.* (1991) led to the characterisation of two other antigens from *M. bovis* AN5 culture filtrate that also reacted with antibodies against MPB70. These antigens, with apparent molecular weights of 25 kDa and 23 kDa in SDS-PAGE, were shown to have carbohydrate associated with them and were reported to be glycosylated forms of MPB70 (Fifis *et al.*, 1991).

The 19 kDa antigen of *M. tuberculosis* has been suggested to be post-translationally modified by *Mycobacterium smegmatis* due to the larger relative molecular weight of the recombinant protein expressed by *M. smegmatis* relative to the product expressed by *E. coli* (Garbe *et al.*, 1993). This posttranslational modification was proposed to be glycosylation as the recombinant 19 kDa antigen produced by *M. smegmatis* bound the lectin Concanavalin A (ConA) whereas no such binding to the recombinant produced by *E. coli* was observed.

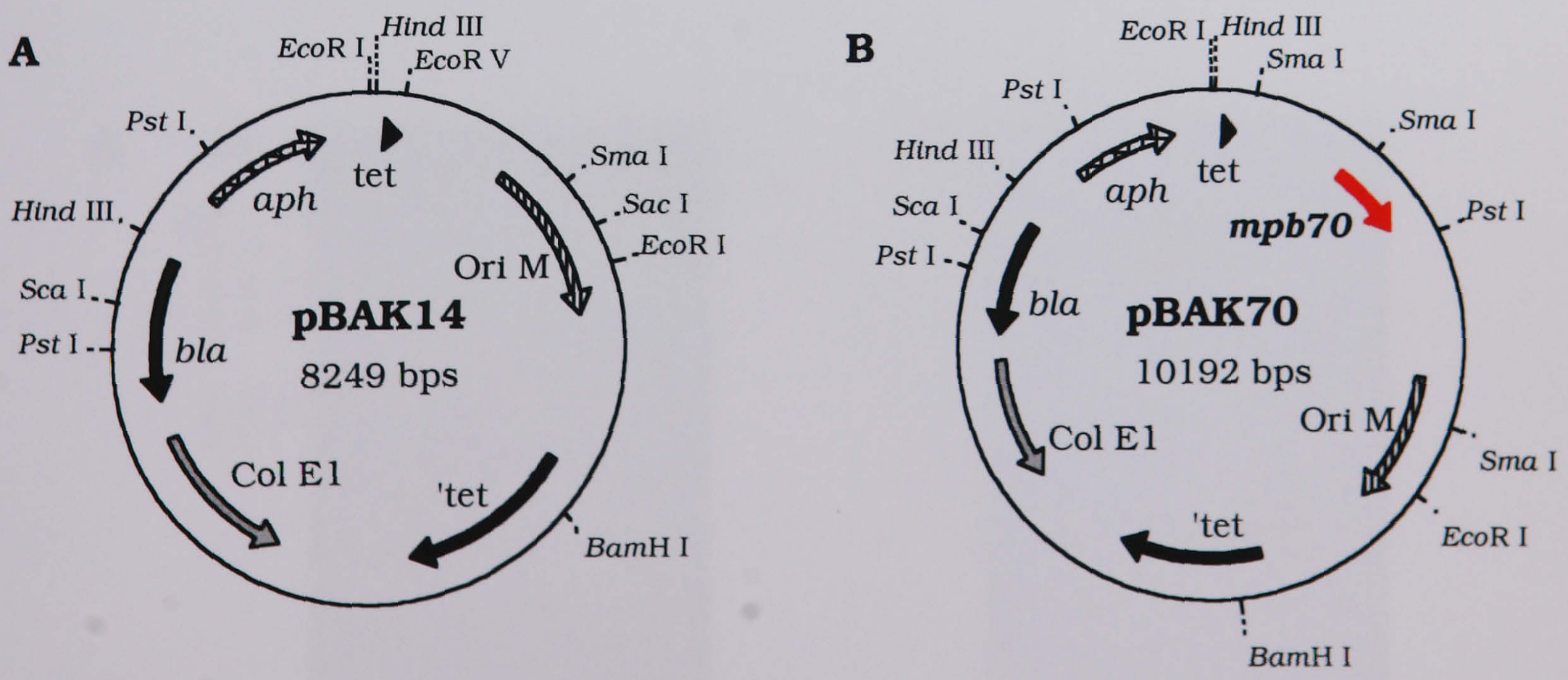
The aim of this part of the study was to identify the gene coding for the 25 kDa antigen by using a similar approach to that of Garbe *et al.* (1993).

2.2 RESULTS

2.2.1 Expression of MPB70

To test the hypothesis that the 25 kDa antigen of *M. bovis* is a post-translationally modified form of MPB70 (Fifis *et al.*, 1991) the gene for *mpb70* was cloned from a λEMBL3 library of *M. bovis* AN5 DNA, into the *Eco*R V site of the mycobacterial shuttle vector pBAK14 (Garbe *et al.*, 1993) (Fig. 2.1A). This construct, pBAK70 (Fig. 2.1B), was transformed into *M. smegmatis* mc^2155 and *E. coli* DH5α. Expression of MPB70 was driven from the tetracycline promoter in both hosts. These recombinants along with *M. smegmatis* mc^2155 and *E. coli* DH5α containing the vector pBAK14 were supplied by Dr. R. G. Hewinson, VLA, Weybridge.

Fig. 2.1 Plasmid maps of pBAK14 (A) and pBAK70 (B).



2.2.1a Characterisation of pBAK70

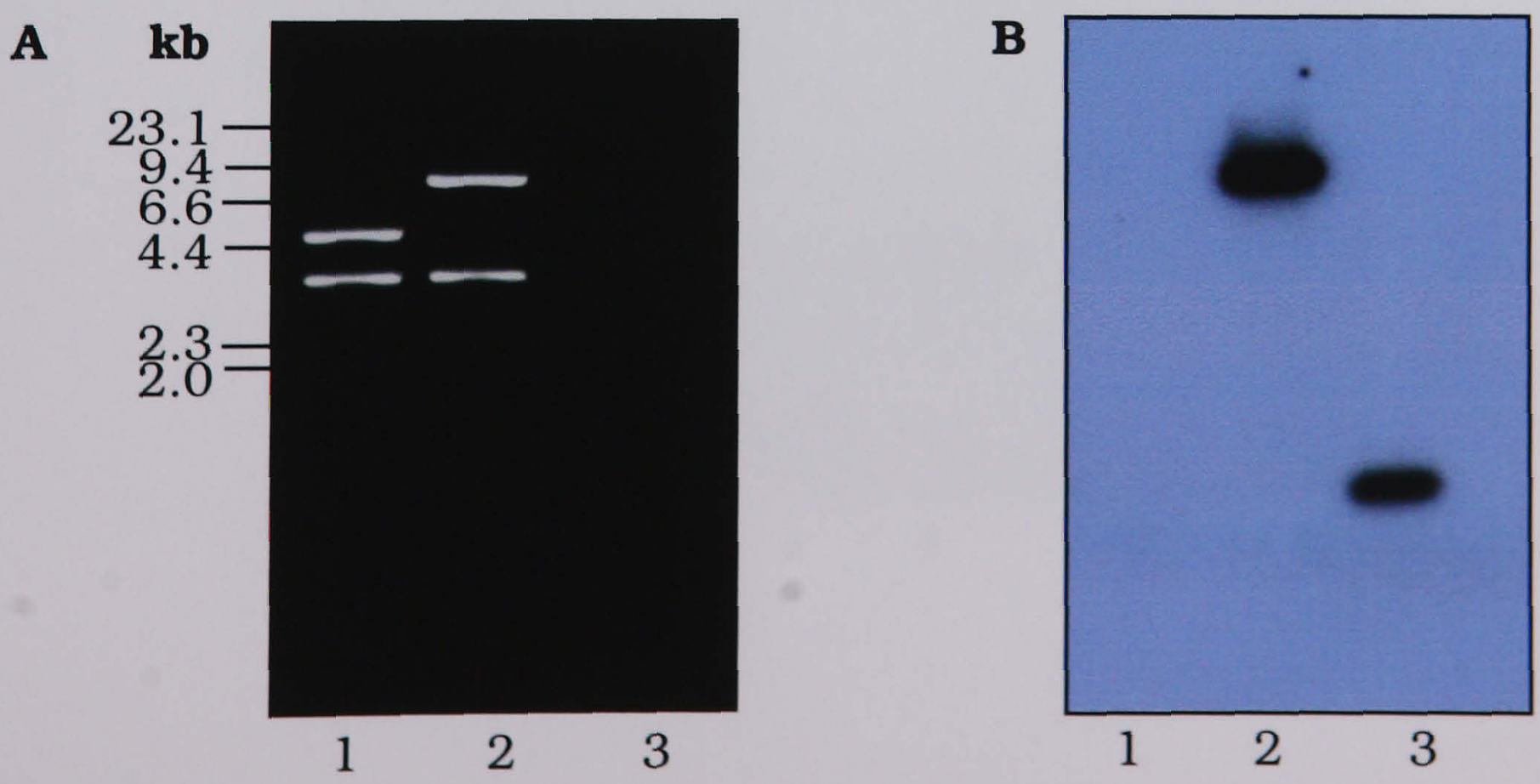
Confirmation that the recombinants contained the correct plasmids was performed by Southern hybridisation and DNA sequencing (as described in section 8.6.2 and 8.7), using the oligonucleotide primers 1, 2, 3 and 4 (appendix 1) designed against the DNA sequence of pBAK14 and *mpb70* (Garbe *et al.*, 1993; Radford *et al.*, 1990).

Both plasmids were isolated from *E. coli* DH5α and *M. smegmatis* mc^2155 as described in section 8.5. Plasmids were then digested with the restriction enzymes *Bam*H I and *Sca* I. Restriction digests were analyzed by agarose gel electrophoresis (Fig. 2.2A), Southern blotted and probed with the *mpb70* gene (Fig. 2.2B) which was produced as described previously (Hewinson and Russell, 1993).

Southern blotting confirmed that pBAK70 contained *mpb70* (Fig. 2.2B, lane 2), and DNA sequencing confirmed that the orientation of *mpb70* in pBAK70 was as shown in Fig. 2.1B.

Fig. 2.2 Southern blot analysis of pBAK14 and pBAK70.

(A) Ethidium bromide stained agarose gel of plasmids digested with *Bam*H I and *Sca* I. Lanes: 1, pBAK14 (0.5μg); 2, pBAK70 (0.5μg); and hybridised with *mpb70* (B). Lane 3, *mpb70* PCR product (50 ng, positive control).

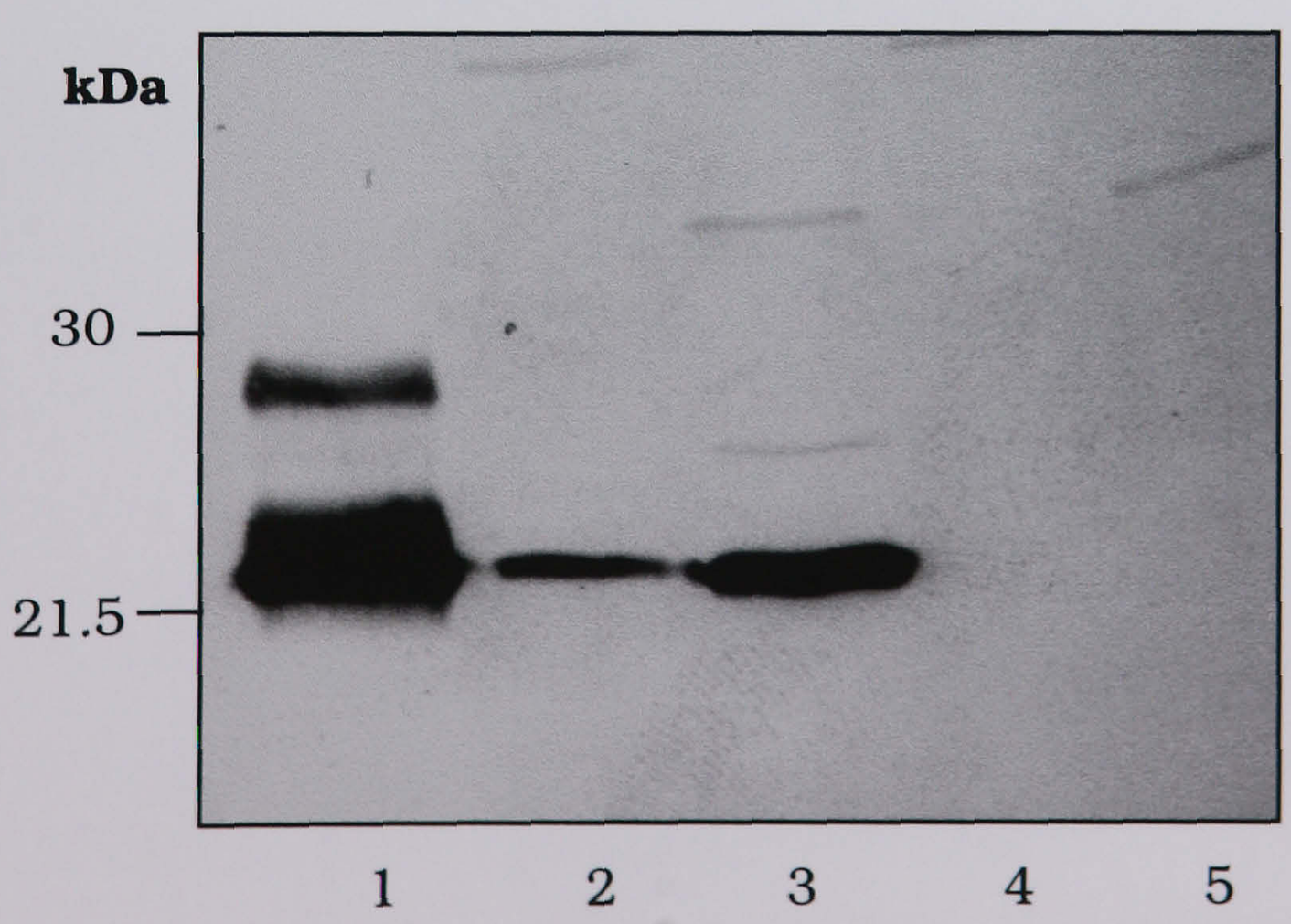


2.2.1b Expression of MPB70 by *Mycobacterium smegmatis* mc²155 and *E. coli* DH5α.

Sonicated extracts of *M. smegmatis* mc²155 and *E. coli* DH5α containing pBAK14 and pBAK70 were separated by SDS-PAGE (section 8.10, 15% separating gel), Western blotted and probed with the MPB70 specific monoclonal antibody, SB10 (Agen Biochemicals, Queensland, Australia; Wood *et al.*, 1988). No size difference was observed between the MPB70 expressed by *M. smegmatis* (Fig. 2.3, lane 2) and that expressed by *E. coli* (Fig. 2.3, lane 3). This demonstrates that unlike the 19 kDa antigen of *M. tuberculosis*, MPB70 is not post-translationally modified by *M. smegmatis* mc²155. It is therefore likely that the 25 kDa antigen is not a glycosylated form of MPB70.

Fig. 2.3 Western blot incubated with monoclonal antibody SB10.

Lanes: 1, *M. bovis* AN5 culture filtrate proteins (2μg); 2, sonicated extract (5μg) *M. smegmatis* mc²155 (pBAK70); 3, total cell protein *E. coli* DH5α (pBAK70); 4, sonicated extract (5μg) *M. smegmatis* mc²155 (pBAK14); 5, total cell protein *E. coli* DH5α (pBAK14).



2.2.2 Expression of the MPB70 cross reactive 25kDa antigen

The 23 kDa, MPB70 cross reactive antigen, has been purified and characterised as the mycobacterial antigen MPB83 (Harboe *et al.*, 1995). Although MPB83 has a similar predicted molecular weight to MPB70, using peptide sequencing Harboe *et al.* (1995) showed that MPB83 contained distinct differences in amino acid sequence compared to MPB70 (Terasaka *et al.*, 1989; Radford *et al.*, 1990; Matsumoto *et al.*, 1995), as shown in Fig. 2.4. These distinct differences indicate that MPB83 and MPB70 are encoded by two homologous but different genes. Subsequently it has been proposed that that the 25 kDa antigen may be a glycosylated form of MPB83 and not a glycosylated form of MPB70 (Wiker *et al.*, 1996).

2.2.2a Expression of pA3 by *Mycobacterium smegmatis*

It has been shown previously that expression of the cross-reactive antigens, MPB70, and MPB83 (23 kDa and 25 kDa), and their *M. tuberculosis* homologues, MPT70 and MPT83, appear to be linked (Wiker *et al.*, 1996; Hewinson *et al.*, 1996). It was therefore reasoned that if these antigens were indeed coded for by different genes, they might be arranged as part of an operon and that this might account for their linked expression. The nucleotide sequences coding for mature MPB70 and MPT70 (Radford *et al.*, 1990; Matsumoto *et al.*, 1995) are identical suggesting conservation of these regions between the chromosomes of *M. bovis* and *M. tuberculosis*. Therefore a cosmid library of genomic DNA from *M. tuberculosis* H37Rv, in the mycobacterial shuttle vector pYUB18, was screened in our laboratory against an *mpb70*-specific gene probe. Of the 1200 colonies screened, nine cosmids hybridizing to *mpb70* were identified. All *mpt70* positive cosmids expressed MPT70 (data not shown), and one, pA3, was used for further study as it contained the smallest DNA insert.

Fig. 2.4 Amino acid alignment of the deduced amino acid sequences of MPB70 with sequenced peptides from MPB83[1].

M. tuberculosis H37Rv MPT70	M K V K N T I A A T S F A A A	15
M. bovis AN5 MPB70	M K V K N T I A A T S F A A A	15
M. bovis BCG Tokyo MPB70	M K V K N T I A A T S F A A A	15
M. bovis BCG Tokyo MPB83	- - - - - - - - - - - - - - -	
M. tuberculosis H37Rv MPT70	G L A A L A V A V S P P A A A	30
M. bovis AN5 MPB70	A V A A L A V A V S P P A A A	30
M. bovis BCG Tokyo MPB70	G L A A L A V A V S P P A A A	30
M. bovis BCG Tokyo MPB83	- - - - - - - - - - - - - - A	1
M. tuberculosis H37Rv MPT70	G D L V G P G C A E Y A A A N	45
M. bovis AN5 MPB70	G D L V G P G C A E Y A A A N	45
M. bovis BCG Tokyo MPB70	G D L V G P G C A E Y A A A N	45
M. bovis BCG Tokyo MPB83	L D L I G X X X A Q Y A T P N	16
M. tuberculosis H37Rv MPT70	P T G P A S V Q G M S Q D P V	60
M. bovis AN5 MPB70	P T G P A S V Q G M S Q D P V	60
M. bovis BCG Tokyo MPB70	P T G P A S V Q G M S Q D P V	60
M. bovis BCG Tokyo MPB83	P T G P G S V A G M - - - - -	26
M. tuberculosis H37Rv MPT70	A V A A S N N P E L T T L T A	75
M. bovis AN5 MPB70	A V A A S N N P E L T T L T A	75
M. bovis BCG Tokyo MPB70	A V A A S N N P E L T T L T A	75
M. bovis BCG Tokyo MPB83	- - - - - - - - M L S T L T S	33
M. tuberculosis H37Rv MPT70	A L S G Q L N P Q V N L V D T	90
M. bovis AN5 MPB70	A L S G Q L N P Q V N L V D T	90
M. bovis BCG Tokyo MPB70	A L S G Q L N P Q V N L V D T	90
M. bovis BCG Tokyo MPB83	A L S G K L N P X V N L V D -	47
M. tuberculosis H37Rv MPT70	L N S G Q Y T V F A P T N A A	105
M. bovis AN5 MPB70	L N S G Q Y T V F A P T N A A	105
M. bovis BCG Tokyo MPB70	L N S G Q Y T V F A P T N A A	105
M. bovis BCG Tokyo MPB83	- - - - - - - - - - - - - - -	
M. tuberculosis H37Rv MPT70	F S K L P A S T I D E L K T N	120
M. bovis AN5 MPB70	F S K L P A S T I D E L K T N	120
M. bovis BCG Tokyo MPB70	F S K L P A S T I D E L K T N	120
M. bovis BCG Tokyo MPB83	- - - - - - - - - - - - - - -	
M. tuberculosis H37Rv MPT70	S S L L T S I L T Y H V V A G	135
M. bovis AN5 MPB70	S S L L T S I L T Y H V V A G	135
M. bovis BCG Tokyo MPB70	S S L L T S I L T Y H V V A G	135
M. bovis BCG Tokyo MPB83	- - - - - - - - - - - - - - -	
M. tuberculosis H37Rv MPT70	Q T S P A N V V G T R Q T L Q	150
M. bovis AN5 MPB70	Q T S P A N V V G T R Q T L Q	150
M. bovis BCG Tokyo MPB70	Q T S P A N V V G T R Q T L Q	150
M. bovis BCG Tokyo MPB83	- - - - - - - - - - - - - - -	
M. tuberculosis H37Rv MPT70	G A S V T V T G Q G N S L K V	165
M. bovis AN5 MPB70	G A S V T V T G Q G N S L K V	165
M. bovis BCG Tokyo MPB70	G A S V T V T G Q G N S L K V	165
M. bovis BCG Tokyo MPB83	- - - - - - - - - - - - - M V	49
M. tuberculosis H37Rv MPT70	G N A D V V C G G V S T A N A	180
M. bovis AN5 MPB70	G N A D V V C G G V S T A N A	180
M. bovis BCG Tokyo MPB70	G N A D V V C G G V S T A N A	180
M. bovis BCG Tokyo MPB83	N N A G L V X G R V H T A N A	64
M. tuberculosis H37Rv MPT70	T V Y M I D S V L M P P A .	194
M. bovis AN5 MPB70	T V Y M I D S V L M P P A .	194
M. bovis BCG Tokyo MPB70	T V Y M I D S V L M P P A .	194
M. bovis BCG Tokyo MPB83	T V Y M - - - - - - - - -	68

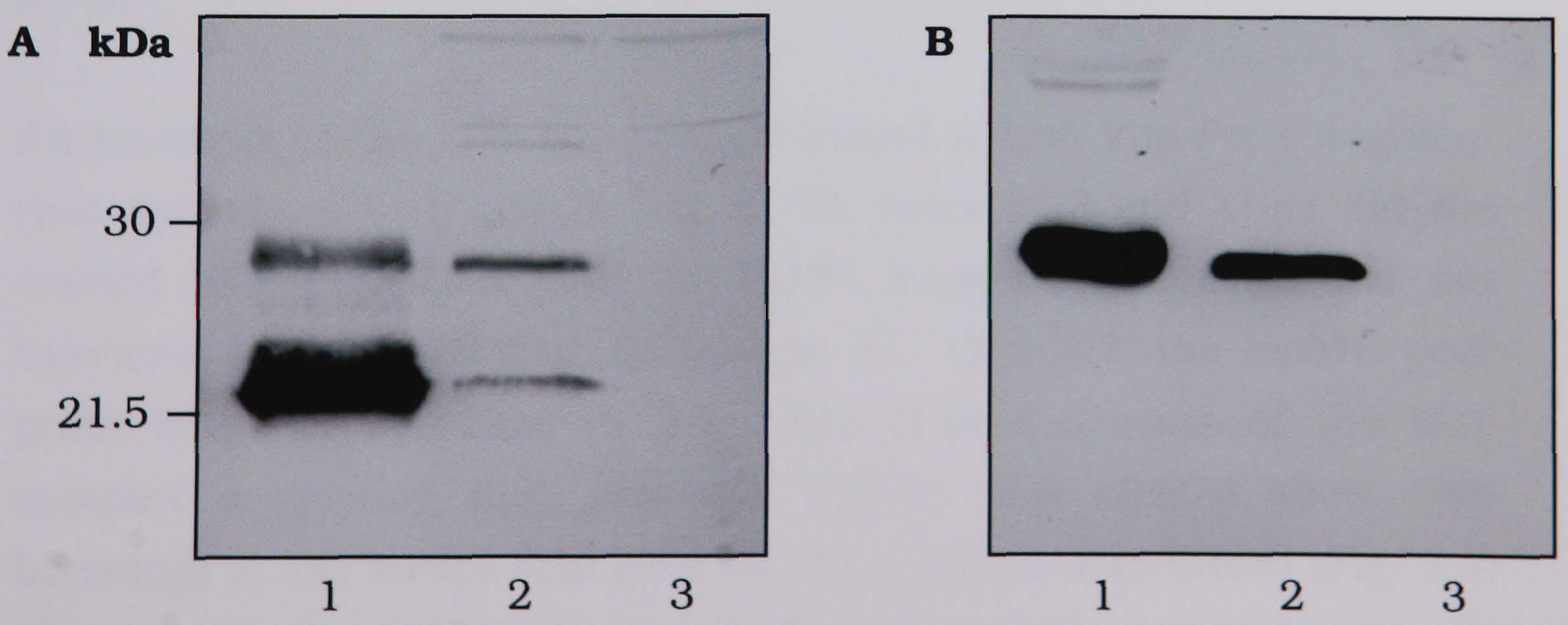
[1]Residues differing from the MPB70 consensus are boxed. - indicates undetermined residues, X denotes uncertainties.

Previous work in our laboratory has also produced a monoclonal antibody, MBS43, that reacts with the 23 kDa and 25 kDa forms of MPB83, but not with MPB70 (Goodger *et al.*, 1994, and Wiker *et al.*, 1996).

Cosmid pA3 was transformed into *M. smegmatis* mc^{2}155 and *E. coli* DH5α as described in section 8.4. Sonicated extracts of *M. smegmatis* containing pA3 were separated by SDS-PAGE (15% separating gel), Western blotted and probed with the monoclonal antibodies SB10 and MBS43 to screen for expression of MPT70[1] and the MPT83 respectively. Two predominant antigenic bands were recognised by SB10 in the sonicated extract of the recombinant *M. smegmatis* containing pA3 (Fig. 2.5A, lane 2), one of 25 kDa and the other of 22 kDa. These bands were not present in *M. smegmatis* containing the control vector (Fig. 2.5A, lane 3). The 25 kDa antigen was recognised by the monoclonal antibody MBS43 (Fig. 2.5B, lane 2), which is specific for the MPB83, confirming that this antigen was the *M. tuberculosis* counterpart of MPB83. Therefore it can be concluded that cosmid pA3 contains the genes encoding both MPT70 and MPT83.

Fig. 2.5 Western blots incubated with SB10 (A) and MBS43 (B).

Lanes: 1, *M. bovis* AN5 culture filtrate proteins (2μg); 2, sonicated extract *M. smegmatis* mc^{2}155 (pA3); 3, sonicated extract (5μg) *M. smegmatis* mc^{2}155 (pYUB18).

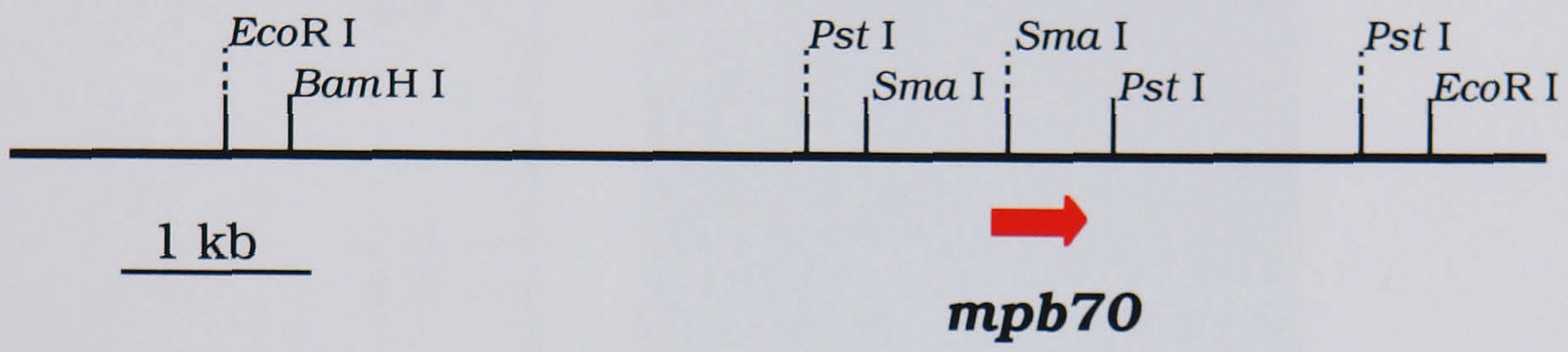


[1] 'T' instead of 'B' as an *M. tuberculosis* H37Rv cosmid was used

2.2.2b Restriction mapping of pA3

The region of the chromosome of *M. bovis* AN5 containing the *mpb70* gene was restriction mapped by Radford *et al.*, (1990) and is reproduced below (Fig. 2.6).

Fig. 2.6 Restriction map of a region of the *M. bovis* chromosome containing the gene *mpb70*.

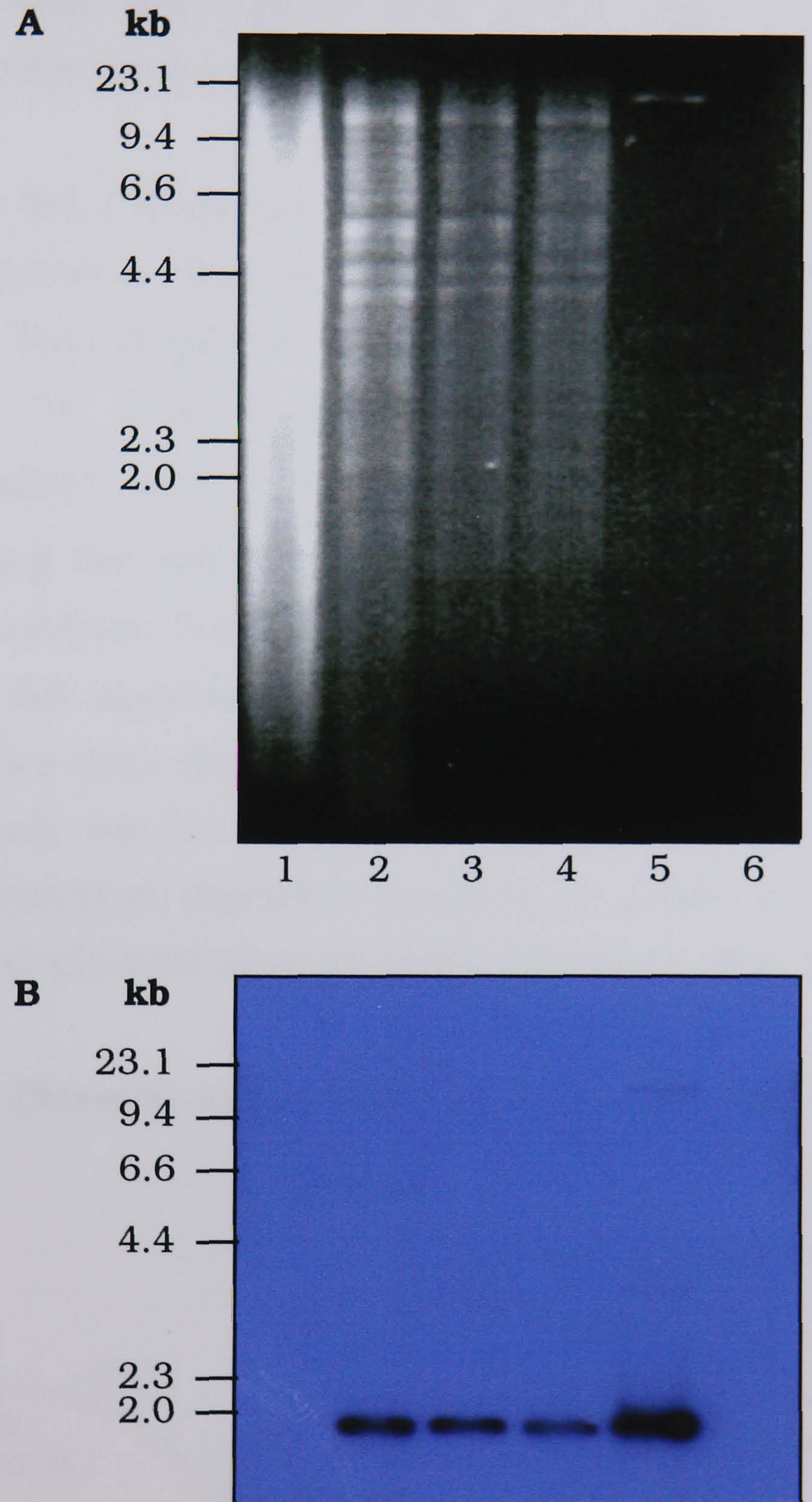


In the first step to determine if the restriction map of the homologous region in *M. tuberculosis* H37Rv was similar to that of *M. bovis* AN5, Southern blot analysis of *Pst* I digested DNA was performed. Genomic DNA was kindly provided by S.L. Hughes (VLA, Weybridge), and cosmid pA3 DNA was isolated from *E. coli* DH5α (pA3) by the midi prep method as described in section 8.6. DNA was digested with the restriction enzyme *Pst* I, visualised by agarose gel electrophoresis (Fig. 2.7A), Southern blotted and probed with the gene probe for *mpb70* (Fig. 2.7B).

All members of the MTb complex contained a 1.85 kbp *Pst* I fragment that hybridised with *mpb70* (Fig. 2.7B, lanes 2, 3 and 4) as did the cosmid pA3 (Fig. 2.7B, lane 5). DNA from *M. smegmatis* did not hybridise with *mpb70* (Fig. 2.7B, lane 1). However, the *mpb70* gene probe failed to hybridise to any other DNA fragments of the MTb complex suggesting that although MPB83 and MPB70 show 68% homology at the amino acid level for the residues determined (Fig. 2.4) (Harboe *et al.*, 1995), the genes for these two antigens may be significantly different.

Fig. 2.7 Southern blot analysis of *mpb70*.

(A) Ethidium bromide stained agarose gel of DNA digested with *Pst* I. Lanes: 1, *M. smegmatis* mc^2155 (4 μg); 2, *M. bovis* BCG Tokyo (2 μg); 3, *M. bovis* AN5 (2 μg); 4, *M. tuberculosis* H37Rv (2 μg); 5, pA3 (0.5 μg). (B) Autoradiograph of gel in panel A Southern blotted and hybridised with *mpb70*. Lane 6, *mpb70* PCR product (50 ng, positive control).



Using the plasmid map of pYUB18 (Fig. 2.8A) and the restriction map produced by Radford *et al.* (Fig. 2.6), the cosmid pA3 was further characterised by restriction digestion with various restriction enzymes (Fig. 2.9A) and Southern hybridisation with the *mpb70* gene probe (Fig. 2.9B). The results were used to generate the map of pA3 shown (Fig. 2.8B). The identification of a single 6.4 kbp *Eco*R I DNA fragment that hybridised with the *mpb70* gene probe (Fig. 2.9B, lane 5) is in agreement with the restriction map of *M. bovis* AN5 (Fig. 2.6).

The 6.3 kbp *Sac* I fragment of pA3 hybridizing with *mpb70* (Fig. 2.9 B, lane 7) contains a disrupted copy of *mpt70* and 6 kbp of upstream sequence. This fragment was subcloned from pA3 into the vector pBluescript SK (Fig. 8.1A) in both orientations. The resulting plasmids, pSM1 and pSM2, were transformed into *E. coli* DH5α, purified using the mini prep method, and orientations confirmed by sequence analysis using the Reverse and M13 -20 primers for pBluescript KS (appendix 1, numbers 5 and 6) as described in section 8.7. Sequence data showed that one end of the 6.3 kbp *Sac* I fragment contained only the first 346 bp of coding sequence of the *mpt70* gene. Further restriction digestion analysis of pSM1 and pSM2 (Fig. 2.10) generated the plasmid map of pSM1 shown in (Fig. 2.11)

Fig. 2.8 Plasmid maps of pYUB18 (A) and pA3 (B).

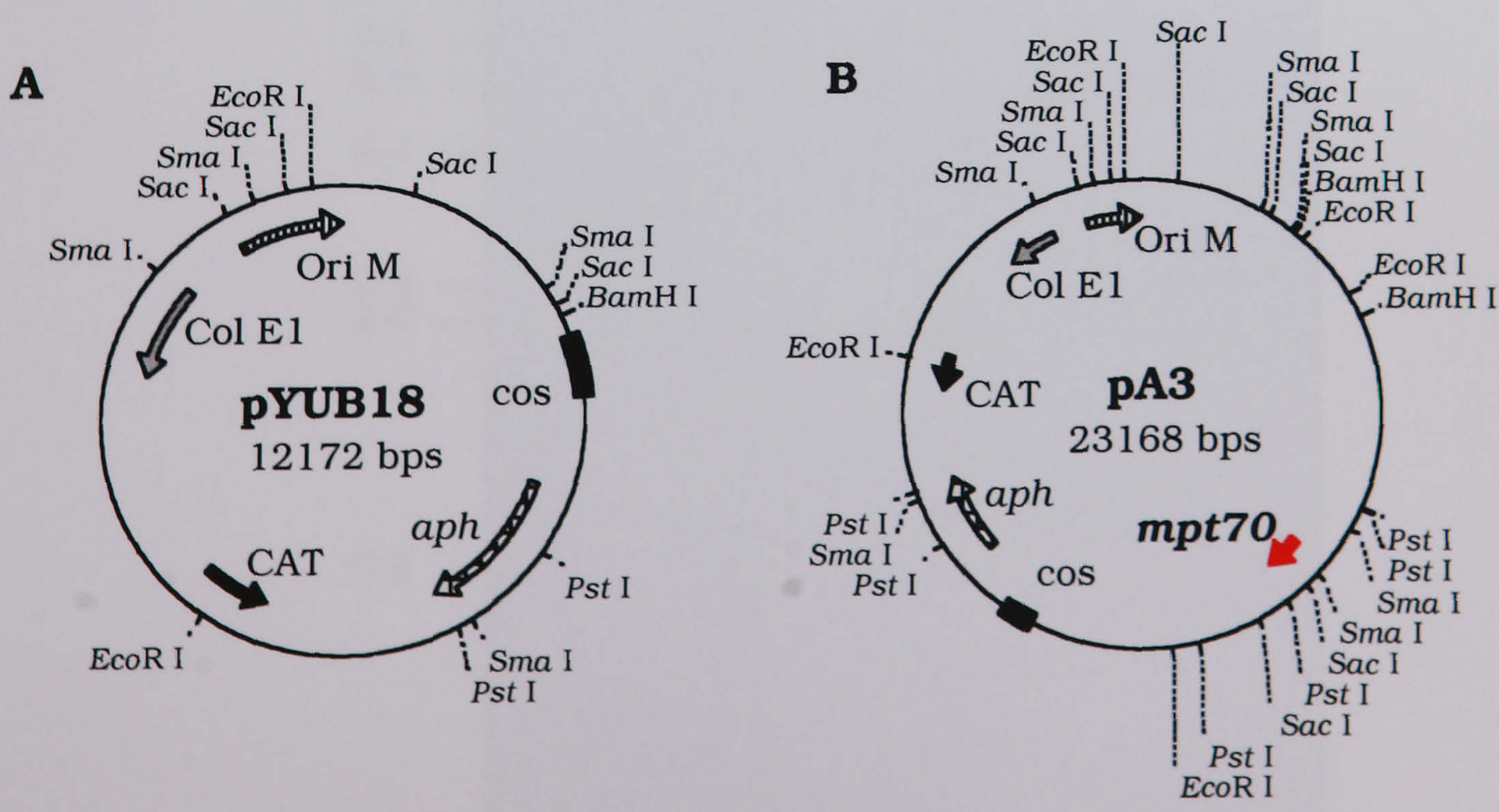


Fig. 2.9 Restriction mapping and Southern blot analysis of pA3.

(A) Ethidium bromide stained agarose gel of lanes: 1, pYUB18 (1μg); 2, pA3 (1μg). pA3 (1μg) digested with, lanes: 3, *Bam*H I; 4, *Eco*R I; 5, *Pst* I; 6, *Sma* I; 7, *Sac* I. (B) Autoradiograph of gel in panel A Southern blotted and hybridised with *mpb70*. Lane 8, *mpb70* PCR product (50ng, positive control).

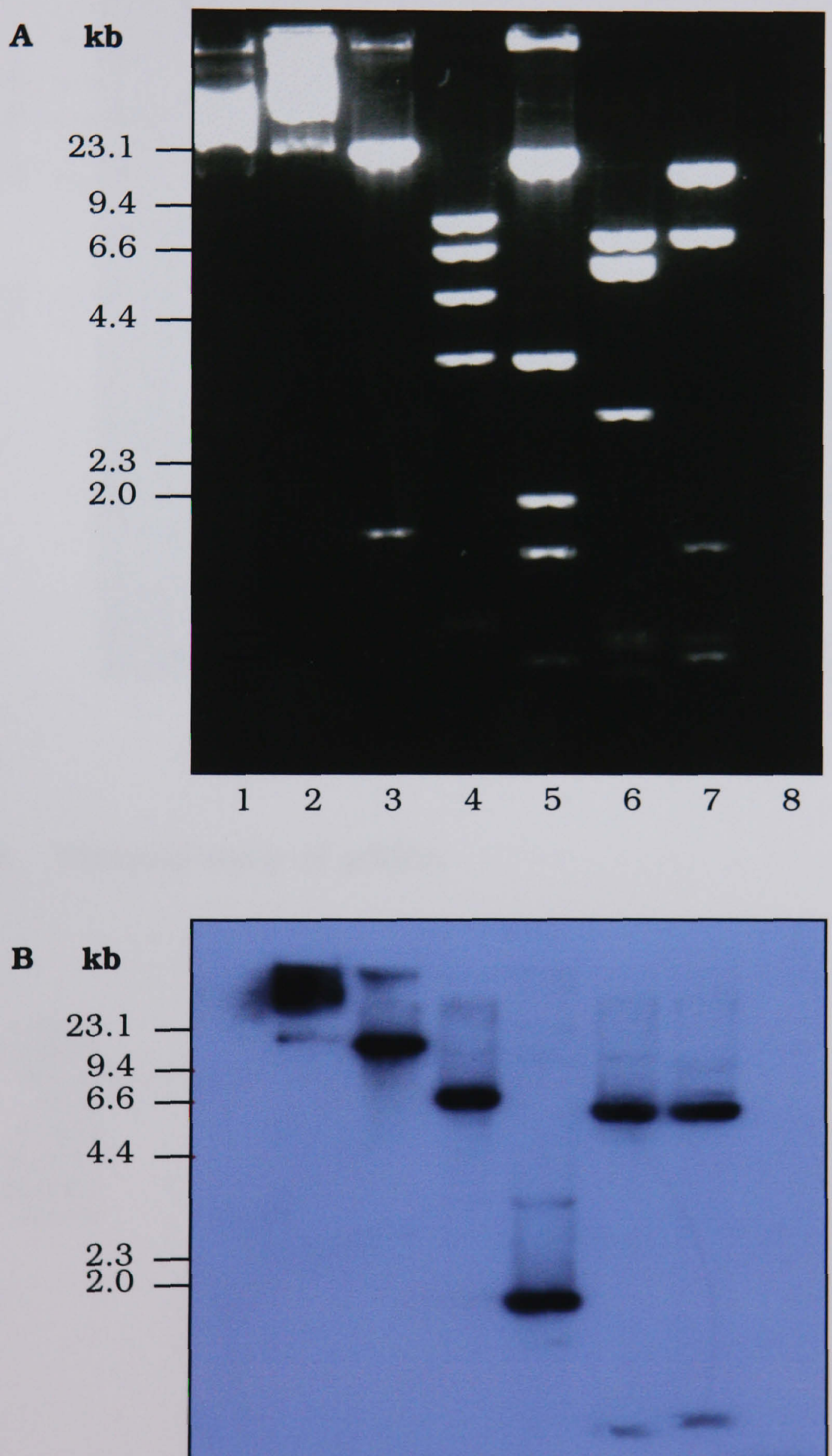


Fig. 2.10 Restriction mapping of pSM1 and pSM2.

Ethidium bromide stained agarose gel of, lane 1, *Sac* I digested pBluescript KS (0.1μg). pSM2 (0.5μg) digested with, lanes: 2, *Sac* I; 3, *Bam*H I; 4, *Eco*R V; 5, *Pst* I; 6, *Spe* I. pSM1 (0.5μg) digested with: lanes: 7, *Sac* I; 8, *Bam*H I; 9, *Eco*R V; 10, *Pst* I; lane 11, *Spe* I.

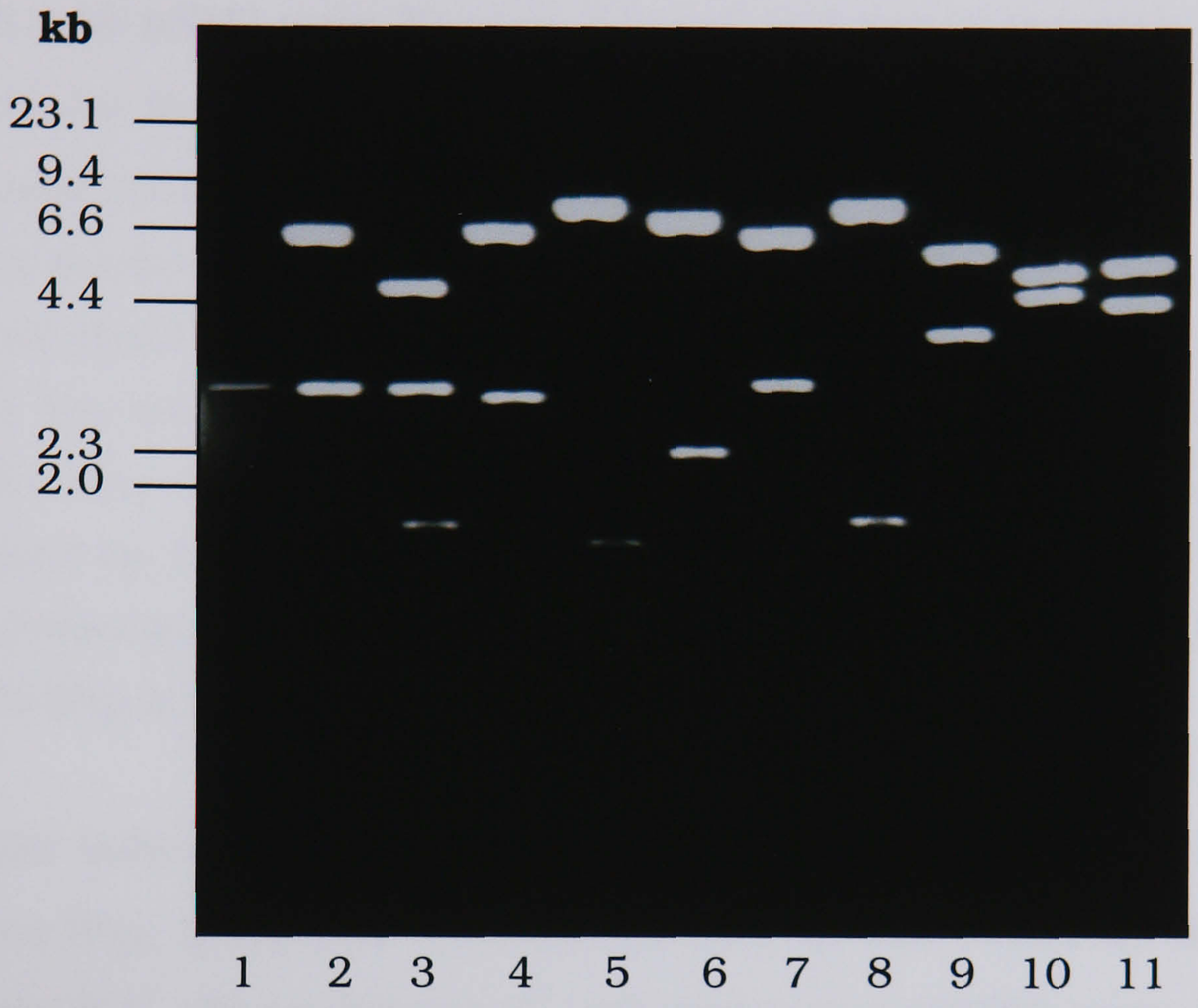
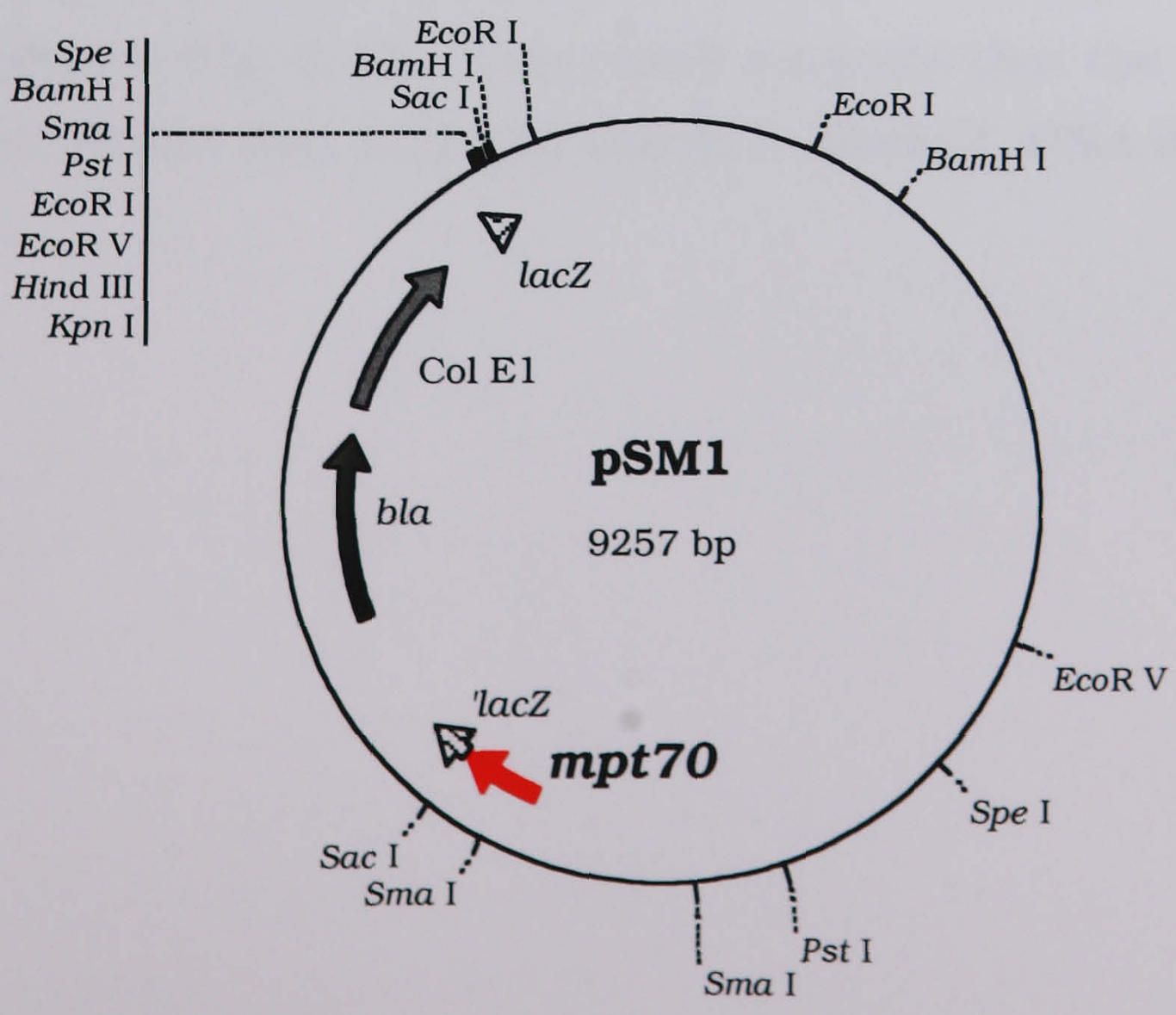


Fig. 2.11 Plasmid map of pSM1.



2.2.2c Expression of MPT83 by *E. coli*

In order to determine if the 6.3 kbp *Sac* I fragment from pA3 encodes for a protein that is recognised by the MPB83 specific monoclonal antibody, MBS43, sonicated extracts of *E. coli* DH5α containing either pSM1 and pSM2 were Western blotted and incubated with MBS43. It was found that *E. coli* DH5α containing pSM1 expressed a protein of apparent molecular weight 23 kDa on SDS-PAGE that was recognised by the monoclonal antibody MBS43 (Fig. 2.13, lane 3), although pSM2 did not (data not shown). Expression of MPT83 by *E. coli* containing pSM1 but not pSM2 confirmed that transcription of the gene encoding MPT83 was directed from the *lacZ* promoter, with expression being achieved by translational coupling (Schoner *et al.*, 1984). This result also demonstrated that *mpt83* is transcribed in the same direction as *mpt70* (Fig 2.11).

Further subclones of pSM1 were constructed in pBluescript KS and SK vectors (Fig. 2.12) and transformed into *E. coli* DH5α as described in section 8.4. Recombinant *E. coli* expressing MPT83 were detected by Western blotting sonicated extracts of these recombinants with the MPT83 specific monoclonal antibody MBS43 (Fig. 2.13). The smallest subclone directing expression of MPT83 in *E. coli* was found to be pSM13 (Fig. 2.13, lane 7) which contained a 1.85 kbp *Bam*H I - *Eco*R V DNA fragment (Fig. 2.12). This result suggests that the gene for MPT83 is present within this 1.85 kbp *Bam*H I - *Eco*R V DNA fragment.

Fig. 2.12 Screening of subclones from pSM1 with the monoclonal antibody MBS43

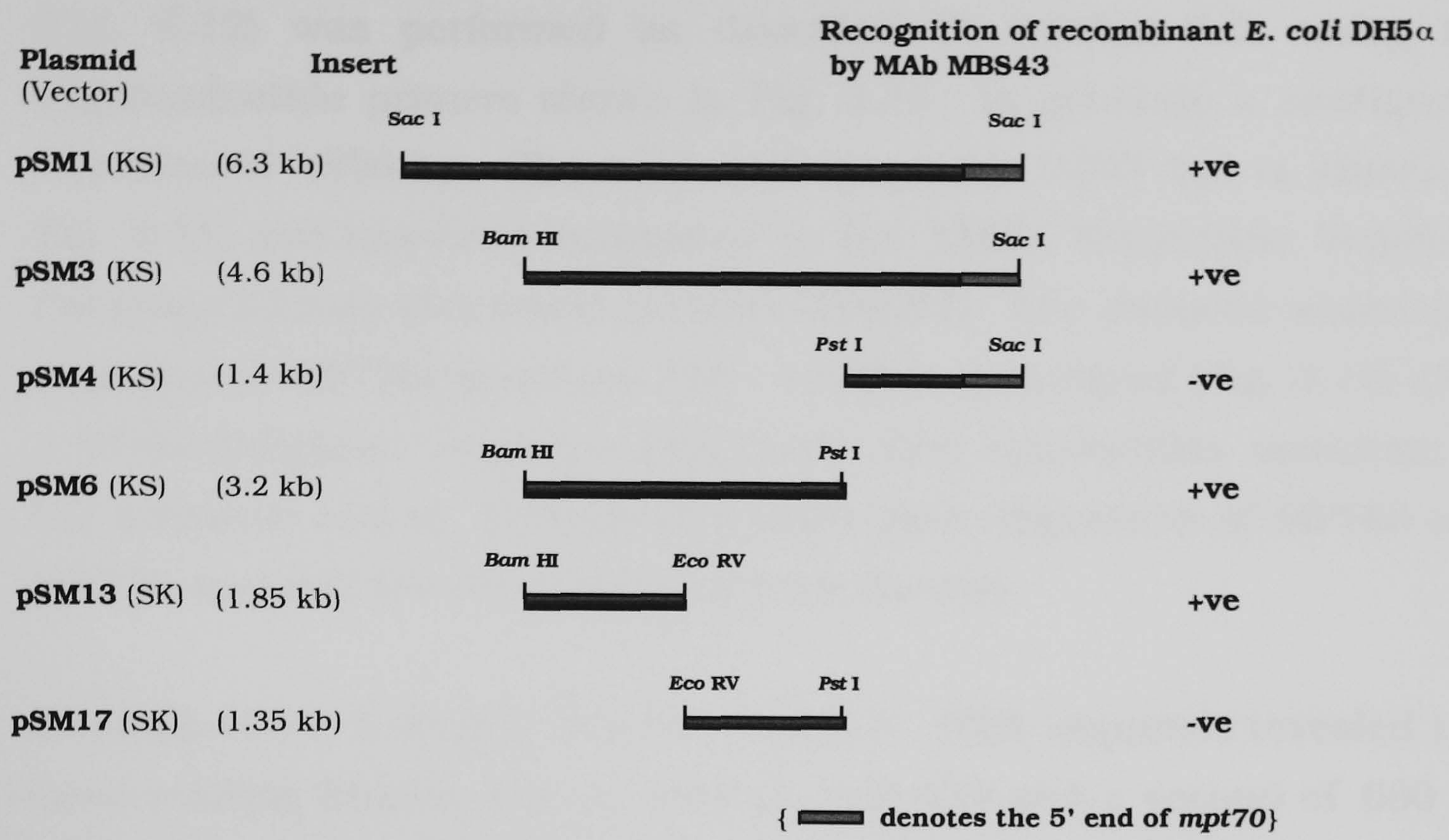
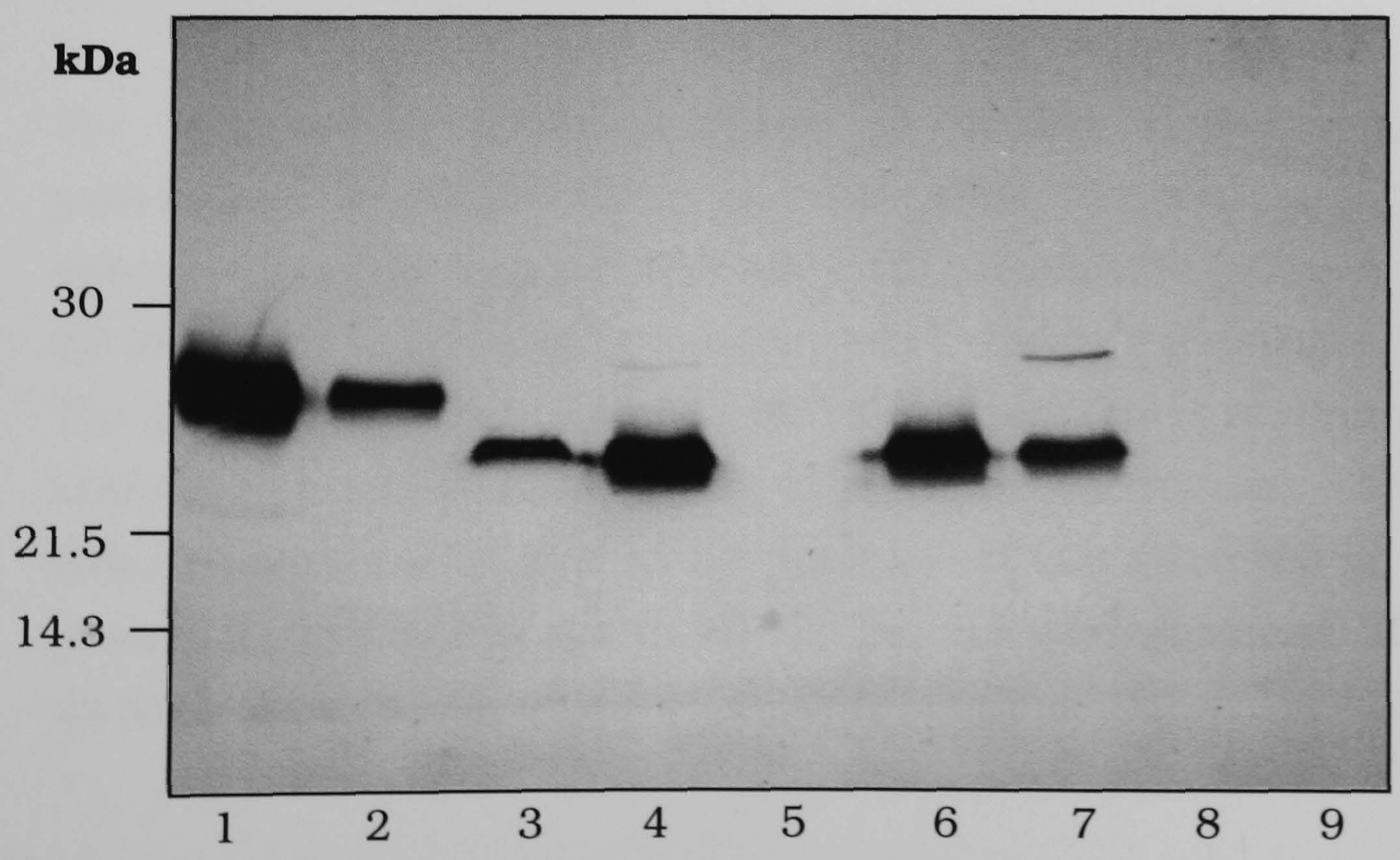


Fig. 2.13 Western blot incubated with the monoclonal antibody MBS43.

Sonicated extracts (5μg) of lanes: 1, *M. bovis* AN5; 2, *M. smegmatis* mc^2155 (pA3); 3, *E. coli* DH5α (pSM1); 4, *E. coli* DH5α (pSM3): 5, *E. coli* DH5α (pSM4); 6, *E. coli* DH5α (pSM6); 7, *E. coli* DH5α (pSM13); 8, *E. coli* DH5α (pSM17): 9, *E. coli* DH5α (pBluescript KS).

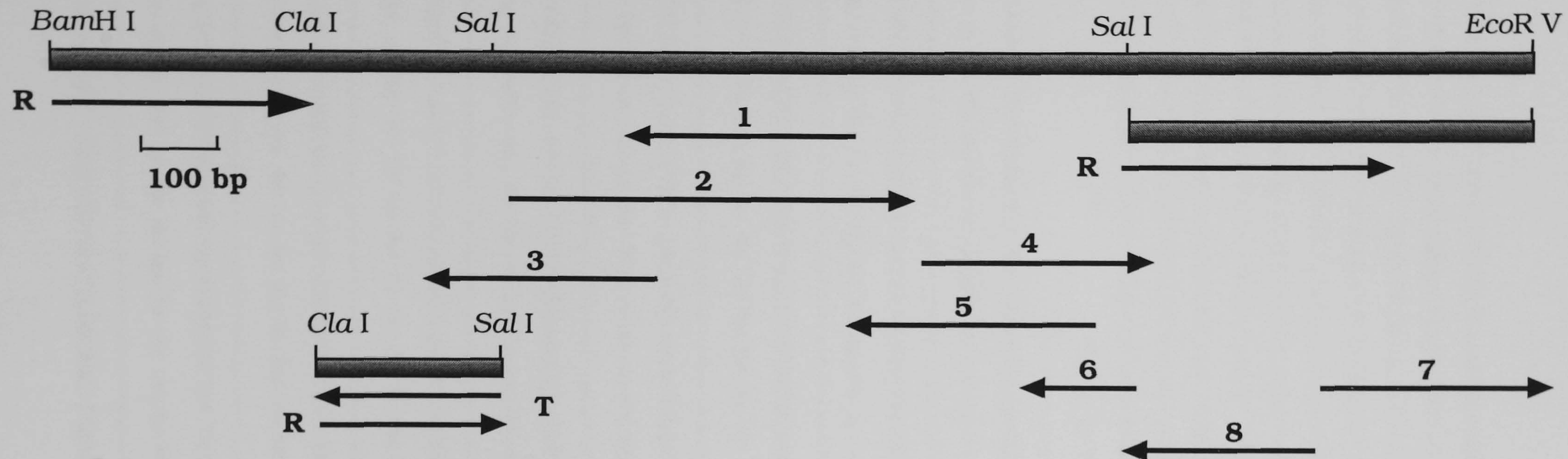


2.2.3 Identification of the gene *mpt83*

DNA sequencing of the 1.85 kbp *Bam*H I - *Eco*R V insert of pSM13 (Fig. 2.12) was performed as described in section 8.7, using the oligonucleotide primers shown in Fig. 2.14, to generate a contiguous sequence of 1896 bp. The nucleotide sequence (1497 bp) is shown in Fig. 2.15, and has been submitted to the EMBL Nucleotide Sequence Database Library (Accession number X94597). The deduced amino acid sequence of MPT83 (positions 750 - 1412) is underlined (Fig. 2.15) as is a Shine-Dalgarno sequence (AAGGAA) nine nucleotides upstream of the initiation codon. Comparison of the gene sequences of MPT83 and MPT70 revealed 68.4% identity between the two.

Analysis of the 1.85 kbp *Bam*H I - *Eco*R V DNA sequence revealed two open reading frames, one at position 106-639 and a second of 660 bp at position 1149-1811. The first open reading frame (ORF) coded for a protein of 177 amino acids which showed only 16% homology with the deduced amino acid sequence for MPT70 (Matsumoto *et al.*, 1995). The deduced amino acid sequence of the second ORF (MPT83) codes for a protein of 220 amino acids (Fig. 2.15), with a predicted molecular weight of 22.1 kDa. MPT83 has 61% identity with the deduced amino acid sequence of MPT70 (Fig. 2.16) and contains the amino acid sequence QDPV, the epitope recognised by the monoclonal antibody SB10 (Radford *et al.*, 1990).

The N-terminal protein sequence of MPT83 contains a typical prokaryotic secretion signal peptide (Von Heijne, 1988) and a lipoprotein signal peptide cleavage motif (FLAGCS) between residues 22-26 (Fig. 2.16) (Young and Garbe, 1991; Sutcliffe and Russell, 1995). The greatest homology between MPT83 and MPT70 is at their carboxy terminus with most dissimilarity at the amino terminus. These latter difference are due in part to the presence of two stretches of residues present in MPT83 but not in MPT70 (amino acid positions 20-40 and 46-55).



Primer	Sequence
1	5'-TGGACAACAGTGATTCGGAG-3'
2	5'-GATGCTGTGCGTTGATGTC-3'
3	5'-TGCCTACCATGCGTCCTGTG-3'
4	5'-ATGATCAACGTTCAGGCCA-3'
5	5'-GTTTCGGGCTGGTGTCTTG-3'
6	5'-TTGTTGGAAGCCGCGGTAG-3'
7	5'-CAGCATCCTGACCTACCAC-3'
8	5'-TGTCGAATGCGGCGTTGGT-3'
R = Reverse	5'-GGAAACAGCTATGACCATG-3'
T = M13 -20	5'-GTAAAACGACGGCCAGT-3'

Fig. 2.14 Sequencing strategy for the *Bam*H I - *Eco*R V insert of pSM13 (Numbered arrows indicate the sequence obtained from the corresponding numbered primers).

Fig. 2.15 Nucleotide sequence of 1497 bp containing *mpt83*

The Shine Dalgarno RBS and deduced amino sequence of MPT83 are underlined.

```
ATCGATGACGAGCTGTACCGCGAGGTGAAAGCAAAGGCCGCTCGTTCCGGGCGTACCGTGGCCGCGGTTCTTGAAGATGC
GGTGCGGCGTGGTCTCAACCCGCCTAAGCCGCAGGCCGCCGGCCGTTATCGAGTCCAGCCGTCGGGTAAGGGCGGCCTGC
GGCCCGGTGTCGATCTATCGTCCAACGCCGCACTTGCCGAAGCGATGAACGACGGCGTGTCGGTCGATGCTGTGCGTTGA
TGTCGACGTGCTCGTTTACGCGCATCGGGCAGACCTACGGGAGCACGCGGACATCGGGGTTTGCTTGAGCGGCTGGCCAA
CGATGACGAGCCGCTGGGTCTACCAGATAGCGTGCTCGCCGGCTTCATCCGGGTGGTTACCAACCGCCGCGTCTTCACCG
AGCCGACGAGCCCACAGGACGCATGGCAGGCAGTCGACGCCCTACTCGCGGCACCCGCAGCCATGCGACTTCGGCCTGGC
GAGCGCCACTGGATGGCCTTTCGGCAGTTAGCGTCCGATGTTGATGCGAACGGCAACGACATTGGGGACGCGCACCTGGC
CGCCTACGCGCTAGAGAACAACGCAACCTGGTTGAGCGCCGACCGCGGCTTTGCCCGTTTCCGTCGACTGCGCTGGCGTC
ATCCGTTGGACGGTCAGACCCATCTATAACCGGCCCCACTCCGAATCACTGGTGTCCACCCAGGAGGACGGCGTTCAACG
CCGCCGCAGAAGCAAAGGAATCGAAGCGATGATCAACGTTCAGGCCAAACCGGCCGCAGCAGCGAGCCTCGCAGCCATCG
                 Met Ile Asn Val Gln Ala Lys Pro Ala Ala Ala Ala Ser Leu Ala Ala Ile
CGATTGCGTTCTTAGCGGGTTGTTCGAGCACCAAACCCGTGTCGCAAGACACCAGCCCGAAACCGGCGACCAGCCCGGCG
Ala Ile Ala Phe Leu Ala Gly Cys Ser Ser Thr Lys Pro Val Ser Gln Asp Thr Ser Pro Lys Pro Ala Thr Ser Pro Ala
GCGCCCGTTACCACGGCGGCAATGGCTGACCCCGCAGCGGACCTGATTGGTCGTGGGTGCGCGCAATACGCGGCGCAAAA
 Ala Pro Val Thr Thr Ala Ala Met Ala Asp Pro Ala Ala Asp Leu Ile Gly Arg Gly Cys Ala Gln Tyr Ala Ala Gln Asn
TCCCACCGGTCCCGGATCGGTGGCCGGAATGGCGCAAGACCCGGTCGCTACCGCGGCTTCCAACAACCCGATGCTCAGTA
 Pro Thr Gly Pro Gly Ser Val Ala Gly Met Ala Gln Asp Pro Val Ala Thr Ala Ala Ser Asn Asn Pro Met Leu Ser
CCCTGACCTCGGCTCTGTCGGGCAAGCTGAACCCGGATGTGAATCTGGTCGACACCCTCAACGGCGGCGAGTACACCGTT
Thr Leu Thr Ser Ala Leu Ser Gly Lys Leu Asn Pro Asp Val Asn Leu Val Asp Thr Leu Asn Gly Gly Glu Tyr Thr Val
TTCGCCCCCACCAACGCCGCATTCGACAAGCTGCCGGCGGCCACTATCGATCAACTCAAGACTGACGCCAAGCTGCTCAG
Phe Ala Pro Thr Asn Ala Ala Phe Asp Lys Leu Pro Ala Ala Thr Ile Asp Gln Leu Lys Thr Asp Ala Lys Leu Leu Ser
CAGCATCCTGACCTACCACGTGATAGCCGGCCAGGCGAGTCCGAGCAGGATCGACGGCACCCATCAGACCCTGCAAGGTG
Ser Ile Leu Thr Tyr His Val Ile Ala Gly Gln Ala Ser Pro Ser Arg Ile Asp Gly Thr His Gln Thr Leu Gln Gly
CCGACCTGACGGTGATAGGCGCCCGCGACGACCTCATGGTCAACAACGCCGGTTTGGTATGTGGCGGAGTTCACACCGCC
Ala Asp Leu Thr Val Ile Gly Ala Arg Asp Asp Leu Met Val Asn Asn Ala Gly Leu Val Cys Gly Gly Val His Thr Ala
AACGCGACGGTGTACATGATCGATACGGTGCTGATGCCCCCGGCACAGTAACGTTCGGCGCGGTCAAGGCGAGGCAGCCC
 Asn Ala Thr Val Tyr Met Ile Asp Thr Val Leu Met Pro Pro Ala Gln •
GTGTAGGCGGTTTGCCTCGCTCATCCGGCGGCTTCGTGCCGATAGATCACGTGATATC
```

Fig. 2.16 Amino acid alignment between MPT83 and MPT70

```
MPT83   M I - - - N V Q A K P A A A A S L A A I  17
MPT70   M K V K N T I A A T S F A A A G L A A L  20

MPT83   A I A F L A G C S S T K P V S Q D T S P  37
MPT70   A V A - - - - - - - - - - - - - - - - -  24

MPT83   K P A T S P A A P V T T A A M A D P A A  57
MPT70   - - - V S P P A A - - - - - - - - - A G  31

MPT83   D L I G R G C A Q Y A A Q N P T G P G S  77
MPT70   D L V G P G C A E Y A A A N P T G P A S  51

MPT83   V A G M A Q D P V A T A A S N N P M L S  97
MPT70   V Q G M S Q D P V A V A A S N N P E L T  71

MPT83   T L T S A L S G K L N P D V N L V D T L  117
MPT70   T L T A A L S G Q L N P Q V N L V D T L  91

MPT83   N G G E Y T V F A P T N A A F D K L P A  137
MPT70   N S G Q Y T V F A P T N A A F S K L P A  111

MPT83   A T I D Q L K T D A K L L S S I L T Y H  157
MPT70   S T I D E L K T N S S L L T S I L T Y H  131

MPT83   V I A G Q A S P S R I D G T H Q T L Q G  177
MPT70   V V A G Q T S P A N V V G T R Q T L Q G  151

MPT83   A D L T V I G A R D D L M V N N A G L V  197
MPT70   A S V T V T G Q G N S L K V G N A D V V  171

MPT83   C G G V H T A N A T V Y M I D T V L M P  217
MPT70   C G G V S T A N A T V Y M I D S V L M P  191

MPT83   P A Q .                                221
MPT70   P A .                                  194
```

2.2.4 Genomic localisation of *mpt83*

The DNA insert of cosmid pA3 was approximately 9.5 kbp (Fig. 2.8). To ensure that no DNA rearrangement had occurred within this cosmid, and to confirm that *mpt83* is situated on a 3.5 kbp *Eco*R I - *Pst* I fragment (Fig. 2.17) of the chromosome in all members of the MTb complex, genomic DNA was digested with *Eco*R I and *Pst* I (Fig. 2.18A) and hybridised with *mpt83* (Fig. 2.18B). The *mpt83* gene probe was generated by PCR using the oligonucleotide primers 7 and 8 (appendix 1) as described in section 8.3.

The gene probe hybridised to a 3.5 kbp *Eco*R I - *Pst* I fragment of chromosomal DNA from *M. tuberculosis* H37Rv, *M. bovis* AN5, *M. bovis* BCG Tokyo and to a 3.5 kbp *Eco*R I - *Pst* I DNA fragment from pA3 (Fig. 2.18B, lanes 2, 3, 4 and 5 respectively). The probe did not hybridise with chromosomal DNA from the non-pathogenic mycobacteria *M. smegmatis* (Fig. 2.18B, lane 1). The *mpt83* gene probe also failed to hybridise to *mpt70*.

The restriction map for the genomic region of H37Rv containing *mpt83* and *mpt70* is shown in Fig. 2.17, and is identical to that found in *M. bovis* AN5 by Radford *et al.*, (1990). Subsequently, the Sanger centre sequencing project of *M. tuberculosis* H37Rv has generated the same map of this region (accession no. Z92699). Thus no chromosomal rearrangement had occurred in cosmid pA3. Futhermore the sequence of *mpb83* from *M. bovis* AN5 has been determined in our laboratory and is identical to that of *mpt83* (data not shown).

Fig. 2.17 Restriction map of a region of the *M. tuberculosis* H37Rv chromosome containing *mpt70* and *mpt83*.

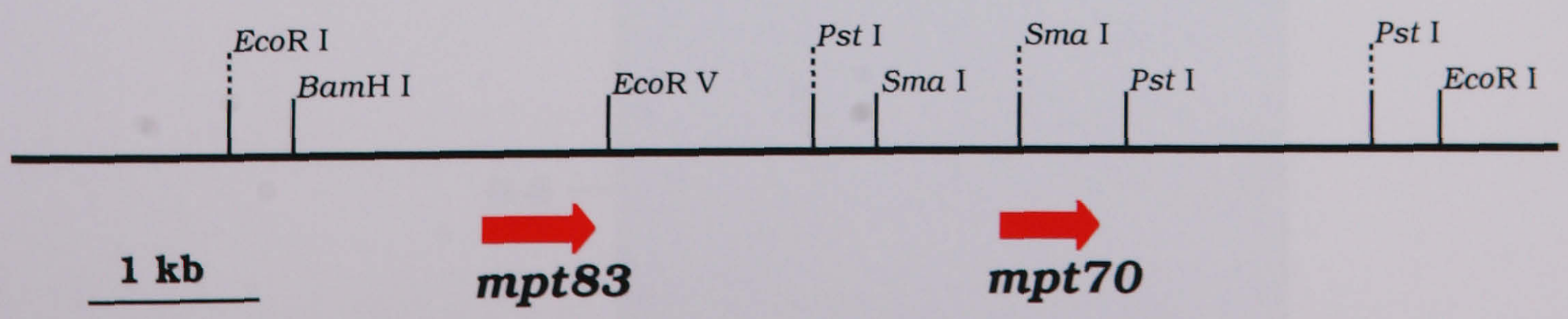
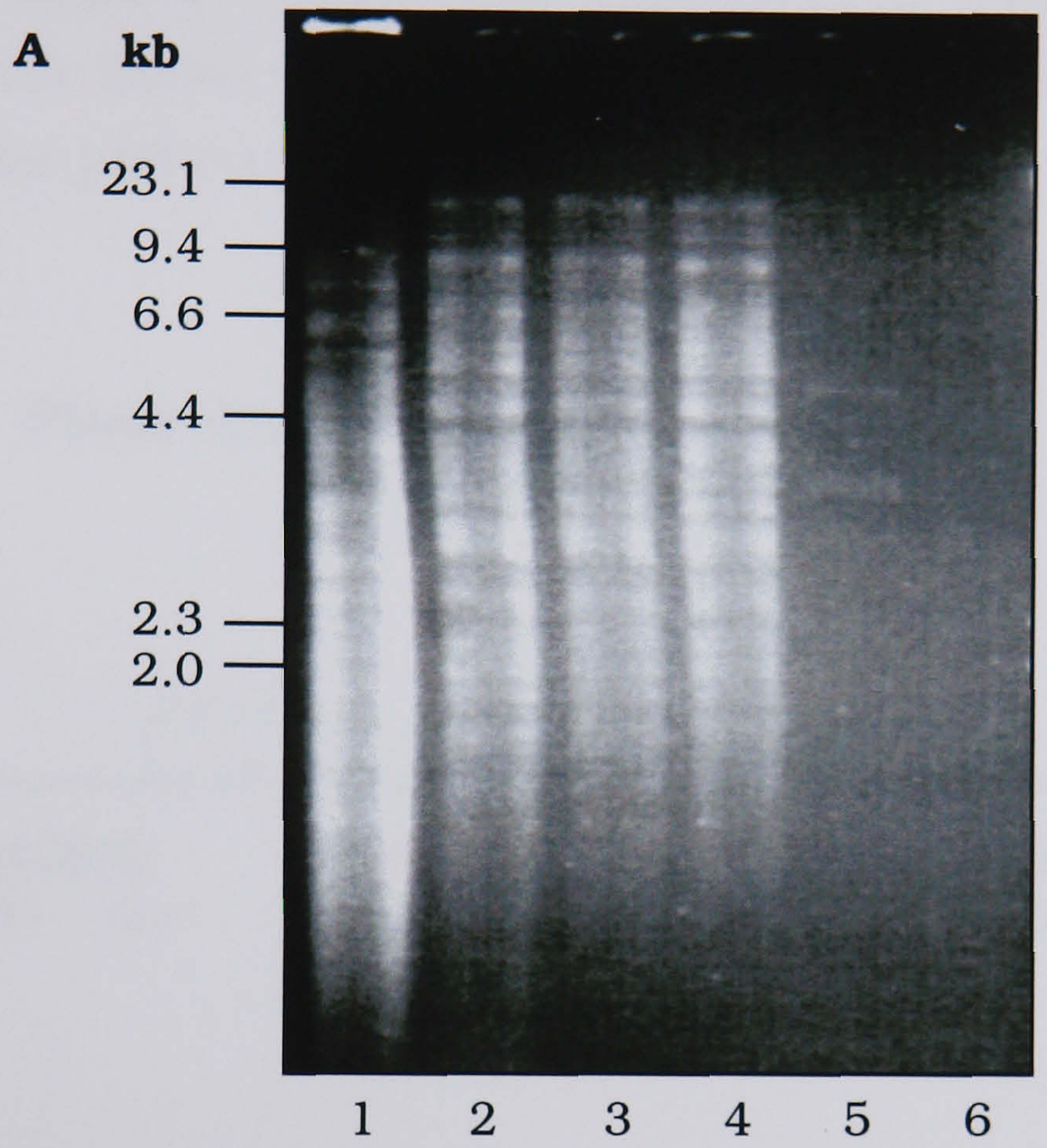
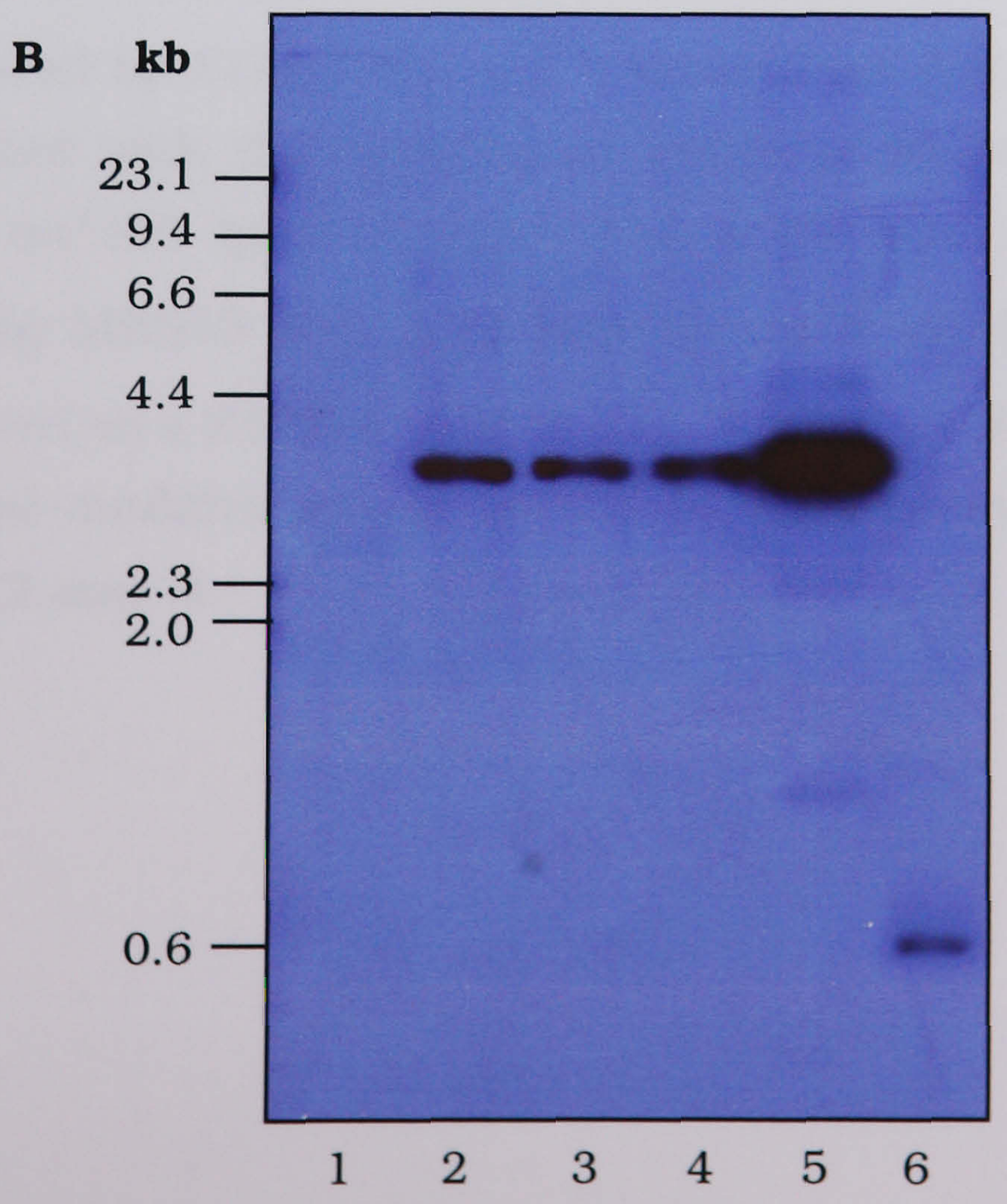


Fig. 2.18 Southern blot analysis of *mpt83*.

(A) Ethidium bromide stained agarose gel of DNA digested with *Eco*R I and *Pst* I. Lanes: 1, *M. smegmatis* mc^{2}155 (2μg); 2, *M. bovis* BCG Tokyo (2μg); 3, *M. bovis* AN5 (2μg); 4, *M. tuberculosis* H37Rv (2μg); 5, pA3 (0.5μg). (B) Autoradiograph of gel in panel A Southern blotted and hybridised with *mpt83*. Lane 6, *mpt83* PCR product (50 ng, positive control).

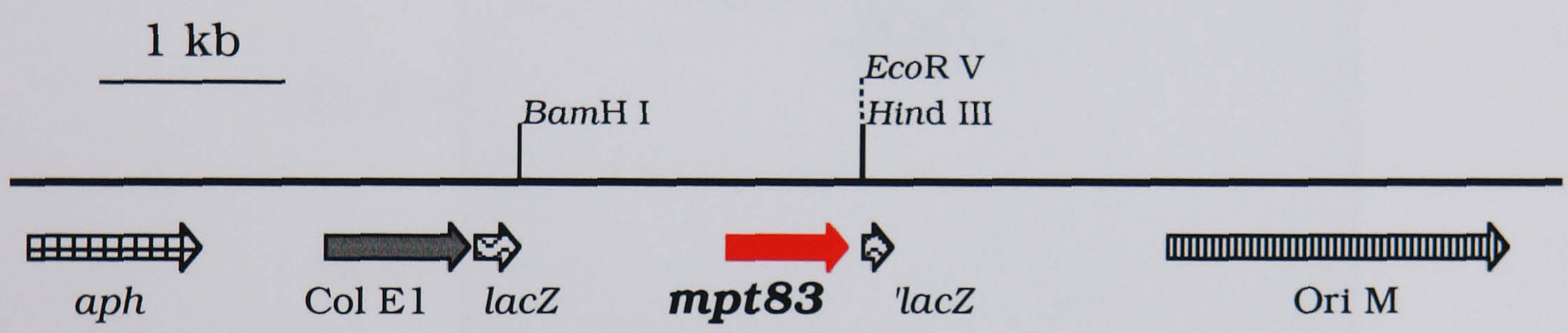




2.2.5 Expression of the *mpt83* gene

In section 2.2.2a *M. smegmatis* mc^{2}155 (pA3) was shown to express a 25 kDa antigen recognised by the monoclonal antibodies SB10 and MBS43 (lane 2, Figs. 2.5 A and B). To confirm that this antigen was encoded for by the gene *mpt83* identified in section 2.2.3, the 1.85 kbp *Bam*H I - *Eco*R V insert was excised from pSM13 (Fig. 2.12) by digestion with *Bam*H I and *Hin*d III and cloned into the mycobacterial shuttle vector pMD31 (Fig. 8.1C) to give the plasmid construct pSM43 (Fig. 2.19).

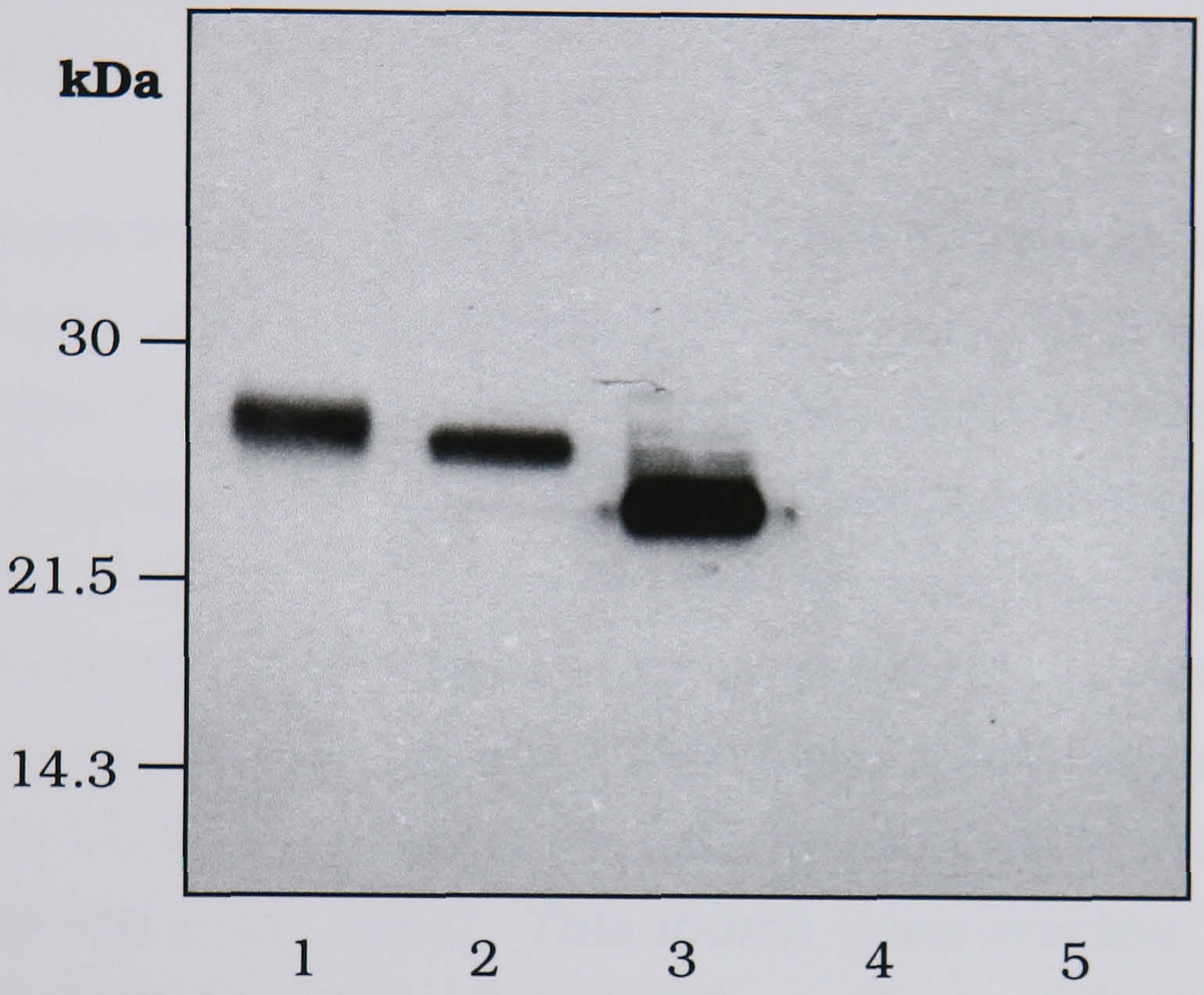
Fig. 2.19 Plasmid map of pSM43.



This construct and control vector were transformed into *M. smegmatis* mc^{2}155 and *E. coli* DH5α. Sonicated extracts of these recombinants were separated by SDS-PAGE (15% separating gel), Western blotted, and incubated with the monoclonal antibody MBS43 (Fig. 2.20). *M. smegmatis* mc^{2}155 (pSM43) expressed a 25 kDa antigen that was recognised by MBS43 (Fig. 2.20, lane 2). In *E. coli* DH5α this protein was expressed as a 23 kDa antigen (Fig. 2.20, lane 3), suggesting post-translational modification of MPT83 by *M. smegmatis* (see also Fig. 2.12, lanes 2 and 7).

Fig. 2.20 Western blot incubated with the monoclonal antibody MBS43.

Sonicated extracts (5 μg) of lanes: 1, *M. bovis* AN5; 2, *M. smegmatis* mc^2155 (pSM43); 3, *E. coli* DH5α (pSM43); 4 *M. smegmatis* mc^2155 (pMD31); 5, *E. coli* DH5α (pMD31).



2.3 Discussion

The presence of several heterogeneous forms of MPB70 has previously been attributed to either post-synthetic changes leading to charge differences in the side chains of amino acid residues (Harboe *et al.*, 1986), or to post-translational modifications such as glycosylation (Fifis *et al.*, 1989; Fifis *et al.*, 1991). In this chapter a gene, *mpt83*, has been identified which is homologous to but distinct from *mpt70*. This gene encodes MPT83, the *M. tuberculosis* homologue of the previously described 25 kDa form of MPB83 (Wiker *et al.*, 1996).

The *mpt83* gene has 65% identity with the *mpt70* gene, is located 2.4 kbp upstream *of mpt70* (Fig. 2.17), and shows significant homology to *mpt70* and *mpb70* upon a BLAST search (Altschul *et al.*, 1990). The genes are co-located on a 7.3 kbp *Eco*R I DNA fragment and are transcribed in the same direction, consistent with the observation that the expression of both genes is linked in an operon (Hewinson *et al.*, 1996). The similarity between the two genes, *mpt70* and *mpt83*, appears to be characteristic of gene duplication in mycobacteria (Cole *et al.*, 1998).

Gene duplication has been observed for other antigenic families of *M. tuberculosis*, such as the antigen 85 complex. This family consists of three cross-reacting members termed 85A, 85B and 85C are all coded for by separate genes that are non clustered (Content *et al.*, 1991). However, a fourth member of the 85 complex, MPT51, has been identified (Nagai *et al.*, 1991). The gene for this protein is co-localised with the gene for 85A on a 5.7 kbp *Eco*R I DNA fragment, in this case the genes for both antigens are also transcribed in the same direction (Rinke de Wit *et al.*, 1993). This fourth cross-reactive member, MPT51, is recognised by the monoclonal antibody HYB76-8 which also recognises the three other members of the antigen 85 complex (Harboe *et al.*, 1996a).

Comparison of the deduced amino acid sequence of MPT83 with the sequence generated from peptides of the 23 kDa form of MPB83 (Harboe *et al.*, 1995) revealed only 4 differences over 68 residues. This raises the possibility that, like the antigen 85 complex, there may be another gene distal to the *mpt83* - *mpt70* gene cluster that codes for the 23 kDa form which would account for the reported amino acid differences. In support of this suggestion, expression of *mpt83* in the mycobacterial host *M. smegmatis* mc^2155 did not produce the 23 kDa form of MPB83. However, no such homologue has been identified within the complete genome sequence of *M. tuberculosis* H37Rv (Cole *et al.*, 1998) and Southern blotting of genomic DNA from the MTb complex with *mpt70* and *mpt83* gene probes also failed to reveal any further homologues.

The 25 kDa form of MPB83 and MPT83 have been shown to be associated with the bacterial sonicate (Hewinson *et al.*, 1996; Vosloo *et al.*, 1997; and Harboe *et al.*, 1998). This can be explained by the presence of a typical prokaryote lipoprotein signal peptide cleavage motif (amino acid residues 22-26) which suggests that these antigens are lipoproteins attached to the cell wall by hydrophobic interactions via a lipid tail attached to the amino terminal cysteine residue through a thioester linkage (Wu and Tokunaga, 1986; Sutcliffe and Russell, 1995). Furthermore, by analysis of monoclonal antibody binding (Vosloo *et al.*, 1997; Harboe *et al.*, 1998), it has been demonstrated that MPB83 of BCG Tokyo is situated at the cell surface. The 23 kDa form of MPB83 purified from the culture filtrate of BCG Tokyo (Harboe *et al.*, 1995) shows no lipophilic nature (Harboe *et al.*, 1998) and its presence may be due to cleavage of the 25 kDa lipoprotein after the cysteine residue, as has been observed for the 38 kDa lipoprotein of *M. tuberculosis*, (Andersen *et al.*, 1990). In contrast, the lack of expression the 23 kDa form of MPT83 by *M. smegmatis* mc^2155 may be due to the absence of a specific endopeptidase which is present in the MTb complex but absent in *M. smegmatis*, as it has been shown that mycobacteria such as *M. vaccae* and *M. smegmatis* vary in their pattern of cleavage of the 19 kDa lipoprotein of *M. tuberculosis* (Herrmann *et al.*, 1996), suggesting heterogeneity of proteases within mycobacteria.

The lipoproteins of Gram positive bacteria have been proposed to be functional equivalents of periplasmic proteins of Gram negative bacteria, the latter being responsible for such functions as substrate transport. The Gram positive counterparts are prevented from extracellular release by their lipid anchor rather than by a selectively impermeable outer membrane (Nielsen and Lampen, 1982). The 38 kDa lipoprotein of *M. tuberculosis* has been shown to be involved in phosphate transport (Andersen *et al.*, 1990; Chang *et al.*, 1994), and like the corresponding transport system of *E. coli*, is part of an operon (Braibant *et al.*, 1996). However, no such function has yet been identified for MPT83 or MPT70 suggesting that these proteins may have some function specific to the pathogenic nature of the MTb complex.

It has been reported previously that MPB70, which shares 67% identity at the amino acid level with MPT83, contains regions with up to 85% identity to the repeat domains of the osteoblast specific factor (OSF-2) from mouse (Ulstrup *et al.*, 1995). These regions of identity in MPT70 are conserved within MPT83 (Fig. 2.21) and the similar spacing between these two regions in all three molecules (71 ± 5 amino acids), suggest that these regions may be of importance in the functional properties of these molecules.

Fig. 2.21 Amino acid alignment between the consensus repeat regions present in OSF-2 and MPT70 and MPT83*.

mOSF-2	G N Y T L F A P T N E A F .....73aa.....	T N G V I H V I D E V L N P
MPT70	94- G Q Y T V F A P T N A A F-106	178- A N A T V Y M I D S V L M P-191
MPT83	120- G E Y T V F A P T N A A F-132	204-A N A T V Y M I D T V L M P-217

(* Amino acids not conserved are underlined)

These repeat regions in OSF-2 also share identity with the protein fascicilin I (Takeshita *et al.*, 1993) the latter of which has been proposed to function as a divalent bridge between cells due to its dimeric ligand binding site properties (Zinn *et al.*, 1988). It is therefore possible that MPT70 and/or MPT83 may function in a similar manner to attach the bacteria to the host or the bacteria to each other.

The 2 kDa size difference between MPT83 expressed in *E. coli* and in both *M. smegmatis* and *M. bovis* (Fig. 2.20) implies that the protein undergoes post-translational modification by mycobacteria. On the basis of previous reports that MPB70 exists as glycosylated forms (Fifis *et al.*, 1991) it is likely that this mycobacterial post-translational modification is due to glycosylation of the protein. Moreover, it has been demonstrated that MPB83 is expressed as 23 kDa and 25 kDa antigens by *M. bovis* BCG and that both forms are associated with carbohydrate (Wiker *et al.*, 1996). This is consistent with previous analysis of these two antigens, where the difference in carbohydrate composition was proposed to be correlated with the difference in molecular mass (Fifis *et al.*, 1991).

The apparent lack of production of a 23 kDa form of MPT83 by recombinant *M. smegmatis* could be due to a lack of certain glycosyltransferases, which may be specific to the MTb complex. The carbohydrate compositions of the 23 and 25 kDa forms of MPB83 purified from *M. bovis* AN5, as determined by gas chromatography - mass spectrometry (GC-MS), showed distinct differences (Fifis *et al.*, 1991) and it is possible that the glycosylation pathway associated with the 23 kDa form is unique to the MTb complex. In support of this hypothesis is the observation that glycoproteins expressed in heterologous hosts such as *Saccharomyces cerevisiae* (Penttilä *et al.*, 1988) or *Streptomyces lividans* (Ong *et al.*, 1994) have attached oligosaccharides that differ in sequence and composition to those that occur naturally.

The structure of the *M. tuberculosis* glycoprotein antigen MPT32 has been fully characterised by Dobos *et al.*, (1996). Initially they reported the characterisation of a glycopeptide from a 45 kDa protein of *M. tuberculosis* which was shown to possess a disaccharide composed of two hexose residues O-glycosidically linked through a threonine residue. The complete sequence of this glycopeptide was DPEPAPPVPTTA (Dobos *et al.*, 1995). Amino acid sequence comparison between MPT83 and MPT70 reveals the presence of a similar proline rich peptide sequence in MPT83 which is not present in MPT70 (Fig. 2.16). A similar proline threonine rich region has been identified as a site for O-glycosylation in the linker region of the β-1,4-glucanase of *Cellulomonas fimi* (Ong *et al.*, 1994), and within the 19 kDa antigen of *M. tuberculosis* (Herrmann *et al.*, 1996). It is therefore possible that this region, i.e. SPKPATSPAAPVTTAA, comprises a signal motif for glycosylation of MPT83.

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It has subsequently been shown that *M. bovis* BCG Tokyo contains a homologous gene, *mpb83*, which codes for a protein identical to the deduced amino acid sequence of MPT83 (Matsuo *et al.*, 1996). Futhermore the sequence of *mpb83* from *M. bovis* AN5 has been determined in our laboratory and is identical to that of *mpt83* (data not shown). The current *M. bovis* sequencing project at the Sanger centre has determined the nucleotide sequence of *mpb83* from *M. bovis* (strain AF2122/97) which is also identical to *mpt83*.

Thus, MPB83 and MPT83 are identical and although the studies in this thesis use the gene *mpt83* which was cloned from *M. tuberculosis* H37Rv, all results obtained during this study are also true for the *M. bovis* homologue MPB83. Part 2 of this thesis describes further investigations into the nature of the posttranslational modifications of MPT83.

Part 2

Posttranslational modification of MPT83

Chapter 3

Posttranslational covalent modifications of proteins

3.1 Introduction

Release of a nascently synthesised polypeptide chain from a ribosome is frequently not the final chemical step in the generation of a protein. Often a variety of modifications are performed on the polypeptide chain either after or during translation of the messenger RNA. Such modifications can be divided into two main groups, namely the proteolytic processing of the polypeptide chain or the modification of amino acid side-chains of the polypeptide.

3.2 Proteolytic processing

The proteolytic cleavage of amino terminal signal peptides by signal peptidases has been described in chapter 1 (p15-16). In addition to the removal of signal peptides proteolytic processing is involved in generating biologically active forms of proteins. For example, the proteolytic enzyme, trypsin, is secreted as an inactive zymogen after cleavage of the signal peptide of the prezymogen. The inactive zymogen of trypsin, called trypsinogen, is activated by proteolytic cleavage by the specific enzyme enterokinase (Sharma and Hopkins, 1981). The cleaving proteases are highly specific so that only the desired proteins are activated at the correct time and in the right location. Similar proteolytic cleavage is used to generate active forms of peptide hormones (Docherty and Steiner, 1982) and in the generation of functional proteins for virus assembly from the long polyprotein made by RNA viruses (Hellen, 1989).

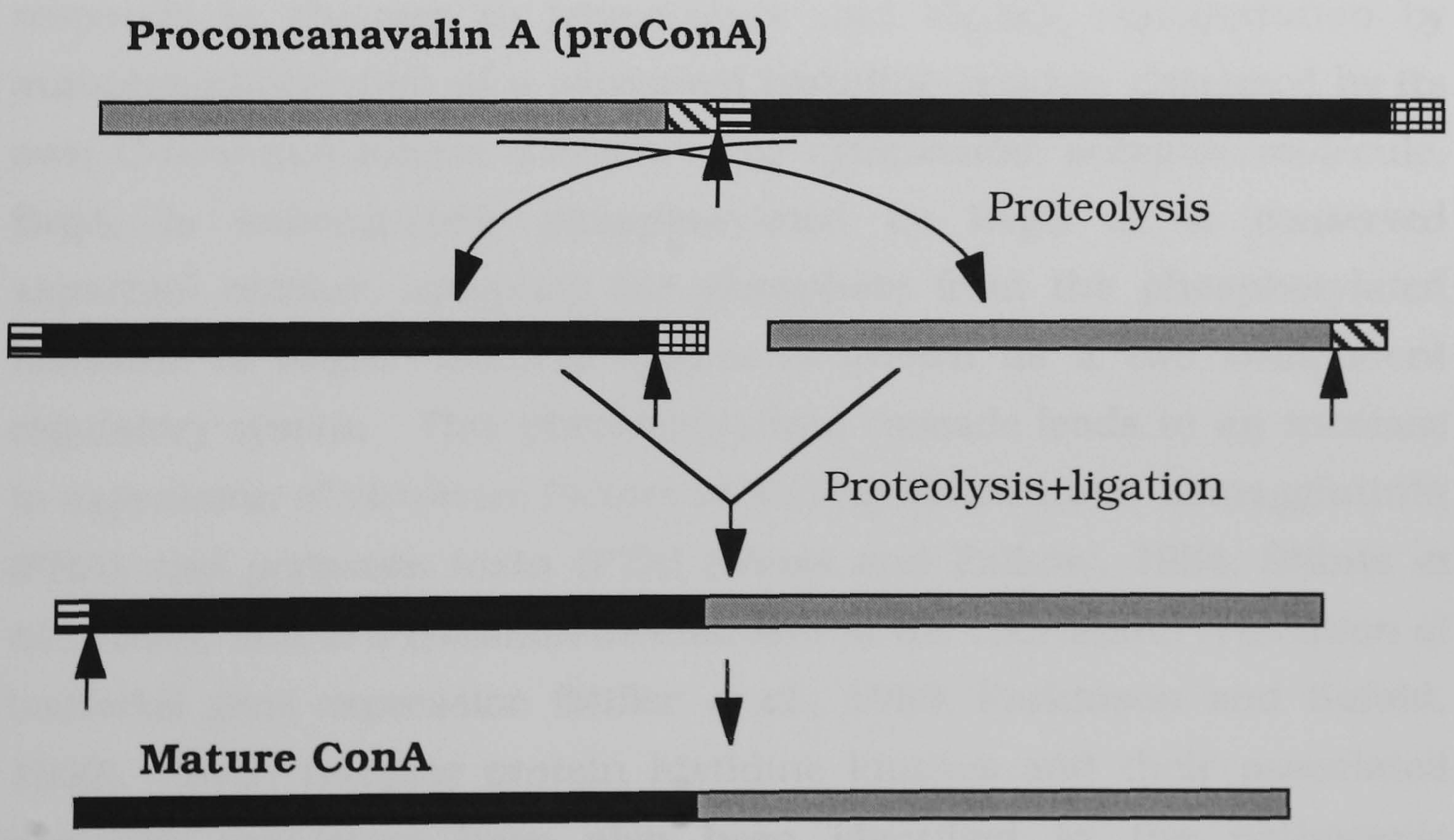
Complex proteolytic processing has been described for a few proteins, including the RecA proteins of *Mycobacterium tuberculosis* and *M. leprae*, which possess inteins or protein introns (Perler *et al.*, 1994; Colston, 1994). The protein intron is a central portion of the polypeptide chain which is removed by proteolysis and the remaining amino and carboxy portions of the polypeptide are spliced together. Whether such an event is autocatalytic is unclear as the intein of RecA from *M. tuberculosis* is cleaved by *E. coli* (Davis *et al.*, 1992)

whereas the intein of RecA from *M. leprae* is not spliced by *E. coli* (Davis *et al.*, 1994).

The proteolytic processing of the plant lectin proconcanavalin A after secretion from preproconcanavalin A is depicted in Fig. 3.1. Cleavage of proconcanavalin A after the asparagine residue at position 131 is followed by cleavage of short peptides from the carboxyl ends of both fragments and an unusual splicing of the α-amino group of the N-terminal fragment and the α-carboxyl of the C-terminal fragment (Carrington *et al.*, 1985; Bowles and Pappin, 1988). This reversal of the two fragments produces an amino acid sequence that is a permuted form of the original sequence.

Fig. 3.1 Proteolytic processing and rearrangement of the primary structure of concanavalin A.

(Proteolytic cleavage after asparagine residues is indicated by the short arrows. The rearrangements of the chain depicted after the first cleavage do not occur physically because the terminal residues of proConA are already in proximity in the 3-D structure of the folded protein (Creighton, 1993)).



3.3 Amino acid side-chain modification

The side-chains of many amino acids are covalently modified by the addition of various chemical groups. The rest of this chapter briefly describes some of the enzymatic modifications made to amino acid-side chains, focusing mainly on the glycosylation of side-chains.

3.3.1 Protein phosphorylation

Phosphorylation of proteins is usually reversible and has important functional implications. The sites of phosphorylation are usually: the hydroxyl groups of serine (Ser), threonine (Thr) or tyrosine (Tyr) residues; the side-chain amino groups of histidine (His) or lysine (Lys); or the side-chain carboxyl group of aspartate (Asp). The addition of phosphate to these residues is catalyzed by protein kinases which have different specificities for different proteins. The major function of protein phosphorylation is in cell signaling mechanisms such as signal transduction.

For example, in *Bordatella pertussis*, the 'sensory' protein, BvgS, responds to changes in temperature and Mg_2SO_4 concentration by autophosphorylation of a conserved histidine residue, catalyzed by its own C-terminal kinase domain. The cytoplasmic acceptor molecule, BvgA, is subsequently phosphorylated by BvgS at a conserved aspartate residue, accepting the phosphate from the phosphorylated histidine of BvgS. Such a system is known as a two component regulatory system. This phosphorylation cascade leads to an increase in expression of virulence factors including filamentous hemagglutinin (FHA) and pertussis toxin (PTX) (Weiss and Falkow, 1984; Stibitz *et al.*, 1988), and is a common mechanism in the coordinate regulation of bacterial gene expression (Miller *et al.*, 1989; Parkinson and Kofoid, 1992). Such receptor protein histidine kinases and their associated response regulators have also been identified in the pathogenic mycobacteria (Via *et al.*, 1996).

In addition to this two component regulatory system, it has also been shown *that M. tuberculosis* possess eukaryotic like serine-threonine protein kinases (STPK's), with suggestions that the latter may play a role in disruption of macrophage activation by interfering with the hosts signal transduction mechanisms (Av-Gay and Davies, 1997).

3.3.2 Lipid attachment

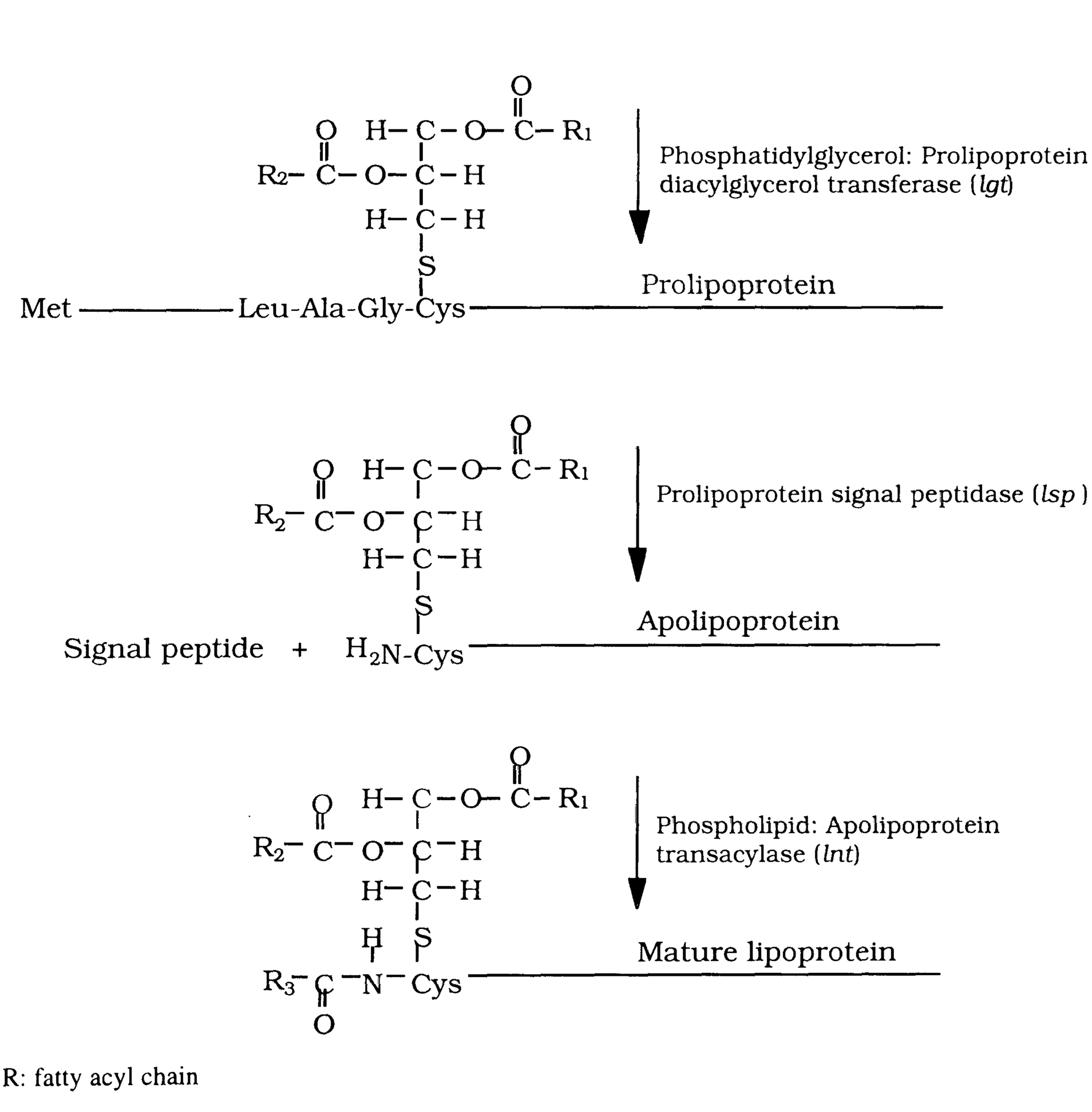
Lipids frequently tether intrinsically soluble proteins to membranes. The polar group of the lipid is covalently attached to the protein while the hydrophobic side chains of the lipid interacts with the lipid membrane in order to retain proteins at this location.

Investigations into the mechanism of bacterial acylation of proteins have used the major lipoprotein of *Escherichia coli* (Braun's lipoprotein (Braun and Rehn, 1969)) as the model for understanding acylation of all bacterial lipoproteins (Tokunaga *et al.*, 1982). Sankaran and Wu (1994) proposed a pathway for the biosynthesis of bacterial lipoproteins, shown in Fig. 3.2, based on *in vitro* studies of Braun's lipoprotein. Cleavage of a bacterial lipoprotein signal peptide cleavage motif (step 2, Fig. 3.2) by prolipoprotein signal peptidase (SPase II) is dependent on prior modification of the side chain of the cysteine residue within the motif (Tokunaga *et al.*, 1984).

In contrast to Chattopadhyay and Wu (1977), who proposed transfer of an unmodified glycerol to the thiol group of the cysteine residue followed by acylation of the glycerol hydroxyl groups by specific O-acyltransferases, Sankaran and Wu (1994) demonstrated that diacylglycerol is transferred from phosphatidylglycerol (PG) to the prolipoprotein in one step (step 1, Fig. 3.2). The latter reaction forming a thioester linkage through the thiol group of the cysteine side-chain. The enzyme catalyzing this transfer is prolipoprotein diacylglycerol transferase, the gene for which (*lgt*) has been characterised (Gan *et al.*, 1993). This enzyme is specific for the source

of diacylglycerol, efficiently transferring diacylglycerol from PG but not from phosphatidylethanolamine (Sankaran and Wu, 1994).

Fig. 3.2 The proposed pathway for lipoprotein maturation.
(Sankaran and Wu, 1994)



Following cleavage of the signal peptide (Step 2, Fig. 3.2) the amino terminal cysteine of the apolipoprotein is further N-acylated by the enzyme apolipoprotein N-acyltransferase which, unlike prolipoprotein diacylglycerol transferase, has no specificity with respect to phospholipids as acyl donors (Gupta *et al.*, 1991).

Based on the presence of a prokaryotic lipoprotein signal peptide cleavage motif (FLAGCS, Sutcliffe and Russell (1995)), 65 putative lipoproteins (*lppA-lprO*) have been identified within the *M. tuberculosis* genome (Cole *et al.*, 1998). However, whether all these proteins are indeed acylated remains to be demonstrated.

3.4 Protein glycosylation

Covalent attachment of carbohydrates to proteins is one of the most prevalent posttranslational modifications, especially of eukaryotic secreted and membrane proteins, though the purpose has no well-defined universal function. The ubiquitous presence of glycoproteins on cell surfaces suggests that the carbohydrates may frequently be involved in interactions with other cells or molecules. Probably two of the most relevant properties of carbohydrates attached to proteins are (a) their variable structure, increased by a degree of flexibility around some glycosidic bonds, which permit specificity in their interactions with other molecules; and (b) their hydrophilic nature, which keep them in aqueous solution.

A detailed compilation of the numerous biological roles of protein glycosylation has been compiled (Varki, 1993), concluding that the underlying roles of the vast functional array of glycoproteins are, "that they mediate 'specific recognition' events or that they provide 'modulation' of biological processes". The rest of this chapter describes the mechanisms of protein glycosylation and highlights the relatively recent discovery of bacterial glycoproteins.

3.4.1 Structure of oligosaccharides

Description of glycoproteins is made difficult by the complexity of the carbohydrate structures attached to the proteins. The eight most common monosaccharides that are found in the glycan chains of eukaryotic glycoproteins are shown in Fig. 3.3 as cyclic Haworth projections. Six membered rings such as glucose (glu*p* Fig. 3.3). are

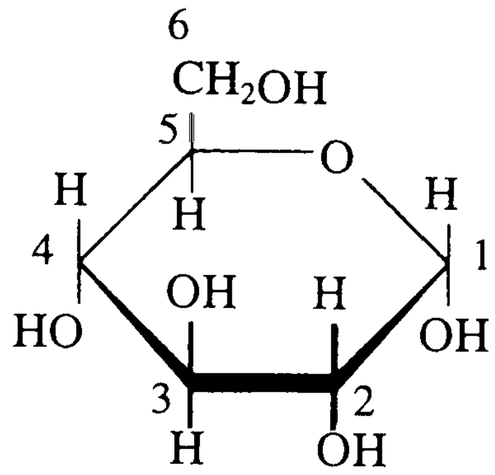
described as pyranoses reflecting their structural similarity to the heterocyclic compound pyran. Similarly, the five membered rings such as xylose (xyl*f* Fig. 3.3), are described as furanoses reflecting their structural similarity to the heterocyclic compound furan.

Monosaccharides containing both 5 carbon atoms (pentoses) and 6 carbon atoms (hexoses) cyclise via internal hemiacetal formation, and both have the ability to form either a furanose or a pyranose ring. Cyclisation creates an asymmetric carbon at position 1, giving rise to a pair of stereoisomers distinguished by the designations α and β. Such isomers, differing in configuration at carbon 1 are called anomers. The α anomer is represented in a Haworth projection as having its hydroxyl group below the plane of the ring as depicted in Fig. 3.3 for glucose. Likewise, the β anomer is depicted in a Haworth projection as having its hydroxyl group above the plane of the ring.

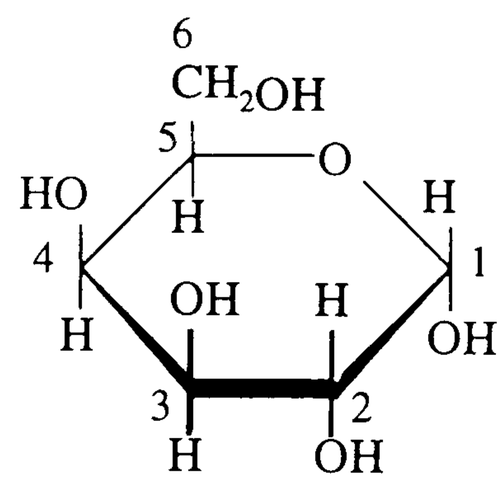
3.4.2 Formation of the glycosidic bond

The glycosidic bond between two monosaccharides is created by a condensation reaction catalyzed by specific glycosyltransferases. The donor molecule contains an activated mono-or oligosaccharide in the form of either a nucleotide sugar, dolichol-phosphate sugar or dolichol-pyrophosphate sugar. The specificity of the glycosyl transferases depends on: (a), the two monosaccharides to be linked; (b), the carbons involved in the linkage; (c) the order of the two monosaccharides; and (d) the anomeric configuration of the hydroxyl group on the carbon 1 of each monosaccharide. The large number of complex oligosaccharide structures that have been described indicates the existence of many different glycosyltransferases.

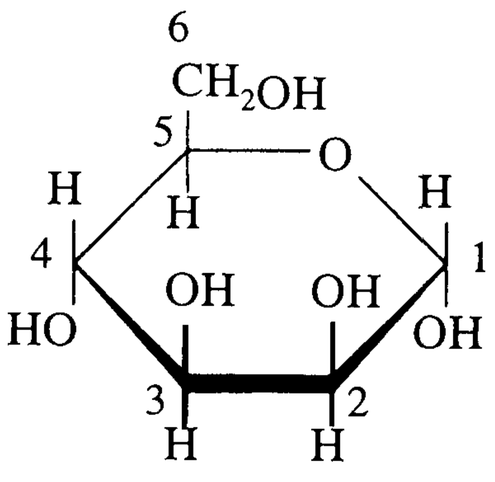
Fig. 3.3 Structures of monosaccharides commonly found in carbohydrate chains of glycoproteins.



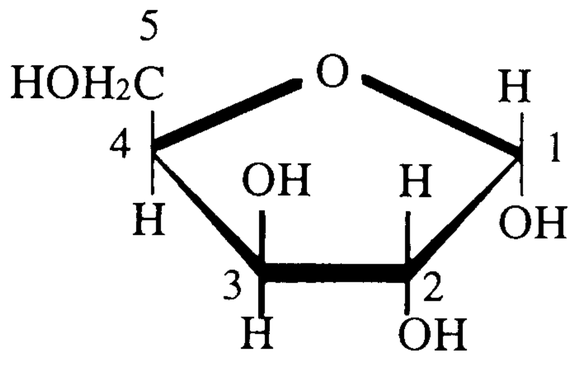
α-glucose (Glc)



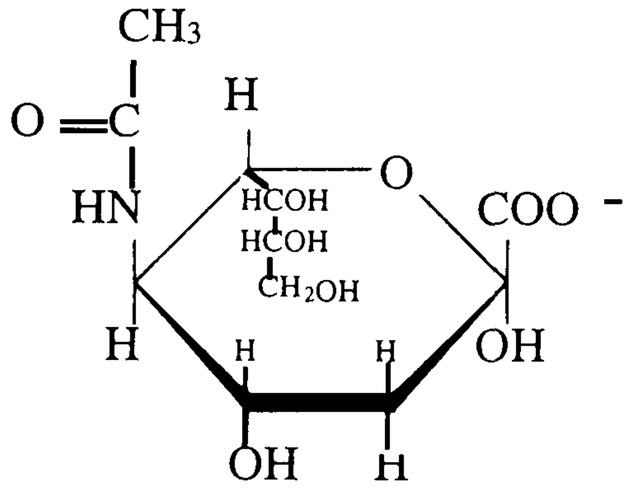
α-galactose (Gal)



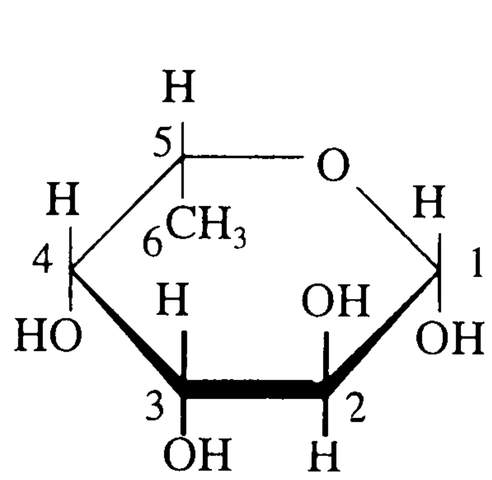
α-mannose (Man)



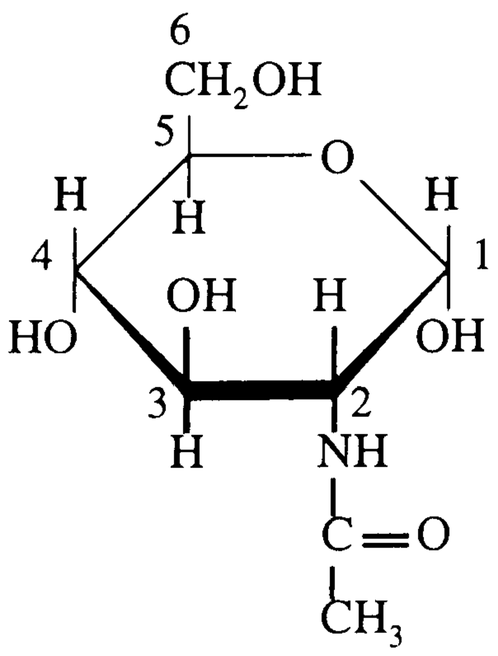
α-xylose (Xyl)



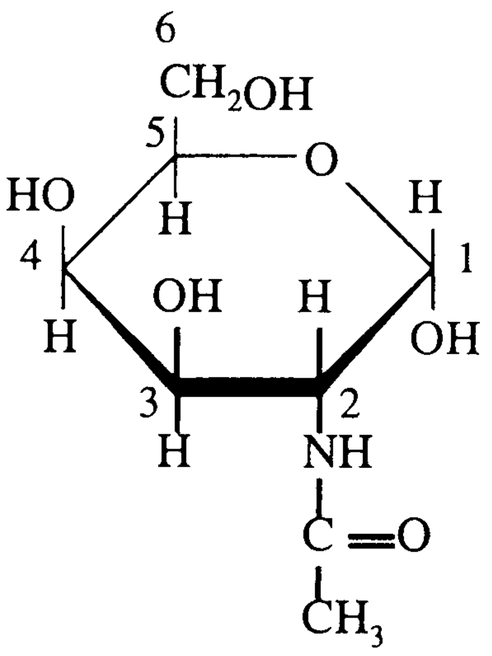
α-N-acetylneuraminic acid
(Sialic acid (Sia))



α-fucose (Fuc)



α-N-acetylglucosamine (GlcNAc)



α-N-acetylgalactosamine (GalNAc)

However, there are exceptions to the "one linkage-one glycosyltransferase" rule. The Lewis blood group-dependent α-1,4/3-fucosyltransferase can transfer fucose to either carbon 3 or 4 of a galactose residue (Kukowska-Lataloo *et al.*, 1990). Conversely, it is also possible for a specific linkage to be made by more than one transferase. For example, there are at least five human α-1,3-fucosyltransferases that can attach fucose to carbon 3 of (GlcNAc) in the Gal (β1→4) GlcNAc moiety (Macher *et al.*, 1991).

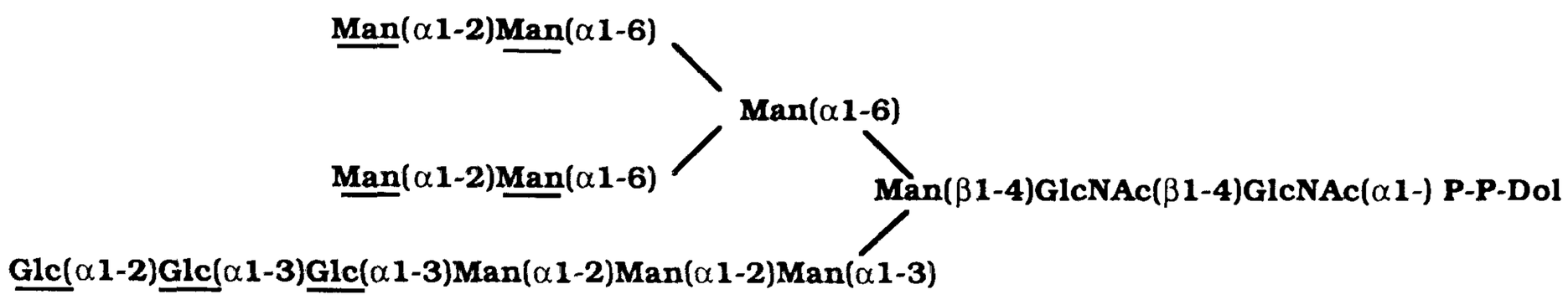
3.5 N-linked glycosylation

The cotranslational modification of polypeptides by N-glycosylation is catalysed by oligosaccharyltransferase (OST), which transfers a preassembled glycan structure from dolicholpyrophosphate (P-P-Dol), a polyisoprenol (H-(-CH_2-$C(CH_3)$=CH-CH_2-)$_n$-OH), to certain asparagine residues which are part of a consensus sequence known as a sequon.

The preassembly of the glycan structure starts at the cytoplasmic face of the endoplasmic reticulum (ER) with the addition of GlcNAc by the tunicamycin sensitive, dolichol phosphate dependent, N-acetylglucosamine-1-phosphate transferase (GPT) (Lehrman, 1991). This enzyme catalyzes the transfer of GlcNAc-1-P from UDP-GlcNAc to dolichol phosphate (Dol-P) to generate GlcNAc-P-P-Dol. Further monosaccharides are added to GlcNAc-P-P-Dol from nucleotide or phosphodolichol activated sugars in a step-wise fashion to generate $Glc_3Man_9GlcNAc_2$-P-P-Dol (Fig. 3.4) (Orlean, 1992; Hemming, 1995).

Transfer is dependent on an asparagine being present in the consensus sequence Asn-Xaa-Thr/Ser, where Xaa can be any amino acid except proline (Bause, 1983). This consensus signal sequence (sequon) for N-glycosylation is highly conserved, although there are reports of cysteine substitution at the Ser/Thr position allowing efficient glycosylation of the asparagine residue (Bause and Legler, 1981).

Fig. 3.4 Structure of the fully glycosylated oligosaccharyl-diphosphodolichol (The underlined sugars are donated from phosphodolichol activated saccharides (Hemming, 1995)).



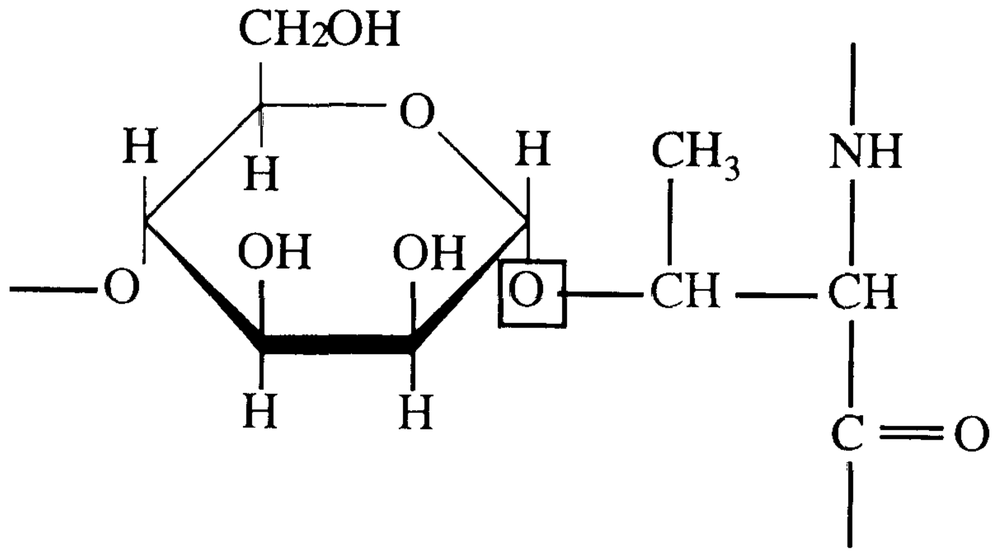
The hydroxyl group of either the serine or threonine residue is proposed to enhance the nucleophilic attack of the amido-nitrogen of the asparagine side-chain at the reducing end of the oligosaccharide donor (Bause and Legler, 1981). The resulting glycosidic linkage formed is shown in the lower panel of Fig. 3.5.

However, the enzymatic mechanism of transfer by the OST is still poorly understood as the multi-subunit enzyme has not yet been fully characterised. The OST from yeast has been purified (Kelleher and Gilmore, 1994) and has recently been shown to consist of 9 subunits (Ost1p (NLT-1), Ost2p, Ost3p, Ost4p, Ost5p, Ost6p, Stt3, Wbp1p, and Swp1p) (Karaoglu *et al.*, 1997). Initial purification of mammalian OST and sedimentary analysis using sucrose gradients revealed that it was also oligomeric (Kelleher *et al.*, 1992). More recent studies on mammalian OST using the yeast two-hybrid system has shown that ribophorin I, ribophorin II, OST48 and Dad1, the mammalian counterparts of Ost1p, Swp1p, Wbp1p and Ost2p respectively, also interact (Fu *et al.*, 1997). Further complete characterisation of the OST will help to elucidate the enzymatic mechanism of protein N-glycosylation.

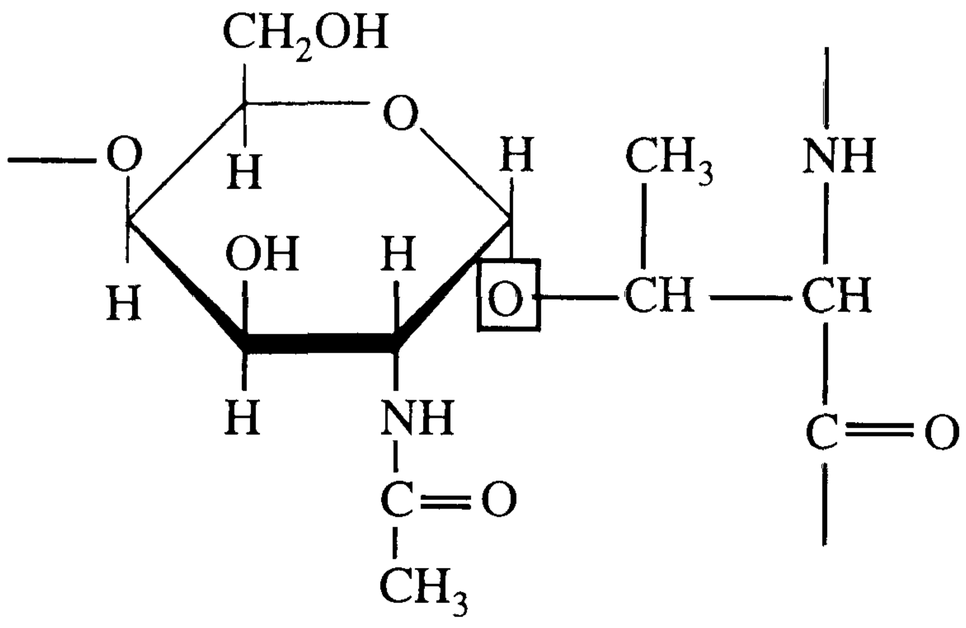
The synthesis of N-glycans in eukaryotes is broadly conserved, with the mechanism of N-glycosylation in humans showing high similarity to that of the yeast *Saccharomyces cerevisiae* (Herscovics and Orlean, 1993). The assembly of the oligosaccharide precursor (Fig. 3.4) is

highly conserved between yeast and other eukaryotes, though the later processing stages vary considerably.

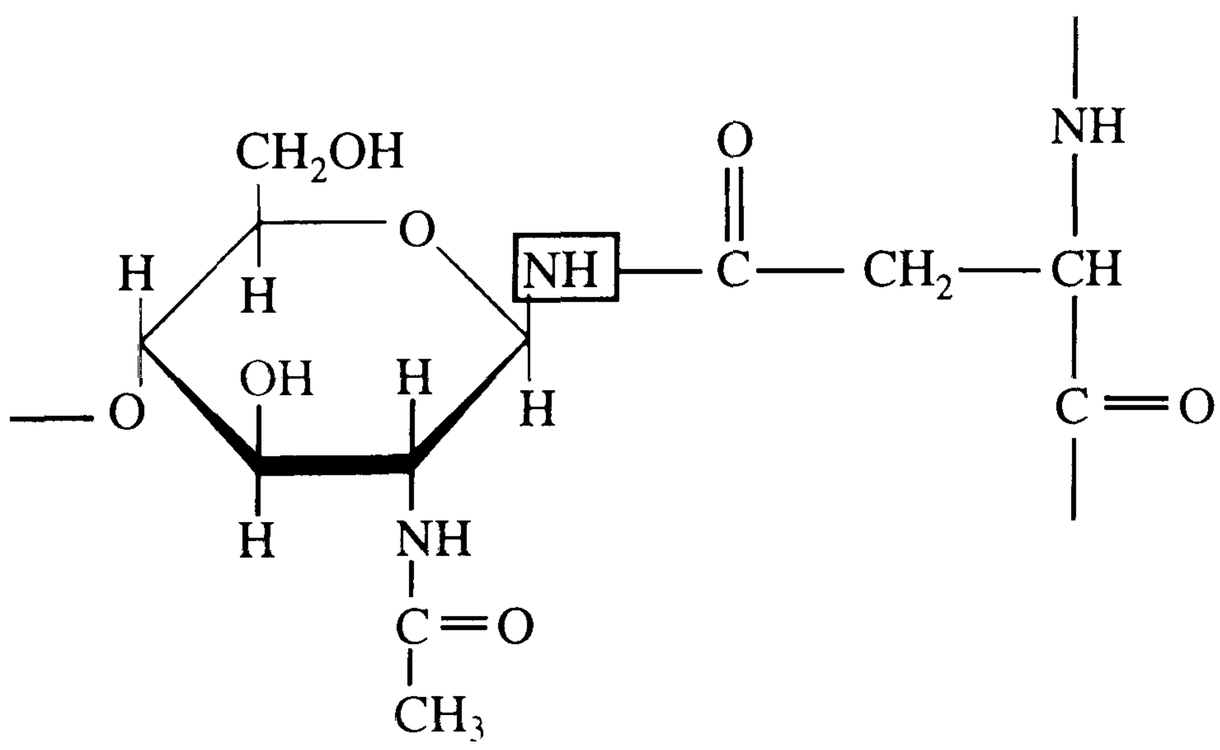
Fig. 3.5 Glycosidic linkages (boxed) formed in O- and N-glycosylation of proteins.



Manα-Thr*



GalNAcα-Thr*



GlcNAcβ-Asn

(* Identical bonds are formed with the hydroxyl group of serine side-chains)

Transfer of the $Glc_3Man_9GlcNAc_2$ moiety to the asparagine side-chain, is followed by deglucosylation of the glycan to $Man_9GlcNAc_2$-Asn-X or $Man_8GlcNAc_2$-Asn-X by the sequential action of glucosidase I and glucosidase II or alternatively by glucosidase, I glucosidase II and endo-α-mannosidase in the mammalian system (Kornfeld and Kornfeld, 1985; Moremen *et al.*, 1994). The processed protein is then transported to the golgi body where it is further processed.

In contrast to the high-mannose, hybrid and complex N-glycan chains found in higher eukaryotes, in yeast, N-glycan chains can be divided into two major groups. N-glycans containing typically $Man_{8\text{-}15}GlcNAc_2$-Asn-X are described as glycoproteins with an inner core mannan (Ballou *et al.*, 1990). This structure can be elongated by the addition of over 50 mannose residues to produce the outer chain mannan containing N-glycans (Lehle and Tanner, 1995). Characterisation of a yeast *mnn* mutant (mannan defective), *mnn9*, revealed that the signal for this chain elongation is the addition of a mannose residue (α1-6) to the α1-3 mannose attached to the β1-4 linked mannose (Fig. 3.4) (Reason *et al.*, 1991).

The elongated outer chain of yeast is composed of a linear α1-6 mannan chain with side-branches of α1-2 linked mannose (Lehle and Tanner, 1995). This structure is similar to the mannan chains of the mycobacterial cell wall lipoarabinomannan (LAM) (Venisse *et al.*, 1995). Yeast N-linked glycoproteins containing an elongated outer chain are often secreted into the periplasmic space and function in maintaining the integrity of the cell wall architecture. Whether LAM shares any functional properties with N-linked glycoproteins remains to be investigated.

It has been proposed that the addition of an α1-2 mannose residue to an α1-6 mannose at the non-reducing terminus of the extending mannose chain functions as a stop signal for chain elongation of N-linked glycoproteins in yeast (Ballou, 1990). The non-reducing ends of

the terminal arabinan chains of LAM from some strains of *Mycobacterium tuberculosis* are capped with a similar α1-2 mannose linkage (ManLAM) (Chatterjee *et al.*, 1992). However, it is unlikely that such capping in mycobacteria is likely to control terminal arabinan chain elongation as uncapped LAM (AraLAM) has the same arabinan chain structure as that of ManLAM (Chatterjee *et al.*, 1991).

It should also be noted that there have been reports of N-glycosylation in eukaryotes that do not contain the classical GlcNAc linkage to the amido nitrogen of an asparagine residue. By a combination of immunological and chemical analyses it has been shown that the protein laminin from rat kidney glomeruli contains a glucose residue, specifically in the β configuration, covalently attached to an asparagine residue (Schreiner *et al.*, 1994). However, to date no mechanism for such a modification has been described.

3.6 O-linked glycosylation

In higher eukaryotes O-linked protein glycosylation is initiated by the transfer of N-acetylgalactosamine (GalNAc) to serine and threonine residues, catalyzed by the family of enzymes UDP-GalNAc: polypeptide N-acetylglactosaminyltransferases (Clausen and Bennett, 1996). The action of UDP-GalNAc: polypeptide N-acetylgalactoaminyltransferase attaches the GalNAc residue to the Ser/Thr via an α-linkage as shown in Fig. 3.5.

In contrast to the N-glycosylation sequon (Asn-Xaa-Ser/Thr) there are no clear-cut motifs for protein O-glycosylation. However, Gooley *et al.*, (1994) have attempted to find motifs for O-linked glycosylation by analyzing the peptide sequences of glycopeptides that have O-linked glycan chains covalently attached to them. Their initial studies proposed four possible motifs for O-glycosylation, although these studies do not take into consideration the tertiary structure of the polypeptide and may be of limited use (Gooley and Williams. 1994).

Similar studies by O'Connell *et al.*, (1991) also showed that proline residues appear to reside in close proximity to both threonine and serine residues, though no rigid consensus for O-glycosylation has been widely accepted.

A major difference between N and O-glycosylation is that whereas transfer of the initial preassembled carbohydrate from the dolichol donor (Fig.3.4) is conserved between higher eukaryotes and yeast, such a conservation is not observed for O-glycosylation. Rather O-linked glycosylation of yeast proteins is characterised by the initial addition of mannose (Herscovics and Orlean, 1993) in contrast to the addition of GalNAc in higher eukaryotic O-glycosylation. Moreover the donor molecule of these different sugars also differs. In yeast the mannose is donated from dolichol-phosphomannose (Dol-P-Man), as opposed to the nucleotide sugar donor UDP-GalNAc used by mammals. The enzyme Dol-P-Man synthase (encoded by the gene *dpm1*) catalyses the transfer of Man from GDP-Man to dolichol, this is essential in yeast as disruption of *dpm1* results in a lethal phenotype (Orlean *et al.*, 1988). However, disruption of *dpm1* will also affect the N-glycosylation pathway as Dol-P-Man is also used as a mannose donor in the assembly of the oligosaccharyl diphosphodolichol (Fig. 3.4)

The transfer of mannose from Dol-P-Man by yeast to either serine or threonine residues is proposed to be catalysed by a family of protein mannosyltransferases coded for by seven genes (PMT1-7), although the involvement in O-glycosylation of only four gene products have so far definitely been demonstrated (*pmt1-4*) (Strahl-Bolsinger and Tanner, 1991; Immervoll *et al.*, 1995; and Lussier *et al.*, 1995). Recently, experiments using *pmt* gene disruption mutants have shown an essential role for protein O-glycosylation affecting cell wall integrity and cell rigidity (Gentzsch and Tanner, 1996). Although major differences have been highlighted between yeast and mammals with respect to their protein O-glycosylation, the presence of a family of four similar UDP-GalNAc: polypeptide N-acetylgalactosaminyl-transferases initiating O-glycosylation in mammals has also been reported (Bennett *et al.*, 1998).

O-linked glycoproteins from yeast typically contain linear glycans consisting of 1 to 5 mannose residues. The second and subsequent mannose residues are transferred directly from GDP-Man in either α-1,2 or α-1,3 linkages (Herscovics and Orlean, 1993). However, the elongation of O-linked glycans in mammals is more complex with the addition of GlcNAc, Gal, or GalNAc onto the initial GalNAc giving rise to seven different core structures (Brockhausen, 1995). These core structures may be elongated by the addition of repeating units of GlcNAc and Gal residues which may in turn be substituted with various terminal carbohydrate structures such as sialic acid or fucose (Brockhausen, 1995).

In addition to the much studied secretory and cell surface glycoproteins significant biochemical evidence has been presented, which is extensively reviewed by Greis and Hart (1997), to confirm the existence of cytoplasmic and nuclear glycoproteins. The majority of these reported to date are O-glycosylated with GalNAc attached to either serine or threonine residues as opposed to the classical GlcNAc-Ser/Thr linkage usually observed in mammalian protein O-glycosylation.

3.7 Bacterial glycoproteins

The omnipresence of glycoproteins in eukaryotic cells is in stark contrast to the relatively few recent reports of prokaryotic protein glycosylation. Conclusive demonstration for the presence of glycoproteins among the prokaryotes was the identification of the linkage unit (amino acid-attached sugar) from the crystalline cell surface protein (S-layer protein) of the archaebacteria *Halobacterium salinarium* (Mescher and Strominger 1983). It has since been demonstrated that an almost universal feature of archaebacteria is the presence of an S-layer that is usually composed of an empirical protein or glycoprotein. Such S-layer glycoproteins have also been identified in eubacteria, of which the Gram positive Bacillaceae been the most

extensively studied of the eubacteria (reviewed by Messner and Sleytr, 1991).

The carbohydrate moieties of the S-layer glycoproteins include a wide range of hexoses, uronic acids along with sulphate and phosphate residues. Little is known about the mechanism of glycosylation of these S-layer proteins by the archae- or eubacteria although the presence of novel linkage types such as aspariginyl-rhamnose and tyrosine-glucose has been reported (Messner and Sleytr, 1988; and Messner *et al.*, 1992). These S-layer glycoproteins form two-dimensional arrays to cover the cell surface completely and in archaebacteria are the only cell wall component external to the cytoplasmic membrane (Beveridge, 1994).

The presence of S-layers on pathogenic bacteria including Chlamydia, Bordatella and Campylobacter has led to the proposal that these structures may prove relevant as virulence factors and may be of use in the development of vaccines (Sleytr and Sára, 1997). However, apart from one observation of an external arrayed layer on BCG (Lounatmaa and Brander, 1989), no other reports on the structure of the mycobacterial cell wall have identified the presence of such S-layers (Brennan and Nikaido, 1995).

In addition to the protomeric glycoproteins of S-layers, non-S-layer glycoproteins have recently been observed in both archae-and eubacteria. Reports of this relatively new phenomenon have been extensively reviewed (Sandercock *et al.*, 1994; Messner, 1997; and Moens and Vanderleyden, 1997). From these reviews it can be seen that the glycosylation of these eubacterial non-S-layer glycoproteins is exclusively O-linked, with the exception of one report by Kuo *et al.* (1996) which describes the presence of an N-linked glycoprotein from chlamydia which is discussed on page 107.

Although the linkage unit of these glycoproteins is of the O-linked genre, the actual carbohydrate residues vary widely from the classical GalNAc-Ser/Thr linkage observed in eukaryotic O-glycosylation (Fig.

3.5). The carbohydrate residues that have been shown to be covalently attached to threonine residues of eubacterial glycoproteins range from simple residues such as mannose on a secreted antigen of *M. tuberculosis* (Dobos *et al.*, 1995; Fig. 3.5) to the more complicated structure of digalactosyl 2,4-diacetamido-2,4,6-trideoxyhexose on the pilin of *Neisseria meningitidis* (Stimson *et al.*, 1995).

The identification of these novel glycoproteins amongst pathogenic bacteria raises the intriguing question as to their function and mechanism of biosynthesis. The eubacterial glycoproteins reported to date (Messner, 1997; Moens and Vanderleyden, 1997) are all surface associated molecules, including flagellin and pilin, or secreted proteins. Whether or not the pathogenic bacteria have developed such mechanisms to aid in adherence and/or invasion of host cells, or to protect themselves from the immune system remains to be seen.

Identification and characterisation of novel glycoproteins from *M. tuberculosis* and *M. bovis* may provide clues as to a common requirement for protein glycosylation by this pathogen. It is hoped that such a study will lead to an improved understanding of the basic biology of *M. tuberculosis* and *M. bovis* and to the elucidation of the function of protein glycosylation. The aim of the work described in chapters 4 and 5 was to investigate the post-translational modification of MPT83 by *M. smegmatis* observed in Chapter 2.

Chapter 4

Characterisation of recombinant MPT83 expressed by *Mycobacterium smegmatis* mc²155

4.1 Introduction

In chapter 2 it was shown that recombinant MPT83 expressed by *M. smegmatis* was posttranslationally modified relative to the expression of the antigen by *E. coli* (Fig. 2.20).

On the basis of [^{3}H] palmitate labeling experiments, Young and Garbe (1991) demonstrated that the 19 kDa antigen of *M. tuberculosis* was a lipoprotein. Garbe *et al.* (1993) demonstrated that this antigen was posttranslationally modified when expressed in *M. smegmatis*, relative to the recombinant form expressed by *E. coli*. The recombinant mycobacterial antigen from *M. smegmatis*, in contrast to the recombinant antigen from *E. coli*, was recognised by the lectin ConA. The lectin Concanavalin A (ConA) is a lectin from the *Canavalia* species and has a high binding affinity for carbohydrate residues such as mannose (Goldstein and Poretz, 1986).

Thus, Garbe *et al.* (1993) proposed that this posttranslational modification by *M. smegmatis* was due to glycosylation of the protein. Further studies of this posttranslational modification of the 19 kDa by *M. smegmatis* by Herrmann *et al.* (1996) supported the observations of Garbe *et al.* (1993). Similarly the 38 kDa antigen of *M. tuberculosis* has also been described as a lipoglycoprotein (Espitia and Mancilla, 1989b; Young and Garbe, 1991).

The presence of a putative lipoprotein signal peptide cleavage motif within MPT83 (Hewinson *et al.*, 1996) (Fig. 2.16) and the observed posttranslational modification of MPT83 by *M. smegmatis* mc^{2}155 (Fig. 2.20) raises the possibility that MPT83 is a lipoglycoprotein. The aim of this part of the study was to test whether recombinant MPT83 expressed by *M. smegmatis* is acylated and glycosylated.

4.2 Results

4.2.1 Immunoblot analysis of MPT83 localisation after phase partitioning with Triton X-114

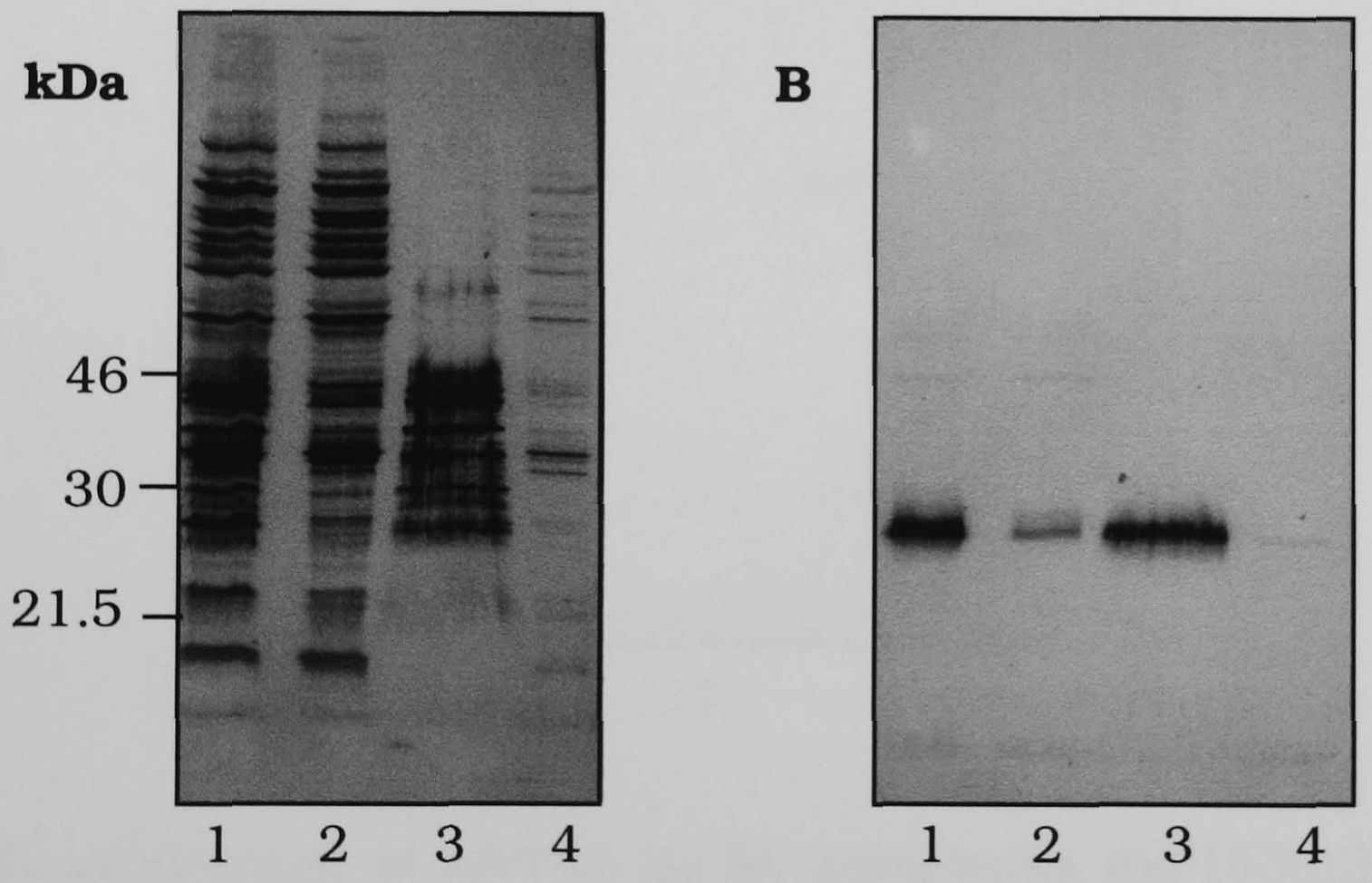
In addition to the classical outer-membrane associated and peptidoglycan bound lipoprotein (Braun's lipoprotein) of Gram negative bacteria (Braun and Rehn, 1969), it has also been reported that both Gram negative (Ichihara *et al.*, 1981) and Gram positive bacteria (Nielsen and Lampen, 1982) possess other lipoproteins. Membrane associated lipoproteins partition to the detergent phase of a solution of the non-ionic detergent Triton X-114 (Bordier, 1981; Radolf *et al.*, 1988; Bricker *et al.*, 1988; and Young and Garbe, 1991). As MPT83 is expressed on the the mycobacterial cell surface and contains a putative lipoprotein signal peptide cleavage motif (Hewinson *et al.*, 1996), it was decided to use phase-partitioning to ascertain if recombinant MPT83 expressed by *M. smegmatis* exhibits the lipophilic properties characteristic of lipoproteins.

Sonicated extracts of *M. smegmatis* (pSM43) were prepared as described in section 8.8.1. A 4% solution of Triton X-114 (500 µl) in PBS (pH 7.0) at 0°C was added to 500 µl of *M. smegmatis* (pSM43) sonicated extract (2.5 mg/ml) at 0°C. The mixture was incubated on ice for 30 min with frequent gentle agitation. Phase separation was performed as described in section 8.9. The detergent phase was washed a second time with PBS to remove the remaining aqueous phase proteins. Detergent and aqueous phases proteins were separated by SDS-PAGE (15% separating gel). Western blot analysis using a polyclonal anti-*M. smegmatis* antibody (K2688, kindly supplied by Morten Harboe, University of Oslo), showed that the majority of proteins from the mycobacterial sonicate efficiently partitioned to the aqueous phase (Fig. 4.1 A, lane 2) with less than 5% of hydrophilic proteins remaining with the re-solubilised detergent phase (as determined by washing the detergent phase with PBS after initial partitioning (Fig. 4.1 A, lane 4). However, this antibody also detected several proteins of *M. smegmatis*

in the detergent phase (Fig. 4 A, lane 3). Western blot analysis of the same samples, using the MPT83 specific monoclonal antibody MBS43, revealed that the majority of recombinant MPT83 partitioned to the detergent phase (Fig 4.1 B, lane 3), at a relative molecular weight of 25 kDa. This partitioning of MPT83 is highly efficient as negligible amounts of MPT83 could be retrieved upon further washing (Fig 4.1 B, lane 4) of the detergent phase.

Fig. 4.1 Western blots incubated with K2688(A), and MBS43 (B).

Lanes: 1, *M. smegmatis* mc^2155 (pSM43); 2, aqueous phase proteins; 3, detergent phase proteins; 4, aqueous proteins from detergent phase wash.

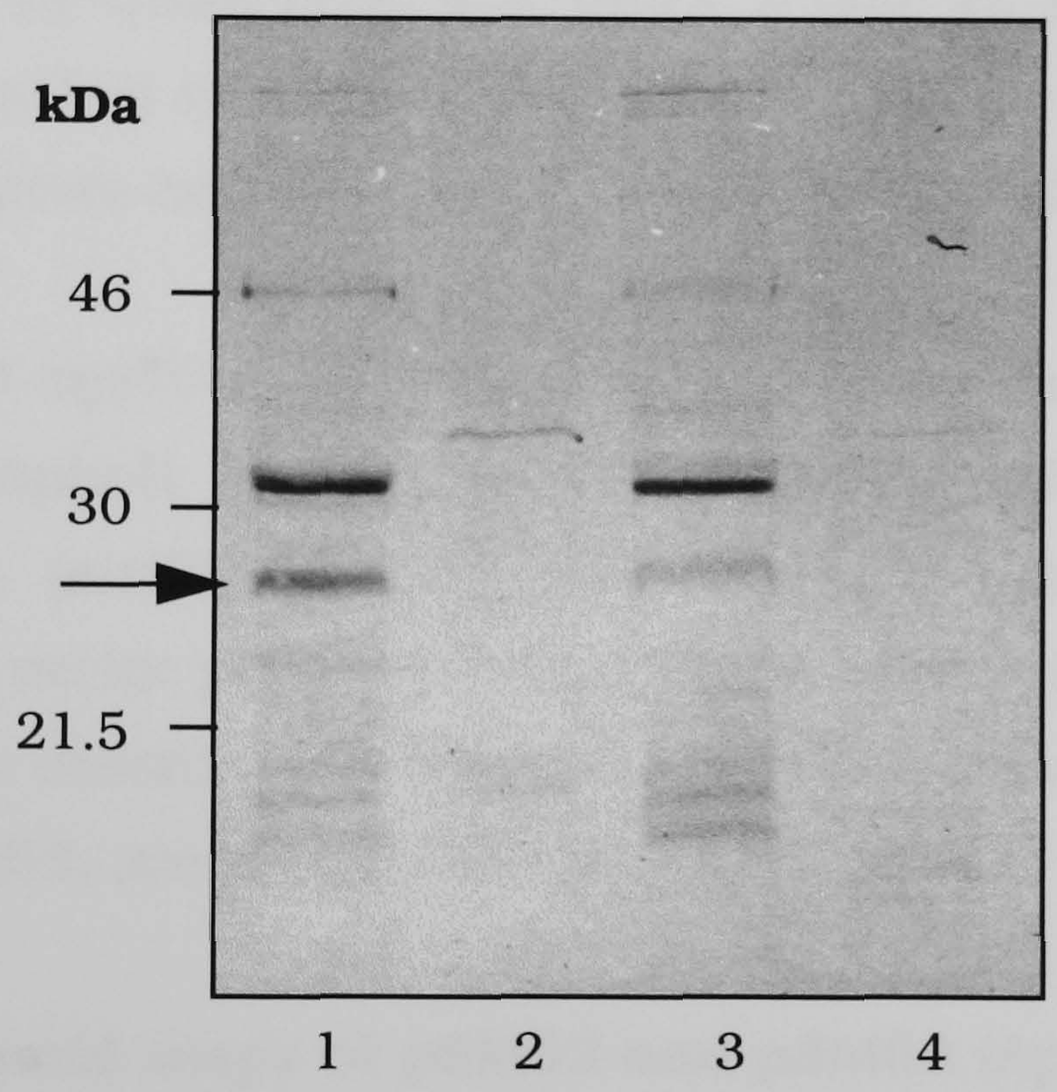


4.2.2 Recognition of recombinant MPT83 expressed by *M. smegmatis* by Concanavalin A

The carbohydrate binding properties of ConA have been developed and used to identify glycoproteins transferred to nitrocellulose membranes (Glass II *et al.*, 1987; Espitia and Mancilla, 1989b; and Garbe *et al.*, 1993). Previous reports that the 25 kDa antigen MPT83 is glycosylated (Fifis *et al.*, 1991) were investigated using ConA blotting.

Sonicated extracts (10μg) of *M. smegmatis* mc²155 and *E. coli* DH5α containing pSM43 (Fig. 2.19) and pMD31 (Fig. 8.1 C) were separated by SDS-PAGE (15% separating gel) and transferred to nitrocellulose. Detection of glycoproteins using peroxidase labelled ConA (Fig. 4.2) was performed as described in section 8.12.

Fig. 4.2 Western blot incubated with peroxidase-labelled ConA.
Sonicated extracts (10 μg) of lanes: 1, *M. smegmatis* mc²155 (pSM43); 2, *E. coli* DH5α (pSM43); 3 *M. smegmatis* mc²155 (pMD31); 4, *E. coli* DH5α (pMD31). Arrow indicates the position of MPT83.



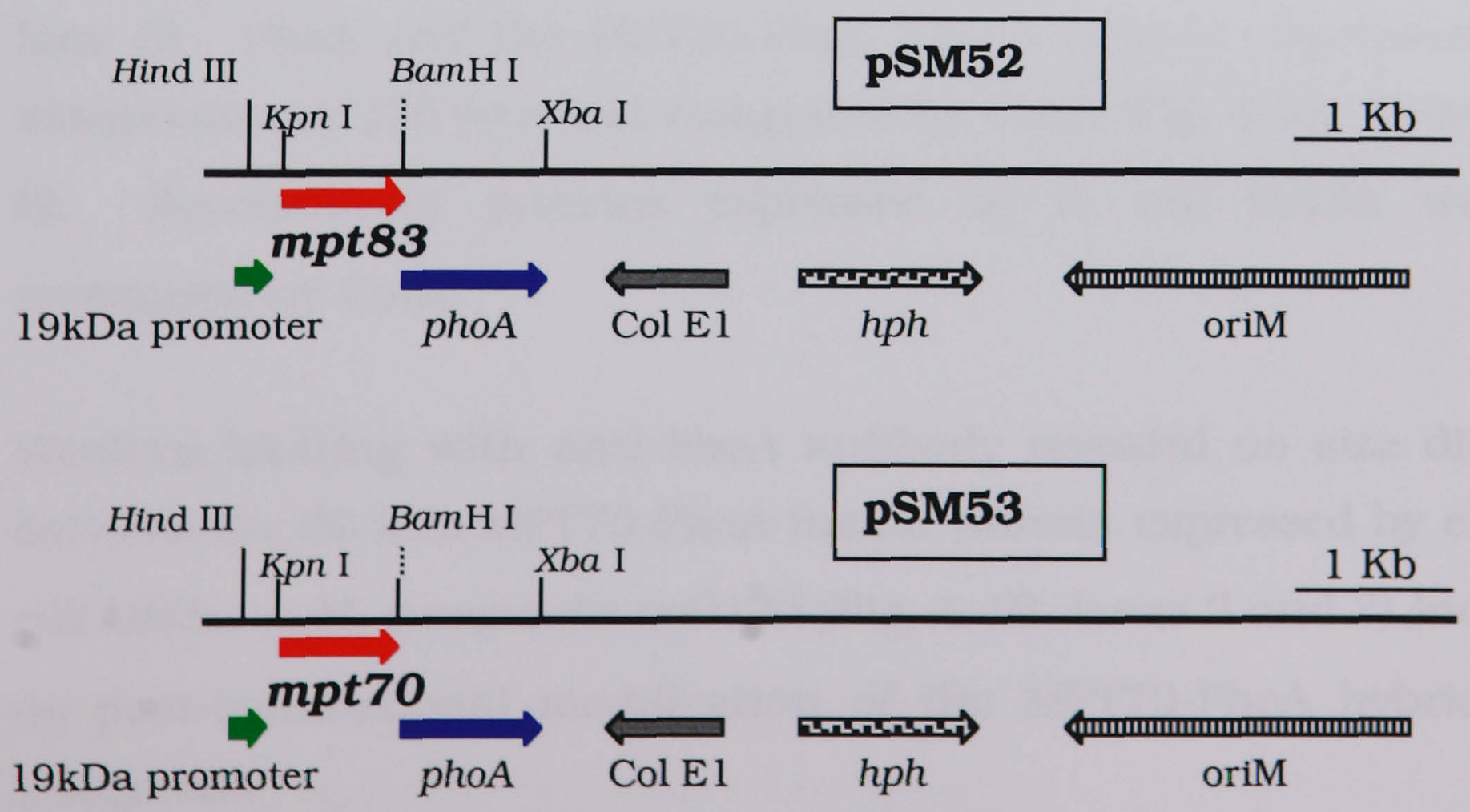
Although production of MPT83 *by M. smegmatis* mc²155(pSM43) was confirmed by Western blotting with the monoclonal antibody MBS43 (Fig. 2.20, lane 2), it was difficult to determine the presence of a 25 kDa protein band that was recognised by ConA in the same sample (Fig. 4.2, lane 1) relative to the bands present in the vector control (Fig. 4.2, lane 3). Several other polypeptides of the mycobacterial sonicates (31 kDa and 48 kDa) were recognised by ConA suggesting that the *M. smegmatis* sonicate may contain other glycoproteins. Only one faint protein band of 32 kDa in the *E. coli* samples, common to both recombinants, was recognised by ConA (Fig. 4.2, lanes 2 and 4). This may be due to some contaminating glycoprotein from the culture media.

4.2.3 Analysis of PhoA hybrid proteins

The *phoA* gene has been used extensively as a reporter for studying protein export in both Gram negative and Gram positive bacteria including the mycobacteria (Manoil *et al.*, 1990; Payne and Jackson, 1991; and Timm *et al.*, 1994), as its product, alkaline phosphatase, is active only when secreted from the cytoplasm. More recently, PhoA has been used as a fusion protein for the study of protein glycosylation, where it was shown that PhoA expressed by mycobacteria is not glycosylated (Herrmann *et al.*, 1996). Since *M. smegmatis* mc^2155 did not produce any proteins above 50 kDa that were recognised by ConA (Fig. 4.2, lanes 2 and 4), the 48 kDa PhoA protein was identified as a suitable fusion partner for ConA analysis of MPT83-PhoA fusions expressed by *M. smegmatis* mc^2155.

Both *mpt83* and *mpt70* were PCR amplified using the oligonucleotide pairs 83phoF/83phoR, and 70phoF/70phoR respectively (numbers 9-12, appendix 1), purified and digested with *Kpn* I and *Bam*H I, and cloned in to the vector pMT3-19-phoA (Fig. 8.1 D) to generate in frame *phoA* fusions as described in section 8.3. The resulting constructs, pSM52 and pSM53, are shown below.

Fig. 4.3 Plasmid maps of pSM52 and pSM53 (Appendix 2).



DNA sequencing of the 19 kDa promoter, the cloned gene and the 5' end of *phoA* gene in the above constructs, using the oligonucleotide primers 3 and 4 and 13-16 (appendix 1), confirmed that both *mpt83* and *mpt70* were in frame with *phoA* and that no mutations had been introduced during the PCR process. Both constructs were transformed into *M. smegmatis* mc^2155. Plating of the transformants on XP indicator plates (section 8.5.2.2) resulted in the formation of blue colonies, $PhoA^+$ phenotype, demonstrating functional activity of the alkaline phosphatase fusion and confirming that both fusion proteins were in frame and translocated across the cytoplasmic membrane.

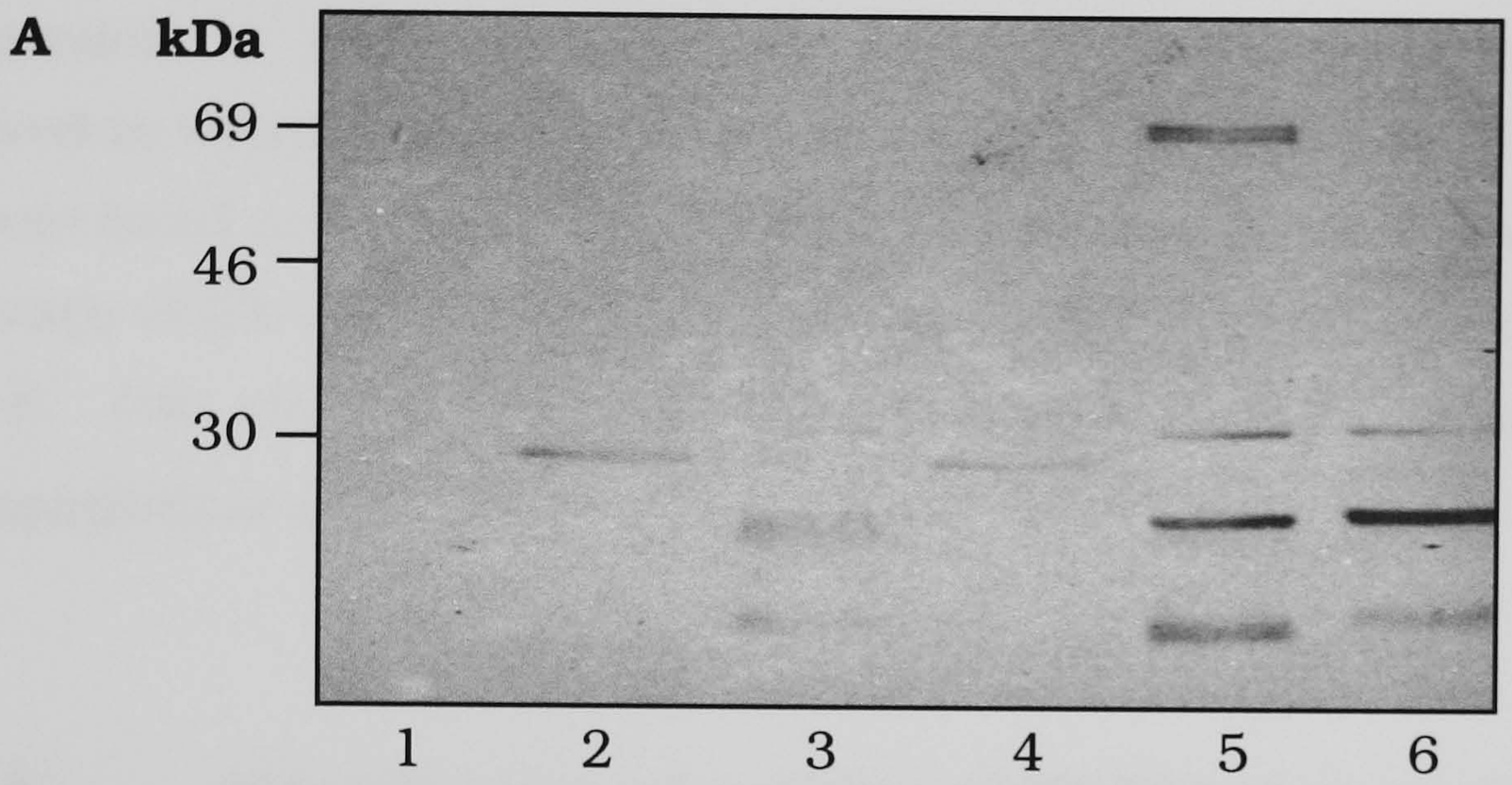
To determine if MPT83 is glycosylated by *M. smegmatis* mc^2155 and to confirm that MPT70 is not, sonicated extracts of *M. smegmatis* mc^2155 containing pSM52 and pSM53 were separated by SDS-PAGE (12.5% separating gel), Western blotted and stained with peroxidase labelled ConA (Fig. 4.4A).

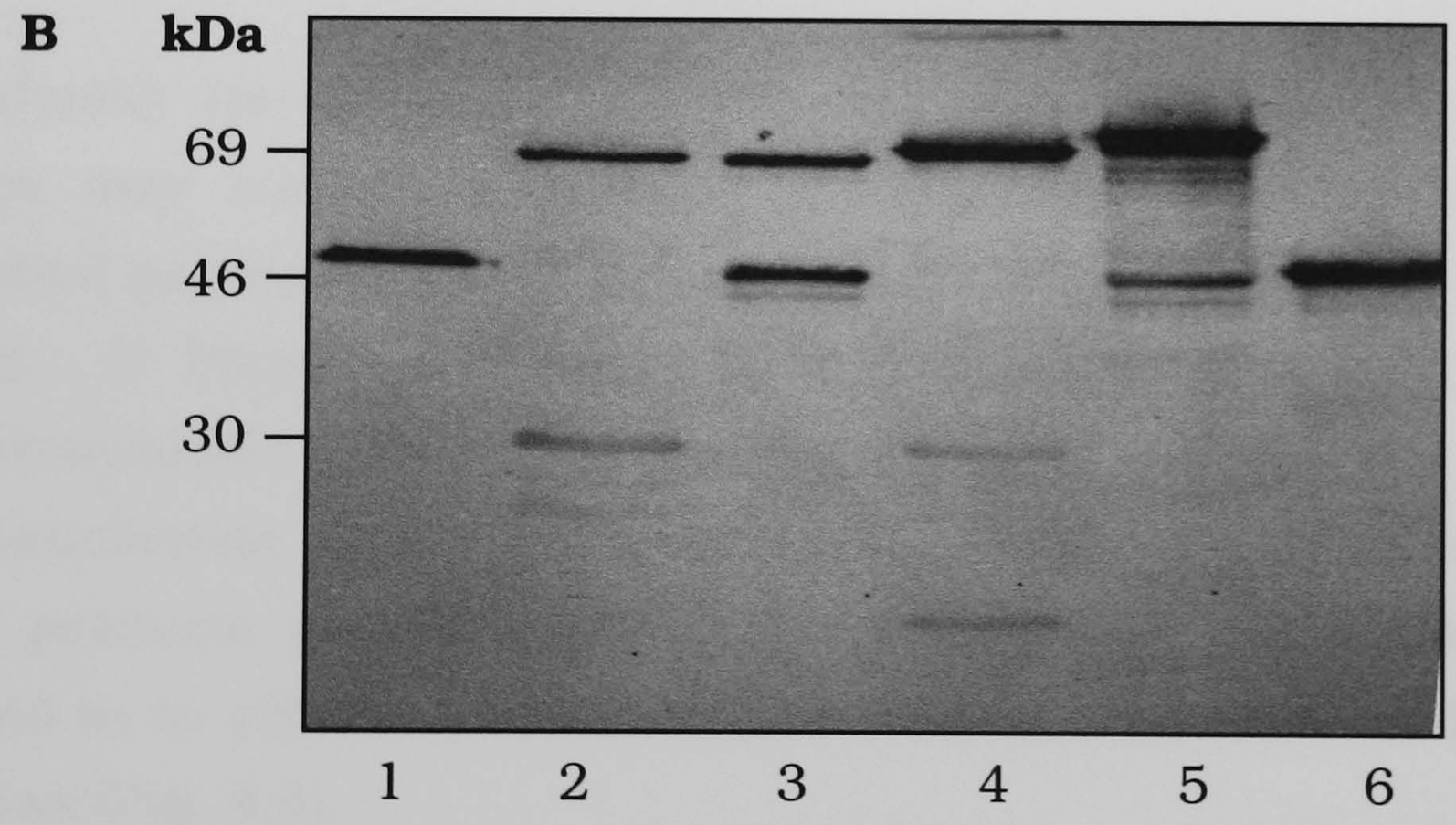
The only ConA reactive PhoA fusion observed was a doublet with a relative molecular weight of about 69 kDa and was found in the sonicated extract of *M. smegmatis* mc^2155 containing the plasmid pSM52, which codes for the MPT83-PhoA fusion (Fig. 4.4A, lane 5). This is approximately equal to the predicted molecular weight (67 kDa) for the MPT83-PhoA fusion and its expression was confirmed by blotting with a polyclonal antibody directed against PhoA (Fig, 4.4B, lane 5). PhoA and the MPT70-PhoA fusion protein expressed *by M. smegmatis* mc^2155 were not recognised by ConA (Fig. 4.4A, lanes 3 and 6). Recombinant proteins expressed by *E. coli* DH5α were not recognised by ConA.

Western blotting with anti-PhoA antibody revealed no size difference between the 66 kDa MPT70-PhoA fusion protein expressed by either *E. coli* DH5α or *M. smegmatis* mc^2155 (Fig. 4.4B, lanes 2 and 3) indicating no post-translational modification of the MPT70-PhoA hybrid by *M. smegmatis*.

Fig. 4.4 Western blots incubated with peroxidase-labelled ConA (A) and anti-PhoA antibody (B).

Sonicated extracts (10 μg) of lanes: 2, *E. coli* DH5α (pSM53); 3, *M. smegmatis* mc²155 (pSM53); 4, *E. coli* DH5α (pSM52); 5, *M. smegmatis* mc²155 (pSM52); 6, *M. smegmatis* mc²155 (pSMT3-19-phoA). Lane 1, 200 ng of purified PhoA.





In contrast, the MPT83-PhoA fusion (pSM52) had a higher relative molecular weight when expressed by *M. smegmatis* mc²155 than *E. coli* DH5α (Fig. 4.4B, lanes 4 and 5). This increase in molecular weight, coupled with the binding of ConA to MPT83 only when expressed in *M. smegmatis* mc²155 suggests glycosylation of MPT83 by the mycobacterial host.

The amount of MPT70-PhoA protein appears to be less than that of the MPT83-PhoA fusion even though both proteins are under the control of the same promoter. This is probably due to the fact more of the MPT70-PhoA fusion is secreted to the extracellular medium. However, no ConA reactive protein band was observed upon increased loading of sonicate containing the MPT70-PhoA fusion (data not shown).

In addition to the apparent glycosylation by *M. smegmatis*, a proportion of both MPT70- and MPT83-PhoA fusion proteins were cleaved to release unfused PhoA as noted by the presence of a 48 kDa protein band recognised by anti-PhoA (Fig. 4.4B, lanes 3 and 5). This cleavage of the fusion proteins is not observed when they are expressed by *E. coli* suggesting that *M. smegmatis* mc^2155 possesses an endopeptidase that is absent in *E. coli* DH5α.

4.2.4 Identification of a region of MPT83 that mediates ConA recognition

To identify the regions of MPT83 that are recognised by ConA, and hence may contain a putative glycosylation signal motif, amino terminal portions of MPT83 of increasing size were fused in frame with PhoA. 5' fragments of *mpt83* were PCR amplified using the forward oligonucleotide primer 83PhoAF (appendix 1) and a series of reverse oligonucleotide primers (numbers 17-20, appendix 1). The resulting PCR products were purified, digested with *Kpn* I and *Bam*H I and cloned in to pSMT3-19-phoA (Fig. 8.1 D), giving rise to in frame *phoA* fusions (Fig. 4.5).

Recombinants were sequenced using the oligonucleotide primers 15 and 16 (appendix 1) to ensure no mutations had been introduced. Sequenced plasmid constructs were subsequently transformed into *E. coli* DH5α and *M. smegmatis* mc^2155. Plating of transformants on XP indicator plates gave rise to blue colonies confirming expression of the in frame PhoA hybrids. Sonicated extracts of the recombinant *M.*

smegmatis were fractionated by SDS-PAGE (12.5% separating gel) as described previously. Glycosylation of the fusion proteins was detected by Western blotting and incubation with peroxidase labelled ConA (Fig 4.6 A). Expression levels of of the PhoA hybrids were monitored using the anti-PhoA antibody (Fig. 4.6 B)

Fig. 4.5 MPT83 amino terminal fusions to PhoA expressed by *M. smegmatis* mc²155.

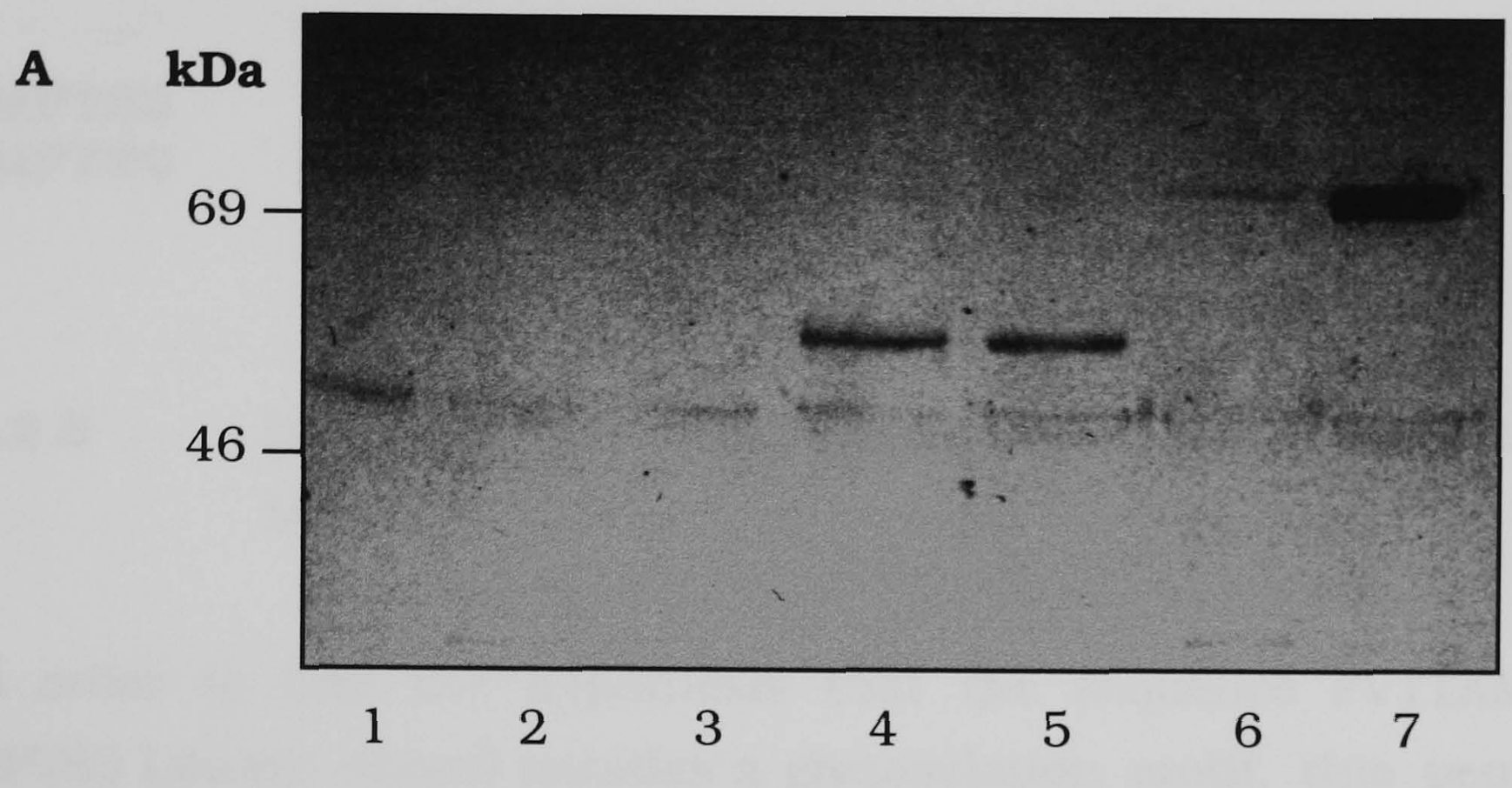
MPT83-PhoA hybrids	Plasmid	Recognition by ConA
1 20 MINVQAKPAAAASLAAIAIAFLAGCSST —— **PhoA**	**pSM54**	-ve
1 —— 20 FLAGCSSTKPVSQDT 35 —— **PhoA**	**pSM55**	-ve
1 —— 20 FLAGCSSTKPVSQDTSPKPATSPAAPVTTAAMADPA 56 —— **PhoA**	**pSM56**	+ve
1 —— 20 FLAGCSSTKPVSQDTSPKPATSPAAPVTTAAMADPAADLIGRG 63 —— **PhoA**	**pSM57**	+ve

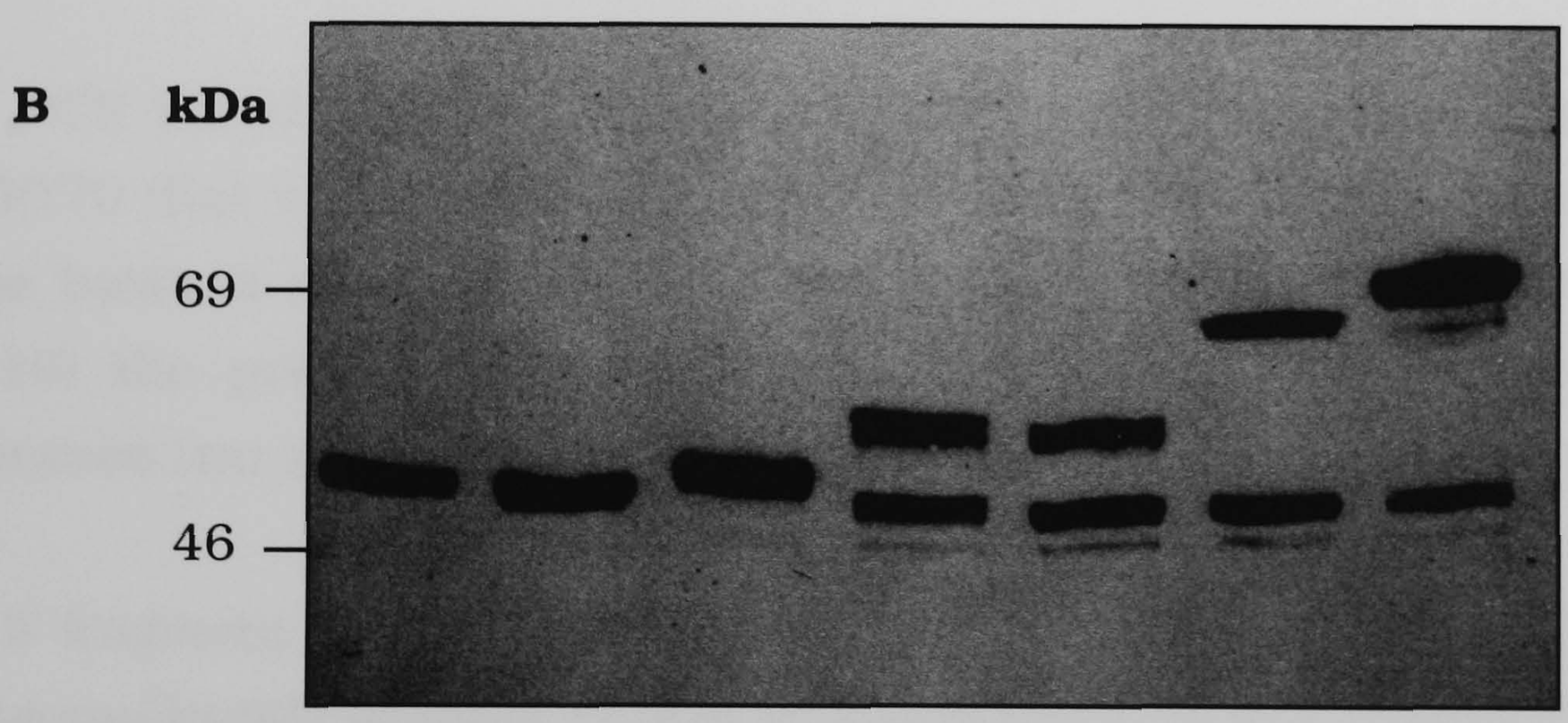
Binding of the anti-PhoA antibody to the PhoA fusion proteins (Fig. 4.6B), confirmed that the recombinant proteins were expressed at equal levels. The first 28 and 35 amino acids of MPT83 when fused to PhoA were not recognised by ConA when expressed in *M. smegmatis* mc²155 (Fig. 4.6A, lanes 2 and 3). Interestingly, no degradation products were observed for these two proteins (Fig. 4.6B, lanes 2 and 3). This was not the case for all fusion proteins containing more amino acid residues of MPT83.

Increasing the number of MPT83 amino terminus amino acid residues fused to PhoA from 35 to 56 resulted in a fusion protein that was recognised by ConA when expressed by *M. smegmatis* mc²155 (Fig. 4.6A, lane 4). Increasing the amino terminus fusion from 56 amino acids to 63 amino acids did not increase the level of binding of Con A (Fig. 4.6A, lane 5).

Fig. 4.6 Western blots incubated with peroxidase-labelled ConA (A) and anti-PhoA antibody (B).

Sonicated extracts (10μg) of *M. smegmatis* mc^{2}155 containing, lanes: 1, pSMT3-19-phoA; 2, pSM54; 3, pSM55; 4, pSM56; 5, pSM57; 6,pSM53; 7, pSM52. Lane 8, 200 ng of purified PhoA. (pSM numbers described on previous page).





Both the 56 and 63 amino acid PhoA hybrids appeared to be cleaved at the PhoA fusion junction to release an unfused 48 kDa PhoA species (Fig. 4.6B, lanes 4 and 5). This was also the case for the MPT83 and MPT70-PhoA fusions (Fig. 4.4B, lanes 3 and 5).

These results suggest that the region of amino acids of MPT83 between positions 35 and 56 mediate ConA binding when expressed in *M. smegmatis*. Comparison of the amino acid sequences of MPT83 and MPT70 within this region, shown below (and Fig. 2.16), reveals a run of 10 amino acids that are present within this region of MPT83 but absent from MPT70 which is not glycosylated by *M. smegmatis*.

MPT83	**S**	**P**	**A**	**A**	**P**	**V**	**T**	**T**	**A**	**A**	**M**	**A**	**D**	**P**	**A**	**A**	**57**
MPT70	**S**	**P**	**P**	**A**	**A**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**A**	**G**	**31**

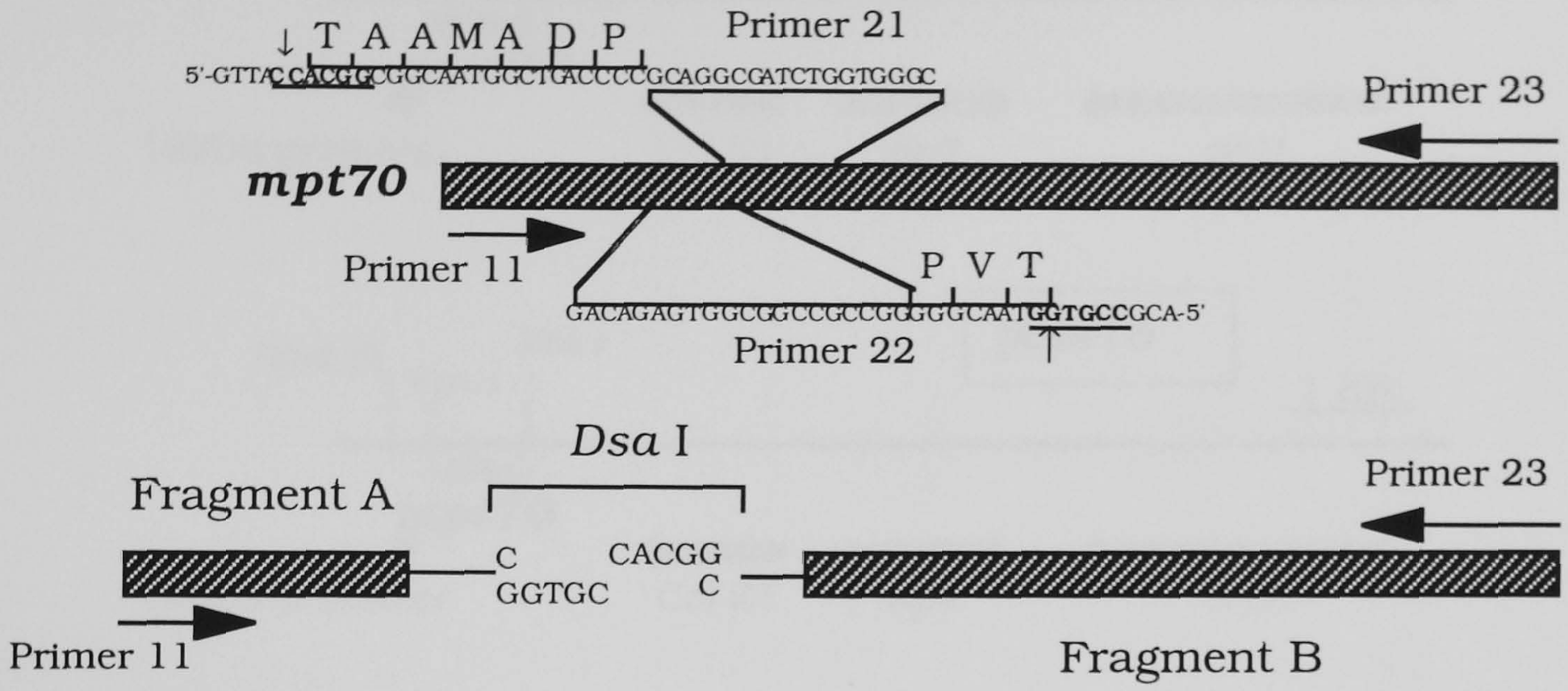
4.2.5 Introduction of a 10 amino acid region of MPT83 into MPT70

In order to test the hypothesis that the sequence PVTTAMADP of MPT83 (shown above) encodes a glycosylation motif, this peptide was introduced into the non-glycosylated MPT70 as described below (Fig. 4.7).

A PCR based strategy was used to construct a modified version of MPT70 that contains the peptide region PVTTAAMADP of MPT83. On the basis of amino acid alignment between MPT83 and MPT70 (Fig. 2.16) the point of introduction for the peptide was chosen to be between two alanine residues (29,30) of MPT70.

A 5' fragment of *mpt70* was PCR amplified from pSM53 (Fig. 4.3) using oligonucleotide primers 11 and 22 (appendix 1) to give fragment A (Fig. 4.7). Primer 22 possesses a 5' extension coding for the amino acids 46-48 of MPT83 and a *Dsa* I restriction site (underlined). Similarly, a 3' fragment of *mpt70* was PCR amplified pSM53 using primers 21 and 23 (appendix 1) to give fragment B (Fig.4.7). The 5' extension on primer 21 codes for amino acids 49-55 of MPT83 and also contains a *Dsa* I restriction site (underlined).

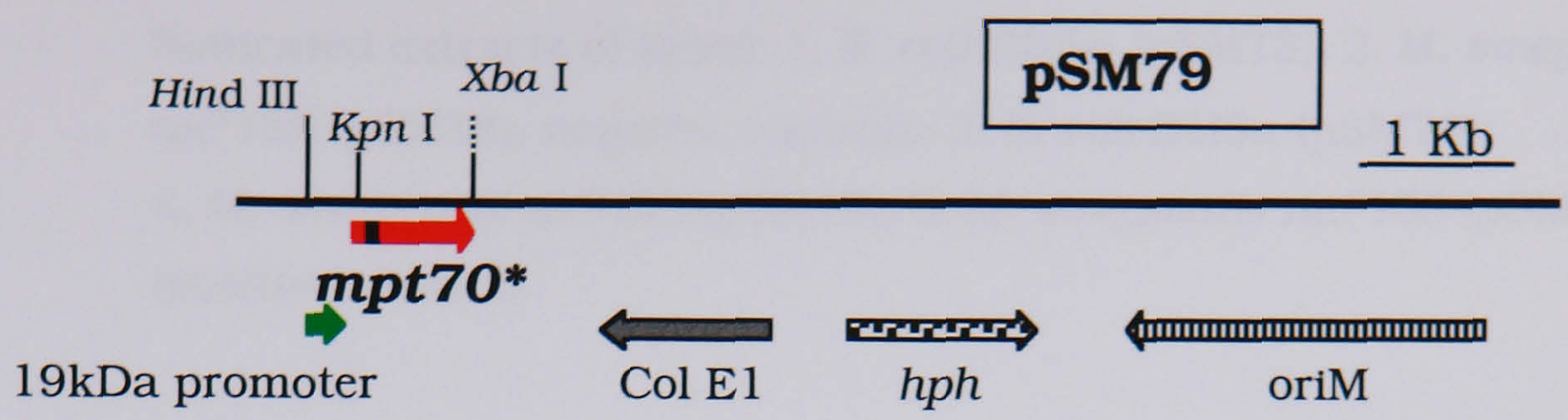
Fig. 4.7 PCR Strategy for introducing amino acids 46-55 of MPT83 into MPT70



Fragments A and B were digested with the restriction enzyme *Dsa* I, purified and ligated (250 ng of each) with T4 DNA ligase in a total volume of 20 µl as described in section 8.3.7. The ligation mixture (1µl) was PCR amplified using primers 11 and 23 (appendix 1) to give a product of 630 bp. This modified *mpt70* gene was digested with *Kpn* I and *Xba* I and cloned into the *Kpn* I and *Xba* I sites of pSMT3-19-phoA (Fig. 8.1D) generating the construct pSM79 (Fig. 4.8). Similarly, an unmodified version of *mpt70* was PCR amplified from pSM53 (Fig. 4.3) using oligonucleotide primers 11 and 23 (appendix 1) and cloned into the *Kpn* I and *Xba I* sites of pSMT3-19-PhoA (Fig. 8.1D), this plasmid was designated pSM75 (Fig. 4.8).

The modified *mpt70* gene in pSM79 was sequenced using the sequencing primers 3, 4 and 15 (appendix 1). Sequence data confirmed that the 10 amino acids had been introduced correctly (appendix 3, A3.1) and that no other mutations had occurred within *mpt70*. The sequenced constructs were then transformed into *M. smegmatis* mc^2155 as described in section 8.5.

Fig. 4.8 Plasmid maps of pSM79 and pSM75.



An attempt was made to observe post-translational modification of the modified MPT70 by *M. smegmatis* on the basis of differences in relative molecular weights of the recombinant protein expressed by *M. smegmatis* and *E. coli*. Sonicated extacts of *M. smegmatis* mc^2155 and *E. coli* DH5α containing pSM79 were fractionated by SDS-PAGE (15% separating gel), Western blotted and incubated with the monoclonal antibody SB10.

However, the monoclonal antibody SB10 failed to detect expression of the modified MPT70 by either *M. smegmatis* mc^2155 or *E. coli* DH5α (Fig. 4.9, lanes 4 and 3 respectively). *M. smegmatis* mc^2155 expressing MPT70 from pSM75 (Fig. 4.8) was detected by SB10 (Fig. 4.9, lane 5). This result suggests that the introduction of amino acids 46-55 of MPT83 into MPT70 leads to the formation of a hybrid protein that is unstable in both *M. smegmatis* mc^2155 *and E. coli* DH5α.

Fig. 4.9 Western blot incubated with the monoclonal antibody SB10

Sonicated extracts of lanes: 1, *E. coli* DH5α (pSMT3); 2, *M. smegmatis* mc^2155 (pSMT3), negative controls; 3, *E. coli* DH5α (pSM79); 4, *M. smegmatis* mc^2155 (pSM79); 5, *M. smegmatis* mc^2155 (pSM75) (positive control)



4.3 Discussion

The processing and secretion of the murein lipoprotein (Braun and Rehn, 1969) is prototypical of those reactions for the non-murein lipoproteins (section 3.3.2) identified in *E. coli* (Ichihara *et al.*, 1981) and is extensively reviewed by Wu and Tokunaga (1986). The presence of proteins from *Bacillus licheniformis* that possess a similar acyl moiety to that of Braun's lipoprotein established the presence of such non-murein lipoproteins in Gram positive bacteria (Nielsen *et al.*, 1981). Young and Garbe (1991) identified 5 lipoproteins of *M. tuberculosis* by metabolic labelling with [^{3}H] palmitate with relative molecular weights of 19, 26, 27, 38 and 65 kDa. The 19 and 38 kDa labelled proteins reacted with monoclonal antibodies HYT 6 and TB71 respectively which are specific for the immunodominant 19 and 38 kDa antigens of *M. tuberculosis* (Jackett *et al.*, 1988). The 26 and 27 kDa

[^{3}H] palmitate labeled proteins of *M. tuberculosis* were also recognised by hyperimmune rabbit sera. It is possible that these antigens correspond to MPT83 (Hewinson *et al.*, 1996) and the 27 kDa lipoprotein identified by Bigi *et al.*, (1997), as both have a consensus prokaryotic lipoprotein signal peptide cleavage motif.

It has been demonstrated in this chapter that MPT83 expressed by *M. smegmatis* partitions to the detergent phase of a solution of the non-ionic detergent Triton X-114. These results demonstrate that this recombinant form of MPT83 has a lipophilic nature and supports previous reports of the lipophilic nature of MPB83 expressed by *M. bovis* BCG (Vosloo *et al.*, 1997; Harboe *et al.*, 1998). The probable cause of this lipophilic nature is through posttranslational acylation of the cysteine residue at amino acid position +25. This residue constitutes part of the typical prokaryote lipoprotein signal peptide cleavage motif consensus, identified within MPT83 (Fig 2.16), and described in section 3.3.2.

The proposed mechanism for this lipid modification in *E. coli* involves the products of at least three genes: (i) *lgt* (prolipoprotein diacylglyceryl transferase), (ii) *lspA* (prolipoprotein signal peptidase/signal peptidase II), and (iii) *lnt* (apolipoprotein N-acyltransferase), (Sankaran and Wu, 1994). The presence of homologues to these three genes identified in the completed sequence of the *M. tuberculosis* genome, (*lgt*, Rv1614; *lspA*, Rv1539; *lnt*, Rv2051c) suggests that a similar mechanism for this modification may be employed by mycobacteria. Moreover, the putative *lspA* gene (signal peptidase II) of *M. tuberculosis* is located downstream of the *ile*S (isoleucyl-tRNA synthetase) gene. This same genomic arrangement has been identified in *Bacillus subtilis* (Prágai *et al.*, 1997) and *E. coli* (Yu *et al.*, 1984), supporting the suggestion that the *lspA* homologue identified in *M. tuberculosis* codes for the mycobacterial lipoprotein signal peptidase.

The functions of lipoproteins identified in Gram positive bacteria are varied, with many having undefined functions, and are reviewed by Sutcliffe and Russell (1995). It has been shown that the 38 kDa

antigen *of M. tuberculosis* is a lipoprotein on the basis of phase partitioning and by labelling with [^{3}H] palmitic acid (Young and Garbe, 1991). This protein has homology to the phosphate binding protein PstS of *E. coli* (Andersen and Hansen, 1989) and its role as a phosphate ion binding protein has been confirmed (Chang *et al.*, 1994; Braibant *et al.*, 1996). The characterised lipoproteins of Gram positive bacteria, appear to have a common role as solute-binding components of the ABC (ATP- binding cassette) transporters (Gilson *et al.*, 1988; Sutcliffe and Russell, 1995).

It has been suggested that the lipid moiety retains the lipoproteins at the cell surface preventing their release to the extracellular medium, a release which would be prevented in Gram negative bacteria by the presence of the outer plasma membrane (Nielsen and Lampen, 1982; Young and Garbe, 1991). However, it is unclear if the lipidation of these molecules by Gram positive bacteria is a prerequisite of their binding function, as the PstS molecule of *Bacillus subtilis* is not a lipoprotein (Takemaru *et al.*, 1996). It has also been noted that some of the lipoproteins of *M. tuberculosis* (e.g. *lppR*) show a degree of homology to the carboxy terminus of the serine-threonine protein kinases (STPK's) identified within the genome (Cole *et al.*, 1997). Whether the lipoproteins have a role in signal transduction has yet to be proved.

The lipoproteins of *M. tuberculosis* identified so far are strongly recognised by the immune system of infected individuals (Jackett *et al.*, 1988; Espitia *et al.*, 1989a; Hewinson *et al.*, 1996; and Bigi *et al.*, 1997). Moreover, synthetic lipopeptide analogues of *E. coli* lipoprotein have been shown to be able to activate B lymphocytes, T lymphocytes and macrophages (Bessler *et al.*, 1985; Deres *et al.*, 1989; and Hauschildt *et al.*, 1990). The ability of synthetic lipopeptides to prime cytotoxic T cells *in vivo* is of particular interest in light of the fact that these cells are thought to play a role in protective immunity to tuberculosis (Zügel and Kaufmann, 1997). It is also interesting to speculate on the role of lipoproteins in eliciting a protective immune response to tuberculosis in light of the observation that clinical

strains of *M. tuberculosis* that do not produce the 19 kDa lipoprotein appear to be more virulent (Lathigra *et al.*, 1996). In contrast, mice immunised with *M. vaccae* expressing the 19 kDa antigen were more susceptible to challenge with *M. tuberculosis* than those immunised with *M. vaccae* alone (Abou-Zeid *et al.*, 1997) Further investigations of the lipoproteins of *M. tuberculosis* may provide clues into their role in TB.

The 19 kDa antigen of *M. tuberculosis* has also been characterised as a glycoprotein by means of a genetic approach. This approach was used to identify regions of the 19 kDa antigen that, when fused to PhoA, directed ConA binding to the hybrid protein expressed by *M. smegmatis*. (Herrmann *et al.*, 1996). Using the same approach, the initial results of this chapter indicate that on the basis of ConA binding the MPT83-PhoA fusion protein is glycosylated. In contrast to previous reports that MPB70 is glycosylated (Fifis *et al.*, 1991), the data in this chapter provide no evidence for this suggestion.

Other antigens of the MTb complex that have been identified as glycoproteins are the 38 kDa antigen (on the basis of ConA binding (Espitia and Mancilla, 1989b) and the 45 kDa antigen (MPT32) for which the glycosidic linkage has been elucidated (Dobos *et al.*, 1996). It is interesting to note that of the glycoproteins mentioned above, three of them are the lipoproteins, i. e. the 19 kDa antigen, the 38 kDa antigen and MPT83. The structurally defined MPT32 is not a lipoprotein but rather has a typical secretion signal peptide of 39 amino acids and an Ala-X-Ala signal peptidase1 cleavage site (Tai, 1986; Laqueyrerie *et al.*, 1995), and also a localisation index distinctive of a secreted antigen (Wiker *et al.*, 1991). These glycoproteins have been shown to be immunodominant during infection (the 19 and 38 kDa antigens following *M. tuberculosis* infection (Havlir *et al.*, (1991); the *M. bovis* BCG counterpart of MPT32 following infection with BCG Pasteur (Romain *et al.*,1993), and MPB83 following infection with *M. bovis* (Fifis *et al.*, (1991)) suggesting a role for these antigens as vaccine candidates.

As well as MPT83, it has been reported that *M. tuberculosis* may contain another 25 kDa antigen that is a lipoglycoprotein. Wadee *et al.* (1987) identified a 25 kDa lipoglycoprotein of *M. tuberculosis* that reduced the intracellular killing ability of polymorphonuclear leukocytes, and inhibitied fusion of phagosomes with lysosomes, as assessed by the transfer of acridine orange from lysosomes to phagosomes. It is unlikely that this protein is MPT83 as the two proteins have strikingly different physio-chemical properties. The 25 kDa protein identified by Wadee *et al.* (1987), was produced in large quantities *in vitro* by *M. tuberculosis* whereas MPT83 is producted at almost undetectable levels by *M. tuberculosis in vitro* (Hewinson *et al.*, 1996). Moreover, the 25 kDa form of MPT83 appears in the void volume fraction after gel filtration (Harboe *et al.*, 1998) as opposed to the bactericidal inhibiting 25 kDa protein which is retained upon gel filtration (Wadee *et al.*, 1987). However, it was reported that removal of either the lipid or carbohydrate portion from the protein identified by Wadee *et al.*, resulted in reversion to normal phagosome-lysosme fusion of polymorphonuclear phagocytes. Whether the carbohydrate or lipid portion of MPT83 possess similar functions remains to be investigated.

The finding that MPT83 is recognised by ConA suggests the presence of mannose residues within the carbohydrate moieties. This suggestion is in agreement with the findings of Fifis *et al.* (1991). whose preliminary chemical analysis of MPB83 identified mannose as a component of the glycan side chains. The structure of the mannose residues attached to the protein backbone of MPT32 (α-D-Man*p*(1→2)-α-D-Man*p*) is akin to the mannose caps identified on the lipoarabinomannan (ManLAM) of *M. tuberculosis* Erdman (Chatterjee *et al.*, 1992), the latter of which have been shown to be involved in phagocytosis mediated by mannose receptors (Schlesinger *et al.*, 1994; Kang and Schlesinger, 1998). This similarity in structure raises the possibility that such glycoproteins may also be involved in attachment and entry to phagocytic cells. The fact that MPT83 is a membrane

bound molecule and is exposed on the surface of tubercle bacilli as determined by antibody binding (Harboe *et al.*, 1998), and its homology with OSF-2 and fascicilin I (Ulstrup *et al.*, 1995), suggest that MPT83 may be involved in cell surface interactions.

The N-terminal mapping experiments described in this chapter identified the amino acid region of MPT83 between residues 35-56 as responsible for ConA binding. The identification of a proline-threonine rich domain within this region suggests the formation of an extended structure containing exposed hydroxyl groups of the threonine residues accessible for O-glycosylation.

Although there is no rigid consensus sequence for O-glycosylation there are general motifs that are present at sites undergoing this modification, including the presence of proximal proline residues (Gooley and Williams, 1994). Comparison of the amino acid regions around the four O-glycosylated threonines of MPT32 (Dobos *et al.*, 1996) with the proline-threonine rich domain of MPT83 (Fig. 4.11) shows that although no amino acid consensus is present, there is striking sequence similarity, supporting the suggestion that this region of MPT83 may be a signal for O-glycosylation with either both or one of the threonine residues at positions 48 and 49 glycosylated.

Fig. 4.11 Amino acid alignment of a proline threonine rich region of MPT83 with glycopeptides of MPT32*

gp1	43	—A P P P V P **T** T A A S P P	——57
gp2	53	— A S P P S **T** A A A P P A P A	——67
gp3	61	— P P A P A **T** P V A P P	——73
gp4	310	— A P T P T **T** P T P Q R	——322
MPT83	42	— P A A P V <u>T T</u> A A M A D P	——55

* Actual sites of O-glycosylation are shown in bold. Hypothetical sites for O-glycosylation are underlined

The use of PhoA as a fusion molecule in the study of protein glycosylation has been used previously to identify similar amino terminal regions of both the 19- and 38 kDa antigen of *M. tuberculosis*, which also contain this proline-threonine rich motif (Herrmann *et al.*, 1996). Their experiments also demonstrated that site directed mutagenesis of the threonine residues present at the amino terminus of the 19 kDa antigen (T→V) abolished ConA binding, suggesting covalent linkage of mannose residues through the hydroxyl groups of those threonine residues.

The noticeable absence of the putative O-glycosylation sequence from MPT70 (Fig. 2.16) raised the possibility that this may be the reason that MPT70 is not glycosylated, as demonstrated by the lack of ConA recognition of the MPT70-PhoA hybrid (page 77). Therefore, it was decided to investigate whether or not the introduction of this region into MPT70 could transform the MPT70-PhoA fusion into a ConA recognizable molecule. The insertion of epitopes into the coding sequences of proteins of both viruses and bacteria has been demonstrated previously, utilizing such molecules as viral coat proteins, toxins and flagellar proteins (Smith, 1985; Gonzalez *et al.*, 1993; Newton *et al.*, 1995; and Chabalgoity *et al.*, 1996). However the results reported here show that insertion of a ten amino acid peptide into the coding sequence of MPT70 led to the degradation of this hybrid molecule in both *E. coli* DH5α and *M. smegmatis* mc^2155 (Fig. 4.9). This degradation may be due to the activity of ATP-dependent proteases such as *La* or *Ti* (Gottesman, 1989).

Degradation of the mycobacterial protein MPB57 by *E. coli* DH5α upon insertion of a nine amino acid epitope within its coding sequence has also been observed (Yamaguchi *et al.*, 1990), suggesting that modification of these proteins may enhance their susceptibility to protease digestion. In contrast, Hetzel *et al.* (1998) have successfully demonstrated the introduction of a variety of epitopes into the secreted protein SOD of *Mycobacterium tuberculosis*, and expression of these chimeric molecules in recombinant mycobacterial systems.

However, the experiments of Hetzel *et al.*, (1998) benefited from knowledge of the complete three-dimensional structure of SOD, allowing the various epitopes to be introduced into an extended loop far from fuctional sites of the protein. In light of the results obtained with SOD, it may well be the case that the successful introduction of such an epitope into MPT70 will require structural knowledge of the protein, such as X-ray crystallography data, which in turn may provide some clues as to the functions of MPT70 and MPT83.

Chapter 5

Identification of amino acid residues within MPT83 that are associated with carbohydrate attachment

5.1 Introduction

It was shown in chapter 4 that ConA binding to MPT83 could be attributed to an amino terminal portion of the protein when expressed in *M. smegmatis* mc^{2}155. This region contained a ten amino acid run containing threonine residues that was absent from the non-glycosylated MPT70. Introduction of this motif into MPT70 resulted in degradation of the recombinant protein when expressed by *M. smegmatis*. Consequently an alternative approach was sought to define the glycosylation sites within MPT83.

The ability to manipulate protein structures by altering individual amino acid residues by site-directed mutagenesis (SDM) has helped to elucidate many classical problems such as the mechanistic basis of enzymatic activity, protein-protein interactions and protein folding, reviewed by (Ackers and Smith, 1985). Site-directed mutagenesis has also been widely used to investigate the function of carbohydrate moieties present on glycoproteins. This has been achieved by altering the amino acid residues to which the carbohydrate is attached, and studying the effects on the non-glycosylated mutants (Bocchinfuso *et al.*, 1992; Delorme *et al.*, 1992; Fan *et al.*, 1997; and Panka, 1997).

Alteration of threonine residues to valine within the 19 kDa antigen of *M. tuberculosis* abolished ConA binding to this molecule, providing strong evidence for O-glycosylation through the hydroxyl group of a threonine residue (Herrmann *et al.*, 1996). This chapter describes the use of SDM in conjunction with ConA blotting to identify amino acids within MPT83 that are involved in its glycosylation

5.2 Results

5.2.1 SDM of threonine residues at positions 48 and 49 of MPT83

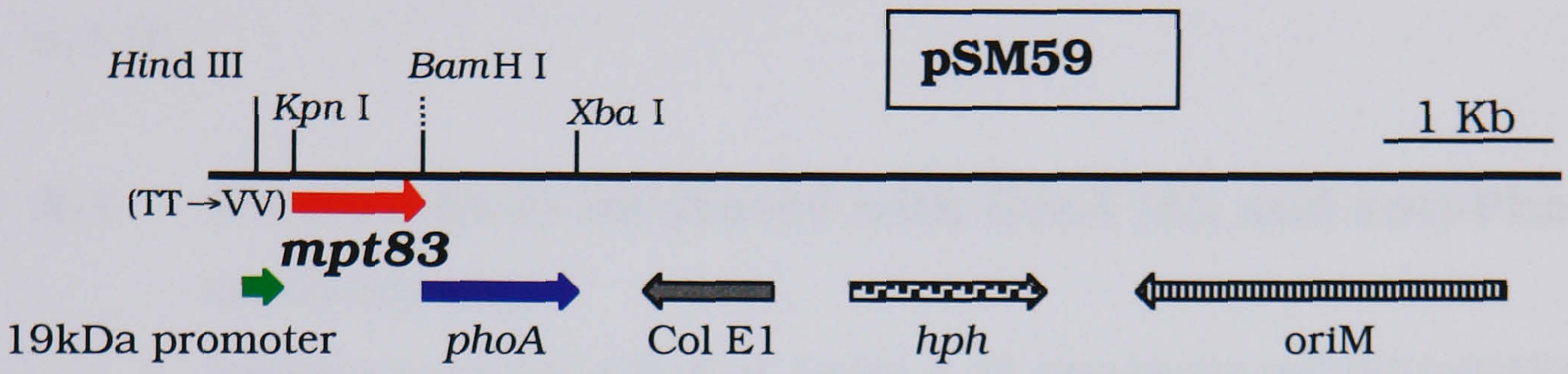
In the previous chapter deletion mapping of MPT83 revealed that the MPT83 63aa-PhoA fusion (pSM57, Fig. 4.5) was recognised by ConA (Fig. 4.6A, lane 5) whereas the 35aa-PhoA fusion (pSM55, Fig. 4.5) was not recognised (Fig. 4.6A, lane 3), indicating that carbohydrate may be covalently attached to an amino acid residue within this region. It was therefore proposed (page 88) that the presence of a ten amino acid region, within amino acid positions 35 and 63 of MPT83, that is absent from the non-glycosylated MPT70 (shown below and Fig. 2.16), was a possible motif for O-glycosylation mediated via a glycosidic bond involving a hydroxyl group on the side-chain of one or more of the threonine residues.

MPT83	**S**	**P**	**A**	**A**	**P**	**V**	**T**	**T**	**A**	**A**	**M**	**A**	**D**	**P**	**A**	**A**	**57**
MPT70	**S**	**P**	**P**	**A**	**A**	-	-	-	-	-	-	-	-	-	**A**	**G**	**31**

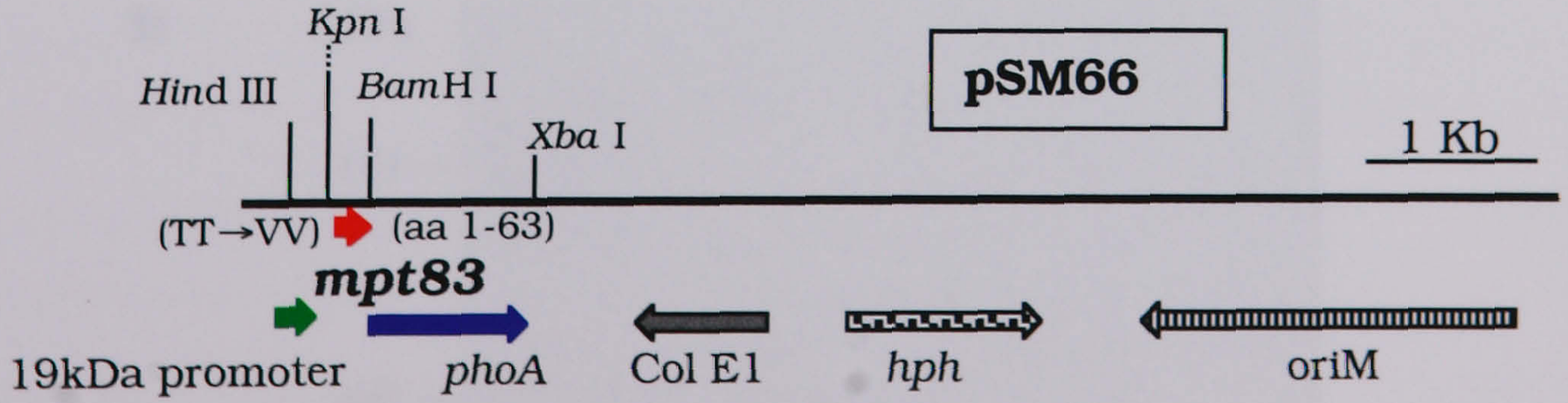
To investigate this possibility the two threonine residues at positions 48 and 49 of MPT83 were mutated to valine residues using the approach of Herrmann *et al.* (1996) as described in section 8.4. This modification replaces the side-chain hydroxyl group with the similarly weighted methyl group which prevents the formation of a glycosidic linkage.

As described in Fig.8.2, the mutagenic primers encoding the double threonine to valine mutation, 24 and 25 (appendix 1), were used in conjunction with reverse and forward primers for *mpt83* primers respectively (Nos. 10 and 9 respectively (appendix 1)) to amplify a 5' and a 3' portion of *mpt83* from pSM52 (Fig. 4.3).

The two amplification products overlapped by 36 bp, which incorporated the mutations. Equimolar amounts of both amplification products were combined and amplified using primers 9 and 10 (appendix 1) to generate a complete *mpt83* gene containing the desired mutation. This mutated gene was digested with *BamH* I and *Kpn* I and cloned into the vector pSMT3-19-PhoA (Fig. 8.1D) to generate the construct pSM59.



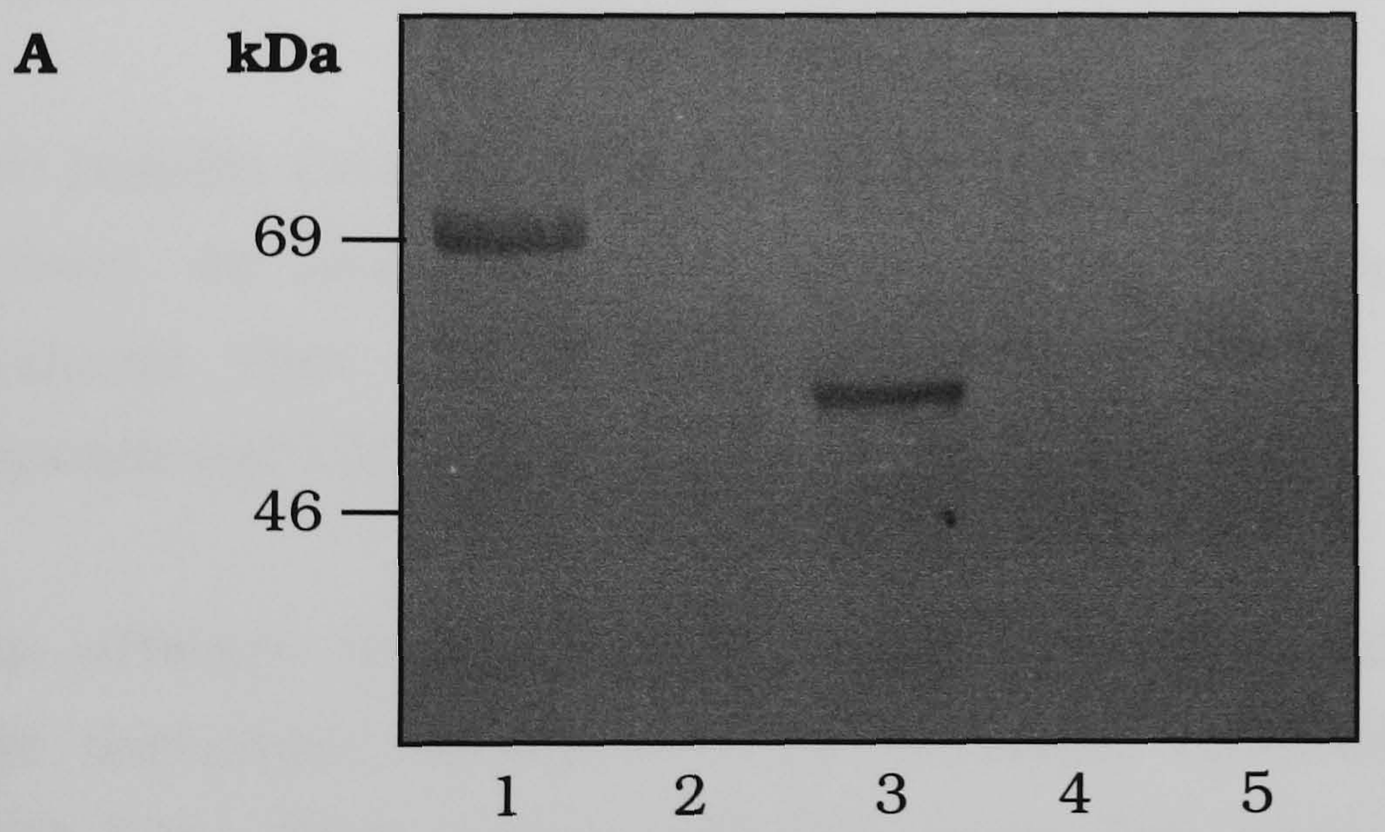
The modified *mpt83* gene was sequenced using the sequencing primers 13-16 (appendix 1), to confirm that the desired mutations had been introduced and that no inadvertant mutations had occurred during the PCR process. This construct (pSM59) was then used as a template to generate a 5' portion of *mpt83*, by PCR amplification using the primres 9 and 20 (appendix 1), which codes for the first 63 amino acids of MPT83 containing the TT(48,49)→VV mutation. This amplification product was digested with *BamH* I and *Kpn* I and cloned into the vector pSMT3-19-PhoA to give the plasmid pSM66 (shown below). This cloned fragment of *mpt83* was sequenced using the primers 15 and 16 (appendix 1) to reaffirm the presence of the threonine to valine mutations within this clone.



This construct (pSM66) encoding the modified MPT83 63aa-PhoA was transformed into *M. smegmatis* mc^{2}155. Transformants appeared as blue colonies on XP indicator plates confirming that the PhoA fusion was in frame with the amino terminus of MPT83. Sonicated extracts of both *M. smegmatis* and *E. coli* containing plasmids encoding the native (pSM57, page 78) and the modified MPT83 63aa-PhoA fusion (pSM66) were separated by SDS-PAGE (12.5% separating gel), Western blotted and incubated with ConA (Fig. 5.1 A) and anti-PhoA antibody (Fig. 5.1 B).

Fig. 5.1 Western blots incubated with ConA (A), and anti-PhoA antibody (B).

Sonicated extracts (10μg) of, lanes: 1, *M. smegmatis* mc^{2}155 (pSM52), positive control; 2, *M. smegmatis* mc^{2}155 (pSM66, MPT83 aa 1-63, TT→VV); 3, *M. smegmatis* mc^{2}155 (pSM57, MPT83 aa 1-63,); 4, *E. coli* DH5α (pSM66) and 5, *E. coli* DH5α (pSM57).



As was observed previously (Fig. 4.6 A, lane 5), the unmutated MPT83 63aa-PhoA fusion expressed by *M. smegmatis* mc^2155 (pSM57) was recognised by ConA (Fig. 5.1A, lane 3). In contrast, mutation of threonine to valine at positions 48 and 49 within the MPT83 63aa-PhoA fusion, (pSM66) resulted in ablation of ConA binding to this fusion protein when expressed by *M. smegmatis* mc^2155 (Fig. 5.1A, lane 2). As observed from the anti-PhoA blot (Fig.5.1B) of these recombinant proteins, the native MPT83 63aa-PhoA fusion also appeared to have a higher relative molecular weight compared to the mutated fusion (Fig. 5.1 B, lanes 2 and 3). This apparent size difference between the native and modified fusion expressed by *M. smegmatis* mc^2155 (Fig. 5.1A, lanes 2 and 3) is not solely attributable to the changes made in the polypeptide backbone (TT→VV) since both fusion proteins expressed by *E. coli* DH5α appear to have more similar relative molecular weights (Fig. 5.1B, lanes 4 and 5) than when expressed in *M. smegmatis*. Moreover, neither fusion protein is recognised by ConA when expressed in *E. coli* (Fig. 5.1A, lanes 4 and 5)

These results confirm an essential role for the threonine residues at positions 48 and 49 of MPT83 in ConA binding, supporting the hypothesis that MPT83 is O-glycosylated when expressed by *M. smegmatis* mc^2155.

In an attempt to determine whether both threonine residues or a single threonine residue was glycosylated, modified versions of the MPT83 63aa-PhoA fusion (pSM57, page 78)) were created in which either the threonine residue at position 48 (pSM67), or the threonine residue at position 49 (pSM68), were mutated to valine. These constructs were generated from pSM52 as described above for pSM66, using the mutagenic primers 26 and 27 (appendix 1) for pSM67, and mutagenic primers 28 and 29 (appendix 1) for pSM68.

Both constructs were sequenced to confirm the introduction of the desired mutation (appendix 3, A3.3) with the sequencing primers primers 15 and 16 (appendix 1). Sequenced constructs were

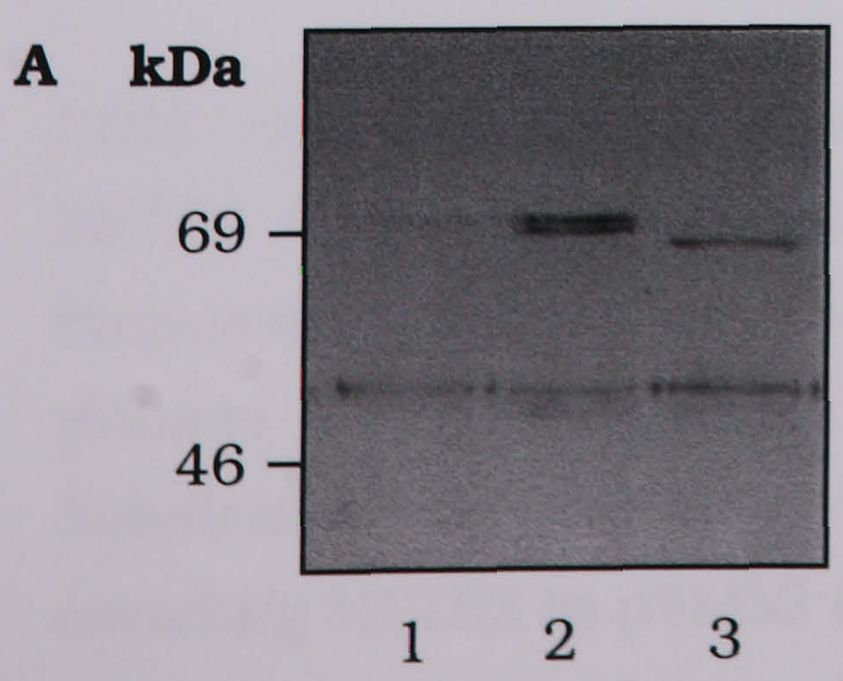
transformed into *M. smegmatis* mc^{2}155 producing blue colonies on XP indicator plates. However, repeated attempts at ConA blotting of sonicated extracts of *M. smegmatis* containing pSM67 and pSM68 failed to generate any ConA reactive bands (data not shown). This suggests that either both threonines are modified or, that if only one is modified there may be a prerequisite for that particular residue to be next to another threonine residue.

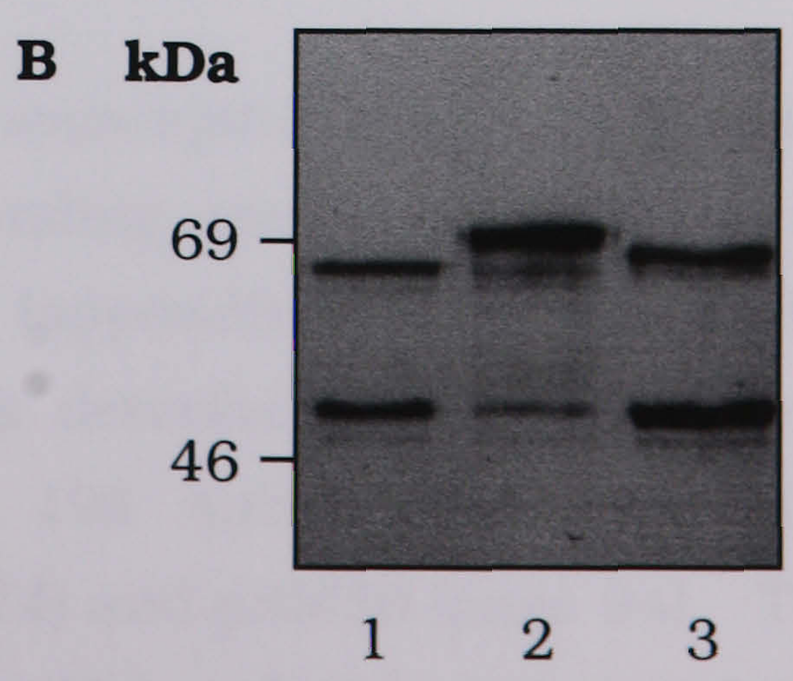
5.2.2 MPT83 contains additional glycosylation sites to the amino terminal O-glycosylation site

In contrast to the inability of the mutated MPT83 63aa-PhoA fusion (TT→VV; pSM66) to bind ConA when expressed by *M. smegmatis* mc^{2}155 (Fig. 5.1A, lane2), the presence of the same mutation in the full length MPT83-PhoA fusion (pSM59, page 94) did not ablate ConA binding. The full length mutated MPT83-PhoA fusion (TT(48,49)→VV) was recognised as a single band by ConA when expressed by the mycobacterial host (Fig. 5.2A, lane 3). This is in contrast to the native MPT83-PhoA fusion which, when expressed by *M. smegmatis* mc^{2}155 (pSM52) is recognised as a doublet by ConA and has a larger relative molecular weight than the mutated form which can be attributed to the lack of glycosylation (Fig. 5.2A, lane 2). Neither of these PhoA fusions were recognised by ConA when expressed by *E. coli* (data not shown).

Fig. 5.2 Western blots incubated with ConA (A), and anti-PhoA antibody (B)

Sonicated extracts (10μg) of, lanes: 1, *M. smegmatis* mc^{2}155 (pSM53); 2, *M. smegmatis* mc^{2}155 (pSM52, MPT83-PhoA); 3, *M. smegmatis* mc^{2}155 (pSM59, MPT83-PhoA, TT(48,49)→VV).





5.2.3 Identification of additional protein glycosylation sites within MPT83

The identification of an O-linked glycosylation site at the amino terminus of MPT83 was initially determined by amino terminal mapping in conjunction with comparison of the amino acid sequences between MPT70 and MPT83 (sections 4.2.5 and 5.2.1). It was therefore reasoned that it might be possible to identify the additional glycosylation sites present in MPT83 by direct comparison of the amino acid sequences of MPT83 and the non-glycosylated MPT70. Such analysis revealed a classical N-glycosylation site (Bause, 1983) at amino acid position 205 of MPT83. However, an identical site was present in MPT70. Thus, it was decided to look for other possible differences between MPT83 and MPT70 that might be responsible for glycosylation and ConA binding.

Further comparisons between the amino acid sequences of MPT83 and MPT70 highlighted three further potential sites for attachment of carbohydrate that are present in MPT83 but absent in MPT70. These are; a threonine residue at amino acid position 88, a serine residue at position 101 and an asparagine residue at position 192. The asparagine residue at position 192 was chosen for initial further investigations for several reasons. Firstly, like the threonine residues at positions 48 and 49 of MPT83, the asparagine at position 192 is located within a region of MPT83 that is least similar to MPT70, and secondly it has been shown that the 19 kDa antigen of *M. tuberculosis* is glycosylated through an asparagine residue at the carboxy terminus (J. L. Herrmann personal communication).

SDM was used to substitute the asparagine residue at position 192 of MPT83 for glycine, the corresponding residue in MPT70, using the mutagenic primers 21 and 22 (appendix 1), in conjunction with primers 3 and 4 (appendix 1), as described in detail in section 8.4. Substrates for the asparagine 192 substitution were the genes encoding MPT83 in pSM52 (page 74) and pSM59 (page 94). The altered genes were recloned into the *BamH* I and *Kpn* I sites of pSMT3-19-

phoA (Fig.8.1D) to generate the plamids pSM64 and pSM65. Fig. 5.3 describes the different recombinant MPT83-PhoA fusions expressed from these plasmids by *M. smegmatis* mc^2155.

Fig. 5.3 Site directed mutations introduced into the MPT83-PhoA fusion protein

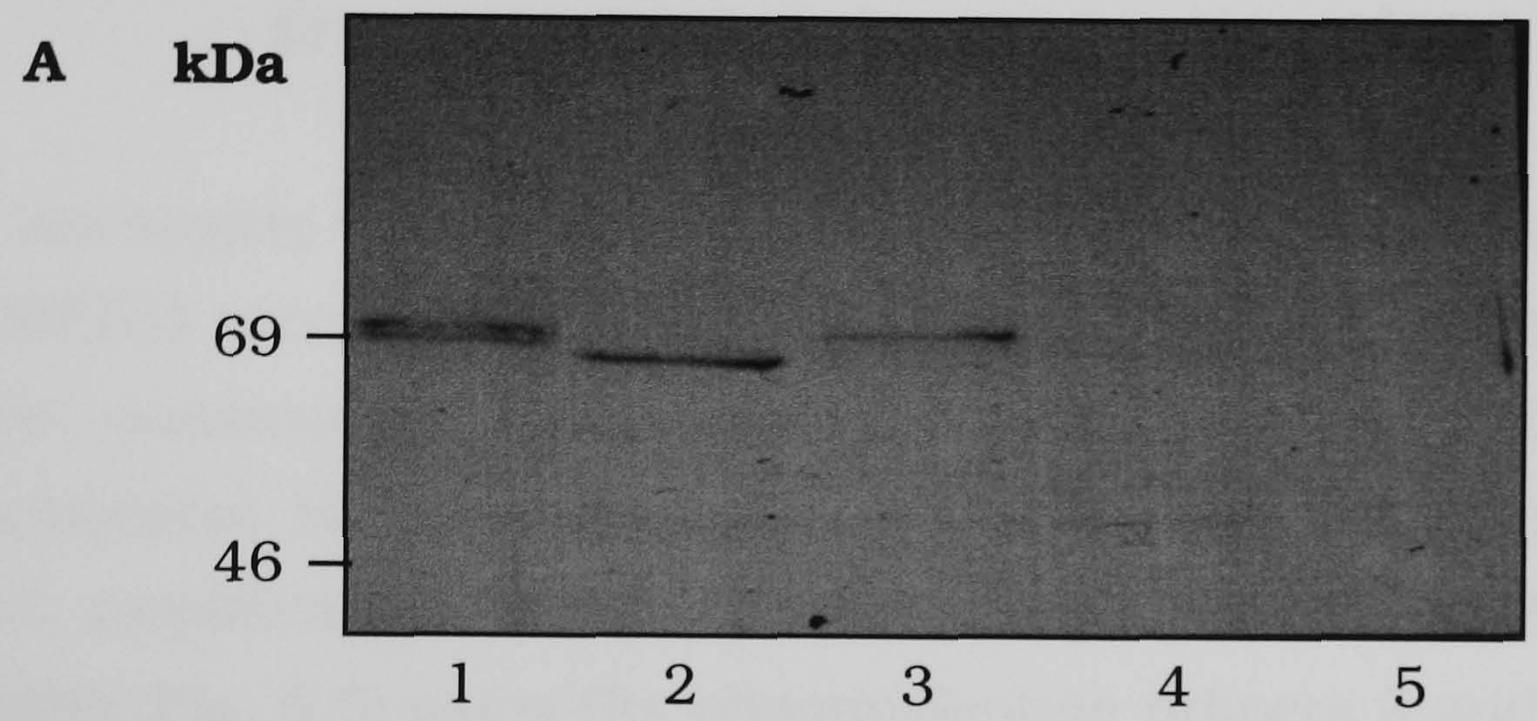
Site-directed mutations within the MPT83-PhoA fusion	Recognition by ConA
pSM52 	+ve
pSM59 	+ve
pSM64 	+ve
pSM65 	-ve

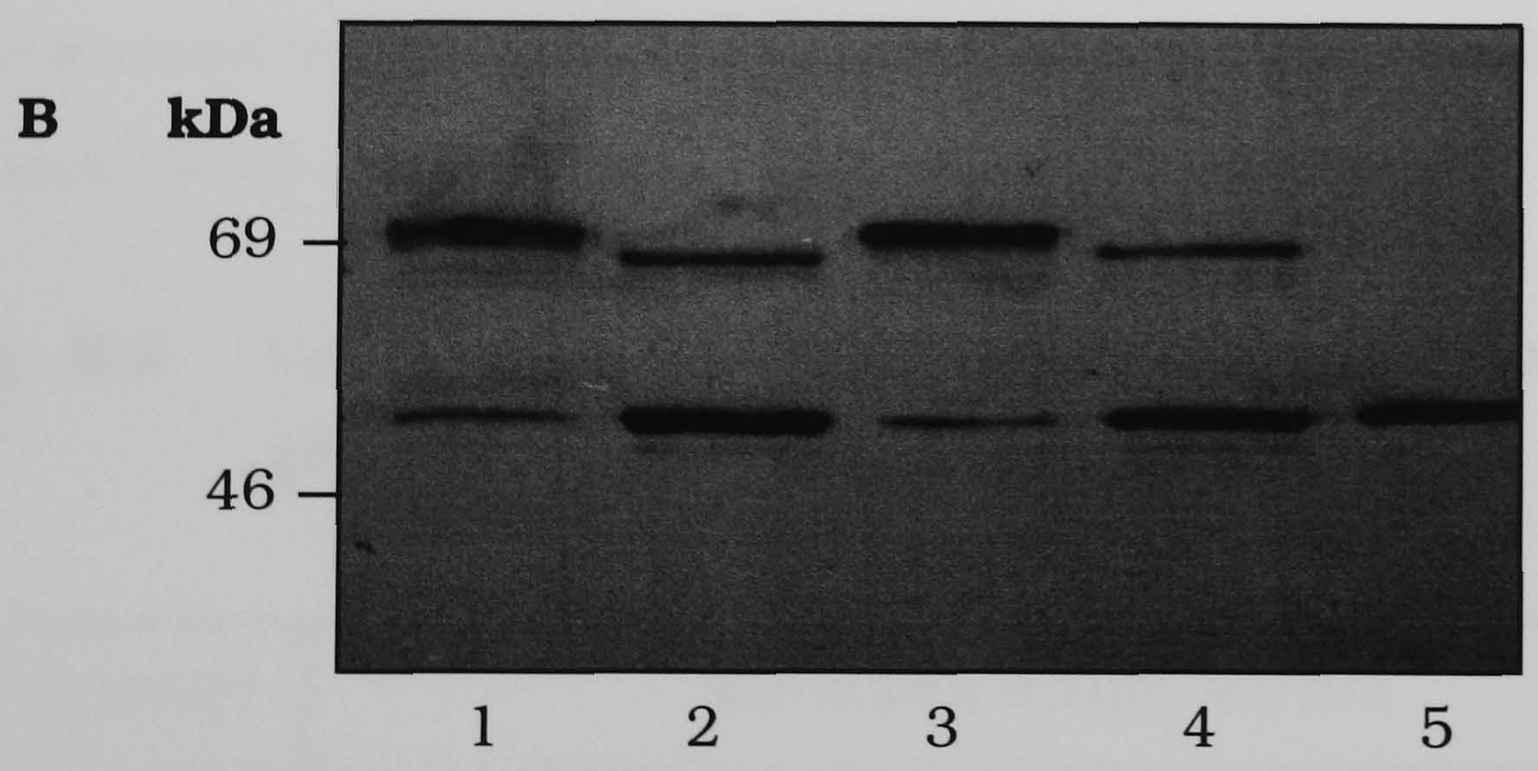
The *mpt83* gene within pSM64 and pSM65 was sequenced using the sequencing primers13-16 (appendix 1), to confirm that the desired mutations had been correctly introduced and that no other mutations had been generated (asparagine to glycine mutations for pSM64 and pSM65 are shown appendix 3, A3.4). Sonicated extracts of *M. smegmatis*, containing the constructs described in in Fig. 5.3 were separated by SDS-PAGE (12.5% separating gel), Western blotted and incubated with ConA (Fig. 5.4A) and anti-PhoA antibody (Fig. 5.4B). The results of the ConA blotting are summarised in Fig. 5.3. All of the PhoA fusions described in Fig. 5.3 were expressed at approximately equal levels as determined by blotting with the anti-PhoA antibody (Fig. 5.4B).

As was previously observed (Fig. 5.2A, lane 2 and 3), the native MPT83-PhoA fusion (pSM52, page 74) expressed by *M. smegmatis* mc^2155 is recognised by ConA as a doublet (Fig. 5.4A, lane 1), whereas mutation of the threonines at positions 48 and 49 to valine within MPT83 (pSM59), resulted in the production of a fusion protein that was recognised by ConA as a single band (Fig. 5.4A, lane 2).

Fig. 5.4 Western blots incubated with ConA (A), and anti-PhoA antibody (B)

Sonicated extracts (10μg) of, lanes: 1, *M. smegmatis* mc²155 (pSM52), positive control; 2, *M. smegmatis* mc²155 (pSM59 TT(48,49)→VV); 3, *M. smegmatis* mc²155 (pSM64 N(192)→G; 4, *M. smegmatis* mc²155 (pSM65 TT(48,49)→VV, N(192)→G) and 5, *M. smegmatis* mc²155 (pSMT3-19-phoA, vector control)





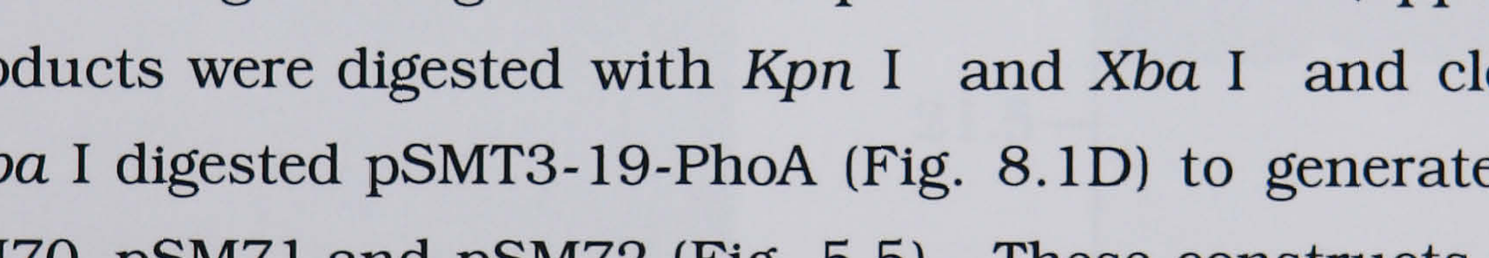
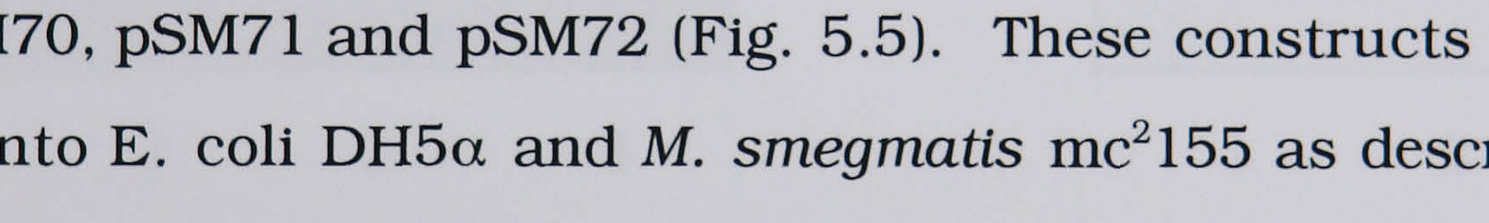
Similarly, substitution of asparagine at position 192 to glycine within MPT83 also resulted in the production of an MPT83-PhoA fusion protein that, when expressed by *M. smegmatis* mc²155, was recognised by ConA as a single band (Fig. 5.4 A, lane 3). These findings demonstrate that mutation of either a threonine doublet at positions 48 and 49, or an asparagine residue at position 192 of MPT83, results

in the reduction of Con A binding to MPT83. This suggests a role for both these regions in the glycosylation of MPT83 by *M. smegmatis* mc^{2}155. This hypothesis is supported by the observation that when both mutations are combined (pSM65, page 99), the recombinant fusion protein produced does not bind ConA indicating that MPT83 is both O- and N-glycosylated by mycobacteria (Fig. 5.4 A, lane 4).

5.2.4 Expression of glycosylated and non-glycosylated forms of MPT83 by *M. smegmatis* mc^{2}155

To investigate whether the relative molecular weights of mutated forms of MPT83 correlated with the proposed lack of glycosylation, plasmids were constructed that express glycosylated MPT83 and non-glycosylated MPT83 without the PhoA fusion. This was achieved by PCR amplification of *mpt83* from the plasmids pSM52, pSM59 and pSM65 (Fig. 5.3) using the oligonucleotide primers 9 and 36 (appendix 1). These products were digested with *Kpn* I and *Xba* I and cloned into *Kpn* I/*Xba* I digested pSMT3-19-PhoA (Fig. 8.1D) to generate the plasmids pSM70, pSM71 and pSM72 (Fig. 5.5). These constructs were transformed into E. coli DH5α and *M. smegmatis* mc^{2}155 as described in section 8.5.

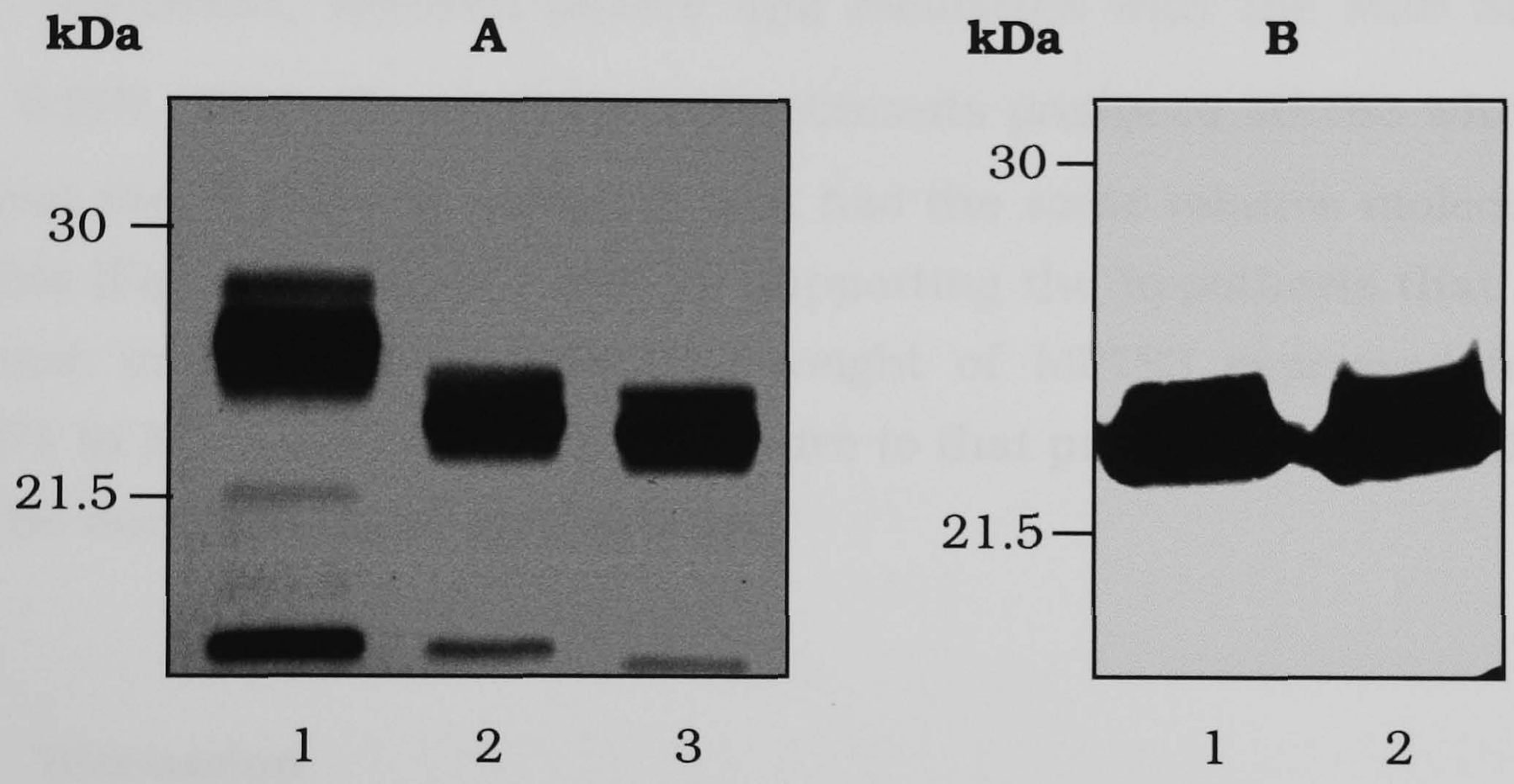
Fig. 5.5 Constructs expressing glycosylated and non-glycosylated MPT83.

Construct	Mutations within MPT83
pSM70	
pSM71	
pSM72	

Sonicated extracts of recombinant *M. smegmatis* mc²155 expressing the constructs shown above were separated by SDS-PAGE (15% separating gel), Western blotted and incubated with the monoclonal antibody SB10 (Fig. 5.6A).

Fig. 5.6 Western blots incubated with MAb SB10.

(A) Sonicated extracts (5 μg) of *M. smegmatis* mc²155 containing, lanes: 1, pSM70 (MPT83); 2, pSM71 (MPT83, TT(48,49)→VV); 3, pSM72, (MPT83, TT(48,49)→VV, N(192)→G. (B) Sonicated extracts (5 μg) of *E. coli* DH5α containing, lanes: 1, pSM71, 2, pSM72.



Introduction of a double threonine to valine substitution at positions 48 and 49 (pSM71) resulted in a form of MPT83 that has a lower relative molecular weight than the unmodified MPT83 (pSM70) when produced in *M. smegmatis* mc²155 (Fig. 5.6A, lanes 1 and 2). This reduction in relative molecular weight correlates with the proposed absence of carbohydrate due to removal of the amino terminal O-glycosylation site of MPT83. Furthermore, introduction of an asparagine to glycine mutation in addition to the double threonine substitution (pSM72) resulted in a further, albeit smaller, reduction in

relative molecular weight of MPT83 when expressed in *M. smegmatis* mc^2155 (Fig. 5.6A, lanes 2 and 3). This small reduction in weight is proposed to be due to the lack of N-glycosylation of MPT83 by *M. smegmatis* mc^2155 effected by alteration of the proposed glycosylated asparagine at position 192.

To confirm that the small size reduction between MPT83, containing the N(192)→ G mutation, expressed by *M. smegmatis* relative to MPT83 without such a mutation (Fig. 5.6A, lanes 2 and 3) was not due to the differences in amino acid substitution, both constructs were expressed in *E. coli* DH5α, Western blotted and incubated with the MAb SB10 (Fig. 5.6B). Both *E. coli* DH5α recombinants produced MPT83 with or without the N(192)→ G mutation that had the same relative molecular weights (Fig. 5.6B, lanes 1 and 2), supporting the hypothesis that the increase in the relative molecular weight of MPT83 expressed from pSM71 in *M. smegmatis* mc^2155, relative to that produced from pSM72, may be due to N-linked glycosylation.

5.3 Discussion

The ablation of ConA binding by site-directed mutation of threonine residues at amino acid positions 48 and 49 to valine within an MPT83 63aa-PhoA fusion (Fig. 5.1A, lane 2), is in agreement with the hypothesis that MPT83 is O-glycosylated by *M. smegmatis* mc^2155. This result is in agreement with the findings of Fifis *et al.* (1991) who purified MPB83 from *M. bovis* AN5 and proposed that it was glycosylated. Their analysis of MPB83 by gas chromatography-mass spectrometry showed that the protein contained mannose (68% wt/wt of total carbohydrate) and arabinose (32% wt/wt).

The preliminary carbohydrate analysis performed by Fifis *et al.* (1991), the recognition of MPT83 by ConA presented in this chapter and the similarity of the amino terminus of MPT83 to the O-linked glycopeptides of MPT32 (Fig. 4.11, Dobos *et al.*, 1996) suggest the

possibility of a Man-Thr O-linkage within MPT83 when expressed by *M. smegmatis* mc^{2}155. In support of this hypothesis, experiments undertaken by Dr. Howard Cooper at St. Mary's hospital, London (personal communication), have demonstrated that membrane fractions of *M. smegmatis* are able to attach radiolabelled mannose ([^{14}C] GDP-mannose) to a peptide of MPT83 containing the identified O-glycosylation site (Fig. 7.3, peptide 5, page124). Furthermore, Fifis *et al.* (1991) also demonstrated a reduction in relative molecular weight of purified MPB83 following treatment with the enzyme α-mannosidase.

Experiments performed by Herrmann *et al.* (1996) showed that removal of the O-glycan chains from the 19 kDa antigen of *M. tuberculosis*, by SDM of a threonine cluster at the amino terminus of this antigen, led to aberrant processing of the proteins signal peptide when expressed by *M. smegmatis* and *M. vaccae*. Consequently the authors proposed that glycosylation by mycobacteria may protect against proteolysis thus acting as a control for the release of surface attached lipoglycoproteins. However, no such degradation was observed for MPT83 expressed by *M. smegmatis* mc^{2}155 when a similar mutation was introduced in to the protein (Fig. 5.6A, lane 2), suggesting that in the case of MPT83 protein glycosylation may serve some other function.

Protein O-glycosyation in yeast results in similar protein-carbohydrate linkages to those characterised for MPT32 (Dobos *et al.*, 1996). The enzymes responsible for this O-mannosylation in yeast have been well characterised and constitute a family of protein mannosyltransferases (PMT) (Gentzsch and Tanner, 1996). Amino acid alignment of PMT 1 and PMT 2 reveal a highly conserved region of 26 amino acids "HTVTRCRLHSHDHKPPVSESSDWQE'. This conserved sequence was used in conjunction with the program 'TBLASTN' (Altschul *et al.*, 1990) to search MycDB (http://kiev.physchem.kth.se/MycDB/seqsearch_form.html) for the presence of any mycobacterial homologues to the

PMT family. The only reasonable match was with the sequence shown over.

Query	4	T R C R L H S H	11
		T R C R H H	
Subject	9	T R C R P H R H	16

The subject sequence was from the EMBL entry U00023_cd2. The matching ORF had been designated a putative glycosyltransferase from *M. leprae*. Amino acid alignment of this translated sequence with the protein sequences of PMT1 and/or PMT2 revealed no further homology (data not shown). However, the DNA sequence of this *M. leprae* gene was found to have homology to a putative rhamnosyltransferase from *M. avium* (accession number AF060183) and two ORF'S from *M. tuberculosis* (accession number MTCY19G5, ORF's 2 and 4c).

The putative rhamnosyltransferase has now been characterised and has been shown to be involved in the transfer of rhamnose to the glycopeptidolipid of the *M. avium* serovar Ser 2 (Eckstein *et al.*, 1998). The authors also noted the presence of the *M. tuberculosis* homologues and have proposed that they may have some role in the synthesis of phenolic glycolipids and not in protein glycosylation although this has yet to be proved.

Nevertheless, the presence of dolichol-linked monosaccharides in the archaebacteria *Haloferax volcanii* (Kuntz *et al.*, 1997), and the presence of mannophospholipids within *M. smegmatis* (Takayama *et al.*, 1973) supports the hypothesis that such molecules may serve as mannose donors for protein 0-mannosylation in mycobacteria.

Substitution of the threonine residues within the MPT83-PhoA fusion caused a reduction in ConA binding to the mutant protein relative to the native MPT83-PhoA fusion protein but did not prevent ConA binding (Fig. 5.4) indicating the presence of other protein glycosylation sites within MPT83. By comparing the amino acid sequence of MPT83

with that of the non-glycosylated MPT70, an asparagine at position 192 of MPT83 was identified as a possible site for N-glycosylation.

Substitution of this asparagine within the MPT83-PhoA fusion led to the production of a protein that, when expressed in *M. smegmatis* mc^2155, bound ConA, although at a lower level than that of the native MPT83-PhoA fusion protein (Fig. 5.4). Combining the asparagine substitution with the double threonine substitution within an MPT83-PhoA fusion abolished ConA binding to this mutant fusion protein expressed by *M. smegmatis* mc^2155. These results support the hypothesis that the asparagine at position 192 of MPT83 is glycosylated. Taken together these results suggest that MPT83 expressed by *M. smegmatis* mc^2155 is both O and N-glycosylated.

Fig. 5.7 Proposed model for the glycosylation of the antigen MPT83



Reports of N-linked glycosylation in bacteria are extremely limited with most restricted to crystalline surface layer (S-layer) proteins of the archaebacteria (Messner and Sleytr, 1988; Messner, 1997; and Moens and Vanderleyen, 1997). The only report of a non S-layer N-glycosylated protein from eubacteria is that of the major outer membrane protein (MOMP) from *Chlamydia trachomatis* (Kuo *et al.*, 1996). *Chlamydia trachomatis* is an obligate intracellular bacterium. The MOMP studied by Kuo *et al.* (1996) was obtained from *C. trachomatis* grown in HeLa cells and was shown to be N-glycosylated with oligosaccharides mainly of the 'high mannose' type (Kobata, 1994). The structure of this oligosaccharide is identical to that of N-linked glycans seen in eukaryotes (Herscovics and Orlean, 1993).

The synthesis of this complex glycan moiety has been studied extensively in higher eukaryotes and the yeast *Saccharomyces cerevisiae*. Attachment of the $Glc_3Man_9GlcNAc_2$ core to the asparagine residue involves many glycosyltransferases and glycosidases which have been shown to act on both the lumenal and cytosolic face of the endoplasmic reticulum (Abeijon and Hirschberg, 1992). The fact that *C. trachomatis* does not possess an endoplasmic reticulum raises the possibility that the glycosylation of the MOMP analyzed by Kuo *et al.* (1996) may have been due to the glycosylation machinery of the HeLa cell and not by the bacteria.

Western blot analysis of recombinant *M. smegmatis* expressing a panel of SDM's of MPT83, with the monoclonal antibody SB10, demonstrated a range of relative molecular weights for the various forms of MPT83 (Fig.5.6 A, page 102). The relative molecular weights of these mutant forms of MPT83 correlates with the lack of proposed glycosylation resulting from the substitution of putative glycosylation sites. Interestingly, substitution of the N-glycosylation site (asparagine residue to glycine at position 192) of MPT83 coupled with the alteration of the O-glycosylation site (TT→VV at positions 48 and 49) only led to a small reduction in molecular weight of MPT83 relative to the threonine mutant alone. This suggests that the carbohydrate moiety present at the N-glycosylation site of MPT83 is very simple.

The experiments described in this chapter support the hypothesis that the asparagine residue at position 192 of MPT83 is glycosylated, although it is acknowledged that this asparagine does not constitute the typical recognition sequence or 'sequon', Asn-Xaa-Thr/Ser, for N-glycosylation. The hydroxyl group present on the amino acid side chain of either the threonine or serine residue, within the sequon for N-glycosylation, has been proposed to participate in the catalytic mechanism of glycosyl transfer by means of a proton-relay system (Bause and Legler, 1981). The proposed essential role for the hydroxy amino acid within the sequon (Bause, 1983) is based on the *in vitro* transfer of [^{14}C] carbohydrate to the hexapeptide Tyr-Asn-Gly-Xaa-

Thr(Ser)-Val. If such a proton relay system as proposed by Bause and Lehler (1981) is required for transfer of the carbohydrate to the amino acid side chain of asparagine it is possible that some other residue within the polypeptide backbone of MPT83 may serve as the proton donor.

The enzyme catalyzing the transfer of the $Glc_3Man_9GlcNAc_2$ core to the asparagine residue is the oligosaccharyltransferase (OST). The OST is a multimeric protein containing at least eight different polypeptide chains (Karaoglu *et al.*, 1997) the interactions of which have not been defined, making it impossible to speculate on the catalytic mechanism of this enzyme *in vivo*. TBLASTN searches (Altschul *et al.*, 1990) of MycDB using the amino acid sequence of these polypeptides constituting the OST, failed to reveal any homologous proteins, suggesting that the mechanism of N-glycosylation by bacteria may be distinct from that observed for eukaryotes and may involve a novel protein-sugar linkage.

It is known that bacteria can differ markedly from eukaryotes in their patterns of protein glycosylation. For example the pilin of *Neisseria meningitidis* is O-glycosylated with a 2,4-diacetamido-2,4,6-trideoxyhexose moiety (Stimson *et al.*, 1995). Moreover, a peptide of MPT83 containing the putative N-glycosylation site (Fig. 7.3, peptide 20) failed to incorporate radiolabeled GlcNAc ([^{14}C] UDP-GlcNAc), when incubated with membrane fractions of *M. smegmatis* (Dr. H. Cooper, St. Mary's hospital, London, personal communication). Furthermore, treatment of sonicated extracts of *M. smegmatis* mc^{2}155::pSM43 (page 44) with the enzyme PNGaseF, which is specific for the linkage unit Asn- GlcNAc, failed to cause a reduction in the relative molecular weight of MPT83 (data not shown). These results support the suggestion that MPT83 may be glycosylated at Asn 192 with a sugar residue other than GlcNAc. Supporting this hypothesis is the observation that an asparagine residue present in laminin is N-glycosylated via a β-glucose-asparagine linkage (Schreiner *et al.*, 1994). It is quite possible that such a case is also applicable to MPT83.

Further chemical analysis of this protein is required to confirm these results and to define the carbohydrate moiety and also to characterise the actual peptide - sugar linkages as has been described for MPT32 (Dobos *et al.*, 1996) Such analysis requires the protein in a highly purified form to ensure that no non-covalently bound carbohydrate is associated with the protein.

Approaches to obtain purified protein from sonicated extracts of *M. smegmatis* mc^2155 expressing MPT83 from the plasmid pSM43 (page 43), and from *M. bovis* BCG Tokyo have met with little success due to apparent co-purification of LAM with MPT83 (data not shown). This may have been due to the initial purification step involving isolation of the protein from the detergent phase of a Triton X-114 extraction performed on sonicated extract of the bacteria. This method was used as MPT83, in contrast to MPT32, is associated with the cell wall and not present in large amounts in the culture filtrate. An alternative srtategy may be to retrieve the protein from a 2-D PAGE gel if sufficient amounts can be resolved.

In parallel with attempts to purify a glycosylated form of MPT83 it was also decided to look at immune responses to this antigen. Part 3 describes some of the effects protein glycosylation can have on antigen presentation and recognition, and describes some preliminary data on the effect of glycosylation by mycobacteria with respect to T cell responses to MPT83.

Part 3

T-cell recognition of MPT83

Chapter 6

T-cell recognition of antigens

6.1 T-Lymphocytes

T-lymphocytes are the effector cells of the cell-mediated immune response to intracellular parasites. They originate from stem cells in the bone marrow and continue their maturation in the thymus gland.

Facultative intracellular bacteria such as the pathogenic mycobacteria, leishmania, salmonella and yersinia, have adapted to replicate within cells, particularly macrophages. Such pathogens have evolved to subvert the innate killing mechanisms of these phagocytic cells and thus, are provided with an intracellular niche which affords protection from the humoral immune response (Kuhn *et al.*, 1988; Chan *et al.*, 1991; Sturgill Koszycki *et al.*, 1994; and Yamamoto *et al.*, 1997). However, presentation of processed antigens of the intracellular bacterium in the context of major-histocompatability complex (MHC) antigens results in recognition of infected cells by T-lymphocytes.

6.1.1 $CD4^+$ T lymphocytes

A sub-population of T cells bearing the CD4 cell surface marker ($CD4^+$), called T-helper (Th) cells, recognise peptides of processed foreign antigens associated with the MHC class II antigen. 'Exogenous antigen' is endocytosed and peptides are generated through proteolytic digestion in a phago-lysosomal compartment. These peptides then associate with MHC class II molecules, and are exported to the cell surface (Engelhard, 1994; Clemens, 1996).

The role of $CD4^+$ Th cells in the immune defence against *M. tuberculosis* infection is well established (Orme and Collins, 1984; Müller *et al.*, 1987; and Ladel *et al.*, 1995). Recognition of processed peptides by these cells results in their secretion of cytokines which in turn activate the bactericidal capabilities of macrophages. This activation of macrophages is attributable to cytokines, such as

interferon gamma (IFN-γ) (Flynn *et al.*, 1993), and is typical of a response which is classified as T_h1.

6.1.2 $CD8^+$ T lymphocytes

T cells recognizing processed antigenic peptides in association with MHC class I molecules and β_2-microglobulin express the T8 cell surface determinant ($CD8^+$). Peptides associated with class I molecules and β_2-microglobulin are mainly derived from proteins present in the cytoplasmic compartment of the target cell (endogenous antigen), although recognition of exogenous antigens has been reported.

The proteolytic processing of antigen for binding to MHC class I molecules is facilitated by an enzymatic complex called the proteosome. The resulting peptides are transported to the lumen of the endoplasmic reticulum by transporter molecules, known as TAP proteins, for association with MHC class I and β2-microglobulin (Engelhard, 1994). Studies with β2-microglobulin knockout mice have shown $CD8^+$ MHC class I-restricted T cells to be an essential part of protective immunity against *Mycobacterium tuberculosis* (Flynn *et al.*, 1992). Human $CD8^+$ T cells recognising mycobacterial antigens have also been identified (Rees *et al.*, 1988; Kaleab *et al.*, 1990; and Turner and Dockrell, 1996).

The effector function most closely attributed to $CD8^+$ T cells has been cytotoxicity, mediated mainly by the perforin granzyme system, and to a lesser degree by fas-mediated cytotoxicity. However, the protective role of cytolysis, mediated by $CD8^+$ T cells, against *M. tuberculosis* infection in mice has been put in doubt recently, as perforin and granzyme gene knockout mice, as well as mice expressing a defective Fas receptor, show no discernible differences in the course of early experimental mycobacterial infection (Laochumroonvorapong *et al.*,

1997; Cooper *et al.*, 1997). These results have been put into perspective by data from Dr. Bloom's laboratory, who demonstrated a significant reduction in the mean survival time of perforin knockout mice at a later time point of experimental infection with *M. tuberculosis* compared to wildtype mice (H.-M. Vordermeier, personal communication). This suggests a role for $CD8^+$ cytotoxic T cells in the containment of disease.

Nevertheless, it has to be emphasised that $CD8^+$ T cells are potent cytokine producers and therefore alternative mechanisms against *M. tuberculosis* infection mediated by $CD8^+$ T cells, perhaps through the secretion of IFN-γ, are highly plausible (Bonato *et al.*, 1998; Tascon *et al.*, 1998). The recent identification of *M. tuberculosis* specific $CD8^+$ T cells in humans that produce IFN-γ in an antigen specific manner supports this hypothesis (Lalvani *et al.*, 1998). The relevance of cytolysis by $CD8^+$ T cells *in vivo* in conferring protection against *M. tuberculosis* infection in humans remains to be determined, although it has been shown *M. tuberculosis* specific human $CD8^+$ T cells have cytolytic activity *in vitro* (Stenger *et al.*, 1997; Lalvani *et al.*, 1998).

6.2 Antigen recognition

6.2.1 MHC-restricted α/β T cells

Classical $CD4^+$ MHC class II-restricted and $CD8^+$ MHC class I-restricted T cells possess antigen-specific receptors (TCR) composed of α and β chains, and are thus described as α/β T cells. The α/β chains possess variable domains which specifically recognise peptides of processed antigens in conjunction with the highly polymorphic MHC class I or II molecules to form a trimolecular complex. The diversity of the T cell α/β heterodimer provides a vast epitope coverage against microbial pathogens.

6.2.2 CD1-restricted α/β T cells

Recently $CD4^-CD8^-$ α/β T cells (also called double negative (DN) T cells), and a subpopulation of $CD8^+$ α/β T cells have been identified that recognise nonproteinacious ligands in a non-MHC restricted manner, thus mitigating the dogma of exclusive peptide-MHC recognition by T cells. The antigens recognised by these T cells are bacterial glycolipids and polysaccharides (Moody *et al.*, 1997; Fairhurst *et al.*, 1998), including the mycobacterial lipids mycolic acid, phosphatidylinositol mannosides (PIMs) and LAM. Stimulation of these T cells by lipoarabinomannan has been shown to produce a cytolytic response (Beckman *et al.*, 1994; Sieling *et al.*, 1995). These mycobacterial lipids are presented to DN and $CD8^+$ T cells by the CD1b molecules, members of the CD1 antigen presenting family. CD1 molecules are non-polymorphic (non-classical) MHC class I molecules which associate with β_2-microglobulin, a distinctive feature they share with classical MHC class I molecules (Porcelli *et al.*, 1998).

The structural requirements for T cell recognition of mycobacterial lipids has been investigated and a general recognition motif has been elucidated for these CD1b-restricted antigens. The fine specificity for T cell recognition of these lipid molecules lies in their hydrophilic components and not in the lipid chains (Moody *et al.*, 1997).

6.2.3 γ/δ T cells

In addition to the αβ T cells, a sub-population of T cells recognise antigen via a TCR composed of γ and δ chains instead of the α/β heterodimer (Brenner *et al.*, 1988). Although γ/δ T cells have been shown to recognise peptides in an MHC dependent manner (Kozbor *et al.*, 1989) they also recognise nonproteinacious antigens. These ligands contain phosphate as an essential component that can be bound to either alkyl, nucleotide or carbohydrate residues (Schoel *et al.*, 1994; Burk *et al.*, 1995; and Tanaka *et al.*, 1995). The

mycobacterial phospholigands such as isopentyl pyrophosphate (IPP) (Tanaka *et al.*, 1995) and phosphorylated molecules containing nucleotide, described as TUBag's (Constant *et al.*, 1994), are stimulatory for γ/δ T cells. The response of γ/δ T cells to IPP is rapid and results in the IFN-γ production by the stimulated cells (García *et al.*, 1997).

The mechanism by which these phospholigands are presented and the determinants which interact with the TCR are as yet unknown. However it has been shown that IPP must be presented on the surface of the host cell, though there appears to be no evidence for the involvement of MHC, MHC-like or CD1 antigen presenting molecules (Morita *et al.*, 1995). Furthermore, recent studies have shown that mycobacteria synthesise a variety of phosphatidyl ligands distinct from IPP which also stimulate γδ T cells whose role in the immune response to *M. tuberculosis* infection remains to be investigated (Schoel and Kaufmann, 1998). Although much remains to be resolved regarding the role of γ/δ T cells, their importance in mycobacterial infections is highlighted by the observation that mice which have the gene for the δ chain of the TCR disrupted, succumb to high-dose *M. tuberculosis* infection. Conversely, such an infection is tolerated by immunocompetent controls (Ladel *et al.*, 1995).

These findings demonstrate that the paradigm of peptides as exclusive ligands for T cells has now changed to encompass non-proteinacious molecules as additional ligands for T cells. This new-found knowledge will allow further investigation into the protective immune response against intracellular microbial pathogens and aid in the development of improved vaccines.

However, the relatively recent discovery of glycoproteins produced by prokaryotes raises interest in the possible T cell recognition of glycopeptides from these antigens. Very little is known about the role of protein-bound carbohydrates with respect to antigen processing via

the MHC class I or MHC class II presentation pathway. It is unlikey that glycoproteins undergo extensive deglycosylation within the processing compartments of antigen presenting cells as many of the enzymes involved in processing are themselves glycoproteins. The question then arises as to whether glycopeptides produced from glycoproteins during antigen processing can bind to MHC molecules and, if so, whether the carbohydrate portion can be recognised specifically by T cell receptors?

Although it has recently been demonstrated that a naturally processed glycoprotein can elicit a T cell response specific to the carbohydrate moiety (Michaëlsson *et al.*, 1996), most research into the recognition of glycoproteins by T cells has focused on the fundamental ability of glycopeptides to associate with MHC molecules and subsequently on their ability to elicit carbohydrate T cell specific responses. The following is an overview of the work performed so far in analyzing the effect of glycopeptides on T cell recognition.

6.3 Effect of carbohydrate on peptide binding to MHC molecules

The peptide binding sites of class I and class II MHC molecules differ mainly in their interactions with peptide. Although both have a characteristic groove, in class I molecules the peptide binds to the cleft by interactions of its amino and carboxy termini with specific pockets at the ends of the binding cleft (Fremont *et al.*, 1992), whereas most of the peptide binding to class II molecules occurs in the centre of the cleft (Stern *et al.*, 1994). In addition to these amino acid side chain interactions, bound peptides possess other exposed side-chains that are recognised by the T cell receptor (Garcia *et al.*, 1996).

Ishioka *et al.* (1992) demonstrated that the lack of MHC restricted T cell responses to pure carbohydrate was due to the inability of a series of synthesised carbohydrate compounds to bind to MHC molecules. Furthermore they showed a variable pattern of MHC binding responses

of N-acetylglucosamine modified asparagine mutants of a known T cell epitope of ovalbumin (OVA). The modifications were best tolerated at the amino terminus, though all modifications failed to increase peptide binding to the MHC class II molecule relative to the native peptide.

In contrast to Ishioka *et al.* (1992), Mouritsen *et al.* (1994) demonstrated that the addition of carbohydrate to a MHC class II binding peptide of hen egg lysozyme (HEL), resulted in a two-fold increase in MHC class II binding. However, in support of Ishioka's (1992) findings such additions were only tolerated at the amino terminus of the peptide and not when attached to a serine residue central to the peptide. These findings, in agreement with the proposed structure of the MHC class II binding cleft, suggest that the addition of large groups to side chains of amino acids lying within the centre of the cleft would prevent the interactions required for stable MHC class II-peptide complexes. Further support for this finding is the observation that the addition of galabiose (Galα1,4Galβ) to an immunodominant petide from HEL (52-61), was only tolerated at the amino terminus of the peptide. If the same carbohydrate was attached to a serine residue introduced at position 56, the resulting glycopeptide was unable to bind to the class II MHC molecule I-A^k (Deck *et al.*, 1995).

Conversely, since interactions with amino and carboxy terminal peptide residues facilitate binding to MHC class I binding sites, peptides that bind to MHC class I molecules should tolerate the addition of carbohydrate to the side chains of amino acids located at the centre of the MHC class I cleft. Indeed, Haurum *et al.*, (1994) showed that an MHC class I restricted peptide of Sendai virus could tolerate the addition of either GlcNAc or GalNAc, two naturally occurring O-linked residues, to a serine residue in the centre of the peptide. However, whether such a substitution could be tolerated at one or other of the termini of this molecule, a modification that would

probably prevent interaction of the peptide with the end pockets of the MHC class I binding cleft, was not investigated.

These findings confirm that glycopeptides associate with MHC molecules and attempts have been made to define their interactions with the well characterised MHC binding clefts. The following summarises some of the work that has been reported on characterizing the specificity of T cell receptors for carbohydrate moieties.

6.4 Carbohydrate specific T cell recognition

The findings that glycopeptides can bind to MHC molecules have been extended to study the interactions of the carbohydrate with the T cell receptor. Studies by Harding *et al.* (1993), and Deck *et al.* (1995), indicated that the addition of galabiose to a T cell determinant of HEL (52-61), generated a carbohydrate specific response only when attached at position 56. In support of this, Haurum *et al.* (1994) demonsrated that cytotoxic T cells could specifically recognise GlcNAc attached to a T cell epitope of Sendai virus. When GlcNAc was substituted with GalNAc recognition of the glycopeptide was abolished. This substitution generated a glycopeptide that differs only in its orientation of a hydroxyl group at position 4 of the pyranose ring, indicating that there is specific recognition of the carbohydrate component of glycopeptides. In support of these findings Jensen *et al.* (1997) have recently identified Th cells recognizing glycopeptides that are specific for the attached GalNAc moiety.

A fine specificity against carbohydrate has also been observed for the non MHC-restricted DN α/β T cells. The ablity of T cells to recognise lipoglycans, especially from mycobacteria, in association with CD1 antigen-presenting molecules is analogous to the TCR-glycopeptide-MHC complex. It has been proposed that the hydrophobic acyl chains of the glycolipids provide the interaction with the antigen presenting CD1 molecule and that the recognition specificity of the TCR is

directed against the hydrophilic head groups (Sieling *et al.*, 1995; Moody *et al.*, 1997). This hypothesis is supported by further studies of Moody *et al.* (1997) who altered the positions of hydroxyl groups on the pyranose ring of the mycobacterial glycolipid glucose monomycolate. Substitution of the glucopyranose ring with either manno- or galactopyranose revealed a structural requirement for the hydroxyl groups at positions 2 and 4 of the pyranose ring for recognition by CTLs.

The data described in this chapter clearly show that nonproteinacious antigens can widen the array of targets for the cell mediated immune response. However very little is known about the effect of carbohydrates attached to proteins in terms of antigen processing. This may be important not only in the generation of glycopeptides for immune recognition but also in determining the pattern of non-glycosylated peptides presented.

Chapter 7

Identification of murine T cell epitopes from the *Mycobacterium tuberculosis* antigen MPT83

7. 1 Introduction

Proteins secreted across the cell wall of mycobacteria have been shown to be important targets for protective immunity against *M. tuberculosis* infection in both mice and guinea pig experimental models (Andersen, 1994; Horwitz *et al.*, 1995; and Roberts *et al.*, 1995). These secreted proteins appear on the cell surface and in the culture media during active replication of the mycobacteria (Andersen *et al.*, 1991; Rambukkana *et al.*, 1991), and their role in protective immunity has been attributed to their ability to stimulate T cell proliferation, and IFN-γ release, from peripheral blood mononuclear cells (PBMC) of tuberculosis patients (Havlir *et al.*, 1991; Roche *et al.*, 1994). As such, the T cell epitopes of many mycobacterial antigens have been identified in an attempt to identify differences in recognition of such epitopes as a possible mechanism for the diminished resistance to tuberculosis in some individuals.

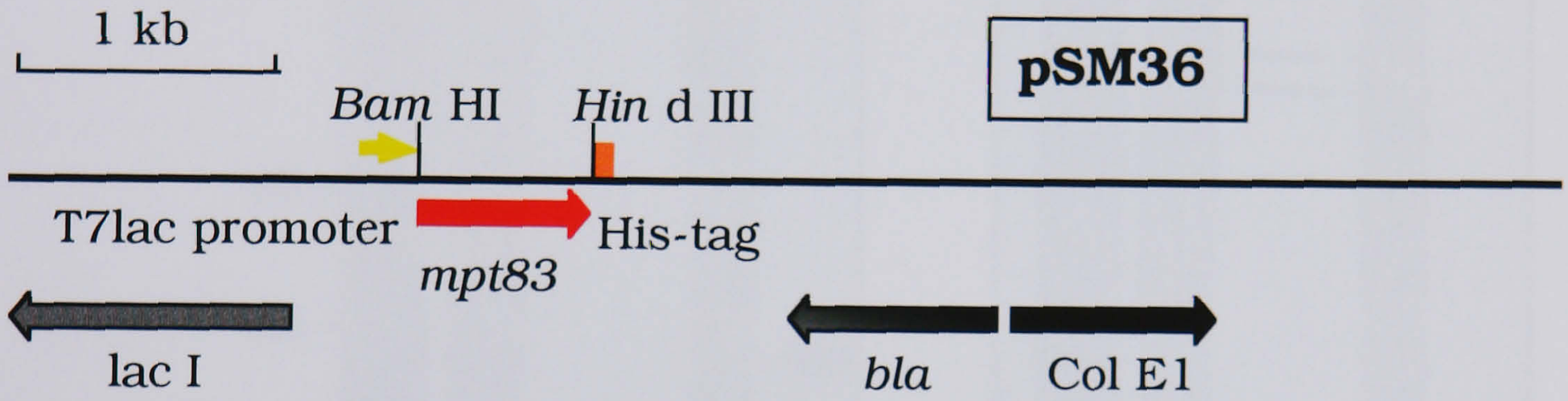
However, to date there have been no studies into the T cell recognition mycobacterial antigens with respect to their glycosylation status. In the light of results from studies on hemagglutinin from the type A influenza virus (Drummer *et al.*, 1993) where removal of an N-glycosylation site led to the recognition of an otherwise hidden T cell determinant, it was decided to investigate whether there is a difference in the recognition of T cell epitopes of MPT83 with respect to its glycosylation status.

7.2 Results

7.2.1 Production of recombinant MPT83 from *E. coli*.

The gene for MPT83 was amplified by PCR from pSM13 (Fig. 2.12, page 37) using the oligonucleotide primers 26 and 27 (appendix 1) and cloned into the expression vector pET-21d (Fig. 8.1B) to generate the plasmid pSM36.

Fig. 7.1 Plasmid map of pSM36.

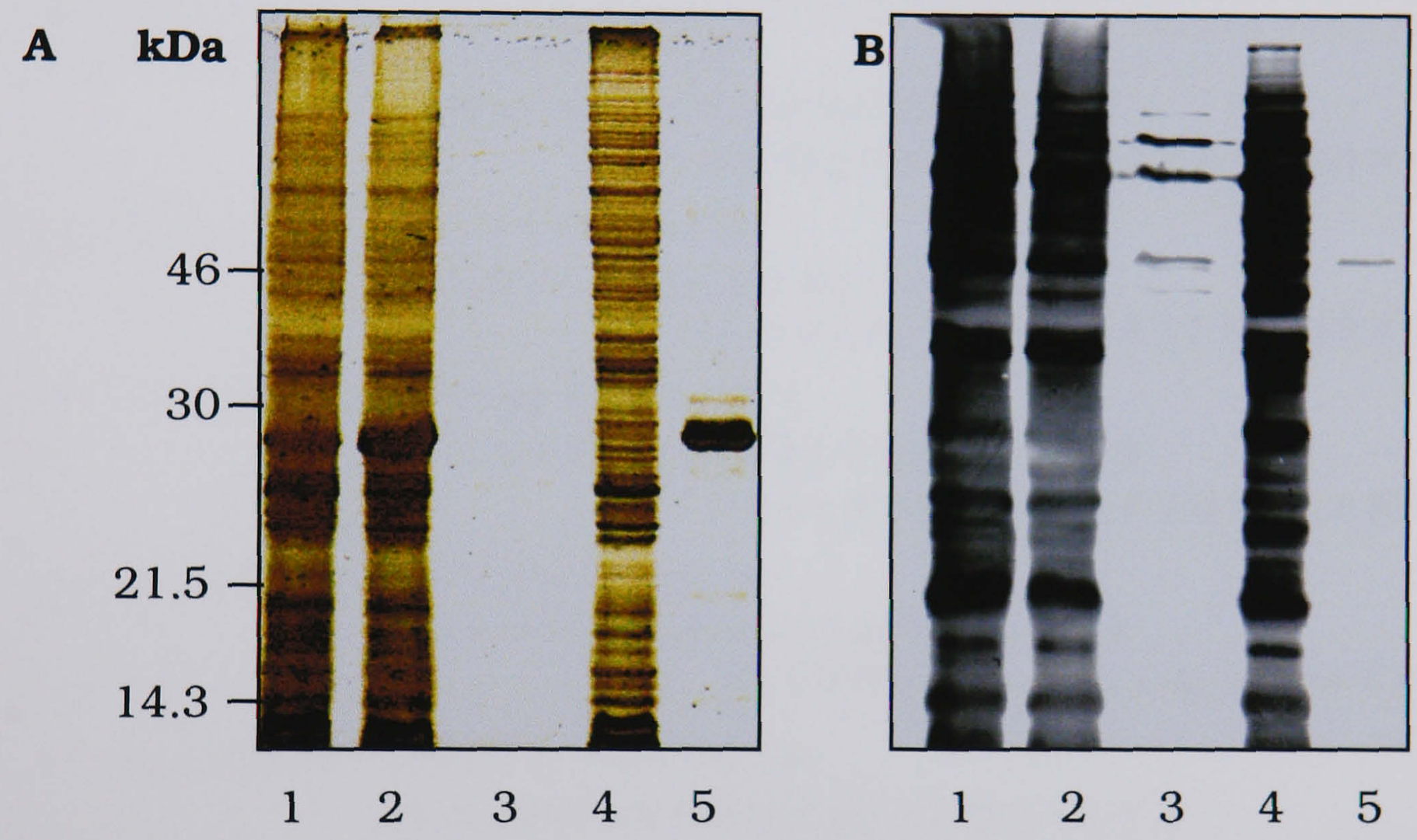


This construct was sequenced using the oligonucleotide primers 9, 10, 28 and 29 (appendix 1) and transformed into *E. coli* BL21(DE3) as described in section 8.5.1. MPT83-His tag was purified from 500 ml LB Broth (100μg/ml ampicillin) four hours after induction of expression, achieved by adding IPTG to a final concentration of 1 mM. Recombinant MPT83 was produced as an inclusion body and retrieved as described in section 8.13.

Purity of the recombinant MPT83-His was determined by silver staining (section 8.10) and Western blotting with anti *E. coli* antibody. (Fig. 7.2 A and B respectively). N-terminal sequencing of the purified product revealed that the recombinant protein was not cleaved at its signal sequence and contained a 14 amino acid amino terminal fusion. This amino terminal fusion and the carboxy terminus His tag fusion resulted in the recombinant migrating slower in SDS-PAGE (15% separating gel) demonstrating a relative molecular weight of 28 kDa (Fig. 7.2 A, lane 5). MPT83-His was not recognised by ConA (data not shown).

Fig 7.2 **(A) Silver stain and (B) Western blot incubated with anti *E. coli* antiserum.**

Lanes: 1, total cell protein of *E. coli* BL21(DE3)::pSM36 uninduced (1 ml); 2, total cell protein of *E. coli* BL21(DE3)::pSM36 induced (1 ml); 3, culture filtrate of *E. coli* BL21(DE3)::pSM36 culture filtrate (1 ml); 4, sonicated extract of *E. coli* BL21(DE3)::pSM36 (5μg); 5, 8M urea treated insoluble pellet (1 μg)



7.2.2 Proliferative responses of lymph node cells from mice immunised with MPT83-His

A complete set of 22 overlapping synthetic peptides covering the sequence of MPT83 (Fig. 2.16) were synthesised in order to identify sites within this antigen that are recognised by murine T cells. Peptides were designated 1 to 22 (Fig. 7.3), were 20 residues long and overlapped each other by 10 amino acids.

Fig. 7.3 Synthetic peptides from the *M. tuberculosis* antigen MPT83. The amino acid sequence is shown by the standard one-letter code. Arrows indicate the position of the signal peptide (residues 1 to 24).



Balb/c mice were immunised in the footpad with approximately 75 μg of purified MPT83-His recombinant protein (Fig. 7.2 A and B, lane 5) emulsified in IFA. Seven days later lymph node cells were harvested and incubated *in vitro* with each of the peptides (25 μg/ml) shown above. The magnitude of the T cell responses against each peptide are shown in Fig. 7.4. The results shown are representative of four separate experiments. Four peptides (2, 16, 17, and 22) induced significant proliferation of LN cells from mice immunised with MPT83-His.

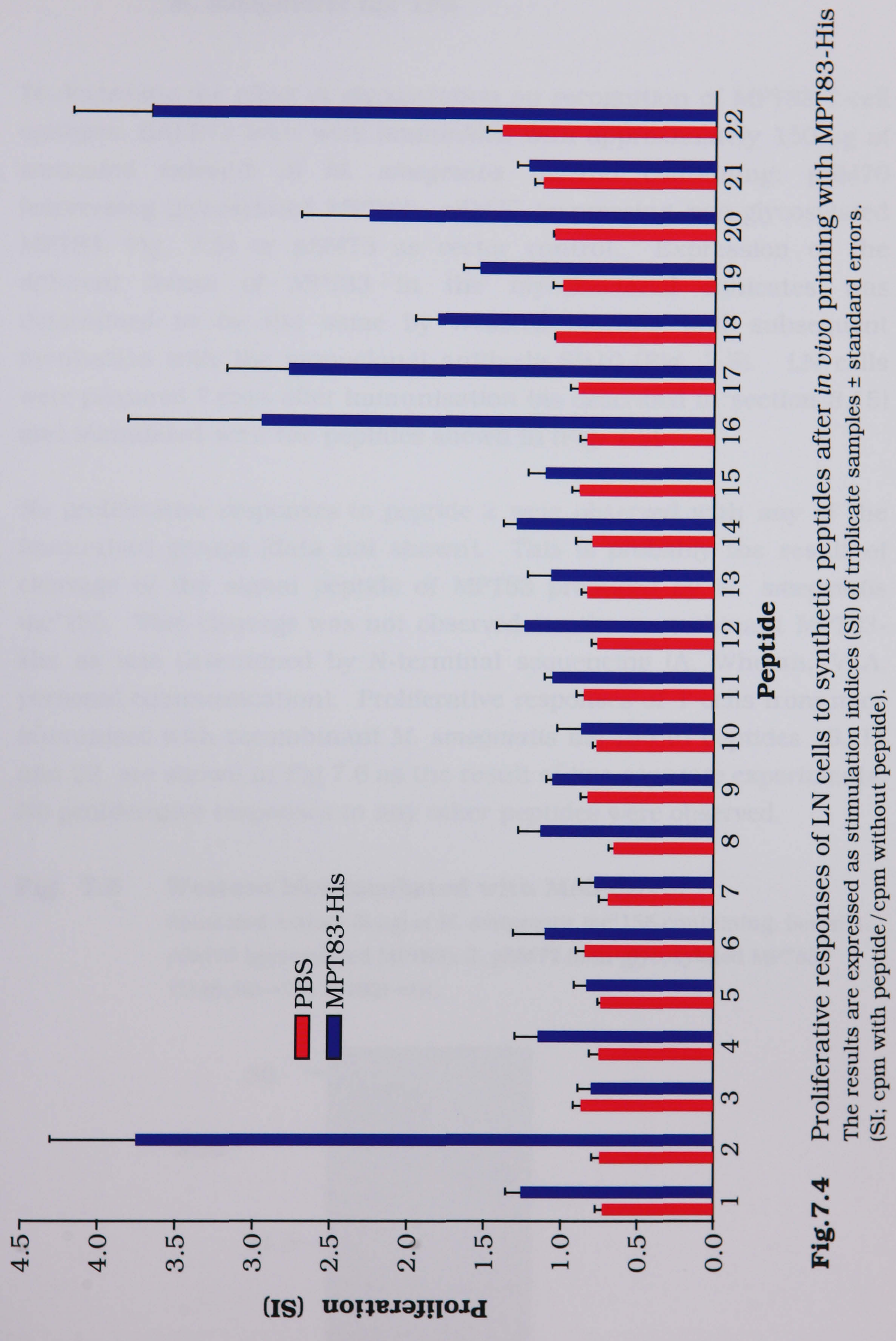


Fig.7.4 Proliferative responses of LN cells to synthetic peptides after *in vivo* priming with MPT83-His. The results are expressed as stimulation indices (SI) of triplicate samples ± standard errors. (SI; cpm with peptide/cpm without peptide).

7.2.4 Proliferative responses of LN cells to glycosylated and non-glycosylated forms of MPT83 produced by *M. smegmatis* mc^{2}155

To determine the effect of glycosylation on recognition of MPT83 T-cell epitopes, BALB/c mice were immunised with approximately 150 μg of sonicated extracts of *M. smegmatis* mc^{2}155 containing: pSM70 (expressing glycosylated MPT83), pSM72 (expressing non-glycosylated MPT83, Fig. 7.5) or pSMT3 as vector control. Expression of the different forms of MPT83 in the mycobacterial sonicates was determined to be the same by Western blotting and subsequent incubation with the monoclonal antibody SB10 (Fig. 7.5). LN cells were prepared 7 days after immunisation (as described in section 8.15) and stimulated with the peptides shown in (Fig. 7.3).

No proliferative responses to peptide 2 were observed with any of the immunised groups (data not shown). This is probably the result of cleavage of the signal peptide of MPT83 produced by *M. smegmatis* mc^{2}155. This cleavage was not observed for the recombinant MPT83-His as was determined by N-terminal sequencing (A. Whelan, VLA, personal communication). Proliferative responses of T cells from mice immunised with recombinant *M. smegmatis* mc^{2}155 to peptides 16, 17 and 22 are shown in Fig 7.6 as the result of two separate experiments. No proliferative responses to any other peptides were observed.

Fig. 7.5 Western blot incubated with MAb SB10.

Sonicated extract (5 μg) of *M. smegmatis* mc^{2}155 containing, lanes: 1, pSM70 (glycosylated MPT83); 2, pSM72 (non-glycosylated MPT83, TT(48,49)→VV, N(192)→G).

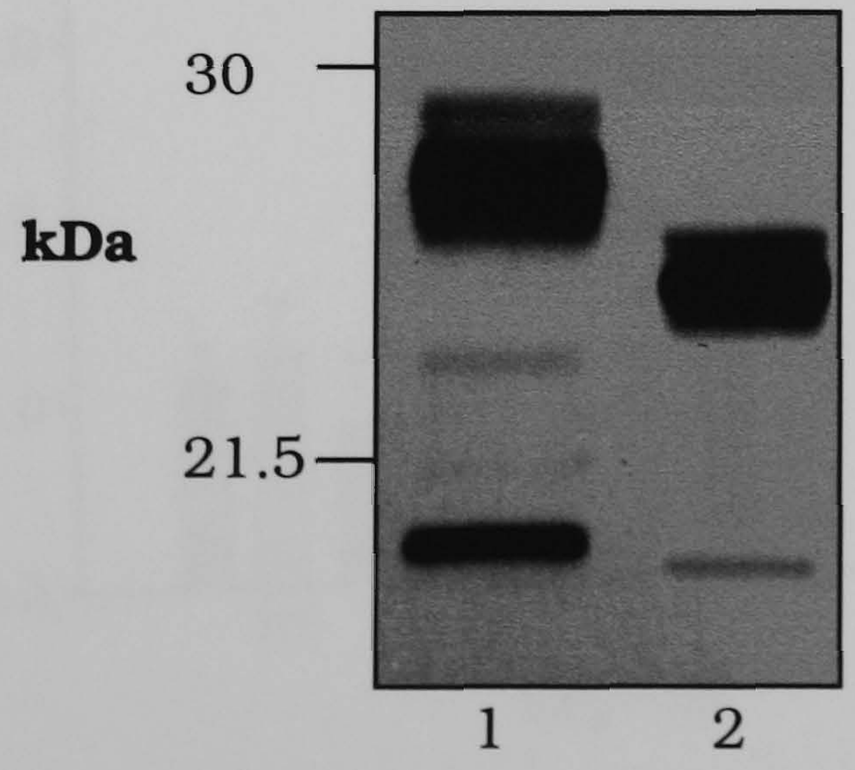
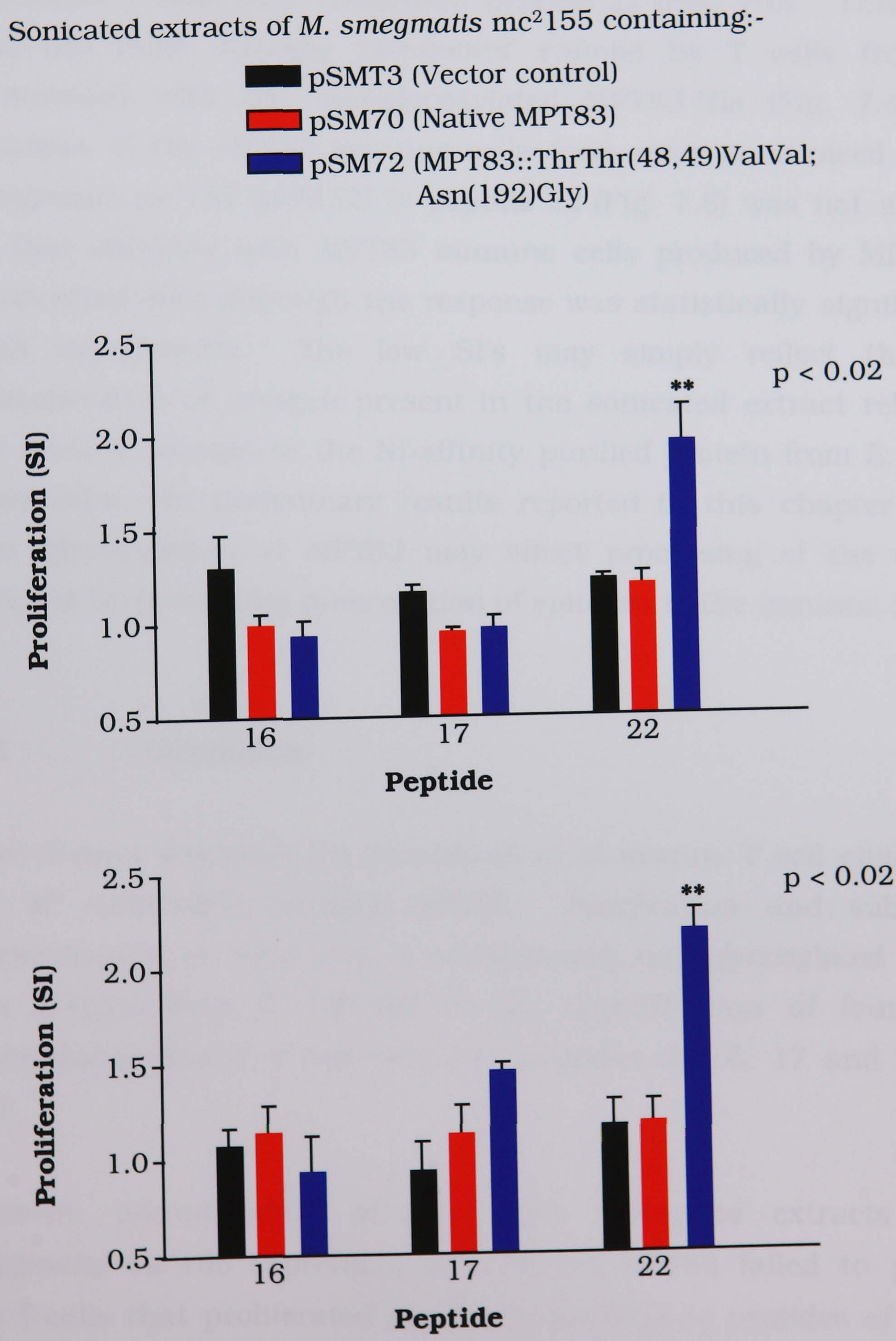


Fig. 7.6 Proliferative responses of LN cells to synthetic peptides after *in vivo* priming with recombinant *M. smegmatis* mc²155. The results are expressed as stimulation indices (SI) of triplicate samples ± standard errors. (SI; cpm with peptide/cpm without peptide). Statistical significance was determined by comparing the proliferation (cpm) of LN from each group of mice to each peptide, with the cpm of the corresponding medium control. An unpaired two tailed t test was used on the two data sets and the confidence interval was set at 95%.



T-cells from mice immunised with *M. smegmatis* mc²155 expressing glycosylated MPT83 (pSM70) failed to recognise peptides 16, 17 and 22 (Fig. 7.6) which were all recognised by T-cells from mice immunised with the non-glycosylated recombinant MPT83-His purified from *E. coli* (Fig. 7.4).

However, mice immunised with sonicated extract of *M. smegmatis* mc²155 expressing a non-glycosylated form of MPT83, (pSM72, Fig. 7.5) generated T cells that recognised peptide 22 (Fig. 7.6). This peptide was the most strongly recognised epitope by T cells from mice immunised with the non-glycosylated MPT83-His (Fig. 7.4). The response of the MPT83 immune cells from mice immunised with *M. smegmatis* mc²155 (pSM72) to peptide 22 (Fig. 7.6) was not as strong as that obtained with MPT83 immune cells produced by MPT83-His immunised mice although the response was statistically significant in both experiments. The low SI's may simply reflect the lower concentration of antigen present in the sonicated extract relative to the amount present in the Ni-affinity purified protein from *E. coli*. In conclusion, the preliminary results reported in this chapter suggest that glycosylation of MPT83 may affect processing of the antigen, perhaps by preventing presentation of epitopes to the immune system.

7.3 Discussion

This chapter describes the identification of murine T cell epitopes for the *M. tuberculosis* antigen MPT83. Purification and subsequent immunisation of mice with a recombinant non-glycosylated form of this antigen from *E. coli* led to the identification of four strong immunostimulatory T cell epitopes (peptides 2, 16, 17 and 22, Fig. 7.4).

However, immunisation of mice with sonicated extracts of *M. smegmatis* mc²155 expressing glycosylated MPT83 failed to generate any T cells that proliferated *in vitro* to overlapping peptides of MPT83. This lack of proliferation could be due to the carbohydrate side chains

protecting the antigen from proteolysis during processing as it has been shown that removal of carbohydrate side chains from fibronectin leads to an increased susceptibility to protease digestion (Bernard *et al.*, 1982). Alternatively antigen presentation pathways may be blocked by mycobacterial lipids (Moreno *et al.*, 1988).

It should be noted that mice immunised with *M. smegmatis* mc^2155 expressing glycosylated MPT83 may produce T cells that recognise glycosylated epitopes that are not stimulated *in vitro* with the non-glycosylated peptides used in this study. Further work is therefore required to characterise the carbohydrate side chains of glycosylated MPT83 so that T cells from mice immunised with glycosylated MPT83 can be tested for their ability to recognise the corresponding glycopeptides *in vitro*.

To investigate the possibility that glycosylation of MPT83 by mycobacteria may have an influence on the presentation of peptides to T cells, mice were immunised with sonicated extract of *M. smegmatis* expressing non-glycosylated MPT83. The results showed that mice immunised with sonicated extracts of *M. smegmatis* mc^2155 expressing non-glycosylated MPT83 produced T cells that proliferated to peptide 22 when stimulated *in vitro* with this peptide (Fig. 7.6). This peptide was also recognised by T cells from mice immunised with the non-glycosylated MPT83-His produced from *E. coli*. Thus, deglycosylation of MPT83 appears to allow processing of the antigen that results in the presentation of a peptide for T cell recognition that is masked when MPT83 is glycosylated. However, the recognition of peptide 22 may have resulted as a consequence of the asparagine to glycine mutation alone or in conjunction with the double threonine to valine substitution. Further studies, such as specific enzymatic removal of the individual carbohydrate chains or preparation of mycobacterial extracts from cultures containing antibiotics that inhibit either specifically O- or N-linked glycosylation are required to confirm these results. Unfortunately these approaches may be of limited use if bacterial glycosylation differs significantly from eukaryotic glycosylation.

Even though there are no proliferative responses to peptides of MPT83 following immunisation with glycosylated MPT83, it is not proposed that glycosylation of MPT83 renders it completely resistant to processing. Indeed a T cell line raised against recombinant MPT83-His from *E. coli* proliferated in response to a sonicated extract of *M. smegmatis* expressing glycosylated MPT83 (data not shown). Studies by other researchers who have used sonicated extracts *of M. tuberculosis* (which contains glycosylated forms of the 19 and 38 kDa antigens) as the immunogen have identified T cell stimulatory peptides of both the 19 kDa (Harris *et al.*, 1991) and the 38 kDa antigen (Vordermeier *et al.*, 1992), confirming that glycosylation by mycobacteria does not result in masking all T cell epitopes of a particular glycoprotein antigen.

However, on the basis of the results in this chapter, it can be proposed that mycobacteria have developed mechanisms of glycosylation in order to modulate the range of peptides that are presented to the immune system. These findings are in agreement with the findings of Drummer *et al.*, (1993), who demonstrated that enzymatic deglycosylation of asparagine 63 within the hemagglutinin of influenza virus led to the recognition of a T cell determinant (^{65}TLIDALLG^{72}) that was not recognised by T cells stimulated with the glycosylated form of this protein. More importantly, this same T cell determinant was recognised by T cells stimulated with hemagglutinin from a mutant strain of influenza that produced hemagglutinin with aspartate substituted for asparagine at position 63, which also led to a non-glycosylated version of this protein at position 63. Studies by Parra-López *et al.*, (1997) also demonstrated a similar phenomenon. In their studies of targeting HEL to the endocytic pathway they found that a glycosylated form of cathepsin D fused to HEL was less efficient in generating a particular T cell epitope of HEL.

Recently, a mechanism for this observed inhibition of antigen processing by glycosylation has been proposed (Manoury *et al.*, 1998). They showed that when a region of the microbial tetanus toxin antigen was incubated with disrupted lysosome, the dominant processing

activity was mediated by an asparaginyl endopeptidase rather than the known lysosomal cathepsins. Moreover, they showed that *in vitro* artificial glycosylation of the asparagine residue within the synthetic peptide (HIDN(N-glucosamine)EEDI) resulted in a loss of hydrolysis of this peptide at the asparagine residue. This finding not only supports the hypothesis that glycosylation may protect antigens against processing within antigen processing cells, but also shows that such glycosylation does not have to be of the typical eukaryotic patterns previously described.

It can thus be speculated that the close proximity of a carbohydrate chain on asparagine at position 192 of MPT83 to peptide 22 (amino acids 205-220) may have a protective effect on the carboxy terminus of MPT83 with consequent masking of the carboxy T cell epitope. It is interesting to note that the dominant T cell epitope of the non-glycosylated MPB70 recognised by cattle infected with *M. bovis* is located at the carboxy terminus of the molecule (Pollock *et al.*, 1994). Moreover, there is only one conservative change from a threonine in MPT83 to a serine in MPB70, between these two epitopes (shown below).

206	N A T V Y M I D T V L M P P - A .	220	MPT83
174	G V S T N A T V Y M I D [S] V L M P P A .	193	MPB70

It will be of great interest to see whether or not peptide 22 of MPT83 is recognised by T cells from cattle immunised with glycosylated MPT83.

Concluding remarks

This thesis describes the genetic characterization of MPT83, the serodominant antigen of *M. bovis*. The identification at the genetic level of novel antigens of the pathogenic mycobacteria is important for several reasons. It allows the techniques of molecular biology and molecular immunology to be employed to investigate the roles of these proteins as putative species specific diagnostic reagents, or as antigens that may be important in conferring protection against tuberculosis.

The research described in this study has utilised a molecular genetic approach to investigate the post-translational modification of MPT83 by mycobacteria. The results identify regions of the antigen to which carbohydrate chains are attached via O and N-linked glycosylation. It can be envisaged that synthetic peptides corresponding to the regions of MPT83 identified as been glycosylated could be used as tools in *in vitro* assays to identify the mycobacterial enzymes responsible for protein glycosylation. Such enzymes are likely to be attractive candidates for inhibition by chemotherapy as the enzymes responsible for protein glycosylation in yeast are essential for survival (Klis, 1994, and Gentzsch and Tanner, 1996,).

In preliminary experiments to investigate the potential role of mycobacterial protein glycosylation with respect to T cell responses we observed a loss of recognition of a T cell stimulatory peptide of MPT83 following immunisation with a mycobacterial sonicate containing a glycosylated form of this antigen. It is therefore conceivable that mycobacteria may glycosylate their proteins in order to protect them from certain proteolytic mechanisms of antigen presenting cells threreby restricting presentation of certain T cell epitopes. Given the importance of T cell responses in the immunopathogenesis of tuberculosis it will be of great interest to study the role that mycobacterial glycosylation of antigens may play in the presentation of T cell epitopes.

Chapter 8

Materials and methods

8.1 Bacterial strains and plasmid vectors

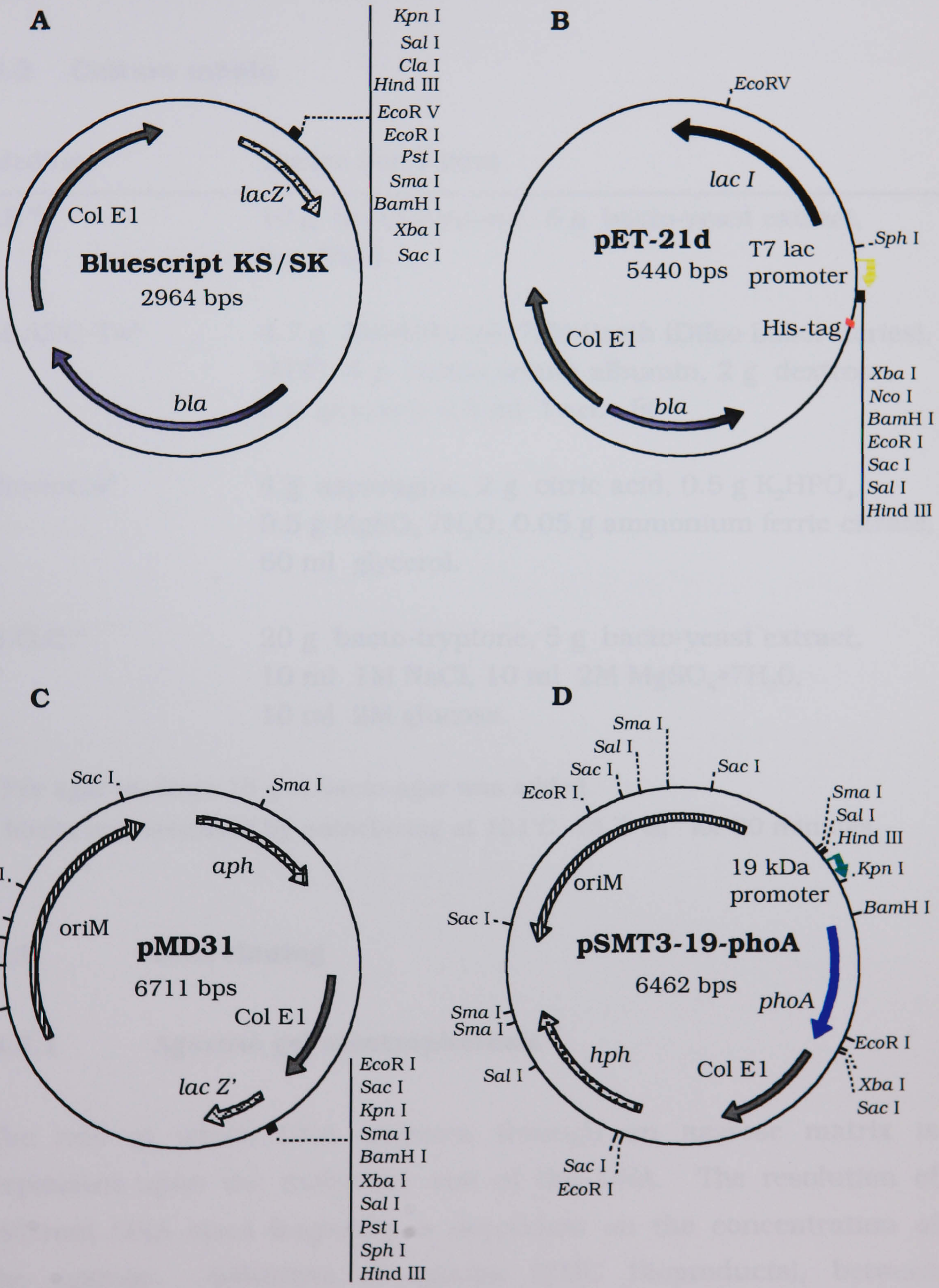
Strain	Genotype
Escherichia coli DH5α (Gibco BRL)	Φ80d*lacZ*ΔM15, *recA*1, *endA*1, *gyrA*96, *thi*-1, *hsdR*17 (r_{K-}.m_{K+}), *supE*44, *relA*1, *deoR*, Δ(*lacZYA-argF*)U169
Escherichia coli BL21(DE3) (Novagen)	F-, *ompT*, $hsdS_B$, (r_{B-}, m_{B-}), *dcm*, *gal*, (λcIts857, *ind*1, Sam7, *nin*5, *lacUV5*-T7 gene 1)
Mycobacterium smegmatis mc^2155 (Gift from W.R. Jacobs Jr III*)	Efficient plasmid transformation mutant of *Mycobacterium smegmatis* $mc^2$6 (Wild-type)

*(Snapper *et al.*, 1990)

Five plasmid vectors, shown in Fig. 8.1, were used in this study. Plasmids pBluescript KS and SK (Fig. 8.1A) were purchased from Stratagene. In plasmid pBluescript KS, transcription is from the *lacZ* promoter in the direction *Sac* I to *Kpn* I and in pBluescript SK, transcription is in the opposite direction due to the reverse orientation of the multiple cloning site (MCS). These vectors were used for general cloning and allowed expression of mycobacterial genes in *E. coli* DH5α via transcription from *the lac Z* promoter. The His-tag fusion vector, pET-21d (Fig. 8.1B), was purchased from Novagen. Genes of mycobacterial antigens were cloned in to this vector in frame with the histidine tag. His-tagged antigens were subsequently expressed in *E. coli* BL21(DE3) from the T7 promoter. The mycobacterial shuttle vector pMD31 (Fig. 8.1C) was a gift from Graham Hatfull, Pittsburgh. This plasmid is capable of extrachromosomal replication in both mycobacteria and *E. coli* as it contains the mycobacterial replicon (Ori M) from the 5.0 kb cryptic plasmid, pAL5000, of *Mycobacterium fortuitum* (Labidi *et al.*, 1985) and the Col E1 origin of replication. Mycobacterial genes cloned into pMD31 were expressed in mycobacteria by transcription from their own promoter, or in *E. coli* with transcription driven from the *lacZ* promoter.

The reporter plasmid pSMT3-19-phoA (Fig. 8.1D) was a gift from Jean Louis Herrmann, London (Herrmann *et al.* 1996). This shuttle vector also contains Ori M and Col E1 and is capable of replcation in both mycobacteria and *E. coli*.

Fig. 8.1 Maps of plasmid vectors used in this study



Coding sequences of DNA were cloned in frame (section 8.3.5) with the *phoA* reporter gene and expression in mycobacteria and *E. coli* was directed via transcription from the promoter of the 19kDa antigen of *M. tuberculosis* (Garbe *et al.* 1993).

8.2 Culture media

Medium	Recipe (for 1 litre)
LB [ab]	10 g bacto-tryptone, 5 g bacto-yeast extract, 5 g NaCl.
M-ADC-Tw[a]	4.7 g Middlebrook 7H9 Broth (Difco Laboratories), (ADC: 5 g bovine serum albumin, 2 g dextrose, 2 g glucose), 0.5 ml Tween 80
Sauton's[a]	4 g asparagine, 2 g citric acid, 0.5 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.05 g ammonium ferric citrate, 60 ml glycerol.
S.O.C.[a]	20 g bacto-tryptone, 5 g bacto-yeast extract, 10 ml 1M NaCl, 10 ml 2M $MgSO_4 \bullet 7H_20$, 10 ml 2M glucose.

[a] For agar medium 15 g of bacto-agar was added.

[b] Media was sterilised by autoclaving at 121°C, 15 lb in^{-2} for 20 minutes.

8.3 DNA cloning

8.3.1 Agarose gel electrophoresis

The rate at which DNA migrates through an agarose matrix is dependent upon the molecular size of the DNA. The resolution of different DNA sized fragments is dependent on the concentration of the agarose. Solutions of agarose (FMC Bioproducts), between 0.8%(w/v) and 1.2%(w/v) In TAE buffer, were melted and cast in Sub-Cell apparatus (Bio-Rad). Typically, DNA molecules ≥10 Kb were

analyzed on gels ≤ 1%(w/v), and DNA molecules ≤ 10 Kb on gels ≥ 1%(w/v). The intercalating dye ethidium bromide (EtBr) (Sigma) was added to gels to give a final concentration of 0.1 μg/ml.

TAE	Recipe for 1 litre of 10x buffer
Tris-acetate EDTA x10	48.4 g Tris-base, 11.4 ml Glacial acetic acid, 20 ml 0.5 M Na_2•EDTA (pH 8.0)

DNA samples were applied to the gels after having been mixed with a gel loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 40% (w/v) sucrose in water) at a ratio of 4 : 1 (DNA:buffer). Samples were electophoresed through agarose at voltages of between 40 V and 120 V until the required resolution was obtained. After electrophoresis, EtBr stained-DNA was visualised by UV transillumination and photographed. DNA size standards, 1Kb Ladder (800 ng, Gibco BRL) or phage λ DNA (500 ng) digested with the restriction enzyme *Hin*d III (Gibco BRL), were applied to each gel to allow estimation of DNA fragment sizes. Quantification of DNA fragments was determined by visual comparison between 0.5 μg of phage λ DNA digested with the restriction enzyme *Hin*d III and the sample fragments.

8.3.2 Polymerase Chain Reaction PCR

The polymerase chain reaction (PCR) is a method that allows for the selective amplification of specific DNA sequences (Sakai *et al.*, 1988). Two oligonucleotide primers are required that delimit the region to be amplified. These primers anneal to their complementary sequences on denatured template DNA upon cooling. Synthesis of complementary DNA molecules, initiated from each primer, is performed by the thermostable *Taq* DNA polymerase from the bacteria *Thermus aquaticus* YT1. The new double stranded DNA is then denatured and the cycle repeated.

The PCR's in this study were perfomed in final reaction volumes of 50µl. 200 ng of genomic DNA, 50 ng of plasmid/cosmid DNA or 10 ng of PCR product were used as template DNA. Reaction conditions were; 50 mM KCl, 10 mM Tris-HCl (pH 8.5), 3-7 mM $MgCl_2$, 200 µM of each dNTP, 0.5 U *Taq* DNA polymerase (Promega) and 20 pmol of each oligonucleotide primer. Two negative control PCR reactions were always performed: one reaction contained no template DNA and the other reaction contained no *Taq* DNA polymerase. PCR's were overlaid with a drop of mineral oil to prevent evaporation. Oligonucleotide primers (Appendix 1) were supplied by Oswel, Southampton UK.

Amplification parameters, performed on a Trio-block thermocycler (Biometra), consisted of an initial denaturation step of 96°C for 2 minutes, followed by 30 amplification cycles of 96°C (denaturation) for 90 seconds, 56°C (annealing) for 90 seconds and 72°C (elongation) for 90 seconds. A final elongation step of 72°C for 5 minutes was performed to ensure the extension of all products. 5 µl of each PCR product was resolved by agarose gel electrophoresis.

8.3.3 Restriction digestion

Genomic DNA (2 - 4 µg), plasmid DNA (0.5 - 1 µg) or PCR products (0.2-1 µg) were digested with appropriate restriction enzymes in final reaction volumes ranging between 20 µl and 50 µl. The restriction enzyme was always present in excess and constituted less than 10% of the final reaction volume. Genomic digests were performed overnight. Digestion of all other DNA substrates was completed after two hours. All restriction enzymes and corresponding reaction buffers were purchased from Promega UK and digestions were performed at the appropriate temperature (usually 37°C). Digested DNA fragments were resolved by agarose gel electrophoresis and visualised by UV transillumination.

8.3.4 Purification of DNA from agarose

DNA fragments (typically 0.5 - 3 µg) that were to be recovered from agarose gels for further manipulation were run on gels made of NuSieve GTG low melting temperature agarose (FMC BioProducts). Two methods for recovery of DNA were used. Fragments of DNA ≥ 200 bp were purified using Wizard PCR Preps DNA Purification System (Promega) and fragments ≤ 200 bp by GELase Agarose Gel-Digesting Preparation (Epicentre Technologies). For DNA preparation using the Wizard PCR Prep method the excised DNA containg agarose fragment was melted at 70°C for 4 minutes, followed by incubation with 1 ml Wizard PCR Preps DNA purification resin for 20 seconds (the resin contains an ion-exchange matrix which selectively binds DNA). The resin/DNA mix was pushed through a Luer-Lok 3 ml syringe with a Wizard Minicolumn attached to the end. The minicolumn was washed with 2 ml of 80% isopropanol, and the DNA eluted with 50 µl of nuclease free distilled water (Promega).

The GELase protocol uses an enzyme that digests the polysaccharide chains of molten agarose and was applied to DNA purification as follows. The agarose slice was melted as described for the Wizard PCR Prep protocol and 1 µl of 50x GELase buffer added per 50 mg of molten agarose. The buffered molten agarose was equlibriated to 45°C and 2 units of GELase enzyme added. The digestion reaction was incubated at 45°C for 30 minutes, and then the DNA was precipitated with one volume of 5 M ammonium acetate and two volumes of room temperature ethanol. The precipitate was pelleted by centrifugation at 14,000 rpm for 30 minutes at room temperature, washed with 70% ethanol and resuspended in an appropriate volume of nuclease free distilled water.

8.3.5 Preparation of DNA fragments for cloning

Plasmid vectors described in Fig. 8.1 were digested with the appropriate restriction enzymes and purified as described above. DNA fragments to be cloned were generated either by restiction digestion of DNA, or if the DNA fragment to be cloned did not contain suitable restriction sites or was required to be transcribed in frame with certain vector sequences PCR was used to generate restriction sites at the desired position. The latter was accomplished by adding the desired restriction enzyme site and when required appropriate bases to the 5' end of the oligonucleotide primer. The restriction site was always 5' with respect to any other base additions to the oligonucleotide. However, certain restriction enzymes cleave their recognition sequences inefficiently if the sequence is located at the end of a DNA fragment. Thus, all oligonucleotide primers containing a 5' restriction site also had three additional bases added to the 5' end to aid restriction digestion.

8.3.6 Dephosphorylation of 5' ends

The phosphate groups from 5' ends of plasmid vectors digested with one restriction enzyme were removed, to prevent self-ligation of the vector, using shrimp alkaline phosphatase (SAP) (Amersham Life Sciences). Up to 2.5 μg of digested vector was treated with; 0.1 units of SAP for vectors with 5'-protruding ends, 0.2 units of SAP for blunt-ended vectors and 0.5 units of SAP for vectors with 5'-recessed ends. All dephosphorylations were performed in reaction conditions of 20 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$ for 1 hour at 37°C. The phosphatase was inactivated by heating at 65°C for 15 minutes. The dephosphorylated vector was then used directly in ligation reactions as described in the manufacturer's instructions.

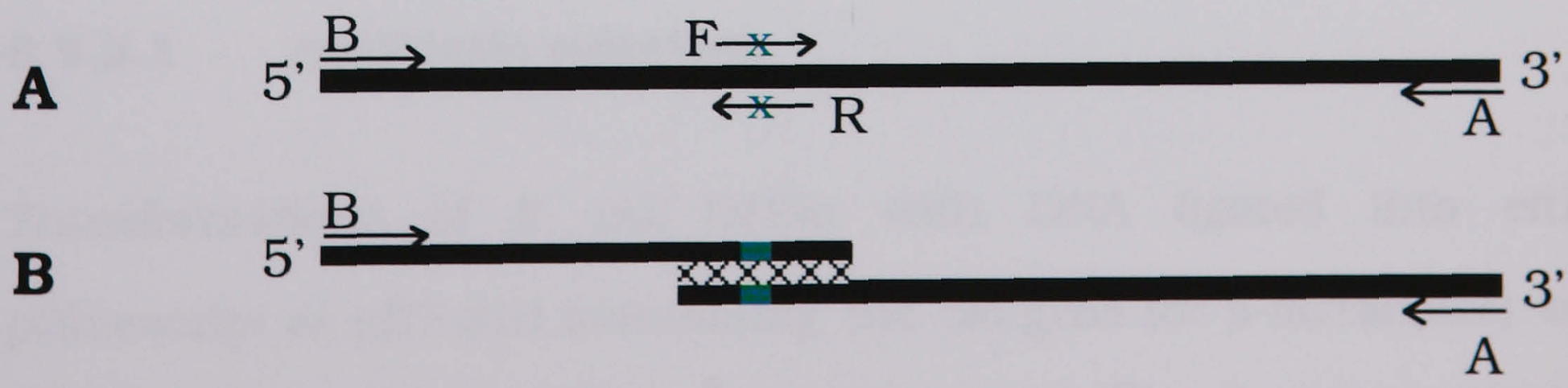
8.3.7 Ligation of plasmid vector and insert DNA

Prepared plasmid vector and insert DNA were quantified as described above. Typically ligation reactions used 50 - 100ng of vector DNA. Vector and insert DNA were ligated together at ratios varying from 1 : 1 to 1 : 3. The two DNA molecules were joined between the 5'-phosphate and 3'- hydroxyl groups using the enzyme T4 DNA ligase (New England Biolabs). The reaction conditions of ligations was 30 mM Tris-HCl pH 7.8, 10 mM $MgCl_2$, 10 mM dthiothreitiol (DTT) and 1 mM ATP, in final rection volumes of 10 μl. Ligations were either performed for 2 hours at 37°C or for 18 hours at 14°C.

8.4 Site-directed mutagenesis (SDM)

Site directed mutagenesis was accomplished using PCR as described by Herrmann *et al.* (1996). PCR's for SDM were performed using the high fidelity proof-reading enzyme *pfu* DNA polymerase (Sratagene) in place of *Taq* DNA polymerase. Complementary forward and reverse oligonucleotide primers containing the desired mutation (F and R, Fig.8.2A) were used in conjunction with oligonucleotide primers for the 3'- end and 5'- end of the gene respectively (A and B, Fig. 8.2A) to generate two PCR products (Fig. 8.2B).

Fig. 8.2 Schematic representation of the method employed for SDM



Following purification, the two PCR products, having an overlap of 30 bp containing the desired mutation, were combined and amplified by PCR using the oligonucleotide primers for the 5' and 3' end of the gene

(primers B and A, Fig.8.2B). The two fragments anneal during the PCR allowing a complete mutated gene to be amplifiied.

8.5 Transformation of DNA.

8.5.1 Transformation of DNA into *E. coli*

All ligations to generate recombinant plasmids were first transformed into *E. coli* DH5α which were purchased as electrocompetent cells (Gibco). Cells were stored at -80°C and thawed on ice prior to use. 50µl of cells were used per transformation with either 2 µl of ligation reaction or 50 ng of plasmid (in a volume ≤ 4 µl). Cells and DNA were incubated on ice for 30 minutes in Falcon 2059 15 ml tubes. The mixture was then heat-shocked at 42°C for 45 seconds and replaced on ice for 2 minutes. 450 µl of pre-warmed S.O.C. Medium (Gibco) was added to the cells which were then allowed to recover by incubation at 37°C for 45 minutes in a shaking incubator at 225 rpm. 200 µl and 20 µl of each transformation were plated on to appropriate selection plates and grown overnight at 37°C. Negative controls were always performed where no DNA was added to the cells.

8.5.2 Selection of transformants

8.5.2.1 Antibiotic selection

Transformations of *E. coli* DH5α with DNA ligated into either pBluescript or pET-21d (containing the *bla* gene for β-lactamase) were plated out on LB agar plates containing ampicillin at a concentration of 100 µg/ml. Transformations with DNA ligated into pMD31 or pSMT3-19-phoA (containing the genes for *aph* (aminoglycocide phosphotransferase) and *hph* (hygromycin B phosphotransferase)

respectively), were plated out on LB plates containing kanamycin sulphate at 25 μg/ml or Hygromycin B at 200 μg/ml respectively.

8.5.2.2 Blue/White colour screening

Colour screening was used to identify recombinants when cloning DNA into pMD31 or pBluescript vectors. These vectors contain the gene *lac Z'* which codes for the α-peptide portion of β-galactosidase. *E. coli* DH5α contains a modified *lac Z* gene which lacks the region coding for the α-peptide and can only synthesise active β-galactosidase when they harbour a plasmid, such as pBluescript, that carries the missing *lac Z'* segment of the gene, a process called α-complementation. Cloning of a DNA fragment into the *lac Z'* gene of pBluescript or pMD31 resulted in the disruption of the α-peptide and such transformants were unable to synthesise functional β-galactosidase. Screening for such recombinants was performed by plating out transformations on LB agar plates containing the appropriate antibiotic, and X-gal and IPTG at final concentrations of 0.1 mM and 40 μg/ml respectively. Non-recombinant transformants were able to synthesise functional β-galactosidase which breaks down the chromogenic substrate X-gal to a deep blue coloured product, producing blue colonies. However, recombinants with a disrupted *lac Z'* gene were are unable to make functional β-galactosidase and appeared as white colonies.

Blue/white colour screening was also used to identify PhoA fusion proteins using the plasmid vector pSMT3-19-phoA. This vector contains a modified version of the gene encoding alkaline phosphatase *phoA*, which is active only when secreted from the cytoplasm. The vector encodes PhoA lacking its own signal sequence. Thus, alkaline phosphatase activity can be restored by fusion of secretion signals to the amino terminus of the protein (Hoffman and Wright, 1985). Amino terminal fusions were created by cloning PCR products in frame with *phoA*. Recombinants were selected for by plating on XP indicator

plates (LB agar plates containing hygromycin and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) p-toluidine salt (Sigma) at 40µg/ml). BCIP is converted by PhoA to an insoluble end-product that is blue-purple in colour such that in frame recombinants appeared as blue colonies. Transformants that were not in frame with the *phoA* gene appeared as white colonies.

8.5.3 Transformation of mycobacteria by electroporation

Recombinant derivatives of pMD31 or pSMT3-19-phoA were introduced into mycobacteria by electroporation (Jacobs *et al.*, 1987). Preparation of cells for electroporation was performed as follows. 100 ml cultures of mycobacteria were grown in M-ADC-Tw at 37°C, 225rpm, to an absorbance (OD_{600}) of approximately 1.0 and incubated on ice for 1 hour. Bacteria were pelleted by centrifugation at 4,800 rpm (Sigma 3K10, rotor 11133) for 10 minutes at 4°C and resuspended in 5 ml of ice-cold 10% glycerol, followed by the addition of 95 ml ice-cold 10% glycerol. This was repeated twice and the cells were finally resuspended in 3.2 ml of ice-cold 10% glycerol.

DNA was electroporated into mycobacterial cells using the Gene Pulser apparatus (Bio-Rad). 200 ng of plasmid DNA was placed in chilled 0.2 cm electroporation cuvettes (Bio-Rad) to which 0.4 ml of chilled electrocompetent mycobacterial cells were added. Conditions for electroporation were 2.5 kV, 25 µF and the resistance set at infinity. Time constants (τ) were typically between 18 and 21. Electroporated cells were resuspended in 4.5 ml M-ADC-Tw and allowed to recover for three hours at 37°C. Transformants, 200 µl and 20 µl, were plated out on LB agar plates containing the appropriate antibiotic (plus BCIP for transformations with pSMT3-19-phoA derivatives) and incubated for 72 hours at 37°C.

8.6 Analysis of recombinant plasmids

Colour and antibiotic selection provide a powerful tool in the identification of recombinants. However, these methods alone are not sufficient to confirm that a correct recombinant plasmid has been produced.

8.6.1 'Mini-Prep' Plasmid DNA preparation from *E. coli*

Putative recombinant colonies were selected as described above and inoculated into 5 ml LB, containing the appropriate antibiotic. These cultures were grown overnight at 37°C in a shaking incubator at 225 rpm. Plasmid DNA was recovered from 1.5 ml of bacterial culture using the Wizard Plus Minipreps DNA purification system (Promega) according to the manufacturer's instructions. Essentially this is a modified version of the technique developed by Birnboim and Doly (1979) which results in the retrieval of clean covalently closed circular (CCC) plasmid DNA.

Briefly, 1.5 ml of bacterial culture was centrifuged at 3,000 rpm (MSE microcentaur) for 5 minutes and the bacterial pellet resuspended in 200 µl of cell resuspension buffer. Cells were then lysed by the addition of 200 µl of cell lysis buffer containing sodium dodecyl sulphate (SDS). Chromosomal DNA, but not CCC plasmid DNA, is denatured at this step due to the high pH of the cell lysis buffer. This mixture was then neutralised by the addition of 200 µl of neutralisation solution. This causes the chromosomal DNA to renature and aggregate forming an insoluble precipitate whereas the CCC plasmid DNA remains soluble. The high acetate content of the neutralisation solution also causes the SDS-protein complexes to precipitate. The insoluble material was separated from the soluble plasmid DNA by centrifugation at 14,000 rpm (MSE microcentaur) for 5 minutes. The supernatant was removed and mixed with 1 ml Wizard Plus Miniprep DNA purification resin (the resin contains an ion-exchange matrix which selectively bind DNA). The plasmid/resin

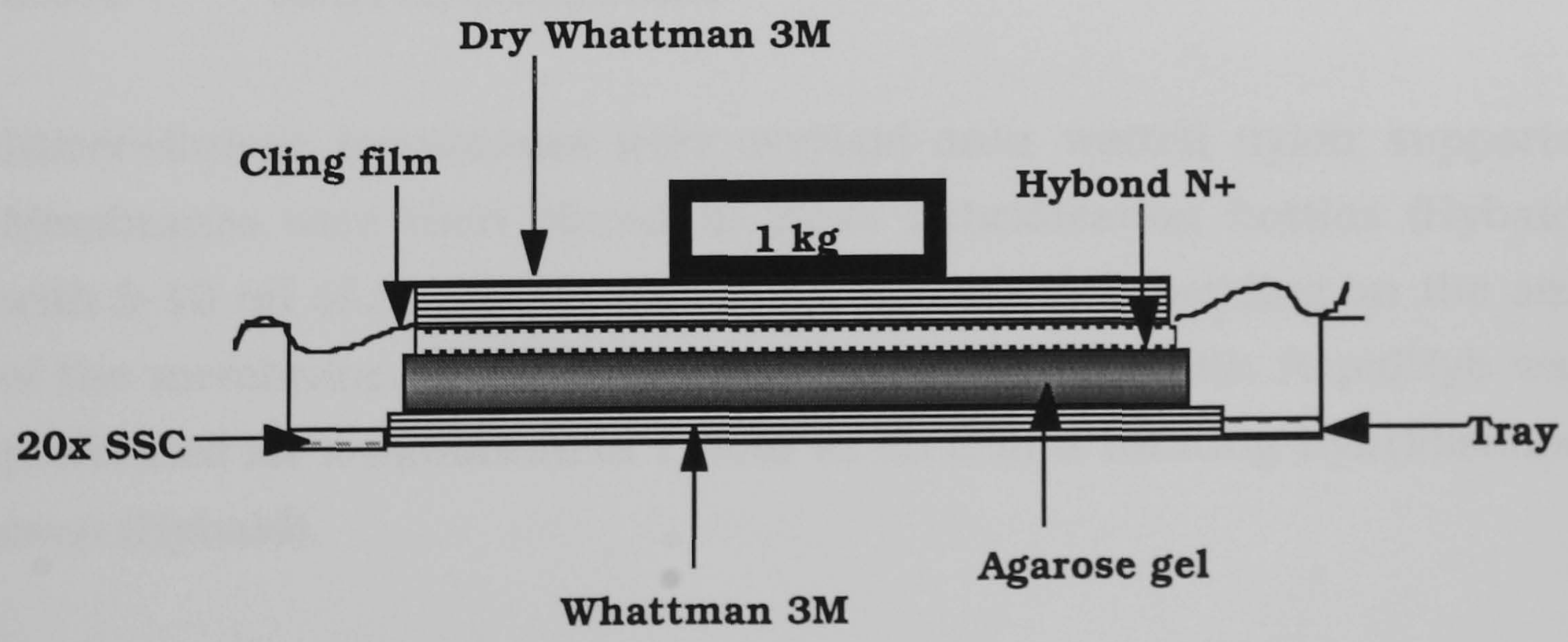
mix was treated as described in section 8.3.4. The resin was washed with 2 ml of column wash solution and DNA eluted with 50 µl Nuclease free distilled water. Plasmid DNA was then digested with appropriate restriction enzymes to confirm the size of the DNA insert of the recombinant plasmid.

8.6.2 Southern blotting

DNA hybridisation was performed as described by Southern *et al.* 1975. Briefly, after gel electrophoresis the DNA was denatured by washing the gel in denaturation solution (0.5 M NaOH, 1.5 M NaCl) for 45 minutes and then neutralised by washing in neutralisation solution (0.5 M Tris-HCl pH 7.4, 1.5 M NaCl) for 45 minutes. Finally the gel was rinsed in 2x SSC solution(3 M NaCl, 0.3 M trisodium citrate).

DNA was transferred to Hybond N+ nylon membrane (Amersham) by capillary action using the technique shown in Fig. 8.3. Care was taken to exclude all air bubbles between the gel and the filter. The cling film has a window the shape of the gel removed to allow the moisture to be drawn upwards. Blotting occured overnight. The blot was then dismantled and the nylon membrane air dried.

Fig. 8.3 The Southern blot technique



8.6.3 Radiolabelling of DNA probes

The method of labelling DNA with radioactivity to high specific activity (up to 10^9 dpm/mg) used in this study was a variation of the method described by Feinberg and Vogelstein (1983). It utilises the ability of DNA polymerase to synthesise a new DNA strand complementary to a template strand, starting from a free 3'-hydroxyl group. Random sequence primers (hexamers) are added to denatured DNA to be labelled. Extension from the primer proceeds, at 37°C, after addition of the Klenow fragment of DNA polymerase I from *E. coli* (which lacks 5'→ 3' exonuclease activity), unlabelled dNTP's and [α^{32}P] dCTP. Following extension the labelling reaction is heat denatured to release single-stranded labelled DNA before addition to a prehybridised membrane.

Typically, 50 - 100 ng of DNA in a volume of 48 µl was denatured at 95°C for three minutes. The denatured DNA was added to a chilled Redi-prime tube (Amersham) containing Klenow, buffer and dNTP's and mixed. 2 µl of [α^{32}P] dCTP (3000Ci/mmol) (Amersham) was then added to the tube which was incubated at 37°C for 15 minutes. The labelled DNA was then denatured at 95°C for 3 minutes before hybridisation to nylon membranes.

8.6.4 DNA Hybridisation

Nitrocellulose membranes were overlaid onto wetted nylon supports. Membranes were then placed in glass hybridisation bottles (Hybaid) with 5-10 ml of RapidHyb solution (Amersham) depending on the size of the membrane. Prehybridisation of membranes with RapidHyb was performed for a minimum of 1 hour at 65°C in a rotating hybridisation oven (Hybaid).

Denatured labelled DNA probe was added to prehybridised membranes and allowed to hybridise, in the presence of the prehybridisation buffer, at 65°C for a minimum of 2 hours. After hybridisation the

probe was decanted and the membranes washed three times for 20 minutes at 65°C to remove non-specfic hybridisations; twice with a 2x SSC solution containing 0.1% (w/v) SDS and once with a 0.1x SSC solution buffer containing 0.1% (w/v) SDS.

Washed membranes were air dried behind a protective screen, wrapped in cling film and placed into cassettes with intensifying screens and exposed to X-ray film at -70°C. Exposure times varied depending on the intensity of the signal.

8.6.5 Colony Hybridisation

This technique was mainly used for screening recombinants when no colour selection was available. Colonies from transformations were picked and streaked on to two gridded agar plates containing the appropriate antibiotic, one having a gridded nylon membrane (Hybond N, Amersham Pharmacia Biotech) on the surface of the agar (replica plate). Typically 200 colonies were plated. Both the master plates and the replica plates were grown at 37°C for 4-6 hours. The master plates were then placed at 4°C and the nylon membranes were subjected to colony lysis.

Briefly, the nylon membranes were placed on filter paper soaked with 5% (w/v) SDS for five minutes, followed by seven minutes on filter paper soaked with denaturation solution (see 8.5.2) and finally for ten minutes on filter paper soaked with neutralisation solution (see 8.5.2). Cell debris was removed from the membranes by rinsing in 20x SSC solution. Membranes were air-dried, fixed by UV crosslinking and then hybridised with labelled insert DNA as described above. The coordinates of colonies that hybridised to the probe were noted and plasmids were retrieved from the living colonies on the master plates by the Wizard Plus Miniprep DNA purification method.

When a recombinant plasmid was shown to be correct, stocks of *E. coli* containing that plasmid were frozen at -80°C at a final concentation of 8% v/v glycerol.

8.7 DNA sequencing

8.7.1 'Midi-Prep' Plasmid DNA preparation from *E. coli*

Midi prep plasmid preparation was performed using Plasmid Midi Kit (Qiagen) according to the manufacturer's instructions. This method employs the same technique described in section 8.5.1. The Midi Prep method was used to purify plasmid DNA from 100 ml of bacterial culture. 100 ml of LB containing the appropriate antibiotic was inoculated from a glycerol stock of recombinant DH5α and grown overnight at 37°C in a shaking incubator at 225 rpm. Bacteria were pelleted by centrifugation at 4,800 rpm (Sigma RC5C+, SLA 1500) for 10 minutes. The cells were resupended in 4 ml of buffer P1 containing RNase A at 100 μg/ml, followed by addition of 4 ml of buffer P2, the mixture was left to incubate for 5 minutes at room temperature. Precipitation of the lysed cells was achieved by addition of 4 ml of buffer P3 and incubation on ice for 15 minutes. The lysate was cleared by centrifugation at 9,000 rpm at 4°C using a Sorvall SLA-1500 rotor. The cleared lysate was applied to an equilibriated Qiagen tip 100 and flowed through under gravity. Plasmid DNA boud to the tip was washed twice with 10 ml of QC wash buffer. Plasmid DNA was eluted with 5 ml of QF elution buffer to which 3.5 ml of isopropanol was added. The precipitated DNA was pelleted by centrifugation at 14,000 rpm , 25°C using a Sorvall SS-34 rotor. The DNA pellet was washed with 5 ml of 70% ethanol and then recentrifuged. DNA was then air-dried and resuspended in 200 μl of nuclease free distilled water. Recovery of plasmid was normally between 1 - 2 μg/μl.

8.7.2 Cycle sequencing

Cycle sequencing uses the dideoxy chain termination method (Sanger *et al.*, 1977). Sequencing reactions were performed using ABI PRISM dRhodamine terminator cycle sequencing ready reaction kit (Perkin-Elmer Applied Biosystems). The terminator ready reaction mix

contains dNTP's and differently dye labelled ddNTP's and the thermostable DNA polymerase, Amplitaq FS.

Cycle sequencing reactions were performed according to the manufacturer's instuctions. Reactions contained between 200 - 500 ng of plasmid DNA from a Midi Prep , 3.2 pmol of oligonucleotide primer, 8 µl of terminator ready reaction mix and deionised water to a final volume of 20 µl. The reaction components were mixed well, briefly spun down and overlaid with mineral oil. Cycle sequencing was performed with a DNA thermal cycler 480 (Perkin-Elmer Applied Biosystems) using the follwing parameters:: 96°C for 30 seconds, 50°C for 15 seconds and 60°C for 4 minutes (25 cycles). Extension products were purified by carefully removing the reaction from beneath the mineral oil and adding it to 2 µl of 3 M sodium acetate and 50 µl of 95% ethanol. The components were vortexed and placed on ice for 10 minutes. Precipitated products were spun for 30 minutes at 14,000 rpm in a microfuge (MSE microcentaur). Pelleted extension products were washed with 250 µl of 70% ethanol, air dried and sent to Oswel (Southampton) for analysis.

8.7.3 DNA sequence analysis

Cycle sequencing reactions were analyzed by Oswel (Southampton) using an ABI 377 DNA Sequencer. For each reaction a sequence analysis document containing information on the sequence was returned. These sequence analysis documents (ABI traces) were analyzed using the computer program, Seqman (DNASTAR Inc.). Contiguous sequences were assembled using Seqman and converted to DNA files in the computer program Editseq (DNASTAR Inc.). DNA files were searched, using Editseq, for open reading frames and when found translated to the corresponding protein. DNA and protein files were aligned using the clustal method of the computer program MegAlign (DNAstar Inc.).

Searches for DNA and protein sequence homology were performed using the 'BLASTP', 'BLASTN' or 'TBLASTN' programs (Altschul *et al.*, 1990) within the National Centre for Biological information (NCBI) home page or MycDB. Retreival of data from DNA sequence databases was acheived using the Entrez browser program at NCBI. Briefly, Genbank reports of required sequence were saved in text format and imported into the Editseq program of DNASTAR as DNA files.

8.8 Preparation of protein samples

Total cell protein samples of *E. coli* were obtained by mixing 800 µl of an overnight culture with 200 µl of 5x SDS gel loading buffer (250 mM Tris-HCl (pH 6.8), 5% (w/v) SDS, 0.1% (w/v) Bromophenol blue, 25% glycerol, 5% β-mercaptoethanol) and heating to 100°C for 3 minutes.

8.8.1 Sonication of bacteria

E. coli were grown from glycerol stocks overnight in 40 ml LB, containing the apropriate antibiotic, at 37°C in a shaking incubator at 225 rpm. Cells were pelleted by centrifugation at 4,800 rpm (Sigma 3K10, rotor 11133) for 10 minutes. The pellet was washed twice with original volumes of PBS (pH 7.4) and resuspended in 3 ml PBS. The cell suspension was sonicated on ice in glass bottles, three times for 30 seconds with 30 second intervals, using a 100-Watt high intensity ultrasonic processor at 10 Watts. After sonication the suspension was centrifuged at 14,000 rpm for 30 minutes (Sigma 3K10, rotor 12153). The supernatant containing soluble proteins was filtered through a 0.22 µm filter unit and stored at -20°C.

Recombinant *M. smegmatis* were grown for 72 hours in 40 ml M-ADC-Tw containing the appropriate antibiotic at 37°C in a shaking incubator at 225 rpm. Cells were washed as described above and

resuspended in 3 ml PBS. The cells were sonicated on ice in glass bottles, five times for 1 minute with 1 minute intervals. Following sonication extracts were prepared in the same way as for *E. coli*. Sonicated extracts, produced by the method of Whipple *et al.*, (1987), and culture filtrate proteins of *M. bovis*, were provided by W. Pressling, VLA, Weybridge.

PBS	Recipe for 1 litre
Phosphate-buffered saline	Dissolve 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4 in 800 ml deionised H_2O. Adjust pH to 7.4 with HCl. Add H_2O to 1litre and autoclave.

8.8.2 Protein concentration estimations

The concentration of total protein present in a sonicated extract was measured using the Bicinchoninic protein assay (BCA) (Pierce). The assay was performed according to the manufacturer's microtitre plate protocol. The assay was calibrated over the range 2 - 0.2 mg/ml by using a range of standards from a 2 mg/ml stock solution of bovine serum albumin (Pierce). Samples, standards and blanks were assayed in triplicate. Samples were also diluted 1 in 5 to ensure a reading was obtained within the limits of the standard curve. The assay was incubated at 37°C for 1 hour and the colorometric change was measured at 560 nm with a microtitre plate reader (Dynex technologies)

8.9 Phase partitioning of lipoproteins

This technique uses the methodology described by Radolf *et al.* (1988). Sonicated extracts of mycobacteria were incubated with an equal volume of a 4% mixture of the non-ionic detergent Triton X-114 (Sigma) in PBS. The resulting mixture was vortexed thoroughly for 2 minutes then incubated on ice for 30 minutes with agitation. The

sample was then warmed to 37°C for 10 minutes and centrifuged at 14,000 rpm for 30 minutes in a 37°C centrifuge (Sigma 3K10. rotor 12153). This resulted in two phases, an organic pellet, consisting of approximately 5% of the starting mixture which contains lipoproteins and other membrane associated molecules, and an aqueous phase. The aqueous phase was re-extracted with another equal volume of PBS. The organic pellets were resolubilised by adding an original volume of 30 mM Tris-HCl (pH 8.7) containing 0.5% (w/v) of the detergent n-octyl β-D-glucopyranoside (Sigma).

8.10 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

The gel system used in this study uses the discontinuous gel method described previously (Laemmli, 1970) in conjunction with the Mini-Protean II system (BioRad). Lower seperating gels of either 12.5 or 15 % acrylamide and an upper gel of 5% were prepared as described in table 8.1. and cast in the mini-protean II apparatus as described by the manufacturer.

8.10.1 Coomassie brilliant blue stain

Following electrophoresis, the fractionated polypeptides were visualised by immersing gels in 20 ml of Coomassie brilliant blue R (CBB) staining solution (5% glacial acetic acid, 0.025% (w/v) CBB, 50 % methanol) and incubated for 3-4 hours on a rotating platform. Stain that was not bound to polypeptide chains was removed from the gel by rinsing it in destaining solution (10% glacial acetic acid, 40 % methanol). When all the background stain had been removed the gel was photographed.

Table 8.1 Formulation for 4 SDS polyacrylamide 7.5 x 10 cm gels

Component	Volume for different acrylamide percentages		
	5%[c]	12.5%	15%
4x lower gel buffer	3.8 ml	5.0 ml	5.0 ml
Water	8.5 ml	6.7 ml	5.0 ml
30% Acrylamide (BioRad)	2.5 ml	8.0 ml	9.8 ml
TEMED[a]	150 µl	200 µl	200 µl
10%APS[b]	15 µl	20 µl	20 µl

[a] N,N,N',N'-tetramethylethylenediene

[b] Ammonium persulphate

[c] Use 4x Upper gel buffer

Solution	Composition
4x lower gel buffer	1.5 M Tris-HCl (pH 6.8), 0.15% SDS
4x upper gel buffer	0.5 M Tris-HCl (pH 8.8), 0.15% SDS
gel running buffer	25 mM Tris,192 mM glycine, 0.035% SDS, pH 8.3

8.10.2 Western blotting

Polypeptides were transferred to a nitrocellulose membrane by the method described by Matsudaira (1987) using Transblot apparatus (BioRad). Briefly, following SDS-PAGE, gels were incubated in CAPS buffer (10 mM 3-(cyclohexylamino)-1 propanesulphonic acid , 10% methanol, pH 11) for 20 minutes. The gel was then placed on Whatman 3M filter paper pre-soaked in CAPS, which in turn was placed on a CAPS pre-soaked sponge support. Nitrocellulose dipped in CAPS was then carefully placed on top of the gel with a symmetrical arrangement on top of the nitrocelluose. The assembly was closed and immersed in CAPS buffer in the Transblot apparatus with the nitrocellulose closest to the anode. The polypeptides were then transferred to the nirocellulose for 2 hours at 300 mA. Under the influence of the current the negatively charged polypeptides migrate

out of the gel towards the anode binding to the nitrocellulose in the process.

8.11 Immunological detection of proteins

Detection of polypeptides immobilised on nitrocellulose with antibodies was performed using the following method. Nitrocellulose filters were first blocked with 3% (w/v) marvel in TBS to saturate non-specific protein binding sites. Blocking solution was then removed by washing the nitrocellulose three times for 10 minutes with TBS containining 0.1% Tween 20 (TBST). The primary antibody was then bound to the nitrocellulose by incubation for 2 hours at 37°C with the primary antibody at the correct dilution in TBST containing 3% marvel. Unbound primary antibody was removed by washing the filter as before. Secondary antibody was either anti-mouse IgG AP conjugate (Sigma) or anti-rabbit IgG AP conjugate (Sigma) depending on whether the primary antibody was a mouse monoclonal or rabbit polyclonal antibody. Binding of secondary antibody was performed under the same conditions as for the primary antibody. Secondary antibody was removed by washing three times with TBS and bound alkaline phosphatase conjugated antibody was detected with SigmaFast tablets (Sigma) dissolved in15 ml water. Colour development was stopped by washing with water.

8.12 Carbohydrate detection by ConA

The lectin ConA has a high specificity for carbohydrate structures such as mannose. ConA binding to immobilised glycoproteins was determined by first blocking the nitrocellulose membranes with 20ml of 5% Bovine serum albumin (Sigma) in PBS with 0.1% Tween 20 (BioRad) for 1 hour with constant shaking at room temperature. After washing the membranes three times for 10 mins with PBS containing 0.5% Tween 20, they were incubated with 20 ml of peroxidase-conjugated ConA at 1 μg/ml in PBS 0.5% Tween 20 without BSA for 1 hour at room temperature. The membranes were then washed twice

with PBS containing 0.5% Tween 20 and finally with PBS. The substrate solution for visualizing peroxidase activity was prepared by dissolving 30 mg 4-chloronaphthol (Sigma) in 12 ml methanol followed by addition of 96 ml of PBS and 17 µl of 30%v/v hydrogen peroxide (Sigma). Colour development was stopped by washing with water.

8.13 Production of recombinant MPT83

Recombinant His-tagged-MPT83 was purified from a 100 ml culture as follows. Briefly, a 100 ml culture of LB broth (100 µg/ml ampicllin) was inoculated with 1 ml of an overnight culture of *E. coli* BL21(DE3) containing pSM36 (Fig 7.1) and grown at 37°C, 225rpm to an A_{600} of between 0.6 and 1.0. Expression of the recombinant protein was induced by the addition of IPTG to a final concentration of 1 mM after which the culture was incubated at 37°C, 225rpm for 4 hours. Cells were pelleted by centrifugation at 4,000 rpm (Sigma RC5C+, SLA 1500) and resuspended in 10 ml distilled water. This suspension was sonicated on ice for 5 mins as described in section 8.8.1. Cell debris was pelleted by centrifugation at 4,000 rpm (Sigma 3K10, rotor 11133) for 10 mins and resuspended in 10 ml distilled water. This solution was sonicated for 5 mins on ice as described above. Following sonication the suspension was centrifuged again at 4,000 rpm for 10 mins. The pellet was resuspended in 1.5 ml of a solution containing, 20 mM Tris-HCl (pH 7.9), 8M Urea and 0.5M NaCl, and left to resolubilise overnight at room temperature. After 24 hours the resolubilised protein was extensively dialyzed against PBS (pH 7.0).

8.14 Immunisation procedure

Female BALB/c mice, 6 - 10 weeks old, were obtained via the Animal Services Unit, VLA, Weybridge. Groups of 5 mice each were sensitised by injection subcutaneously in the footpads with 100 µg of recombinant MPB83 or 1 mg of sonicated mycobacterial extract emulsified in incomplete Freund's adjuvant (IFA). Popliteal lymph

nodes were removed aseptically 7 days later and single cell suspensions prepared.

8.15 Lymphocyte proliferation assay

Lymphocyte proliferation assays were performed as described previously (Vordermeier *et al.*, 1995). Briefly, LN cells (4 x 10^5/well of flat-bottomed 96-well microtitre plates) were kept in triplicate in culture medium (RPMI 1640 (GIBCO) supplemented with 10% heat-inactivated foetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 5 x 10^{-5} M 2-mercaptoethanol) in the presence of recombinant MPB83 (10 µg/ml) or synthetic peptides (25 µg/ml). Concanavalin A (5 µg/ml) was used routinely as positive control. Plates were incubated for 3 days at 37° C in 5% CO_2, then pulsed with 18.5 kBq of [^{3}H]-thymidine (Amersham) and the incorporated radioactivity determined 6 h later in a direct beta-counter (Canberra Packard). The results are expressed as stimulation indices SI (cpm with antigen/cpm without antigen).

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Appendix 1. Oligonucleotide primers used for DNA amplification¶‡

Oligonucleotide	No.	Sequence
pBAK14F	1	5'- ACCCTGGATGCTGTAGGCATAG
pBAK14R	2	5'-GTCTCCGAATCCAACTGGCT
70 Mid Forward	3	5'-CTCGGTGCAGGGAATGT
70 Mid Reverse	4	5'-ACATTCCCTGCACCGAG
M13 -20 primer	5	5'-GTAAAACGACGGCCAAGT
Reverse primer	6	5'-GGAAACAGCTATGACCATG
83 Forward	7	5'- ATGATCAACGTTCAGGCCA
83 Reverse	8	5'- TTACTGTGCCGGGGGATCA
83 phoAF	9	5'-ACGGGTACCATGATCAACGTTCAGGCCAA
83 phoAR	10	5'-TGAGGATCCCTGTGCCGGGGGCATCAGCA
70 phoAF	11	5'-ACGGGTACCATGAAGGTAAAGAACACAAT
70 phoAR	12	5'-TGAGGATCCCGCCGGAGGCATTAGCACGC
83 Mid Forward	13	5'-ACGATCCTGACCTACCACGTGATA
83 Mid Reverse	14	5'-TGAACGTTGATCATCGCTTCGATT
19kDa prom F	15	5'-ACCCAAGCTTGGTTTGTGTTCCATCGGCAC
phoA R Seq	16	5'-CAGTCTGATCACCCGTTAAACGGCG
27aa-83 phoAR	17	5'-TGAGGATCCGGTGCTCGAACAACCCGCTA
35aa-83 phoAR	18	5'-TGAGGATCCGGTGTCTTGCGACACGGGTT
56aa-83 phoAR	19	5'-TGAGGATCCTGCGGGGTCAGCCATTGCCG
63aa-83 phoAR	20	5'-TGAGGATCCCCCACGACCAATCAGGTCCG
70g F	21	5'-GTTACCACGGCGGCAATGGCTGACCCCGCAGGCGATC TGGTGGGC
70g R	22	5'-ACGCCGTGGTAACGGGGGCCGCCGGCGGTGAGACAG
Reverse 70	23	5'-TCCTCTAGATTACGCCGGAGGCATTAGCACGC
TT-VV Forward	24	5'-GCCCGGCGGCGCCCGTT**GTAGTA**GCGGCAATGGCTG
TT-VV Reverse	25	5'-CAGCCATTGCCGC**TACTAC**AACGGGCGCCGCCGGGC
TT-VT Forward	26	5'-GCCCGGCGGCGCCCGTT**GTA**ACGGCGGCAATGGCTG
TT-VT Reverse	27	5'-CAGCCATTGCCGCCGT**TAC**AACGGGCGCCGCCGGGC
TT-TV Forward	28	5'-GCCCGGCGGCGCCCGTTACC**GTA**GCGGCAATGGCTG
TT-TV Reverse	29	5'-CAGCCATTGCCGC**TAC**GGTAACGGGCGCCGCCGGGC
N-G Forward	30	5'-GCGACGACCTCATGGTC**GGC**AACGCCGGTTTGGTAT
N-G Reverse	31	5'-ATACCAAACCGGCCGTT**GCC**GACCATGAGGTCGTCGC
83 His-tagF	32	5'-GCGGGATCCACATGATCAACGTTCAGGCCAA
83 His-tagR	33	5'-ACGCAAGCTTCTGTGCCGGGGGCATCAGCA
pET21d MCS F	34	5'-TGTGAGCGGATAACAATT
pET21d MCS R	35	5'-TATGCTAGTTATTGCTCA
Reverse 83	36	5'-ATATCTAGATTACTGTGCCGGGGGCATCAGCA

¶ Restriction endonuclease sites are underlined

‡ Nucleotides encoding amino acid changes are shown in bold

Appendix 2. DNA Sequence of 19 kDa promoter and *mpt83* in pSM52.

```
pSM52 Consensus   A A G C T T G G T T T G T G T T C C A T  20
19 kDa promoter   - - - - - - G G T T T G T G T T C C A T  14
mpt83             - - - - - - - - - - - - - - - - - - - -  0

pSM52 Consensus   C G G C A C T A C A T T G C C A C T A C  40
19 kDa promoter   C G G C A C T A C A T T G C C A C T A C  34
mpt83             - - - - - - - - - - - - - - - - - - - -  0

pSM52 Consensus   T A C G G T G C A C G C C G G T A G A T  60
19 kDa promoter   T A C G G T G C A C G C C G G T A G A T  54
mpt83             - - - - - - - - - - - - - - - - - - - -  0

pSM52 Consensus   G C C G T T G G C G A A C C A C G C T A  80
19 kDa promoter   G C C G T T G G C G A A C C A C G C T A  74
mpt83             - - - - - - - - - - - - - - - - - - - -  0

pSM52 Consensus   C C G A C C A G A A A G A G A G A A T T  100
19 kDa promoter   C C G A C C A G A A A G A G A G A A T T  94
mpt83             - - - - - - - - - - - - - - - - - - - -  0

pSM52 Consensus   T T C C G C C G C A C C T A G A C C T C  120
19 kDa promoter   T T C C G C C G C A C C T A G A C C T C  114
mpt83             - - - - - - - - - - - - - - - - - - - -  0

pSM52 Consensus   G G G C C C T G C T A A C G C G C A T A  140
19 kDa promoter   G G G C C C T G C T A A C G C G C A T A  134
mpt83             - - - - - - - - - - - - - - - - - - - -  0

pSM52 Consensus   C T G C C G A A G C G G T C C T C A A T  160
19 kDa promoter   C T G C C G A A G C G G T C C T C A A T  154
mpt83             - - - - - - - - - - - - - - - - - - - -  0

pSM52 Consensus   G C C G A T G G A C C G C T A C G A C A  180
19 kDa promoter   G C C G A T G G A C C G C T A C G A C A  174
mpt83             - - - - - - - - - - - - - - - - - - - -  0

pSM52 Consensus   G G C A A A G G A G C A C A G G T A C C  200
19 kDa promoter   G G C A A A G G A G C A C A G G           190
mpt83             - - - - - - - - - - - - - - - - - - - -  0

pSM52 Consensus   A T G A T C A A C G T T C A G G C C A A  220
mpt83 gene        A T G A T C A A C G T T C A G G C C A A  20
                  Met  Ile  Asn  Val  Gln  Ala  Lys

pSM52 Consensus   A C C G G C C G C A G C A G C G A G C C  240
mpt83             A C C G G C C G C A G C A G C G A G C C  40
                  Pro  Ala  Ala  Ala  Ala  Ser
```

This diagram shows the relationship between the DNA sequence of the 19 kDa promoter with the start codon of genes cloned downstream at the *Kpn* I site indicated (boxed). The Shine Dalgarno RBS is highlighted in green. This arrangement is identical for all genes cloned downstream of the 19kDa promoter.

Appendix 3. Electrophoretograms of sequenced mutations

A3.1 DNA sequence data of pSM79

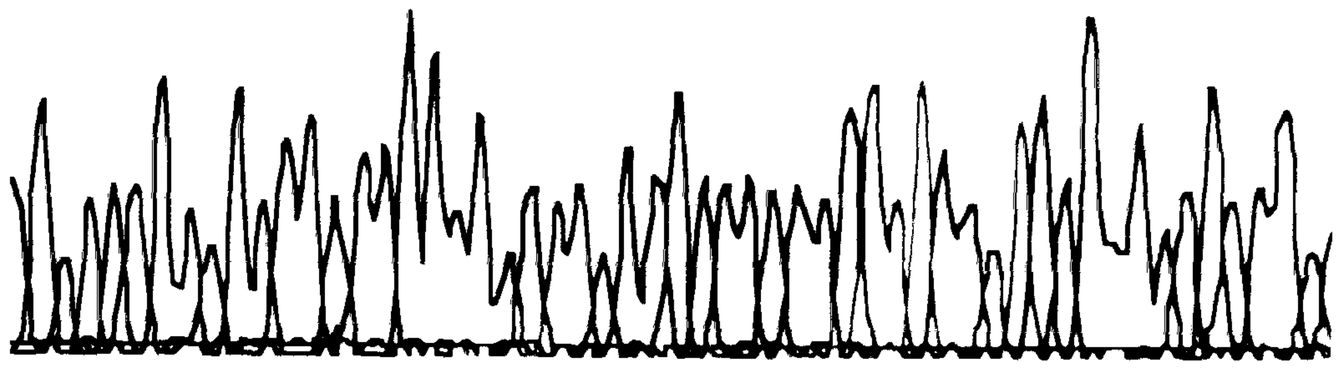
(Inserted peptide shown in bold).

Consensus

GTC TCAC CGC CGGCG GCC CCCGT TAC CACG GCG GCAA TGGC T GAC CCCG CAGG C

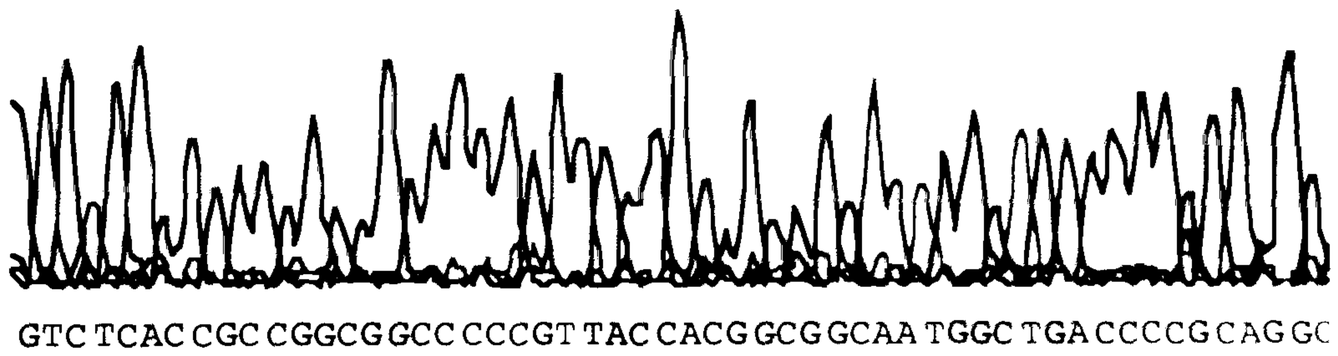
V S P P A A **P V T T A A M A D P** A G

pSM79 (Primer 4)



GTC TCAC CGC CGGCG GCC CCCGT TAC CACG GCG GCAA TGGC TGA CCCCG CAG GC

pSM79 (Primer 15)



GTC TCAC CGC CGGCG GCC CCCGT TAC CACG GCG GCAA TGGC TGA CCCCG CAG GC

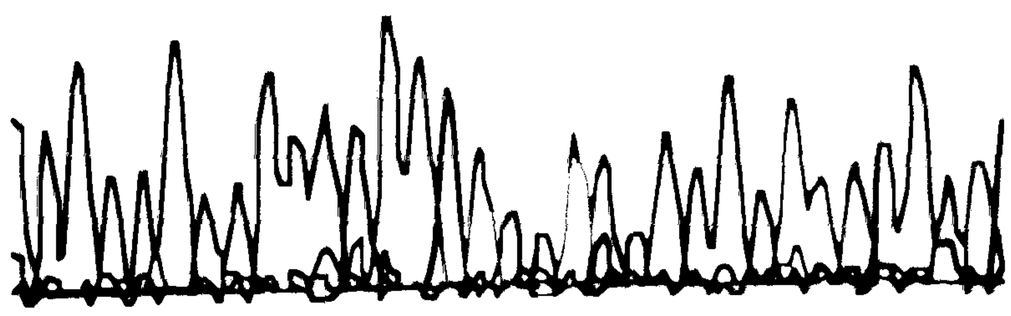
A3.2 DNA sequence data of pSM66 (SDM underlined).

Consensus

C GG C GG CG C CC GT T GT A GT A GC GG C AA T GG C T

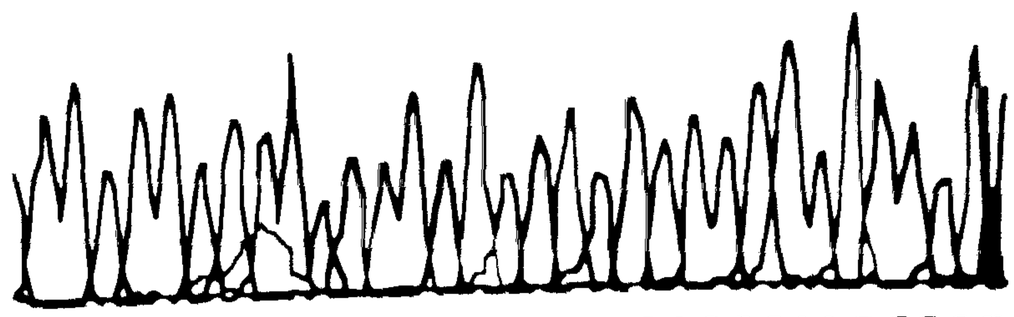
A A P V V V A A M A

pSM66 (Primer 15)



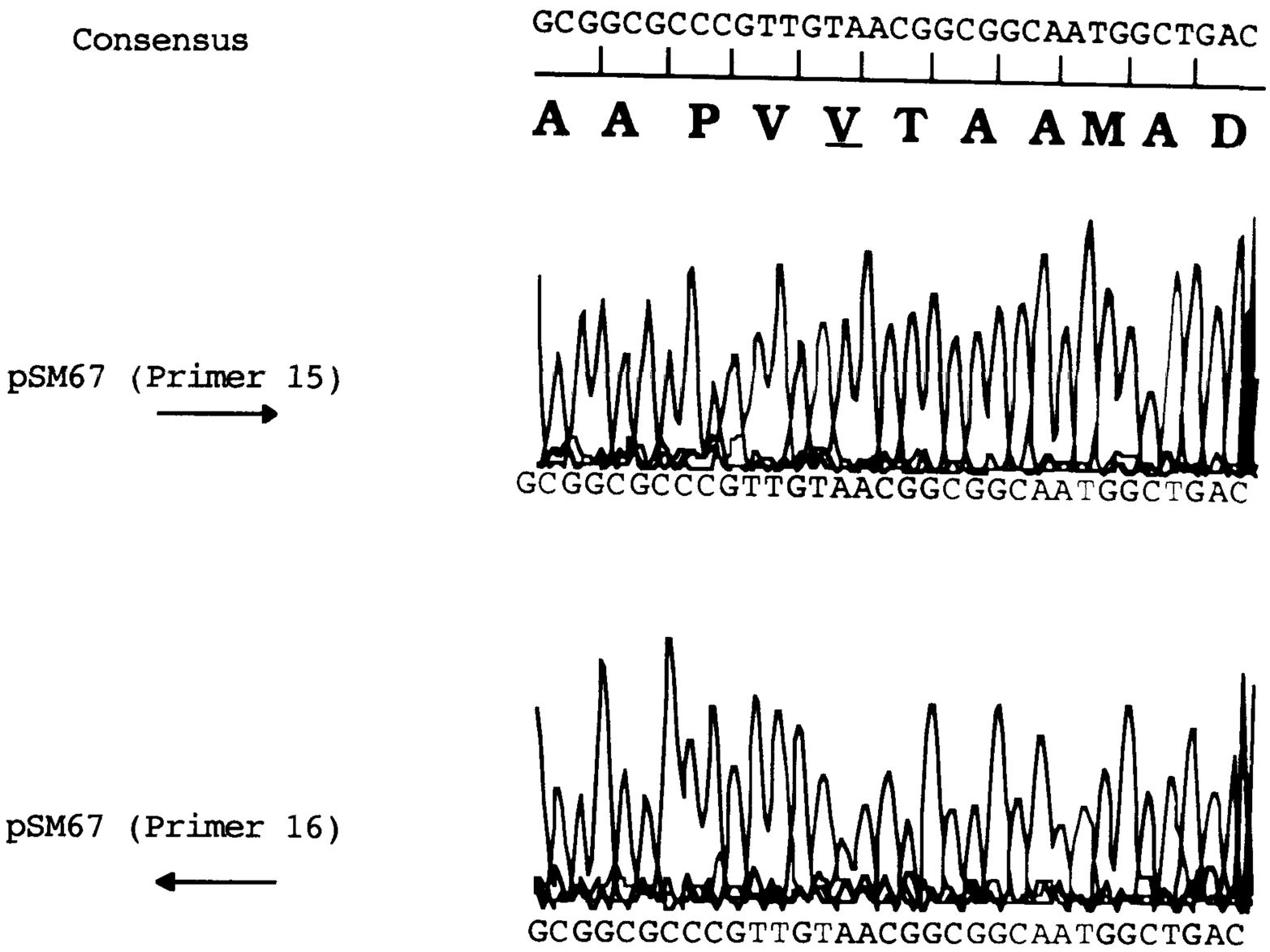
CGGCGGCGC CCGTTGT AGT AGCGGCAATGGCT

pSM66 (Primer 16)

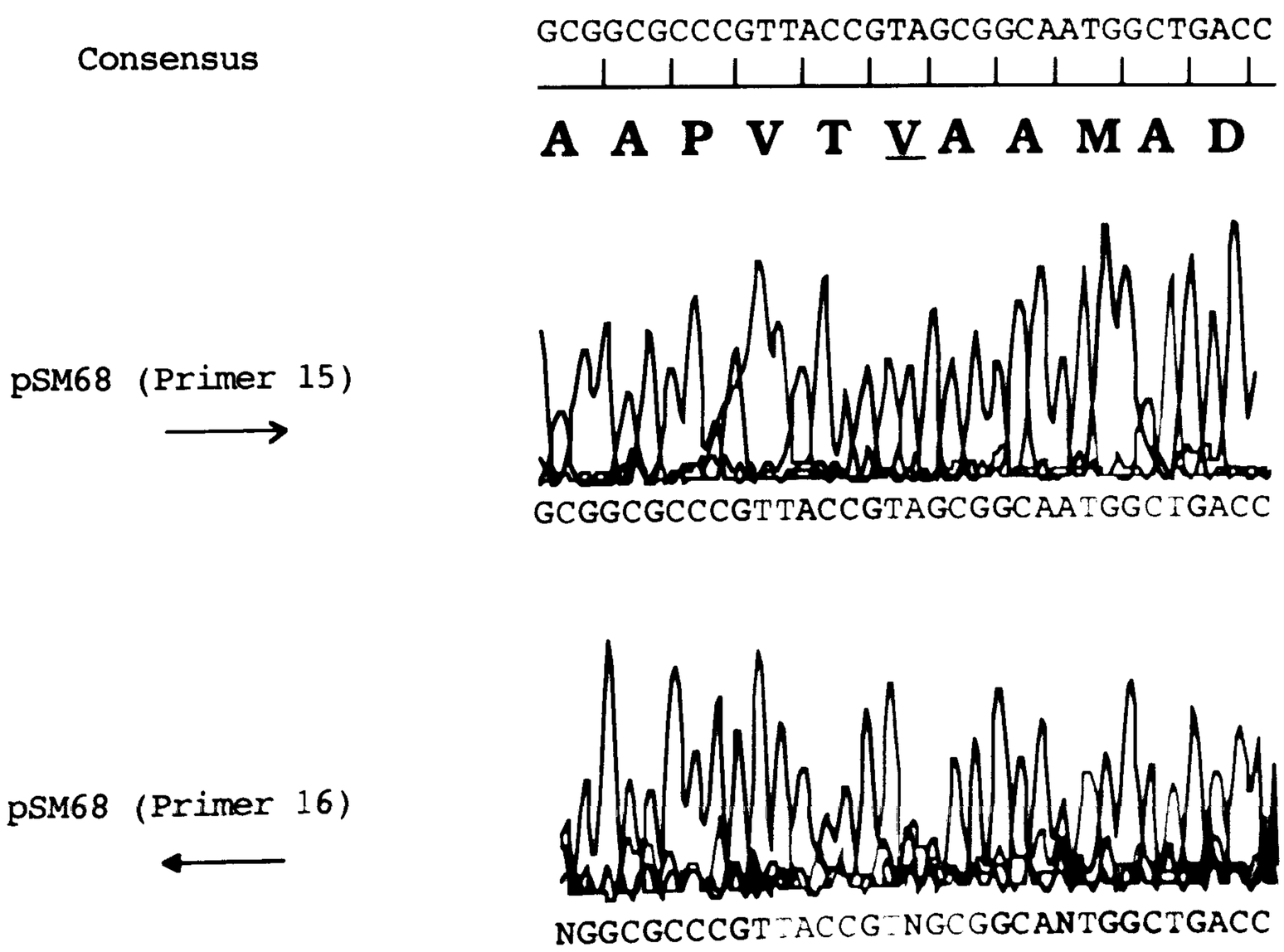


CGGCGGCGC CCGTTGT AGTAGCGGCAA T GGCT

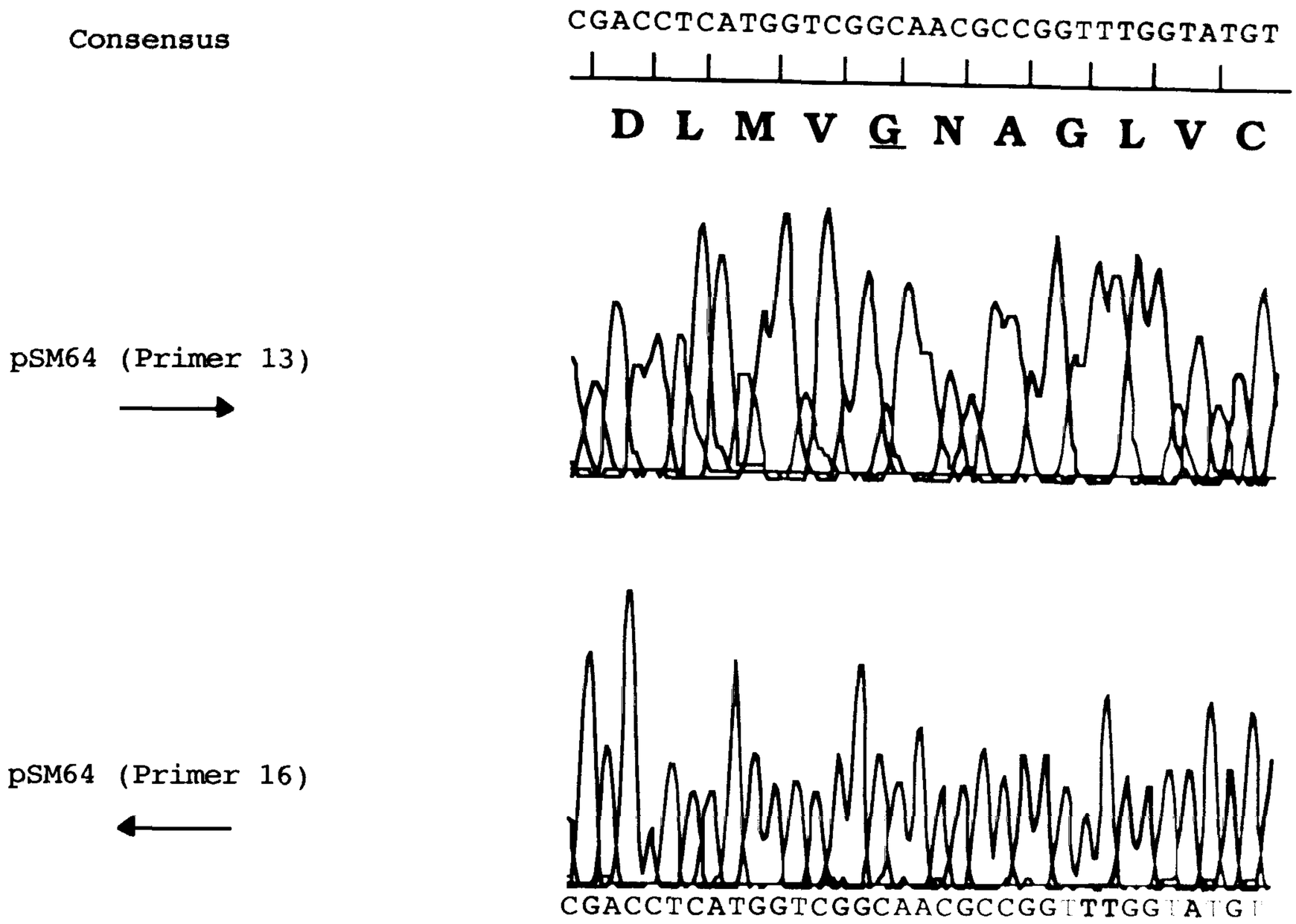
A3.3a DNA sequence data of pSM67 (SDM underlined).



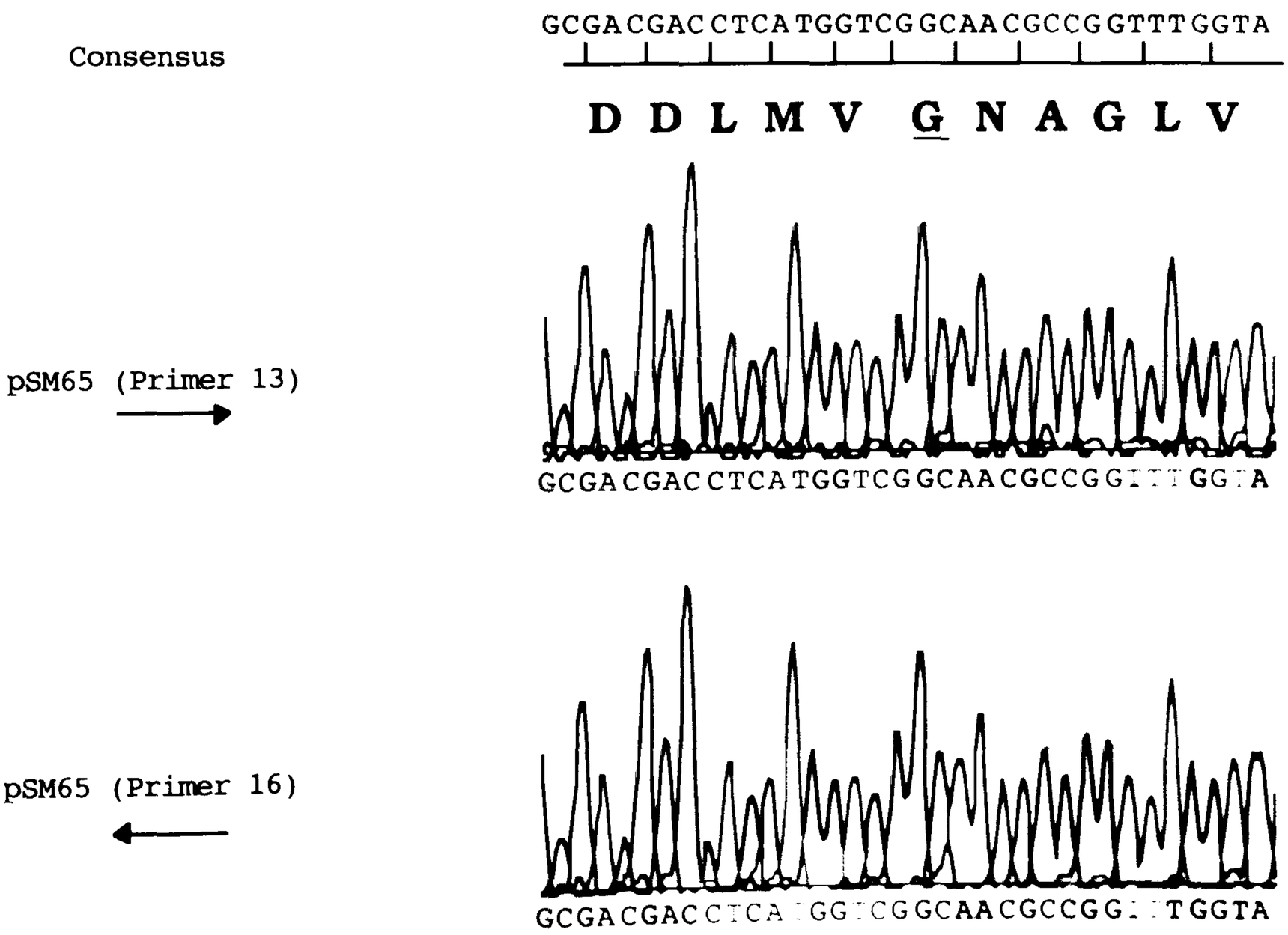
A3.3b DNA sequence data of pSM68 (SDM underlined).



A3.4a DNA sequence data of pSM64 (SDM underlined).



A3.4b DNA sequence data of pSM65 (SDM underlined).



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