Identification of expression signals of the mycobacteriophages Bxb1, L1 and TM4 using the *Escherichia*–*Mycobacterium* shuttle plasmids pYUB75 and pYUB76 designed to create translational fusions to the *lacZ* gene

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Mycobacterial expression signals were cloned using specially constructed gene fusion shuttle plasmid probes carrying a truncated *Escherichia coli lacZ* (β -galactosidase) gene which lacked a promoter, a ribosome binding site, and an ATG start codon. Libraries of mycobacteriophage Bxb1, L1 and TM4 DNAs were constructed, and introduced by electroporation into *Mycobacterium smegmatis* and the 'bacille Calmette-Guérin' (BCG). Clones carrying mycobacterial expression sequences were detected by their blue colour or characteristic fluorescence when plated on media containing chromogenic or fluorogenic substrates. Varying degrees of β -galactosidase expression were observed, and one Bxb1 expression signal was identified where β -galactosidase expression is repressed in phage lysogens.

Introduction

Infections by *Mycobacterium tuberculosis*, *Mycobacterium leprae* and *Mycobacterium avium* continue to cause major health problems in developing countries (Joint International Union Against Tuberculosis & World Health Organization Study Group, 1982). Acquired Immunodeficiency Syndrome (AIDS) patients very often succumb to mycobacterial infections even in developed countries (Centers for Disease Control, 1987). Protection against tuberculosis can be provided by vaccination with a live attenuated *Mycobacterium* strain called 'bacille Calmette-Guérin' (BCG), which is the most widely used vaccine in the world (Fine, 1988), and may be an attractive host for a recombinant multivaccine vehicle (Aldovini & Young, 1991; Stover *et al.*, 1991). No proven vaccines against *M. leprae* or *M. avium* are available yet.

Genetic tools to study mycobacterial pathogenesis and to express foreign antigens in BCG have been developed. Recombinant DNA was first propagated in the mycobacteria by shuttle phasmid vectors derived from virulent mycobacteriophages (Jacobs *et al.*, 1987).

Subsequently, a kanamycin-resistance marker was expressed in mycobacteria using both temperate shuttle phasmids and shuttle plasmid vectors (Snapper *et al.*, 1988).

In addition, however, expression sequences must be supplied to drive the transcription and translation of foreign genes, particularly for the expression of eukaryotic and viral antigens. Since these recognition sequences may differ in mycobacteria and *E. coli*, a strategy was developed to clone both transcriptional and translational initiation signals simultaneously. Since phages usually have strong promoters, we chose to study the expression sequences of mycobacteriophages Bxb1, L1 and TM4, which have genomes of approximately 50 kb.

Mycobacterium smegmatis mc²155 (Snapper *et al.*, 1990) was used as the host strain for the initial cloning experiments because it is readily cultivable, non-pathogenic, fast-growing, and because it can be 'electro-transformed' with high efficiency with plasmid DNA (Snapper *et al.*, 1990). BCG, on the other hand, is very slow growing, requiring about three weeks to form visible colonies after transformation.

Our strategy for cloning gene expression signals exploited the *E. coli lacZ* gene, coding for β -galactosidase, which has been successfully used as a reporter gene in mycobacteria (Barletta *et al.*, 1990), allowing the identification of recombinant clones which express

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enzymically active β -galactosidase fusion proteins by the use of chromogenic substrates (Silhavy *et al.*, 1984). Furthermore, recombinant BCG expressing β -galactosidase fusions has elicited long-lasting humoral and cellular immune responses (Stover *et al.*, 1991). In this study, we describe the construction of mycobacteriophage Bxb1, L1 and TM4 expression libraries, using β -galactosidase gene fusion shuttle plasmids.

Methods

Bacteria and phages. These are described in Table 1. Phage lysates were prepared and their titres determined as described by Jacobs *et al.* (1991). *M. smegmatis* lysogenic for Bxb1 or L1 were purified from colonies growing at the cross-streaks of the mycobacterial host and the respective phage lysates. Maintenance of the lysogenic state was verified by immunity to superinfection by the homologous phage, and by Southern hybridization analysis of chromosomal DNA from the lysogenic strains with radiolabelled phage DNA.

Media and growth of bacterial strains. *Mycobacterium* strains were grown as described by Jacobs *et al.* (1991). Transformed mycobacteria were selected on 7H10 agar supplemented with albumin dextrose complex enrichment and containing 10 μ g kanamycin sulphate ml⁻¹. Transformed *E. coli* cells were selected on Lennox base agar (Lennox, 1955) with 50 μ g kanamycin sulphate ml⁻¹. When necessary the tempered agar was supplemented with 40 μ g X-Gal ml⁻¹, 40 μ g Bluo-gal ml⁻¹ (Bethesda Research Laboratories), or 150 μ g 4-methylumbelliferyl β -D-galactoside ml⁻¹ (Sigma). In these experiments, X-Gal was the best substrate since blue colonies were readily visible after incubation for four days at 37 °C. On Bluo-gal, blue colour increased in intensity upon storage of plates at 4 °C. The umbelliferyl galactoside diffused to some extent, and the corresponding plates showed a higher background.

DNA isolation and manipulation procedures. Plasmid DNA from *E. coli* was isolated, quantified and analysed by standard methods (Maniatis *et al.*, 1982). Plasmid DNA was isolated from *M. smegmatis* by a modification of the standard alkaline lysis procedure for *E. coli* (Jacobs *et al.*, 1991).

Mycobacteriophage DNA was isolated from purified phage particles as previously described (Jacobs *et al.*, 1991). Briefly, phage lysates were purified through CsCl step gradients with a lower layer of 5.0 M-CsCl and an upper layer of 3.0 M-CsCl. Pure mycobacteriophage preparations were incubated overnight at 55 °C with a mixture of proteinase K (2.0 mg ml⁻¹), EDTA (0.05 M), and sodium dodecyl sarcosinate (1.0% w/v). The lysed phage preparation was then extracted with phenol/chloroform, precipitated with ethanol, and resuspended and extensively dialysed against TE buffer (10 mM-Tris hydrochloride, 1 mM-EDTA; pH 8.0).

Restriction enzyme digestions, and ligations with T4 DNA ligase, were performed as recommended by the suppliers or as described by Davis *et al.*, (1980). For *Bal*I digestion, plasmid DNA was isolated from *E. coli* GM119 (Arraj & Marinus, 1983). DNA was separated by electrophoresis in 0.8% horizontal agarose gels in running buffer with 40 mM-Tris hydrochloride, 5.0 mM-sodium acetate, and 1.0 mM EDTA adjusted to pH 7.6 with glacial acetic acid. Extraction of DNA from agarose gels was performed as described by Maniatis *et al.* (1982).

Transformation of bacterial strains. *M. smegmatis* and BCG cells were transformed by electroporation basically as described by Jacobs *et al.* (1991) and Lugosi *et al.* (1989), using 10% (w/v) glycerol as electroporation buffer. Cells were concentrated 10- to 100-fold and kept

Table 1. *Bacterial strains and phages*

Description		Source or reference
<i>M. smegmatis</i> mc ² 6	Wild type	(Jacobs <i>et al.</i> , 1987)
mc ² 155	High-efficiency transformation mutant of mc ² 6	(Snapper <i>et al.</i> , 1990)
<i>E. coli</i> DH5 α	<i>recA lacZ</i> Δ M15	Bethesda Research Laboratories
GM119	<i>dcm-6 dam-3</i>	(Arraj & Marinus, 1983)
Phages		
Bxb1	Phage temperate for <i>M. smegmatis</i> and BCG	W. R. Jacobs, Jr, isolated from Bronx soil
L1	Phage temperate for <i>M. smegmatis</i> and BCG	(Snapper <i>et al.</i> , 1990)
TM4	Phage lytic for <i>M. smegmatis</i> and BCG	(Timme <i>et al.</i> , 1984; Jacobs <i>et al.</i> , 1987)

on ice for 3–7 h prior to electroporation. The Gene Pulser (Bio-Rad) was set at 2.5 kV, 25 μ F and the pulse controller resistance was set at 1000 Ohms. DNAs were then added to 0.4 ml of mycobacterial cells in 0.2 cm electrode gap cuvettes. Samples were pulsed, diluted into 4.5 ml of broth, incubated for 2 h at 37 °C, and plated.

E. coli cells were electroporated following a modification of the standard Bio-Rad procedure (Jacobs *et al.*, 1991). Alternatively, competent DH5 α cells were purchased from Bethesda Research Laboratories and transformed as recommended by the supplier. Strain GM119 was transformed by the CaCl₂ procedure (Dagert & Erlich, 1979).

β -Galactosidase assays. Assay for β -galactosidase was performed basically as described by Miller (1972). Ten ml of middle to late exponential phase cultures (OD₆₀₀ = 0.4–1.0) were harvested, resuspended in 5.0 ml Z buffer (Miller, 1972) and permeabilized with toluene (0.5%, v/v). Assays were carried out at 28 °C. β -Galactosidase activity was measured by hydrolysis of *o*-nitrophenyl- β -D-galactoside (Sigma). The reaction was followed by measuring the A₄₂₀ and correcting for light scattering. Final calculation of β -galactosidase activity in Miller units was made using at least two time points when the hydrolysis of the substrate was linear with respect to time and cell concentration.

Toluene permeabilization has been shown to be a reliable procedure for *E. coli* (Miller, 1972), though there are no further studies described for mycobacteria. Therefore, the units of β -galactosidase measured in these assays are valuable as a relative comparison of the activities of the various clones described here, but these measurements do not necessarily represent the actual number of β -galactosidase units synthesized by these clones.

Southern analysis and colony hybridization. DNA from agarose gels was transferred to Biotrans nylon membranes, hybridized at 65 °C for at least 20 h, and washed at 55 °C with buffer (pH 8.3) composed of 1.0% (w/v) sodium dodecyl sulphate, 18 mM-NaCl, 1.0 mM-sodium phosphate, and 0.1 mM-EDTA. Probes were radiolabelled by nick-translation (Rigby *et al.*, 1977) with [α -³²P]dCTP. Colonies of *M. smegmatis* were lysed on nylon membranes according to a modified *E. coli* protocol (Maniatis *et al.*, 1982; unpublished results).

Fig. 1. Construction of β -galactosidase expression probe vectors pYUB75 and pYUB76. Symbols: shaded line, DNA fragment from pAL5000, ORF1 to ORF5 shown are those proposed by Rauzier *et al.* (1988); solid line, DNA fragments from the *E. coli* plasmid pBR322 (Bolivar, 1977), and the *E. coli lacZ* gene truncated at the N-terminus. The genes encoding ampicillin resistance (*bla*) and kanamycin resistance (*aph*) from Tn903 are also shown, as well as the *E. coli* origin of replication (*ColE1ori*). Arrows indicate the direction of transcription for the various genes. Only relevant restriction endonuclease sites are indicated.

Results

Construction of β -galactosidase expression probe vectors

Plasmid pMLB1034 (Weinstock *et al.*, 1983) which carries a truncated β -galactosidase gene (*lacZ*) devoid of promoter, ribosome-binding site, and initiation codon was digested with *Pst*I and ligated to a *Pst*I fragment carrying the aminoglycoside 3'-phosphotransferase gene (*aph*) from Tn903 (Oka *et al.*, 1981). The *aph* gene provides a selectable kanamycin-resistance marker for both *E. coli* and mycobacteria. One of the recombinant plasmids carrying the kanamycin gene, pYUB71 (Fig. 1), was further digested with either *Sca*I or *Bal*I, and blunt-end ligated to a 2590 bp *Eco*RV-*Hpa*I fragment of pAL5000 (Labidi *et al.*, 1984) which carries the mycobacterial origin of replication (*oriM*; Snapper *et al.*, 1990). Recombinant shuttle plasmids carrying *oriM* were

obtained by selecting kanamycin-resistant transformants in *M. smegmatis* mc²155. Plasmid pYUB75 carried the *oriM* insertion at the *Bal*I site, and plasmid pYUB76 at the *Sca*I site (Fig. 1). Since the relative positions of the pAL5000 putative ORFs (Rauzier *et al.*, 1988) are different in pYUB75 and pYUB76, these two vectors provide independent tests for the presence of expression signals. Neither pYUB75 nor pYUB76 expressed β -galactosidase activity in *E. coli*, *M. smegmatis* or BCG.

Cloning of mycobacteriophage DNA fragments into pYUB76

*Bam*HI fragments of the mycobacteriophages Bxb1 or L1 DNA or *Sau*3AI of Bxb1, L1 and TM4, were inserted into the unique *Bam*HI site of pYUB76 (Fig. 1), and

Table 2. Analysis of recombinants from mycobacteriophage expression libraries

Expression library*	Number of transformants†	Number of recombinants‡	Number of blue recombinants§	Fraction of blue clones
Bxb1/ <i>Bam</i> HI	1500	30	5	0.17
Bxb1/ <i>Sau</i> 3AI	4600	900	29	0.03
L1/ <i>Bam</i> HI	4600	900	163	0.18
L1/ <i>Sau</i> 3AI	6000	1200	53	0.04
TM4/ <i>Sau</i> 3A	1500	ND	10	—

ND Not determined.

* Mycobacteriophage DNA was digested with either *Bam*HI or *Sau*3A, and resulting fragments were ligated at the *Bam*HI site of plasmid pYUB76.

† Strain mc²155 was electrottransformed with ligation mixtures as described in Materials and Methods. Number of transformants shown were obtained from approximately 50 ng of plasmid vector pYUB76 DNA in the ligation mixtures. Approximately 1.0×10^7 transformants were obtained per µg of supercoiled plasmid vector.

‡ Determined by colony hybridization with radiolabelled mycobacteriophage DNA as probe.

§ Determined on Middlebrook 7H10 medium with 40 µg X-Gal ml⁻¹.

introduced by transformation into *M. smegmatis* mc²155. Kanamycin-resistant transformants were selected on Middlebrook medium containing the chromogenic substrates X-Gal or Blue-gal, or the fluorogenic dye 4-methylumbelliferyl-β-D-galactoside. Results from a typical experiment are shown in Table 2. In general, the proportion of blue recombinants was greater in the libraries generated by *Bam*HI digestion than with *Sau*3AI digestion. This is to be expected, as the greater frequency of cleavage by *Sau*3AI will generate smaller fragments not containing expression signals. However, the total number of recombinants was higher with the *Sau*3AI digestion, presumably owing to greater efficiency of ligation with the smaller fragments. Representative colonies expressing β-galactosidase activity were analysed further.

Digestion of mycobacteriophage Bxb1 DNA with *Bam*HI results in four DNA fragments of approximately 29, 13, 5.8 and 2.4 kb. All five analysed Bxb1-derived blue *Bam*HI clones carried the 2.4 kb *Bam*HI fragment from Bxb1 DNA. The purified 2.4 kb fragment was also cloned into the *Bam*HI site of pYUB75, and produced blue colonies in mc²155. Since insertion of the 2.4 kb fragment activated the truncated *lacZ* gene in both pYUB75 and pYUB76, transcriptional and translational initiation signals should be provided by the Bxb1 insert.

A mycobacteriophage L1 library generated by digestion of mycobacteriophage DNA with *Bam*HI was analysed by digestion of the recombinant plasmids with *Bam*HI, which released the vector and the mycobacteriophage DNA inserts. Digestion of mycobacteriophage L1 DNA with *Bam*HI gave a greater number of fragments, ranging in size from 10.6 to less than 0.5 kb. The presence of greater numbers of smaller fragments in this digest than in the corresponding digestion of Bxb1 DNA

increased the probability of obtaining different recombinant clones. Comparison of the ethidium-bromide-stained gel with the bands hybridizing with the L1 probe in the Southern analysis (data not shown) allowed us to correlate the DNA fragments driving β-galactosidase expression in *M. smegmatis* with fragments of a particular size in the *Bam*HI restriction digest of L1. *M. smegmatis* clones expressing β-galactosidase contained single inserts of approximately 6.7, 1.9, 1.5, 1.0, and 0.7 kb, as well as multiple inserts. The 1.9 kb L1 insert from the recombinant clone mc²382 (Table 3) was purified and ligated into the unique *Bam*HI sites of pYUB75 and pYUB76. The recombinant clones obtained with both vectors expressed similar levels of β-galactosidase in mc²155, indicating that the transcriptional and translational start signals were contained in the 1.9 kb insert.

β-Galactosidase expression in *E. coli*, *M. smegmatis*, and BCG

Plasmid DNA was isolated from representative blue *M. smegmatis* clones and introduced by transformation into *E. coli* DH5α (*lacZ* ΔM15). The colour of the various recombinant clones in *E. coli* and *M. smegmatis* was scored as light, medium and dark. A total of 36 recombinant plasmids isolated from all mycobacteriophage libraries (a minimum of five clones from each library) were tested for expression in *E. coli*. Twenty one (60%) of the recombinant plasmids which gave blue colonies in mycobacteria resulted in white *E. coli* transformants, while the remaining plasmids gave blue *E. coli* transformants. This is consistent with previous studies (Kieser *et al.*, 1986). Table 3 shows the results for some representative clones. The ratio of the number of expression signals expressed only in *M. smegmatis* to the

Table 3. Properties of representative *M. smegmatis* clones producing β -galactosidase

Strain	Insert description*	β -Galactosidase activity†		
		Mycobacteria (colour)	<i>M. smegmatis</i> (Miller units)	<i>E. coli</i> (colour)
mc ² 340	None	White	<0.1	White
mc ² 368	Bxb1, <i>Bam</i> HI, 2.4 kb	Medium blue	ND	White
mc ² 373	L1, <i>Bam</i> HI, 1.0 kb	Medium blue	3.9	White
mc ² 374	L1, <i>Bam</i> HI, 1.5 kb‡	Dark blue	56	Dark blue
mc ² 378	L1, <i>Bam</i> HI, 0.7 kb	Medium blue	8.3	Dark blue
mc ² 382§	L1, <i>Bam</i> HI, 1.9 kb	Medium blue	3.9	Light blue
mc ² 383	L1, <i>Bam</i> HI, 6.7 kb	Medium blue	5.7	White
mc ² 390§,	Bxb1, <i>Sau</i> 3AI, 0.6 kb	Medium blue	26	White
mc ² 391	Bxb1, <i>Sau</i> 3AI, <0.6 kb	Medium blue	24	White

ND, not determined.

* Description includes the mycobacteriophage DNA source, the restriction endonuclease used to generate the expression library, and the size of the corresponding insert cloned at the *Bam*HI site of plasmid vector pYUB76.

† β -Galactosidase activity was evaluated qualitatively by the appearance of the colonies on X-Gal plates. The intensity of blue colour for mycobacterial clones was determined on Middlebrook 7H10 medium with 40 μ g X-Gal ml⁻¹. For *M. smegmatis* clones the number of β -galactosidase Miller units was determined. Mycobacterial strains were grown and assayed as described in Methods. Plasmid DNA was isolated from these mycobacterial clones, and transformed into *E. coli*. The appearance of *E. coli* transformants was observed on Lennox agar with 40 μ g X-Gal ml⁻¹.

‡ This fragment resulted from the ligation of two terminal *Bam*HI fragments by the phage L1 cohesive ends.

§ Plasmids from these strains were introduced in BCG and gave β -galactosidase activity.

|| Plasmid from this strain gave low β -galactosidase activity in *M. smegmatis* lysogenic for Bxb1 (see text).

number of signals expressed in both *M. smegmatis* and *E. coli* could also be affected by the relative stabilities of the fusion proteins in these hosts.

Levels of β -galactosidase activity were also measured in representative clones (Table 3). The results confirmed the various levels of gene expression previously visualized as colonies with increasing intensities of blue colour on plates containing X-Gal. The possibility that the various protein fusions may have different specific activities may also have contributed to this effect.

Two recombinant plasmids expressing β -galactosidase in *M. smegmatis* (derived from strains mc²382 and mc²390) were tested in BCG, and both expressed β -galactosidase activity.

Evidence for a Bxb1 regulatable expression signal

The Bxb1 expression library constructed by *Sau*3AI digestion was analysed further. Plasmid DNA isolated from five mycobacterial clones expressing β -galactosidase was introduced into the original host (mc²155) and also into the corresponding Bxb1 and L1 lysogens. All recombinant plasmids, except one, gave rise to blue transformants with similar intensities in all three strains, when plated on medium containing X-Gal. However, one recombinant plasmid, designated pYUB76::Bxb1-I (recombinant plasmid in mc²390), gave medium to dark

Table 4. Evidence for a mycobacteriophage Bxb1-regulated promoter

Mycobacterial strains were grown and assayed as described in Methods

Plasmid	β -Galactosidase activity (Miller units) in mc ² 155 lysogenic for		
	none	Bxb1	L1
pYUB76	<0.1	<0.1	<0.1
pYUB76::Bxb1-I	26	1	26
pYUB76::Bxb1-II	24	27	17

blue transformants in mc²155 and in the corresponding L1 lysogen, but only light blue transformants in the Bxb1 lysogen. Measurement of β -galactosidase activity (Table 4) revealed no enzyme activity in strains carrying the vector pYUB76, and a moderate level of activity in non-lysogenic strains or L1 lysogens carrying the recombinant plasmid pYUB76::Bxb1-I. However, this recombinant plasmid gave a very low level (above background) of β -galactosidase activity in the Bxb1 lysogen. This effect was plasmid-specific, since another recombinant plasmid from the Bxb1 library (pYUB76::Bxb1-II, recombinant plasmid in mc²391) gave a substantially higher level of activity in the Bxb1 lysogen, similar to the level of

activity obtained in the other mycobacterial hosts which did not carry the Bxb1 prophage. Since approximately the same amount of plasmid DNA was recovered from transformants of mc²155 and the corresponding Bxb1 and L1 lysogens, the reduced expression level of β -galactosidase could not be ascribed to a lower copy number of plasmid pYUB76::Bxb1-I in the Bxb1 lysogen (data not shown). Furthermore, plasmid pYUB76::Bxb1-I isolated from the Bxb1 lysogen gave rise to medium to dark blue transformants with mc²155 and its L1 lysogen as hosts, but light blue transformants with the Bxb1 lysogen as host. Hence, the reduced level of β -galactosidase activity was not due to a spontaneous mutation in the plasmid DNA upon transformation of the Bxb1 lysogenic strain.

The above data suggested that β -galactosidase expression from the insert in pYUB76::Bxb1-I was repressed by the lysogenic Bxb1 strain.

Discussion

These studies have established that mycobacteriophage expression signals are capable of driving the expression of the *E. coli* β -galactosidase reporter gene in the translational fusion vectors pYUB75 and pYUB76. A promoter, a ribosome binding site, and a start codon in the correct reading frame must be provided by the cloned DNA to generate β -galactosidase activity. Previous studies of mycobacterial promoters have used *E. coli* vectors carrying the *galK* reporter gene (Sirakova *et al.*, 1989) or *Streptomyces lividans*, a bacterium from a closely related genus, as the host system (Kieser *et al.*, 1986). The vectors described herein allow the direct search for expression signals in mycobacteria. Furthermore, these bifunctional vectors can also replicate and be used as probes in *E. coli*. Since the consensus sequence and the strengths of mycobacterial ribosome-binding sites are not fully characterized, we used a translational rather than a transcriptional probe.

Since promoters are only expected to function in one orientation, and ligations of mycobacteriophage fragments must be in frame to activate the truncated *lacZ* gene in pYUB75 or pYUB76, the actual number of expression signals obtained seems unusually high. This raises the question whether the signals detected by this methodology correlate with real expression signals directing mycobacteriophage gene expression. Sequence analysis of mycobacterial clones expressing β -galactosidase coupled with nuclease S1 protection experiments will be needed to analyse this problem. Furthermore, the complete nucleotide sequence of the mycobacteriophage L5 (a close relative of phage L1) has been determined (unpublished results cited by Lee *et al.*, 1991) and is

expected to be available for further sequence comparison. The 6.7 kb fragment from the mycobacteriophage L1 *Bam*HI library, identified by this methodology as an expression signal, may contain sequences necessary for the expression of the *int* gene (Lee *et al.*, 1991). The identification of a regulatable expression signal from the *Sau*3A Bxb1 library strengthens the correlation between the expression signals identified by this methodology and true expression signals *in vivo*. The unusually high number of expression signals (especially those producing low levels of β -galactosidase activity) may be due to the existence of weak and secondary promoters within the mycobacteriophage genomes which are used to fine tune the level of gene expression in special situations.

Mycobacteriophage expression sequences with varying levels of gene expression were detected, providing evidence for the existence of promoters and/or ribosome-binding sites of different strengths. Expression signals functional in *E. coli* and mycobacteria might have either the consensus sequence for the *E. coli* σ^{70} subunit of the RNA polymerase (Rosenberg & Court, 1979; Hawley & McClure, 1983), or more likely the heat-shock *E. coli* σ^{37} factor (Grossman, *et al.*, 1984), and the *E. coli* ribosome-binding site coding sequence (Shine & Dalgarno, 1974). Expression sequences which are functional in mycobacteria, but not in *E. coli* are impaired either in transcription or translation. Previous studies suggest that most mycobacterial genes are expressed only weakly at best from their own signals in *E. coli* (Clark-Curtiss *et al.*, 1985; Thole *et al.*, 1985). Expression of major mycobacterial protein antigens in *E. coli* systems was achieved by the use of expression vectors with regulatory signals functional in *E. coli* (Jacobs *et al.*, 1986; Young *et al.*, 1985a, b). However, Kieser *et al.* (1986) found that mycobacterial promoters work well in *Streptomyces* (73% G + C) which, like *Mycobacterium* (56–65% G + C), has a higher G + C content than *E. coli*.

If the impairment of mycobacterial expression in *E. coli* were at the transcriptional level, the presence of a specific σ factor encoded by the mycobacterial host would remain an attractive possibility. The screening method utilized herein would not favour detection of promoters requiring a mycobacteriophage-encoded σ factor.

Of particular interest was a Bxb1 expression sequence repressed in *M. smegmatis* lysogenic for phage Bxb1. Most likely, this expression sequence is regulated at the transcriptional level through the binding of a repressor at the promoter sequence. The repressor would turn off the expression of genes involved in the earlier stages of mycobacteriophage gene expression leading to a lysogenic response. This type of regulation has been found in all temperate phage-host systems studied such as the regulation of P_R and P_L promoters by repressor in the

lambda-*E. coli* system (Ptashne *et al.*, 1976; Gussin *et al.*, 1983). A regulated promoter in mycobacteria should facilitate the cloning of foreign genes which would otherwise be lethal for the host.

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