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Cloning of the DNA of mycobacterium tuberculosis and analysis of the expressed protein antigens

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Rabson, 1987; Kalish, Radin, Phair, et al., 1983; Stroebel, Daniel, Lau, et al., 1982) have recently been developed for the diagnosis of both mycobacterial antigens, and antibodies to mycobacteria, in serum, pleural, synovial, and cerebrospinal fluid. These techniques are reliable and may differentiate active tuberculosis from a variety of other diseases.

With very few exceptions, however, studies on the serology of tuberculosis have shown that sera from a proportion of patients with non-mycobacterial diseases, and healthy individuals, contain antibodies which react with mycobacterial antigens. There are several reasons for this (Grange, 1980);

i. antibodies produced in response to contact with environmental mycobacteria;
ii. antibodies produced to antigens common to mycobacteria and other bacteria as a result of contact with the latter;
iii. cryptic infection by environmental mycobacteria in other diseases;
iv. unproven mycobacterial aetiology of certain other diseases;
v. an increase in the background level of antibodies due to polyclonal stimulation of antibody production;
vi. a "non-immune" reaction from serum factors.

1.5.4 The tuberculin skin test

Persons who develop tuberculous disease or become infected with tubercle bacilli demonstrate delayed hypersensitivity to certain mycobacterial antigens (Youmans, 1986). Such hypersensitivity can be detected by introducing small amounts of tuberculoprotein (tuberculin; PPD) into the skin (Drutz and Graybill, 1987). In the face of competent cell-mediated immunity, however, the tuberculin skin test provides no diagnostic information relative to acute illness unless it can be established that the skin test has converted from negative to positive in temporal relation to the illness. A positive skin test therefore only indicates that the patient has experienced tuberculosis or a closely related mycobacterial infection at some time in the past (Drutz and Graybill, 1987).

Positive and negative tuberculin reactions must however be interpreted with caution. A false positive reaction may occur as a result of conversion to tuberculin hypersensitivity by persons vaccinated with BCG, and due to possible cross-reaction of PPD, the exact antigenic composition of which is unknown, with the proteins of other mycobacteria (Youmans, 1986). A negative skin test can signify no tuberculosis, or an anergic immune state (Daniel, Oxtoby, Pinto, et al., 1981; Ellner, 1986), caused by advanced and disseminated tuberculosis, or immune suppression due to viral

infections, lymphoreticular malignancies, Hodgkin's disease, sarcoidosis, and immunosuppressive therapy (Drutz and Graybill, 1987; Youmans, 1986).

Characterisation of the M.tuberculosis antigens which are responsible for eliciting antibody responses and delayed hypersensitivity in infected persons is therefore necessary in order to reduce the low sensitivity and non-specificity of currently available diagnostic tests. Ideally, mycobacterial antigens which can be characterised to the level of protein or polypeptide, and which are unique to M.tuberculosis, could act as specific tools for the diagnosis of mycobacterial infection.

1.6 PREVENTION AND VACCINATION PROGRAMMES

The basic principle involved in the prevention of tuberculosis focuses on procedures that prevent contact between susceptible persons and the diseased carrier (Youmans, 1986). Intensive efforts have been made to control tuberculosis by these means. Mass chest roenterogram screening programmes have been conducted in order to detect persons with pathologic pulmonary findings (Youmans, 1986). Diseased persons diagnosed in this way have then been intensively treated with chemotherapeutic agents to render them non-infectious (Youmans, 1986). In many communities, tuberculin testing surveys of populations are conducted

regularly. Those found to be tuberculin positive are then further tested and treated if found to be infected with M.tuberculosis (Drutz and Graybill, 1987).

The application of these procedures for the detection, isolation and treatment of tuberculosis has resulted in a steady drop in the incidence and prevalence of, and mortality from, tuberculosis in the Western world (Youmans, 1986). However, most cases of tuberculosis have been found in areas where there is a low level of education, lack of sanitation, overpopulation, and overcrowding. The preventive programmes described above are enormously expensive, present problems with the full cooperation of everyone in the targeted population groups, and require facilities for the detection of tuberculosis and the treatment of known cases. These programmes are therefore of limited benefit to those populations most at risk. Other means of control in such populations must therefore be found.

An important approach to the prevention of tuberculosis is vaccination against the disease. A vaccine is available in the form of BCG (Bacillus Calmette-Guerin). BCG is an attenuated mutant of a virulent strain of M.bovis, developed in the early years of the twentieth century (Calmette, 1931). BCG vaccination against tuberculosis has been used for many years in practically all national programmes (Youmans, 1986). A high level of immunity can be induced, which persists for

as long as 10 years after a single vaccination with BCG (Youmans, 1986). Although it has been instrumental in controlling tuberculosis in the Western world, BCG has, however, proved completely inefficient in providing protection against M.tuberculosis infections in a large-scale clinical trial in rural Southern India (Ten Dam, 1984). In addition, vaccination with BCG induces tuberculin hypersensitivity in the recipient, and as such a valuable diagnostic tool may be lost.

It is thus of considerable importance that a standardised, specific and effective vaccine be found against M.tuberculosis.

1.7 MYCOBACTERIAL ANTIGENS

An important feature in the pathogenesis of tuberculosis is the nature and specificity of the response of the immune system to antigens of tubercle bacilli. These antigens are numerous, but as yet have been poorly defined. A variety of polysaccharide and protein antigens from mycobacteria have been described (Young, 1988; Chaparas, 1982; Daniel and Janicki, 1978). There is currently no standard nomenclature for mycobacterial antigens; different investigators use different codes based on various reference systems. The methods used to isolate mycobacterial antigens to date have largely employed physiochemical fractionation, including ion-exchange chromatography, molecular exclusion chromatography, density gradient ultracentrifugation, isoelectric focussing, and zonal electrophoresis (Daniel and Janicki, 1978). Salt or solvent solubility has often been used in combination with these techniques (Daniel and Janicki, 1978).

It was not until the work of Janicki and his collaborators (1971) that a widely and readily applied system of identification and nomenclature for individual mycobacterial antigens became available. Their work was based on a relatively simple immunoelectrophoresis technique, offering the advantage of ready applicability with easily obtainable materials (Daniel and Janicki, 1978). Such

immunoelectrophoresis techniques have been used to identify 11 major mycobacterial antigens (Daniel and Janicki, 1978). A second reference system based on crossed immuno-electrophoresis (CIE) was more recently introduced (Closs, Harboe, Axelsen, et al., 1980) It has certain advantages over other characterisation systems in that it covers a large range of antigen molecules; it has been particularly useful for identifying antigens present in mycobacterial culture filtrates. Significant progress has been made in the purification and analysis of several of the antigens originally defined by CIE (antigens MPB64, MPB70, and MPB80 of M.bovis BCG), and partial or complete sequence data has been obtained in some cases (Wiker, Harboe, Bennedsen, et al., 1988; Harboe, Nagai, Patarroyo, et al., 1986). Disadvantages of the CIE system however, include low reproducibility of results, and the identification of single precipitin lines corresponding to multi-molecular structures (for example, line 7 of the M.leprae CIE system has been shown to represent not one, but 3 antigens subsequently identified using monoclonal antibodies) (Engers, Abe, Bloom, et al., 1985).

Cell wall polysaccharides, proteins and peptides have all been shown to be antigenic under different experimental conditions (Daniel and Janicki, 1978). With respect to polysaccharide antigens (Table 1), however, the ability to elicit delayed hypersensitivity reactions remains doubtful

TABLE 1 Mycobacterial carbohydrate antigens (Young, 1988)

Antigen class	Distribution	Location
Glycopeptidolipids	M.avium M.intracellulare M.scrofulaceum M.lepraemurium M.paratuberculosis (species specific)	capsule
Phenolic glycolipids	M.leprae M.kansasii M.bovis M.tuberculosis (canetti) (species specific)	capsule
Lipooligosaccharides	M.kansasii M.gordonae M.szulgai M.xenopi (species specific)	capsule
Phosphatidyl inositol mannosides	all mycobacteria	membrane
Arabinogalactan	all mycobacteria	cell wall
Lipoarabinomannan	all mycobacteria	cell wall, capsule

and may be limited to guinea pig models only (Daniel and Janicki, 1978). In addition, these antigens have been shown to be non-specific, being shared by all species of mycobacteria, and by nocardia and corynebacteria as well (Young, 1988). Cell wall arabinogalactan and arabinomannan have been found to be excellent antigens in serological systems, and the antigenic determinant of arabinogalactan has been identified to be a major arabinose side chain (Daniel and Janicki, 1978).

Some mycobacterial antigens have been shown to be species-specific (Daniel and Janicki, 1978; Young, 1988). Antigens 1 and 2 (arabinogalactan 2) have been identified to be polysaccharide antigens and have been found to be widely distributed among the mycobacteria (Daniel and Janicki, 1978). Antigens 6, 7, and 8 have also found to be present in culture filtrates from the majority of mycobacterial species studied (Daniel and Janicki, 1978). Antigen 5, a cytoplasmic protein antigen, has been demonstrated to be limited to M.tuberculosis and M.bovis (Daniel and Janicki, 1978). Antigen 6 has been shown to contain at least 2 antigenic determinants, one of which appears to be specific for M.tuberculosis, and the other of which is present on several other mycobacteria (Daniel and Janicki, 1978).

It is unreasonable to expect real success in antigen purification using physiochemical techniques, as the antigens

purified by such means have a greater or lesser extent of molecular heterogeneity. All antigenic materials are derived from physically disrupted or lysed cells, and there is no reason to expect that their release is a uniform process; many antigens are probably derived from the cell wall, where repeating units abound, and individual antigenic determinants may be expected to be present on various-sized fragments in varying combinations and numbers (Daniel and Janicki, 1978).

Purified, well characterised, and standardisable antigens are necessary for the accurate diagnosis of tuberculosis, and also for the assessment of cell-mediated immunological responses to tuberculous infection. A means to survey all of the protein antigens of M.tuberculosis, without carrying out a rigorous biochemical isolation of each component, could facilitate the identification of specific diagnostic tools, and would be a useful prerequisite for the selection of a polypeptide vaccine candidate for further study.

1.8 MYCOBACTERIAL ANTIGENS IDENTIFIED WITH MONOCLONAL ANTIBODY AND RECOMBINANT DNA TECHNOLOGY

Recombinant DNA technology offers an effective strategy to thoroughly and systematically examine the antigens encoded in linear segments of the M.tuberculosis genome, and the development of monoclonal antibody technology has provided a means for producing monospecific antibody reagents which can be used to dissect mycobacterial antigens (Kolk, Ho, Klatser, et al., 1984). Application of these techniques for the analysis of mycobacterial antigens has resulted in the identification and detailed structural characterisation of a variety of protein antigens from M.tuberculosis, M.leprae, and M.bovis, in addition to the previously described carbohydrate and glycolipid components. DNA from M.tuberculosis, M.leprae, and M.bovis was first cloned into Escherichia coli (E.coli) using standard plasmid, cosmid, or λ bacteriophage vectors (Jacobs, Docherty, Curtiss, et al., 1986; Clark-Curtiss, Jacobs, Docherty, et al., 1985; Bhattacharya and Bhattacharya, 1984; Thole, Dauwerse, Das, et al., 1985). These studies showed that mycobacterial promoters were (mostly) recognised weakly, if at all, by the E.coli transcriptional machinery, but that mycobacterial translational signals, however, were expressed satisfactorily. More recent experiments, however, have indicated that although most of the recombinant mycobacterial proteins identified to date have been fusion proteins, some

recombinant proteins were capable of being expressed from their own signals (Shinnick, 1987; Cohen, Mayer, Rumschlag, et al., 1987; Lamb, Kingston, Estrada-Garcia, et al., 1988). These latter studies suggest that some mycobacterial promoters are functional in E.coli.

The combination of monoclonal antibodies and the λ gt 11 expression system has been a powerful influence on the progress made in the identification of novel mycobacterial antigens over the past few years, and recombinant clones expressing antigenic determinants recognised by monoclonal antibodies have been isolated (Young, 1988) (Table 2). The presence of shared epitopes among mycobacterial antigens has been demonstrated using monoclonal antibodies (Daniel and Olds, 1985; Ivanyi, Sinha, Aston, et al., 1983; Husson and Young, 1987; Anderson, Barry, and Buchanan, 1988). A panel of monoclonal antibodies now exists which are able to recognise antigens unique to M.leprae (Engers, Abe, Bloom, et al., 1985). Less success has been achieved in the development of species-specific probes for M.tuberculosis. Although some antigenic diversity has been demonstrated, (Coates, Allen, Hewitt et al., 1981), the majority of the monoclonal antibodies used failed to distinguish between M.tuberculosis and M.bovis BCG (Young, 1988).

TABLE 2 M.tuberculosis proteins identified using antibodies

antigen molecular weight (daltons)	origin of protein	gene sequence known	type of antibody	references
71 000	culture filtrate; λgt 11 library	yes	polyclonal; monoclonal	Young et al., 1987 Shinnick et al., 1987 Andersen et al., 1986 Lu et al., 1987
68 000	culture filtrate	no	polyclonal; monoclonal	Collins et al., 1988
65 000	cell sonicate; λgt 11 library	yes	polyclonal; monoclonal	Oftung et al., 1987 Shinnick et al, 1987a Laab et al., 1986 Shinnick, 1987 Shinnick et al., 1987 Andersen et al., 1988 Shinnick et al., 1988 Young et al., 1987 Daalani et al., 1988 Lu et al., 1987
53 000	recombinant plasmid	no	polyclonal	Cohen et al., 1987
39 000	culture filtrate	no	polyclonal; monoclonal	Worsaae et al., 1987
38 000	cell sonicate; culture filtrate; λgt 11 library	yes	polyclonal; monoclonal	Young et al., 1986 Andersen et al., 1988 Young et al., 1987 Andersen et al., 1986

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TABLE 2 (continued)

antigen molecular weight (daltons)	origin of protein	gene sequence known	type of antibody	references
35 000	cell sonicate; culture filtrate; λgt 11 library; recombinant plasaid	no	polyclonal; monoclonal	Damiani et al., 1988 Olds et al., 1987 Cohen et al., 1987
32 000-33 000	culture filtrate	no	polyclonal; monoclonal	Worsaae et al., 1987 Andersen et al., 1986
19 000	culture filtrate; λgt 11 library	yes	polyclonal; monoclonal	Andersen et al., 1988 Young, 1988 Lamb et al., 1986 Oftung et al., 1987 Shinnick et al.,1987a Lu et al., 1987
14 000	λgt 11 library	yes	polyclonal; monoclonal	Young et al., 1987 Kingston et al., 1987 Shinnick et al.,1987a Lu et al., 1987
12 000	λgt 11 library	no	monoclonal	Shinnick et al.,1987a

In addition to screening antigens with monoclonal antibodies, mycobacterial antigens have also been identified using polyclonal antisera (Thole, Dauwerse, Das, et al., 1985; Young, Kent, and Young, 1987; Young and Davis, 1983). The majority of the antigens identified with polyclonal antibodies have been shown to overlap with the proteins identified using monoclonal antibodies. The mycobacterial antigens identified to date using antibody probes are shown in Table 2.

It is important to note that the use of antibodies as probes may result in a focus of attention on antigens which are important in the humoral response, but which may play only a minor role in the T cell response. The human T cell response has been assessed using purified mycobacterial antigens (Young, Kent, Rees, et al., 1986; Ottenhof, Klatser, Ivanyi, et al., 1986), or antigens expressed in recombinant DNA clones (Emmrich, Thole, van Embden, et al., 1986; Mustafa, Gill, Nerland, et al., 1986; Oftung, Mustafa, Husson, et al., 1987; Thole, van Schooten, Keulen, et al., 1988). T cell recognition, as judged by lymphocyte proliferation, has been demonstrated for these antigens (Table 3). Limiting dilution analysis has shown that T cells which recognise the 65 kDa protein make up a major portion (20%) of the total antimycobacterial T cell response following immunisation of mice with M.tuberculosis (Kaufmann, Vath, Thole, et al., 1987).

TABLE 3 M.tuberculosis proteins which stimulate the proliferation of T lyephocytes

antigen molecular weight (daltons)	T lyephocyte populations	antigen source	references
68 000	polyclonal	purified antigen	Collins et al., 1988
65 000	polyclonal; clone	SDS blot; recombinant clone	Damiani et al., 1988 Laab et al., 1986 Oftung et al., 1987 Kaufmann et al., 1987
52 000-55 000	clone	SDS blot	Laab and Young, 1987
39 000	polyclonal	purified antigen	Worsaae et al., 1987 Laab et al., 1986
38 000	polyclonal	purified antigen	Young et al., 1986
35 000	polyclonal	SDS blot	Damiani et al., 1988
32 000-33 000	polyclonal	purified antigen	Kingston et al., 1987 Young et al., 1987
28 000	polyclonal	SDS blot	Damiani et al., 1988
19 000	polyclonal; clone	recombinant clone	Laab et al., 1986 Oftung et al., 1987 Worsaae et al., 1987

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TABLE 3 (continued)

antigen molecular weight (daltons)	T lymphocyte populations	antigen source	references
16 000-18 000	clone	recombinant clone	Mustafa et al., 1986 Lamb and Young, 1987
14 000	polyclonal; clone	purified antigen; recombinant clone	Oftung et al., 1987 Kaufmann et al., 1987 Kingston et al., 1987

Techniques have also been developed to allow the identification of antigens by direct screening with T cells. Antigens separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes have been assessed for recognition by cloned or polyclonal T cell populations (Young and Lamb, 1986; Abou-Zeid, Filley, Steele et al., 1987) (Table 3). Analysis of T cell clones in this way is analogous to the identification of antigens using a panel of monoclonal antibodies, and has resulted in the identification of several novel antigenic specificities (Young, 1988).

Screening of SDS-PAGE fractionated antigens using polyclonal T cell populations has provided a unique important approach to the identification of immunodominant, biologically active antigens of important biological function (Young, 1988). Comparison of recognition patterns from patients, BCG vaccinees, and healthy individuals may provide a means for assessment of the relative contribution of individual polypeptides to the overall cellular immune response (Young, 1988).

Recombinant DNA technology, monoclonal and polyclonal antibody, and T cell-based assays provide a novel source of mycobacterial antigens. These can be subsequently purified using the appropriate antibodies, and can be analysed by T cell recognition assays. In addition, sequence analysis,

epitope mapping, and analysis of protein function (Young, 1988) for the antigens are now possible with use of such technology. The applications of these antigens include:

- taxonomic assignment of mycobacteria, based on antigen and DNA characteristics;
- identification and isolation of antigens with diagnostic and/or protective potential;
- large-scale production of such antigens, and the production of synthetic peptides produced on the basis of identified nucleotide and amino acid sequences.

1.9 RECOMBINANT DNA TECHNOLOGY

Recombinant DNA technology has been defined as "the formation of new combinations of heritable material by the insertion of nucleic acid molecules, produced by whatever means outside the cell, into any virus, bacterial plasmid, or other vector system so as to allow their incorporation into a host organism in which they do not naturally occur, but in which they are capable of continued propagation" (Seckl, 1985). Cloning involves the *in vitro* joining of passenger DNA to vector DNA, and the propagation of the resulting hybrid molecules into suitable host cells to obtain a clone of identical cells harbouring recombinant DNA, and derived from a single parental cell.

1.9.1 Vector-host systems for molecular cloning

Three types of vectors have generally been used to clone fragments of foreign DNA and to propagate them in the host cell of choice, which has usually been *E.coli* (Maniatis, Fritsch, and Sambrook, 1982). These are plasmids, bacteriophage λ, and cosmids. These vectors are different in size and structure, but share the following properties:

i. they can replicate autonomously in *E.coli*, even when in recombinant form;
ii. they can be easily separated from bacterial nucleic

acid;

iii. they contain regions of DNA which are not essential for their propagation in host bacteria. Foreign DNA inserted into these regions is replicated and propagated as if it were vector DNA.

The various types of available cloning vectors - plasmids, bacteriophage λ, cosmids, and single-stranded bacteriophages - have particular biological and physiological properties that make each vector suitable for different cloning purposes (Maniatis, Fritsch, and Sambrook, 1982).

1.9.2 Single-stranded DNA vectors

Single-stranded DNA cloning vectors have been used chiefly as sources of templates for sequencing, and as sources of strand-specific probes for nucleic acid hybridisation (Maniatis, Fritsch, and Sambrook, 1982). The relative instability of DNA inserts that are larger than about 1 kilobase (kb) effectively eliminates their usefulness for most other cloning purposes.

1.9.3 Plasmids

Plasmids have been described as extrachromosomal genetic elements present in a variety of bacterial species (Perbal, 1984; Maniatis, Fritsch, and Sambrook, 1982). They are

double-stranded, closed circular DNA molecules that range in size from 1 kb to greater than 200 kb. In most cases, plasmid cloning vectors have been derived from naturally occurring plasmids that have a "relaxed" kind of replication control, implying that they are found in multiple (10-200) copy number inside the bacterial cell (Maniatis, Fritsch, and Sambrook, 1982). Plasmids with a "stringent" replication control have been found to be present in a single copy per bacterial cell (Maniatis, Fritsch, and Sambrook, 1982).

The demonstration of single sites for several restriction endonucleases, and of drug resistance markers in plasmid DNA has simplified the introduction of foreign DNA into plasmids, and the selection of the resultant recombinant plasmids (Perbal, 1984). Plasmid vectors are well adapted for cloning DNA fragments whose sizes range from a few hundred base pairs (bp) up to approximately 9 000 bp (Perbal, 1984). The choice of a particular plasmid depends essentially on the availability of restriction sites compatible with those present in the fragment to be cloned, and of the drug resistance markers for the selection of recombinants. The ability of plasmids to accept moderately sized fragments of DNA has made them the vectors of choice for subcloning from genomic DNA libraries constructed in cosmids or bacteriophage λ . They have also been described as being the only vectors suitable for the cloning of complimentary DNA (cDNA) (Perbal, 1984).

1.9.4 Bacteriophage λ (Maniatis, Fritsch, Sambrook, 1982)

Bacteriophage λ has been described as a double stranded DNA virus with a genome size of approximately 50 kb. λ DNA is in the form of a linear duplex molecule with single-stranded complementary ends 12 nucleotides in length (cohesive ends). After entering the host cell, the DNA circularises through pairing of the cohesive ends. λ became a basic vector for molecular cloning because of an interesting feature of its molecular organization; λ can not only replicate using lytic functions, but can also exist in an integrated form in the bacterial genome (Maniatis, Fritsch, and Sambrook, 1982). During lytic growth, the circular DNA is replicated manyfold in the cell, phage gene products are synthesised, progeny phage particles are formed and mature, and the cell eventually lyses, releasing many new infectious particles. Alternatively, during lysogenic growth, the λ genome is integrated into the bacterial host DNA and is subsequently replicated and transmitted to progeny bacteria as any other chromosomal gene.

Many of the λ genes involved in recombination and lysogenisation are not essential for phage multiplication, and can therefore be deleted and replaced by foreign DNA without impairment of the replicative functions. This provides the basis for construction of λ-derived cloning vectors. There is no single λ vector suitable for cloning

all DNA fragments, and the choice of the cloning vector is influenced by the restriction enzyme(s) that is to be employed, and the size of the fragment of foreign DNA that is to be inserted. Only about 60% of the viral genome (the left arm, approximately 20 kb in length, including the head and tail genes, and the right arm) is necessary for lytic propagation of the phage. The centre one-third of the phage DNA, termed the "stuffer" region, can be replaced by foreign DNA. It has been well documented that the viability of λ decreases dramatically when DNA longer than 105% (53 kb) or shorter than 78% (38 kb) is packaged (Maniatis, Fritsch, and Sambrook, 1982). It is therefore important to choose a combination of vector and foreign DNA such that the size of the recombinant phage falls within acceptable limits.

Cloning of the DNA into λ vectors involves the following 3 steps:

i. elimination of the stuffer region DNA after digestion with restriction endonucleases;

ii. ligation of the foreign DNA to the λ arms;

iii. packaging and multiplication of the recombinant DNA molecules to give rise to infectious recombinant phage.

1.9.5 Cosmids (Perbal, 1984; Maniatis, Fritsch, and Sambrook, 1982)

The limited size capacity of λ vectors is a disadvantage in some situations, particularly for the cloning of eukaryotic DNA fragments. Cosmid vectors were thus specifically designed for cloning large fragments of DNA. The essential components of cosmid vectors are:

i. a drug-resistance marker and a plasmid origin of replication;
ii. a small size, so that DNA fragments up to 45 kb in length can be accommodated;
iii. a DNA fragment that carries the ligated cohesive ends (cos sites) of bacteriophage λ.

Cosmids can circularise like phage DNA and replicate as normal plasmids without the expression of the λ phage functions. The use of cosmid vectors, however, presents several problems, including

i. vector to vector ligation whereby intramolecular recombination between 2 or more vector molecules can lead to a faster-replicating cosmid vector and eventually to loss of the cosmid by segregation;
ii. "scrambling" caused by insertion into the same vector molecule of 2 or more foreign fragments that were not adjacent to one another in the original genome;

iii. difficulty in screening large numbers of host colonies.

1.9.6 Bacteriophage λ gt 11

The bacteriophage λ gt 11 has several unique properties which make it the vector of choice for the generation of large recombinant genomic libraries. These include (Young and Davis, 1983):

i. λ gt 11 is an expression vector, and can therefore express foreign genetic material in addition to containing the foreign genetic material in an integrated form;

ii. the recombinant DNA can be propagated in the λ gt 11 host as a single copy genomic insert, thus enhancing its stability and facilitating re-expression;

iii. λ gt 11 can respond to induction with a rapid increase in copy number, and high level transcription of the foreign DNA;

iv. λ gt 11 and its bacterial hosts include features that minimise degradation of foreign proteins expressed in the cells.

In addition, this vector has been demonstrated to be efficient in generating many clones from small amounts of DNA (Davis, Dibner, and Battey, 1986), and to have an advantage over plasmid vectors for library formation in that the efficiency and reproducibility of *in vitro* packaging of λ DNA

is high (Huynh, Young, and Davis, 1985). Furthermore, screening and manipulating phage libraries has been shown to offer many technical advantages over screening bacterial colonies (Davis, Dibner, and Battey, 1986).

The structure of λ gt 11 is shown in Figure 1. The site of insertion of foreign DNA has been described as a unique Eco R1 cleavage site located within the LacZ gene, 53 base pairs upstream from the β-galactosidase translation termination codon (Young and Davis, 1983). 11 was constructed to accommodate up to 8.2 kb of in DNA, assuming a maximum wild type packageable DNA length of 105% (Young and Davis, 1983). The vector has been shown to produce a temperature-sensitive repressor which is inactive at 42°C, and to contain an amber mutation which renders it lysis-defective in host bacteria that lack the amber suppressor (Huynh, Young, and Davis, 1985). The site of insertion of foreign DNA in λ gt 11 was chosen to be within the structural gene for β-galactosidase, and thus foreign DNA sequences in the vector have the potential to be expressed as fusion proteins with β-galactosidase (Huynh, Young, and Davis, 1985).

Proper expression of foreign DNA in recombinant lysogens has been shown to depend on the orientation and reading frame of the insert DNA with respect to those of lacZ. Thus, it has been suggested that one-sixth of λ gt 11 recombinants

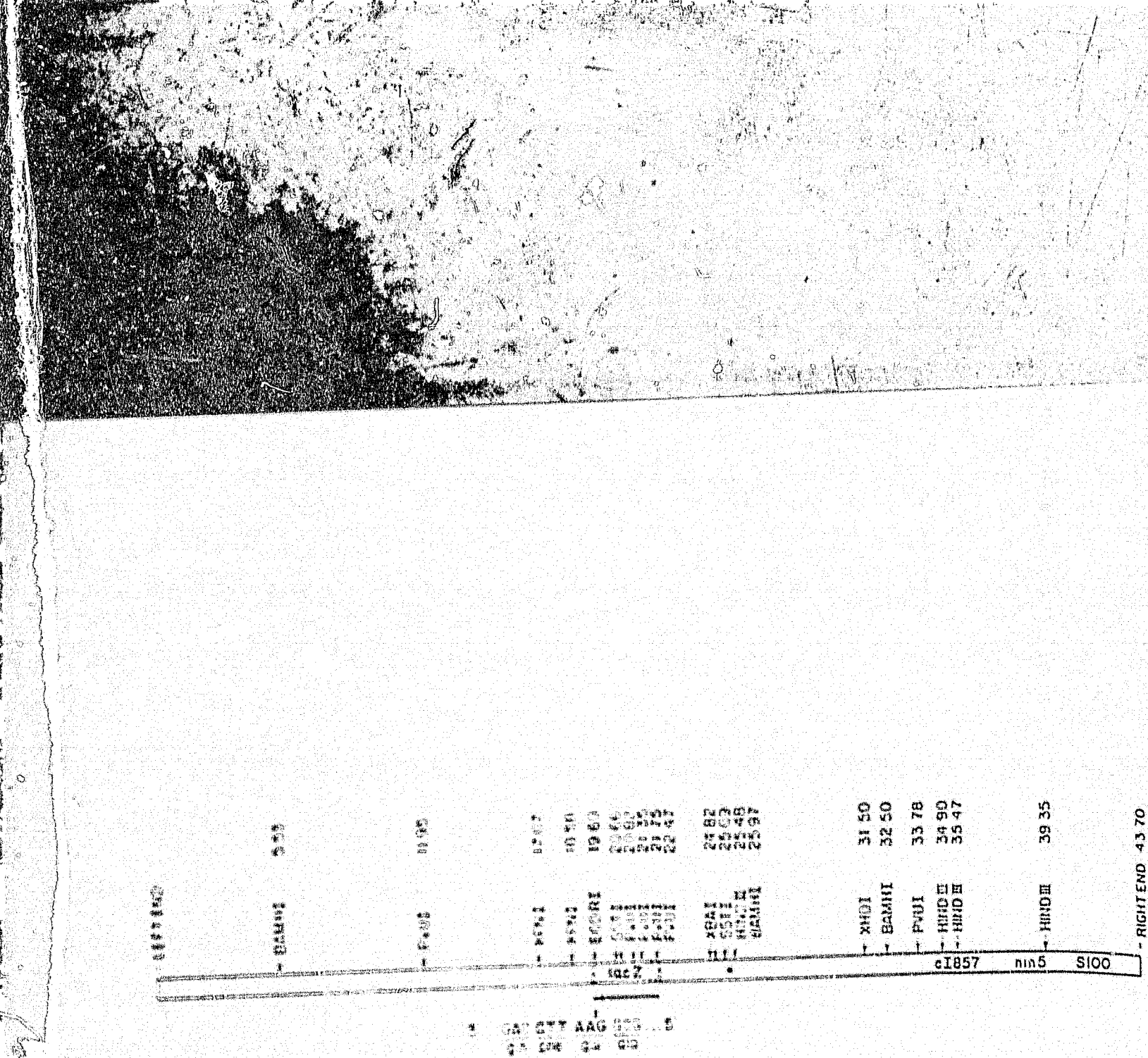


FIG. 1. Features of λgt11. Restriction endonuclease cleavage sites are designated in kb from the left end *. A attachment site. The transcription termination signals (t_R1 and t_R2) are given by the arrowheads. The sequence of the unique *EcoRI* site (boldface letters), the nucleotides that immediately surround it, and the amino acids encoded are shown below the phage map (Young and Davis, 1983)

containing a specific foreign insert would be expected to produce β-galactosidase fused to the protein of interest (Young and Davis, 1983).

Since λ gt 11 was designed as an expression vector, recombinant DNA libraries constructed in λ gt 11 can be screened with antibody probes for antigens produced by recombinant clones (Young and Davis, 1983). Individual recombinants are screened in the form of phage plaques in a lawn of protease-deficient E.coli cells (Young and Davis, 1983 b). Proteins released by the lysis of cells within the plaques are immobilised on nitrocellulose filters, and the bound proteins are then probed with antibodies specific for the antigen of interest (Young and Davis, 1983; Young and Davis, 1983 b). Antibody binding is revealed by subsequently probing the filters with radioactively-labelled secondary antibodies (Young and Davis, 1983; Young and Davis, 1983 b), or with enzyme-labelled secondary antibodies (Young, Bloom, Grosskinsky, et al., 1985).

Several problems have been shown to be associated with the production of foreign proteins in E.coli (Young and Davis, 1983); the λ gt 11 vector was designed specifically to minimise these difficulties (Young and Davis, 1983). The first problem is the achievement of an adequate level of expression of the foreign DNA sequence. It was found that fusing these DNA sequences to β-galactosidase gene sequences

ensured that the foreign DNA would be expressed efficiently in E.coli, which has a strong lacZ promoter (Young and Davis, 1983). The second problem is the ability to control the production of foreign proteins, which have often been shown to be toxic to the host cell and kill it before sufficient amounts of antigens are produced (Young and Davis, 1983). It was found that this could be minimised by using host cells which produced large amounts of the lac operon repressor to prevent lacZ-directed expression of the fusion protein until the number of infected cells was sufficiently large (Young and Davis, 1983). LacZ-directed expression could then be induced by inactivating the repressor with isopropyl-β thiogalactopyranoside (IPTG). The third problem associated with the production of fusion proteins in E.coli is the instability of the fusion proteins themselves. The position chosen for fusion with the β-galactosidase gene, corresponding to a region close to the carboxyl terminus of the β-galactosidase protein, appeared to aid the stability of the fusion protein and hence overcome this problem to a certain extent (Young and Davis, 1983). The use of lon protease deficient host cells for the screening procedure was also found to aid in maintaining the stability of fusion proteins (Huynh, Young, and Davis, 1985).

λ gt 11 recombinant libraries can therefore be used to produce preparative amounts of recombinant proteins in the form of fusion polypeptides (Hyunh, Young, and Davis, 1985).

Hybrid proteins can be produced by λ gt 11 clones as lysogens in E.coli. Lysogens can be grown to high cell density, lacZ-directed fusion protein production induced, and the cells are harvested and lysed (Huynh, Young, and Davis, 1985). Recombinant proteins can then be resolved from other proteins in the cell lysates by polyacrylamide gel electrophoresis, and can be further purified by classical column chromatography.

The ability to clone and express mycobacterial genes in E.coli has provided an approach towards major progress in the characterisation of mycobacterial antigens. As discussed in Section 1.8, the combination of the λ gt 11 expression system, monoclonal antibodies, and the establishment of T cell clones reactive to M.tuberculosis, has enabled the identification of several novel antigens of potential clinical importance. In order to obtain the maximum benefit from the advances described in the latter two sections, it is important that the investigation of antigen structure should be closely integrated with study of the microbiology and immunology of tuberculous disease (Young, 1988).

AIM

Cloning of the antigens encoded by the M.tuberculosis genome provides an initial starting point whereby large quantities of recombinant antigens can be produced easily, using standardisable techniques. Once this has been achieved, detailed characterisation of these antigens can then be made. The aim of the present study, therefore, is to generate recombinant M.tuberculosis antigens utilising the λ gt 11 expression system, and to characterise these antigens by polyacrylamide gel electrophoresis and Western blotting.

CHAPTER 2 MATERIALS AND METHODS

2.1 PREPARATION OF M.tuberculosis SONICATE

M.tuberculosis organisms were collected by scraping colonies (from positive sputum cultures) off Lowenstein-Jensen slopes into physiological saline. The suspensions were centrifuged at 300 g for 5 minutes to remove any contaminating medium. The supernatant was collected and centrifuged at 2 000 g for 30 minutes at 4°C, followed by 3 successive washings of the pellets in saline. Mycobacteria were then heat-killed by autoclaving, washed 3 more times as before, counted, and made up to 10 X 10^6 /ml in saline. The organisms were sonicated (18 µm peak to peak) at 4°C in a MSE Soniprep 150 sonicator. The sonicated material was centrifuged at 2 000 g for 15 minutes, the supernatant carefully collected, and the protein concentration of the sonicate determined using the Biorad protein estimation kit (Biorad Laboratories, Richmond, CA) according to the manufacturer's instructions. The mycobacterial sonicate was aliquoted and stored at -20°C until required.

2.2 PREPARATION OF ANTIBODIES TO M.tuberculosis

Rabbits were immunised intramuscularly with 1 ml each of M.tuberculosis sonicate, 0.5 mg/ml in saline. Immunisations were repeated at 7-day intervals for 4 consecutive weeks. Blood was obtained from the rabbits by bleeding from the ear

veins. The serum was separated and tested for the presence of antibodies to M.tuberculosis using the ELISA assay of Wadee, Cohen, and Rabson (1987). Serum from 3 healthy, saline-immmunised rabbits was used as a negative control. All sera were aliquoted and stored at -20^{0}C until required.

The ELISA was performed in flat-bottomed 96-well plates (Nunc, Denmark), the wells of which were coated with 100 ul of 10 ug/ml M.tuberculosis sonicate in sodium carbonate buffer, pH 9.6 (Appendix 8), for 1 hour at room temperature. The plates were washed 4 times with PBS-0.05% Tween, pH 7.2 (appendix 8) and the unbound sites then blocked with 100 ul per well of 0.5% bovine serum albumin (BSA; Seravac, SA) in sodium carbonate buffer for 1 hour at room temperature. Plates were washed as before, 100 ul of a 1/40 dilution of anti-M.tuberculosis serum in PBS-Tween were added to each well, and the plates were incubated at room temperature for 2 hours. After washing as before, 100 ul of peroxidase-conjugated swine anti-rabbit IgG (Dako Immunoglobulins, Denmark), diluted 1/2 000 with PBS-Tween, were added to each well, and the plates were incubated at room temperature for 1 hour. The plates were washed again, 100 ul of peroxidase substrate (appendix 5) were added to each well, and the colour reaction terminated after 10 minutes by the addition of 2.5 M H_2SO_4 (50 ul per well). The absorbance of each well was read at 492 nm in a Titertek Multiskan MC plate reader (Flow Laboratories, Sweden).

In order to ascertain that the antibodies to M.tuberculosis were able to recognise specific M tuberculosis antigens immobilised on nitrocellulose, Western blots of the M.tuberculosis sonicate were screened with the anti-M.tuberculosis antibodies. Proteins and polypeptides in the mycobacterial sonicate were resolved by gel electrophoresis on 10% polyacrylamide-SDS Laemmli gels (Laemmli, 1970; appendix 7). The mycobacterial sonicate was solubilised in 1/3 volume splitting solution (see section 2.9), boiled for 5 minutes, and applied to the gels at a concentration of 100 ug per well. Conditions for electrophoresis and immunoblotting were the same as those described in Section 2.9 and Section 2.10.

2.3 ANTIBODIES TO β-GALACTOSIDASE

Monoclonal antibodies to β-galactosidase, in the form of ascitic fluid, were obtained as a gift from Professor E.Dowdle, Department of Clinical Science and Immunology, University of Cape Town, South Africa. They were stored in 0.1% sodium azide at 4°C.

2.4 ISOLATION OF M.tuberculosis DNA

Mycobacteria from positive sputum cultures were harvested from Lowenstein-Jensen slopes, and washed extensively with saline. To 2 ml of pelleted mycobacterial cells, 4 ml of

40 mM Tris-HCl, pH 8.0, were added, followed by lysozyme (Boehringer Mannheim, W.Germany) and EDTA, pH 8.0, to final concentrations of 2 mg/ml and 40 mM respectively. The mycobacteria were incubated on a rotating wheel for 2 hours at 37°C. Sodium dodecyl sulphate (SDS; BDH, Poole, England), pH 7.2, was then added to a final concentration of 2%, and the incubation was continued for a further 30 minutes at 37°C.

An equal volume of a 1:1 (volume/volume) mixture of chloroform (chloroform/isoamylalcohol 24:1) (Merck, Darmstadt, W.Germany) and Tris-saturated phenol (Merck, Darmstadt, W.Germany) was then added, and the aqueous phase was extracted with gentle agitation for 10 minutes. The phases were separated by centrifugation at 1 500 g for 10 minutes. Chloroform/phenol extraction of the aqueous phase was repeated twice more. The aqueous phase was then extracted once with an equal volume of 24:1 (volume/volume) chloroform/isoamylalcohol with gentle agitation for 5 minutes, followed by centrifugation at 1 500 g for 5 minutes. Sodium acetate (Univar, S.A.) was added to 0.3 M to the aqueous phase, and DNA was precipitated with 2.5 volumes of ice-cold absolute ethanol (Merck, Darmstadt, W.Germany). The DNA was collected by centrifugation at 5 000 g for 30 minutes at 0°C, and resuspended in 2 ml TE buffer (appendix 8).

DNase - free RNase (Boehringer Mannheim, W.Germany) was added

to the DNA solution to a concentration of 50 ug/ml, and the solution was incubated at 37°C for 60 minutes. Proteinase K (Boehringer Mannheim, W.Germany) was then added to 100 ug/ml, and the solution incubated for a further 30 minutes at 37°C. The DNA was then extracted once with chloroform/isoamyl-alcohol, precipitated with 2.5 volumes of ice-cold absolute ethanol, and collected by centrifugation as before. The DNA was dried at room temperature, resuspended in 1 ml TE buffer, pH 8.0 (appendix 8), and quantified spectrophotometrically (appendix 3).

2.5 RECOMBINANT DNA LIBRARY CONSTRUCTION

2.5.1 Introduction

Libraries of genomic DNA may be prepared in 2 ways (Maniatis, Fritsch, and Sambrook, 1982). The first approach involves the digestion of genomic DNA to completion with a restriction enzyme, and insertion of the resulting fragments in an appropriate λ bacteriophage vector. This method suffers from 2 drawbacks: first, if the sequence of interest contains recognition site(s) for the particular restriction enzyme chosen, it will be cloned in 2 or more pieces. In addition, the sequence may not be cloned at all, if, for example, it is contained in a larger DNA fragment than the vector can accept. Second, if very large genomic DNA is used initially - the genome of *M.tuberculosis* is 2.1×10^9 bp in size (Imaeda,

Barksdale, and Kirchheimer, 1982), large compared to that of E.coli, which is 4 X 10^6 bp in size, and the human genome, which is approximately 3.9 X 10^9 bp in size (Stryer, 1988) - the average size of the fragments generated by cleavage of the DNA with many restriction enzymes is relatively small (approximately 4 kb), and an entire library therefore contains a very large number of recombinant bacteriophages, and screening thus becomes laborious and expensive.

Problems in library construction can be avoided by cloning fragments that are generated by random shearing of genomic DNA (Maniatis, Fritsch, and Sambrook, 1982). This method ensures that there is no systematic exclusion of sequences from the cloned library merely because of an unfortunate distribution of restriction sites. In addition, knowledge of the distribution of restriction sites in or around the sequence of interest is not required before cloning is attempted.

The basic steps for the construction of a M.tuberculosis genomic library in the λ gt 11 vector (Young and Davis, 1985) are shown in Figure 2, and are summarised as follows:

1. The DNA of λ gt 11, which accepts inserts up to 3.2 kb in length, is digested with Eco R1 to to produce left and right arms, which carry all the genetic information for lytic growth of the virus.

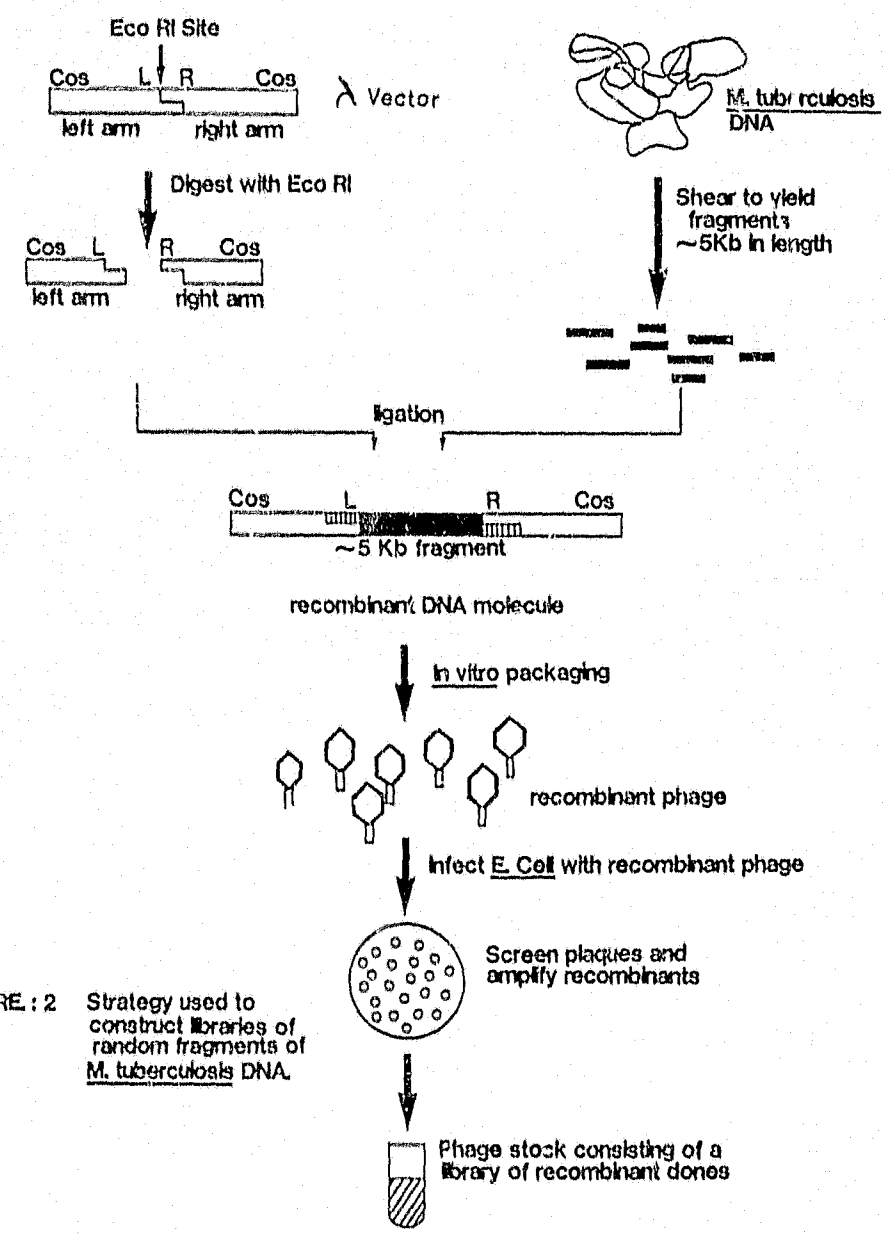


FIGURE : 2 Strategy used to construct libraries of random fragments of M. tuberculosis DNA.

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