Identification of *Mycobacterium paratuberculosis* gene expression signals

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***Mycobacterium paratuberculosis* promoter-containing clones were isolated from a genomic DNA library constructed in the transcriptional-translational fusion vector pYUB76. The promoter-containing DNA fragments were identified in the surrogate host *Mycobacterium smegmatis* by expression of the promoterless *lacZ* reporter gene of pYUB76. The expression signals exhibited a wide range of strengths, as indicated by their corresponding β -galactosidase activities. Eight clones were sequenced and characterized further. Predicted open reading frames and codon usage were identified by computer analysis. Database searching for related sequences using the BLAST method revealed no homologies. Transcriptional activity was measured by slot-blot hybridization with steady-state RNA isolated from *lacZ*⁺ *M. smegmatis* clones. Primer extension analysis identified the transcription start sites within the cloned fragments. The promoter regions characterized in this study were used to establish a consensus promoter sequence for *M. paratuberculosis*. *M. paratuberculosis* consensus hexanucleotide sequences of TGMCGT and CGGCCS centred approximately 35 and 10 bp upstream from the transcription startpoints do not correspond to the consensus hexanucleotides of *Escherichia coli* promoters.**

Keywords: *Mycobacterium*, promoter, gene expression, codon usage, primer extension

INTRODUCTION

Despite the medical importance of members in the genus *Mycobacterium*, characterization of mycobacterial gene expression signals has lagged behind other bacterial systems. Genetic manipulation is difficult because pathogenic mycobacteria are slow growing, have undeveloped methods of genetic exchange, and are refractile to chemical or enzymic lysis. For these reasons, *Mycobacterium paratuberculosis* (*M. avium* subsp. *paratuberculosis*), the aetiological agent of Johne's disease, has not been well characterized at the molecular level. Few genes have been cloned and sequenced (Stevenson *et al.*, 1991; Gilot *et al.*, 1993; Green *et al.*, 1989; Hance *et al.*, 1989; El-Zaatari *et al.*, 1994) and only two promoters have been characterized in this species (Murray *et al.*, 1992; Thomas *et al.*, 1992). No composite class trans-

posons have been discovered and a method of genetic exchange has only recently been established (Foley-Thomas *et al.*, 1995).

Recently, several mycobacterial promoters have been cloned (Sela & Clark-Curtiss, 1991; Barletta *et al.*, 1992; DasGupta *et al.*, 1993; Thomas *et al.*, 1992; Timm *et al.*, 1994). In these studies the cloned promoter fragments were not sequenced (Barletta *et al.*, 1992; Timm *et al.*, 1994) and transcription start site mapping was either performed in recombinant *Escherichia coli* clones (Sela & Clark-Curtiss, 1991) or not at all (Barletta *et al.*, 1992; DasGupta *et al.*, 1993). Very recently, a thorough analysis of *Mycobacterium smegmatis* and *M. tuberculosis* promoter elements has been performed (Bashyam *et al.*, 1996).

The transcriptional-translational fusion vector pYUB76 has been used to clone expression signals from mycobacteriophages (Barletta *et al.*, 1992). We used this vector to extend the current knowledge of promoter architecture and gene expression in *M. paratuberculosis* by cloning and characterizing chromosomal promoter elements.

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The Genome Sequence Database accession numbers for the *M. paratuberculosis* sequences determined in this work are L76360, L76361, L76363, L76364, L76365, L76373, L76374 and L76378.

METHODS

Bacterial strains and plasmids. *M. paratuberculosis* ATCC 19698 was provided by D. Whipple, NADC, Ames, IA, USA. *Escherichia coli* DH10B (Gibco-BRL) served as the strain for routine plasmid maintenance and isolation. *M. smegmatis* mc²155 (Snapper *et al.*, 1990) was the surrogate host for the *M. paratuberculosis* gene expression experiments. The transcriptional-translational *lacZ* fusion vector pYUB76 (Barletta *et al.*, 1992) replicates in *E. coli* and *M. smegmatis*.

Growth conditions and extraction of *M. paratuberculosis* DNA. For genomic DNA isolation, *M. paratuberculosis* was grown in Middlebrook 7H9 broth (Difco) containing 0.5% Tween-80, OADC enrichment (Difco) and 2 mg l⁻¹ mycobactin J (Allied Monitor). The cells were harvested after 5 weeks of growth in tissue-culture flasks (Corning) at 37 °C. *M. paratuberculosis* pellets (1–3 g) were frozen at –70 °C and genomic DNA was extracted by the method of Whipple *et al.* (1987).

DNA cloning. Genomic DNA from *M. paratuberculosis* was partially digested with *Sau*3AI and size-fractionated by agarose gel electrophoresis. Fragments in the 0.2–2 kb size range were excised from the gel and purified by electroelution (Sambrook *et al.*, 1989). Approximately 0.5 µg purified genomic DNA fragments was ligated to 1 µg dephosphorylated, *Bam*HI-cleaved pYUB76 in a final volume of 10 µl. The ligation mixture was used to transform *M. smegmatis* mc²155 or *E. coli* DH10B by electroporation. *E. coli* transformants were selected on Luria–Bertani (LB) medium containing 50 µg kanamycin ml⁻¹ and X-Gal (45 µg ml⁻¹). *M. smegmatis* electrotransformants were selected on Middlebrook 7H10 agar (Difco) with 50 µg kanamycin ml⁻¹ and X-Gal. Blue colonies on this medium were considered candidate *M. paratuberculosis* promoter sequences.

Quantitative β -galactosidase assays. β -Galactosidase assays for *M. smegmatis* were performed as described by Barletta *et al.* (1992). For all experiments, β -galactosidase activity is expressed in Miller units (Miller, 1972) and normalized by dividing by the OD₆₀₀ readings of the respective cultures, which ranged from 0.2 to 0.3.

RNA extraction. Total cell RNA was extracted from *M. smegmatis* using the protocol described by Bashyam & Tyagi (1994).

DNA sequencing. Sequencing reactions were performed manually with the Sequenase version 2.0 sequencing kit (United States Biochemical) for generating size markers to resolve the length of the primer extension products. The *lacZ*⁺ *M. paratuberculosis* inserts in pYUB76 were sequenced with the *Taq* DyeDeoxy Terminator Cycle Sequencing Kit (Perkin Elmer/Applied Biosystems Division: PE/ABD). Each sequencing reaction included 100 ng template DNA and 10 pmol sequencing primer in a total volume of 20 µl. Each reaction underwent 25 cycles (98 °C for 15 s, 50 °C for 2 s, 60 °C for 4 min) in the GeneAmp PCR System 9600 thermal cycler (Perkin-Elmer). The sequencing products were purified through Centri-Sep columns (Princeton Separations) and were loaded onto 6% denaturing polyacrylamide gels in an automated sequencer (373A DNA Sequencer; PE/ABD). Template DNA for automated sequencing was prepared by one of two methods: PEG preparation (Tartof & Hobbs, 1987) or Qiagen miniprep extraction.

To sequence *lacZ*⁺ inserts from pYUB76, synthetic oligonucleotides (5'-CATTATTATCATGACATTAACC-3' and 5'-GTGCTGCAAGGCGATTAAGTTG-3') that flank the

cloning site and hybridize to the vector were employed. When needed, internal primers were designed to sequence further into the cloned inserts.

Computer analysis of DNA sequences. Sequence alignments were performed with the software package SeqEd (Version 1.0.3; PE/ABD). Basic Local Alignment Search Tool (BLAST; Altschul *et al.*, 1990) was used for database homology searches. The ORFs were identified with either Gene Finder, Sequencher 3.0, or the Wisconsin package GCG (Devereux *et al.*, 1984).

Slot-blot hybridization analysis. Aliquots (5 µg) of *M. smegmatis* total cell RNA were heated and chemically denatured prior to application to a Hybond-N nylon membrane (Amersham). RNA samples were applied with a filtration manifold consisting of a lucite block containing a number of slots (slot-blotting apparatus; Gibco-BRL). Prehybridization, hybridization and washes were performed by routine methods (Sambrook *et al.*, 1989). The radioactive probes used for hybridization consisted of [α -³²P]dATP-labelled 3.2 kb *Eco*RI fragment from pCCLac5 (Cupples & Miller, 1988) and 16S and 23S rRNA from *M. smegmatis*. Plasmid pCCLac5 contains the 3.2 kb *Eco*RI fragment carrying *lacZ*. Unincorporated nucleotides were separated from labelled probe by two consecutive precipitations with ethanol in the presence of 7.5 M ammonium acetate. Slot-blot signals were quantified by scanning the autoradiogram into the shareware computer program NIH Image and performing an internal calibration. The levels of RNA were adjusted by first normalizing the relative amounts of RNA used in the rRNA slots then similarly adjusting the RNA levels in the *lacZ* slots.

Primer extension analysis. Total cell RNA from *M. smegmatis* strains harbouring the *lacZ* fusions was used as a template for the synthesis of cDNA products from 5'-end-labelled synthetic oligonucleotide primers. The primer (5'-AGTCACGACGT-TGTAACACGACG-3'), which corresponds to nucleotides internal to *lacZ* in pYUB76, was end-labelled by T4 polynucleotide kinase (Promega) with [γ -³²P]ATP (Amersham). The annealing and reverse transcription reactions were performed with the AMV reverse transcriptase primer extension system following the instructions of the manufacturer (Promega). The products of the reactions were visualized by autoradiography after separation in 6% urea-polyacrylamide gels.

RESULTS

Cloning *M. paratuberculosis* promoters in pYUB76

To define *M. paratuberculosis* promoter elements, a genomic DNA library was constructed in the transcriptional-translational fusion vector pYUB76 (Barletta *et al.*, 1992) and transformed into *E. coli* DH10B. This library was pooled, amplified and transformed by electroporation into *M. smegmatis*. In a representative experiment, 24 *lacZ*⁺ colonies were obtained from 1774 kanamycin-resistant transformants. Eight clones displaying varying intensities in the chromogenic assay (e.g. dark to light blue on X-Gal plates) were selected for further study.

Relative β -galactosidase activity and mRNA levels of the *M. smegmatis lacZ*-fusion constructs

Fig. 1 shows quantitative β -galactosidase activities and steady-state mRNA levels of *M. smegmatis* transformants harbouring the pYUB76 vector with and

Fig. 1. Schematic diagram of the pYUB76::lacZ fusions with corresponding mRNA levels and β -galactosidase activities. The construct maps are drawn to show relative sizes of the ORFs and the upstream sequences. The transcriptional (mRNA levels, percentage density of *lacZ* message) and translational (β -galactosidase activities in Miller units) efficiencies are shown. The constructs are listed from highest to lowest amount of *lacZ* mRNA in Fig. 2. Also indicated are the normalized ratio of mRNA levels and β -galactosidase activities [*lacZ* mRNA/maximum *lacZ* mRNA (pAJB303)]/[β -galactosidase activity/maximum β -galactosidase activity (pAJB300)].

without promoter-containing inserts. Also shown are the schematic maps of the *lacZ* fusions in this study. The *lacZ* fusions pAJB300, pAJB301 and pAJB303 displayed high expression of the reporter gene; fusions pAJB86, pAJB125, and pAJB305 gave medium levels of β -galactosidase activity; and fusions pAJB73 and pAJB304 showed low levels. No detectable activity was observed with the strain carrying the vector pYUB76. The *M. smegmatis* (pAJB73) *lacZ* fusion exhibited a blue colour on X-Gal-containing media, but had little β -galactosidase activity when quantitatively determined using ONPG as the substrate. When these constructs were transformed into *E. coli* DH10B, only pAJB73, pAJB86 and pAJB125 expressed β -galactosidase, at moderate levels (data not shown).

To quantify transcriptional activity from the pYUB76::lacZ fusions, slot-blot hybridization experiments were performed. Steady-state RNA from *lacZ*-positive *M. smegmatis* strains was applied to nylon membranes and hybridized (Fig. 2). Comparing the relative amounts of *lacZ* fusion message, *M. smegmatis* cells harbouring pAJB300, pAJB301, pAJB303 and pAJB304 showed the highest level of transcriptional activity. No transcriptional activity was detected in *M. smegmatis* harbouring pYUB76 by either Northern hybridization analysis (data not shown) or slot-blot analysis (Fig. 2). As indicated by the normalized ratio of mRNA levels and β -galactosidase activities, the cor-

Fig. 2. Quantification of steady-state RNA from *M. smegmatis* harbouring pYUB76::lacZ fusions. Total cell RNA from *M. smegmatis* was quantitatively spotted onto a nylon membrane and hybridized with either radiolabelled rRNA from *M. smegmatis* (left column) or a *lacZ*-specific fragment from pCCLac5 (right column). The strength of the signals was determined using an internal calibration in the computer program NIH Image. The results are reported as % density: (intensity of *lacZ* mRNA/intensity of rRNA) $\times$ 100.

Table 1. Sequence data from *lacZ*⁺ fusion constructs

Construct	Insert size (bp)	G + C content (mol %)	No. of aa derived from the mycobacterial gene
pAJB303	610	67.2	95
pAJB300	412	66.1	24
pAJB301	292	70.9	61
pAJB304	663	68.2	7
pAJB73	104	69.2	15
pAJB305	559	71.0	10
pAJB86	> 1800	66.3	20
pAJB125	1660	60.8	81

relation between these two measurements is not complete. The pAJB303 construct shows the most steady-state mRNA, but β -galactosidase activity ranks it third strongest. Similarly, pAJB300 has the highest β -galactosidase activity and is second to pAJB303 in transcriptional activity. pAJB125 shows the least correlation, displaying medium levels of β -galactosidase activity, but very low *lacZ* mRNA levels. The reverse situation occurs with pAJB73. This lack of correlation is probably attributable to several factors, such as mRNA stability and translational efficiency.

DNA sequence analysis

The nucleotide sequence was determined for each of the *lacZ*⁺ *M. paratuberculosis* inserts. Insert sizes, mol % G + C, and number of amino acids derived from each mycobacterial fusion, are listed in Table 1. The mol % G + C of sequenced inserts agrees well with the published figure of 66–67 mol % for *M. paratuberculosis* DNA (McFadden *et al.*, 1987). The putative ORFs were identified by computer analysis and confirmed for conditions specific to *lacZ* expression in pYUB76 [i.e. all cloned sequences must contain a promoter, ribosome-binding site (RBS), and a start codon that must be in-frame with the ninth codon (GUC) of *lacZ*]. The pAJB303 construct has two possible in-frame start codons (AUG and GUG). Since the GUG codon is separated from the RBS by only two nucleotides, we chose the AUG start codon which is separated by 8 nucleotides, a more prototypical spacing seen in prokaryotes. BLAST searches of nucleic acid databases showed no significant homology to any of the *M. paratuberculosis* sequences in this study. However, pAJB73 and pAJB304 have a *M. paratuberculosis* nucleotide or protein sequence of insufficient length to reveal meaningful homologies in the database.

Primer extension analysis

To localize the promoter region within the cloned *M. paratuberculosis* inserts, the transcription start sites were mapped. The mRNA initiation sites of the cloned *lacZ*-fusion transcripts were determined by primer

Fig. 3. Primer extension analysis of *lacZ* fusion transcripts from pAJB300 (lane 1), pAJB301 (lane 2), pAJB303 (lane 3), pAJB86 (lane 4), pAJB305 (lane 5), pAJB125 (lane 6), and pAJB304 (lane 7). The positions of the primer extension products are indicated by arrows along with the nucleotide (nt) length of the product. An M13 sequence ladder was used to size the extension products with the potential limitation of a 1–2 nucleotide inaccuracy. Details of the experiment are described in Methods.

extension analysis. Primer extension products were generated for the pAJB73 (data not shown), pAJB86, pAJB125, pAJB300, pAJB301, pAJB303, pAJB304 and

Table 2. Nucleotide sequences of predicted *M. paratuberculosis* ribosome-binding sites

Predicted RBSs are underlined, with nucleotides that may base-pair with rRNA shown in bold. Potential AUG and GUG initiation codons are also underlined.

Construct	Nucleotide sequence	ΔG (kcal mol ⁻¹)	Position of start codon
pAJB303	UGGGCUGGGCGGGCUUGCGGAA UACCG AAAGAUAGUGAA AUG	-6.0	+94
pAJB300	GGACGAUUUCACGAACCUUG UACGCAAGUUA CAGUUAUCUCC AUG	-4.8	+96
pAJB301	CGACCAGGGUUUCAUGCA ACGGCGUG GCGACCAUCAGCCUGCGGUG	-5.6	+59
pAJB304	GAGCUGGACG GGAGGU CGUCGA AUG	-15	+22
pAJB73	UGCUCGAGCUC GAGCU CGGUG AUG	-3.6	+15
pAJB305	GCGACAAGGCAAUGUUGUCGGUACCG GGAUGAGAU GGUCUCU AUG	-10	+79
pAJB86	GGCUACCGCGUCG AAGCCGGAACCAUGA CUGGCGGUUCGGCAGAA AUG	-8.0	+118
pAJB125	AGGAGGU CGCCGGCAUCAGCGCCGGCACACGUUGUUGCGCCAC GUG	-19	+76
16S rRNA 3'	AUUCCUCCACUA		

Table 3. -35 and -10 promoter regions and their spacings in mycobacteria

Construct	-35 region		-10 region		+1	Spacing*	Initial codon
<i>M. paratuberculosis</i> promoters from this study†							
pAJB303	GG TGGCGT	CGCCGGTGTAGCCGAA	CGGCAC	GTGCGCG	T	92	ATG
pAJB300	CC TGACGT	TGGCCGACCTCGGCGCG	CAGCCG	ACCGCGC	A	195	ATG
pAJB301	GG TCCAGT	ACACCGCGAGTTCGCGCACG	CTGGCC	GGCAGCGTCT		55	GTG
pAJB304	CC AAGGACA	AACGGCCGTATCCGGTA	CCAACG	GGTGTGC	G	21	ATG
pAJB73	TG TGCCGC	TGAACCGGCCAGCTCCCG	CTCCAG	GGTGACG	T	13	GTG
pAJB305	GT TGTTGG	AGTTTCTGTCGGACGGT	TGGTTG	GCGGCAT	T	77	ATG
pAJB86	GAT TGACGT	TGCACGGCTGGGATTAA	CGGTCC	GCGTGC	T	116	ATG
pAJB125	CAT TAAAG	ATCGANGGCGCCGGNT	CATGTC	CCCTTCAC	C	74	GTG
Consensus‡	TGMCGT	--N ₁₆₋₂₀ --	CGGCCS				
Other mycobacterial promoters§							
<i>hsp60</i> BCG	<u>TTGCAC</u>	17*	TAAGAA	6*	T	183	ATG
<i>P_{AN}</i> <i>M. ptb.</i>	<u>TCGACA</u>	17	TACACT	7	A	40	ATG
16S rRNA <i>leprae</i>	<u>TTGACT</u>	16	ATTAAT	7	G		
16S rRNA <i>M. tub.</i>	<u>TTGACT</u>	18	TAGACT	7	T		
<i>cpn60</i> <i>M. tub.</i>	<u>TGCTCA</u>	17	GGCGGC	7	A	29	ATG
<i>mpb70</i> BCG	<u>CCGATC</u>	17	CATCAG	6	G	176	ATG
<i>bla fortuitum</i>	<u>TTCAAA</u>	19	TACGCT	7	A	0	ATG
<i>ask smegmatis</i>	<u>CCCACG</u>	17	ACGCTG	7	G	250	GTG
85A <i>M. tub.</i>	<u>TTGACT</u>	22	CGCCTG	7	A	63	ATG

*Number of nucleotides between indicated regions.

†Nucleotides in bold represent the -35 and -10 consensus regions. Underlined nucleotides match the consensus at that position.

‡M represents C or A; S represents G or C. pAJB125 and pAJB304 were excluded when calculating the consensus.

§*Mycobacterium* sp. promoter regions as compiled by Kremer *et al.* (1995). *M. ptb.*, *M. paratuberculosis*; *M. tub.*, *M. tuberculosis*. Only promoters for which the transcription start sites are known were included. Underlined nucleotides indicate a match to the *M. paratuberculosis* consensus sequence.

pAJB305 constructs (Fig. 3). The startpoint of the pAJB303 transcript was not determined to single base-pair accuracy due to the large size of the cDNA product. Therefore, an estimate of the extension product was made using radiolabelled ϕ X174 DNA markers digested with *HinfI* (data not shown).

Putative ribosome-binding sites in *M. paratuberculosis* mRNA

A region of homology to the 3' terminus of *M. leprae* 16S rRNA followed after a spacing of 4-37 bp by an initiation codon and an ORF for translation was

observed for all the *lacZ* fusions. Nucleotides that may base-pair with rRNA are shown in bold in Table 2. The predicted free energy of base-pairing (ΔG) of these RBSs with 16S rRNA, calculated according to the rules of Tinoco *et al.* (1973), ranged from -3.6 to -19 kcal mol $^{-1}$ (-15.5 to -79.5 kJ mol $^{-1}$).

Codon usage

A compilation of the codons employed in the N-terminal mycobacterial ORFs which participated in the *lacZ* fusions was performed (data not shown). The 309 codons compiled for *M. paratuberculosis* in this study show a characteristic GC bias, with 73% of the codons containing a G or C in the third position, whereas codons ending in T and A are represented at frequencies of 15 and 12%, respectively. This codon usage is remarkably similar to that obtained for *M. leprae* (Honoré *et al.*, 1993) and the *M. tuberculosis* complex (Andersson & Sharp, 1996).

Determination of a consensus promoter sequence

The eight *M. paratuberculosis* promoters that had their transcription start sites identified by primer extension analysis were aligned as shown in Table 3. The nucleotides shown in bold in Table 3 represent the putative -35 and -10 regions. Based on these data, a *M. paratuberculosis* consensus sequence for the -35 region was established as TGMCGT. No strong consensus was established for any of the positions in the -10 region. However, for the aligned sequences in Table 3, a cytosine nucleotide was present in the first position of the highlighted -10 region in 87% of cases. Additionally, the occurrence at the other positions was 37% G, 62% G, 37% C, 50% C and 100% S. The putative promoter regions from pAJB125 and pAJB304 were excluded from the calculation of the consensus since those two promoter regions differed significantly from the other six. For comparison, the -35 and -10 regions of all mycobacterial promoters that contain an experimentally determined transcription start site were also compiled; these are listed at the bottom of Table 3. Nucleotides that match the consensus are underlined. None of these promoter regions display significant homology with the *M. paratuberculosis* consensus.

DISCUSSION

Eight authentic *M. paratuberculosis* promoter elements were identified in this study based on transcriptional/translational *lacZ* fusions and primer extension analysis using *M. smegmatis* as a surrogate host. Ideally, gene expression studies should be performed in the native host. However, a genetic system for *M. paratuberculosis* has only recently been developed (Foley-Thomas *et al.*, 1995) and the expression of the *lacZ* reporter gene in *M. paratuberculosis* has not been documented. Nevertheless, a homologous mycobacterial system such as *M. smegmatis* offers clear advantages to the use of an *E. coli* system that is unable to recognize many mycobacterial

expression sequences (Barletta *et al.*, 1992; Clark-Curtiss *et al.*, 1985; Thole *et al.*, 1985).

Strong β -galactosidase activity was observed in the *lacZ* fusion constructs of pAJB300, pAJB301 and pAJB303. The Miller units obtained in the quantitative β -galactosidase assay indicated that pAJB300 is stronger than most of the mycobacteriophage promoters cloned in pYUB76 (Barletta *et al.*, 1992). In contrast, pAJB301 and pAJB303 were slightly weaker than the cloned mycobacteriophage promoters. The cloned pAJB300 or pAJB303 inserts may be good candidates for expression of heterologous antigens in *M. bovis* BCG for recombinant vaccine development (Stover *et al.*, 1991, 1993) because of their high level of expression.

The slot-blot hybridization of *M. smegmatis* RNA was performed to quantify steady-state levels of the *lacZ* fusion message. Thus, pAJB303 might contain the strongest promoter. Alternatively, the pAJB303 message might be more stable. The 5' leader sequence of some stable transcripts such as the T4 bacteriophage gene 32 or the *E. coli ompA* can stabilize other sequences fused downstream from them (Ehretsmann *et al.*, 1992). For example, the *lacZ* transcript has a half-life of 90 s, but a gene 32::*lacZ* fusion has a half-life of 20 min.

Although we have not identified initiation codons directly by amino acid sequencing, all of the *lacZ* fusion mRNAs display a putative RBS complementary to the 3' region of *M. leprae* 16S rRNA followed by a possible initiation codon and an open reading frame. The free energies of interaction (ΔG) for the proposed RBSs vary from -3.6 to -19 kcal mol $^{-1}$ (-15.5 to -79.5 kJ mol $^{-1}$) as compared to -9.4 kcal mol $^{-1}$ (-39.3 kJ mol $^{-1}$) for the prototype *E. coli* RBS AGGA (McLaughlin *et al.*, 1981).

Six of the promoters transcriptionally mapped in this study display some degree of conformity in their -35 regions and, less so, in the -10 regions, with the canonical sequence including matches in at least half of the positions for each region. The intervening sequence between the -35 and -10 regions (16–20 bp) corresponds to the preferred interval for prokaryotic promoters. The -35 promoter regions from pAJB304 (no position conserved) and pAJB125 (two positions conserved) differ significantly. pAJB125 also differs significantly at the -10 box (two positions conserved). Significant differences are observed when we compare the *M. paratuberculosis* consensus sequence with nine previously studied promoters (Table 3). These deviations suggest that these promoters may be under the control of different sigma factors, a well-known situation in other microbial systems (Hawley & McClure, 1983). Since *M. paratuberculosis* promoters were identified *in vitro* under standard growing conditions, it is likely that the consensus sequence established in this study corresponds to the principal sigma factor involved in the transcription of housekeeping genes. In addition, since *M. smegmatis* was the surrogate host for these promoter studies, there may exist a bias toward *M. paratuberculosis* promoters that are highly active in that

host. Conversely, some of the previously studied promoters may require special sigma factors related to stress conditions, as exemplified by the heat-shock promoters *hsp60* and *mpb70*.

Recently, Bashyam *et al.* (1996) reported a thorough study on the consensus sequences of *M. smegmatis* and *M. tuberculosis* promoters. In that study, a fairly well-conserved -10 box was found which resembled the *E. coli* Pribnow box. In contrast, we failed to find any conserved sequence of this kind. These differences may be attributable to the use of different reporter genes and selection markers, which may have led to the isolation of a different set of promoters. The observation that a bacterial sigma factor can recognize promoters with diverse -10 regions is unusual but not unique. Studies showing diverse -10 hexamers have been described for *Caulobacter crescentus* (Malakooti *et al.*, 1995) and *Chlamydia trachomatis* (Douglas & Hatch, 1996).

Additional promoter sequences must be compared to test and refine the consensus regions. The -35 region of the paratuberculosis consensus bears some resemblance to *E. coli* whereas the -10 region does not. Upstream regions of mycobacterial genes have been shown to have a higher G+C content than their *E. coli* counterparts (Dale & Patki, 1990). This observation was confirmed in the present study, as the conserved regions have a G+C content of 66.7 mol% compared with 16.7 mol% for the *E. coli* consensus. As was pointed out by Dale & Patki (1990), analysis of the sequence of many mycobacterial genes does not reveal any regions upstream from the putative translational start position that resemble an *E. coli*-like consensus promoter. The *M. paratuberculosis* promoters defined in this study are more closely related to each other than they are to the other compiled mycobacterial promoters in Table 3. The results of this study have, for the first time, revealed a potential paratuberculosis consensus promoter.

ACKNOWLEDGEMENTS

We thank the DNA sequencing and synthesis facility (1184 Molecular Biology Building, Iowa State University) for performing the automated sequencing of the pYUB76::lacZ fusion inserts. We also thank Greg Phillips for providing plasmids and thoughtful discussions.

This is journal paper no. J-16741 of the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa. The work is part of Project no. 3222, and was supported by Hatch Act and State of Iowa Funds. R.G.B. was supported by Cooperative State Research Service Project NEB 14-077.

REFERENCES

- Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* 215, 403–410.
- Andersson, S. G. E. & Sharp, P. M. (1996). Codon usage in the *Mycobacterium tuberculosis* complex. *Microbiology* 142, 915–925.
- Barletta, R. G., Kim, D. D., Snapper, S. B., Bloom, B. R. & Jacobs, W. R., Jr (1992). 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. *J Gen Microbiol* 138, 23–30.
- Bashyam, M. D. & Tyagi, A. K. (1994). An efficient and high yielding method for isolation of RNA from mycobacteria. *BioTechniques* 17, 834–836.
- Bashyam, M. D., Kaushal, D., DasGupta, S. K. & Tyagi, A. K. (1996). A study of the mycobacterial transcriptional apparatus: identification of novel features in promoter elements. *J Bacteriol* 178, 4847–4853.
- Clark-Curtiss, J. E., Jacobs, W. R., Doherty, M. A., Ritchie, L. R. & Curtiss, R., III (1985). Molecular analysis of DNA and construction of genomic libraries of *M. leprae*. *J Bacteriol* 161, 1093–1102.
- Cupples, C. G. & Miller, J. H. (1988). Effect of amino acid substitutions at the active site in *Escherichia coli* β -galactosidase. *Genetics* 120, 637–644.
- Dale, J. W. & Patki, A. (1990). Mycobacterial gene expression and regulation. In *Molecular Biology of the Mycobacteria*, pp. 173–198. Edited by J. J. McFadden. London: Surrey University Press.
- DasGupta, S. K., Bashyam, M. D. & Tyagi, A. K. (1993). Cloning and assessment of mycobacterial promoters by using a plasmid shuttle vector. *J Bacteriol* 175, 5186–5192.
- Devereux, J., Haeberli, P. & Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res* 12, 387–395.
- Douglas, A. L. & Hatch, T. P. (1996). Mutagenesis of the P2 promoter of the major outer membrane protein gene of *Chlamydia trachomatis*. *J Bacteriol* 178, 5573–5578.
- Ehretsmann, C. P., Carpousis, A. J. & Krisch, H. M. (1992). mRNA degradation in procaryotes. *FASEB (Fed Am Soc Exp Biol) J* 6, 3186–3192.
- El-Zaatar, F. A. K., Naser, S. A., Engstrand, L., Hachem, C. Y., Graham, D. Y. (1994). Identification and characterization of *Mycobacterium paratuberculosis* recombinant proteins expressed in *E. coli*. *Curr Microbiol* 29, 177–184.
- Estrada-G, I. C. E., Lamb, F. I., Colston, M. J. & Cox, R. A. (1988). Partial nucleotide sequence of 16S ribosomal RNA isolated from armadillo-grown *Mycobacterium leprae*. *J Gen Microbiol* 134, 1449–1453.
- Foley-Thomas, E. M., Whipple, D. L., Bermudez, L. E. & Barletta, R. G. (1995). Phage infection, transfection, and transformation of *Mycobacterium avium* complex and *Mycobacterium paratuberculosis*. *Microbiology* 141, 1173–1181.
- Gilot, P., De Kesel, M., Machtelincx, L., Coene, M. & Cocito, C. (1993). Isolation and sequencing of the gene coding for an antigenic 34-kilodalton protein of *Mycobacterium paratuberculosis*. *J Bacteriol* 175, 4930–4935.
- Green, E. P., Tizard, M. L. V., Moss, M. T., Thompson, J., Winterbourne, D. J., McFadden, J. J. & Hermon-Taylor, J. (1989). Sequence and characteristics of IS900, an insertion element identified in a human Crohn's disease isolate of *Mycobacterium paratuberculosis*. *Nucleic Acids Res* 17, 9063–9073.
- Hance, A. J., Grandchamp, B., Levy-Frebault, V., Lecossier, D., Rauzier, J., Bocart, D. & Gicquel, B. (1989). Detection and identification of mycobacteria by amplification of mycobacterial DNA. *Mol Microbiol* 3, 843–849.
- Hawley, D. K. & McClure, W. R. (1983). Compilation and analysis of *Escherichia coli* promoter DNA sequences. *Nucleic Acids Res* 11, 2237–2255.
- Honoré, N., Bergh, S., Chanteau, S. & 15 other authors (1993). Nucleotide sequence of the first cosmid from the *Mycobacterium*

- leprae* genome project: structure and function of the Rif-Str regions. *Mol Microbiol* 7, 207–214.
- Kremer, L., Baulard, A., Estaquier, J., Content, J., Capron, A. & Loch, C. (1995). Analysis of the *Mycobacterium tuberculosis* 85A antigen promoter region. *J Bacteriol* 177, 642–653.
- McFadden, J. J., Butcher, P. D., Chiodini, R. J. & Hermon-Taylor, J. (1987). Determination of genome size and DNA homology between unclassified *Mycobacterium* species isolated from patients with Crohn's disease and other mycobacteria. *J Gen Microbiol* 133, 211–214.
- McLaughlin, J. R., Murray, C. L. & Rabinowitz, J. C. (1981). Unique features in the ribosome binding site sequence of the Gram-positive *Staphylococcus aureus* β -lactamase gene. *J Biol Chem* 256, 11283–11291.
- Malakooti, J., Wang, S. P. & Ely, B. (1995). A consensus promoter sequence for *Caulobacter crescentus* genes involved in bio-synthetic and housekeeping function. *J Bacteriol* 177, 4372–4376.
- Miller, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Moran, C. P., Jr, Lang, N., LeGrice, S. F. J., Lee, G., Stephens, M., Sonenshein, A. L., Pero, J. & Losick, R. (1982). Nucleotide sequences that signal the initiation of transcription and translation in *Bacillus subtilis*. *Mol Gen Genet* 186, 339–346.
- Murray, A., Winter, N., Lagranderie, M., Hill, D. F., Rauzier, J., Timm, J., Leclerc, C., Moriarty, K. M., Gheorghiu, M. & Gicquel, B. (1992). Expression of *Escherichia coli* β -galactosidase in *Mycobacterium bovis* BCG using an expression system isolated from *Mycobacterium paratuberculosis* which induced humoral and cellular immune responses. *Mol Microbiol* 6, 3331–3342.
- Muto, A. & Osawa, S. (1987). The guanine and cytosine content of genomic DNA and bacterial evolution. *Proc Natl Acad Sci USA* 84, 166–169.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sela, S. & Clark-Curtiss, J. (1991). Cloning and characterization of the *Mycobacterium leprae* ribosomal RNA promoter in *Escherichia coli*. *Gene* 98, 123–127.
- Snapper, S. B., Melton, R. E., Mustafa, S., Kieser, T. & Jacobs, W. R. (1990). Isolation and characterization of efficient plasmid transformation mutants of *Mycobacterium smegmatis*. *Mol Microbiol* 4, 1911–1919.
- Stevenson, K., Inglis, N. F., Rae, B., Donachie, W. & Sharp, J. M. (1991). Complete nucleotide sequence of a gene encoding the 70 kd heat shock protein of *Mycobacterium paratuberculosis*. *Nucleic Acids Res* 19, 4552.
- Stover, C. K., de la Cruz, V. F., Fuerst, T. R. & 11 other authors (1991). New use of BCG for recombinant vaccines. *Nature* 351, 456–460.
- Stover, K., Bansal, G. P., Hanson, M. S. & 7 other authors (1993). Protective immunity elicited by recombinant bacille-Calmette-Guérin (BCG) expressing outer surface protein A (Osp A) lipoprotein: a candidate lyme disease vaccine. *J Exp Med* 178, 197–209.
- Tartof, K. D. & Hobbs, C. A. (1987). Improved media for growing plasmid and cosmid clones. *Bethesda Res Lab Focus* 9, 12.
- Thole, J. E. R., Dauwerse, H. G., Das, P. K., Groothuis, D. G., Schouls, L. M. & van Embden, J. D. A. (1985). Cloning of *Mycobacterium bovis* BCG DNA and expression of antigens in *Escherichia coli*. *Infect Immun* 50, 800–806.
- Thomas, T. J., Thoen, C. O. & Andrews, R. E., Jr (1992). Molecular cloning and characterization of *Mycobacterium paratuberculosis* promoters in *Escherichia coli*. *Vet Microbiol* 32, 351–362.
- Timm, J. M., Perilli, G., Uez, C. & 9 other authors (1994). Transcription and expression analysis using *lacZ* and *phoA* gene fusions of *Mycobacterium fortuitum* β -lactamase genes cloned from a natural isolate and a high level β -lactamase producer. *Mol Microbiol* 12, 491–504.
- Tinoco, I., Borer, P. N., Dengler, B., Levine, M. D., Uhlenbeck, O. C., Crothers, D. M. & Gralla, J. (1973). Improved estimation of secondary structure in ribonucleic acids. *Nature New Biol* 246, 40–41.
- Whipple, D. L., Le Febvre, R. B., Andrews, R. E., Jr & Thiermann, A. B. (1987). Isolation and analysis of restriction endonuclease digestive patterns of chromosomal DNA from *Mycobacterium paratuberculosis* and other *Mycobacterium* species. *J Clin Microbiol* 25, 1511–1515.

Received 9 August 1996; revised 25 October 1996; accepted 28 October 1996.