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# Cloning and Characterization of the Promoters of Temperate Mycobacteriophage L1

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**Four putative promoters of the temperate mycobacteriophage L1 were cloned by detecting the β-galactosidase reporter expression in *E. coli* transformants that carried L1 specific operon-fusion library. All of the four L1 promoters were also found to express differentially in the homologous environment of mycobacteria. Of the four promoters, two were suggested to be the putative early promoters of L1 since they express within 0 to 10 min of the initiation of the lytic growth of L1. One of the putative early promoters showed a relatively better and almost identical activity in both *E. coli* and *M. smegmatis*. By a sequence analysis, we suggest that the L1 insert that contained the stronger early promoter possibly carries two convergent *E. coli* $\sigma^{70}$-like L1 promoters, which are separated from each other by about 300 nucleotides. One of them is the early promoter of L1 as it showed a 100% similarity with the early $P_{left}$ promoter of the homoimmune phage L5. The second promoter, designated *P4*, was suggested for its appreciable level of reporter activity in the absence of the –10 element of the $P_{left}$ equivalent of L1. By analyzing most of the best characterized mycobacteriophages-specific promoters, including the L1 promoter *P4*, we suggest that both the –10 and –35 hexamers of the mycobacteriophage promoters are highly conserved and almost similar to the consensus –10 and –35 hexamers of the *E. coli* $\sigma^{70}$ promoters.**

**Keywords:** *β-galactosidase* reporter, Consensus, Early promoter, Mycobacteriophage L1

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## Introduction

The gene regulatory elements of several bacteriophages (e.g., lambda, T7, T4 etc.) have been exploited extensively for generating tightly-regulated expression vectors. In turn, they eased the genetic studies in their hosts (Sambrook *et al*., 1989). Since the late 1980s, several mycobacteriophages were studied in order to identify their gene regulatory elements (such as promoters, transcription terminator, repressor, *cos* site, *att*P site, integrase, etc.) so that they could be assembled to form various types of vectors, including expression vectors. Some promoters of bacteriophage K11 (Han *et al*., 2002) and those of the temperate mycobacteriophage I3 (Ramesh and Gopinathan, 1995), Bxb1 (Jain and Hatfull, 2000), and L5 (Nesbit *et al*., 1995) were recently cloned and characterized. Also, using the *att*P site of phage L5, some mycobacteria-specific integration vectors were constructed (Pashley and Stoker, 2000). The early $P_{left}$ promoter of L5 has been shown to be negatively regulated by its own repressor (Nesbit *et al*., 1995). No information, however, is yet available on a mycobacteriaspecific expression vector.

The L1 phage, a sister homoimmune phage of L5, has a 50-kb double-stranded DNA genome, and it lysogenizes *M. smegmatis* by integrating at a specific site into the host chromosome (Snapper *et al*., 1988). By genetic analyses, the gene coding for the L1 repressor and 28 other genes that are involved in the lytic development of L1 have been mapped. Some were also to an extent characterized (Chaudhuri *et al.*, 1993). Of the 28 genes, the *G27* gene was shown to be an early positive regulator that controls the expression of both the delayed early and late genes at the transcriptional level (Datta and Mandal, 1998). A few promoters of L1 have been cloned in a promoter-cloning vector that has ß-galactosidase as the reporter gene, but further characterization of these promoters is yet to be reported (Barletta *et al*., 1992). In this communication, as a prerequisite to developing the suitable mycobacteria-specific expression vectors, we report the cloning and characterization of four promoters of the L1 temperate mycobacteriophage. Furthermore, we present the

deletion analysis of one L1 promoter clone, which showed identical activities in both *E. coli* and *M. smegmatis*.

## Materials and Methods

**Bacterial and phage strains, vectors, and growth conditions** The *E. coli* DH5α cells were grown in a Luria broth. *M. smegmatis* $mc^26$ and LR222, obtained from B. Bloom (Albert Einstein College of Medicine, Bronx, NY, USA) and Anil Tyagi (University of Delhi, South Campus, Delhi, India) respectively, were grown in a Middlebrook 7H9 medium. The composition of the 7H9 medium was given in Chaudhuri *et al*. (1993). The L1*c*I$^-$ and L1*c*I*ts*391 mycobacteriophage strains were described in Chaudhuri *et al*. (1993). The L1*cIts*391 lysogen of LR222 was purified from the turbid growth at the center of the plaque that formed at 32°C (Chaudhuri *et al*., 1993). The *E. coli* promoter-probe vector pTAC3734 was obtained from Dr. T. Atlung (Technical University of Denmark, Denmark), and the *E. coli-M. smegmatis* shuttle vector pSD5S30 from Dr. Anil Tyagi.

**DNA isolations and manipulations** All of the molecular biological techniques (like the plasmid DNA isolation from *E. coli*, restriction enzyme digestion, agarose gel electrophoresis, ligation, transformation, etc.) were performed according to the methods described by Sambrook *et al.*, (1989). Mycobacteriophage L1 DNA was isolated as described by Chaudhuri *et al.*, (1993). The isolation of the plasmid DNA from *M. smegmatis* and the electro-transformation of LR222 were done according to the standard methods of Das Gupta *et al*., (1993).

**Construction of a L1 promoter library** The *Bam*HI-*Bgl*II double-digested L1 DNA fragments were ligated with the *Bgl*II digested-promoter probe vector, pTAC3734, that carried *β-galactosidase* as the reporter gene. After transformation of the ligated DNAs into competent *E. coli* DH5α cells, the culture was plated on Luria agar (containing 100 µg/ml of ampicillin, 40 µg/ml of X-gal). After 16-18 h incubation at 37°C, the transformants, which appeared as blue colonies, were picked up for analysis.

**Construction of an *E. coli-M. smegmatis* promoter probe shuttle vector** The 1.2 kb *Pst*I fragment of the *E. coli* plasmid pUC4K (Pharmacia, Buckinghamshire, UK) that carried the kanamycin resistance gene was cloned into the *Pst*I site of pMZ240 plasmid to obtain the pNCM1 plasmid. The pMZ240 vector was a derivative of pKO that contained the *Plac* promoter at the upstream of *galK* (Rosenberg *et al*., 1983). The pNCM2 plasmid was obtained by replacing the 600 bp *Bam*HI- *Sca*I fragment of pNCM1 with the 3.6 kb *Bam*HI-*Sca*I fragment of pTAC3734 that carried the promoterless *β-galactosidase* reporter gene and multiple cloning sites. The pTAC3734 plasmid is similar to the pTAC3953 vector without a transcriptional terminator at the *Xho*I site (Brøndsted and Atlung, 1994). A 3.27 kb *Dra*I fragment of pSD5S30, carrying the mycobacteria-specific origin of replication, was cloned into the *Sca*I site of pNCM2 to generate the pLG plasmid (Fig. 1). The pSD5S30 vector was constructed by cloning a mycobacteria-specific promoter *S30* into the multiple-cloning site of pSD5 (Bashyam *et al*., 1996; Jain *et al*., 1997). This newly-constructed promoter probe vector, pLG, can replicate in both *E. coli* and *M. smegmatis*, and showed very low *lacZ* activity in both species (Table 1).

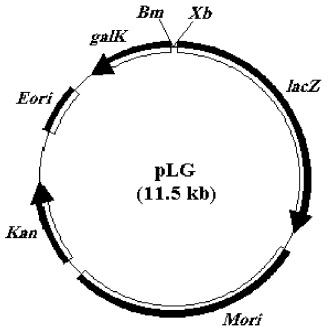


**Fig. 1.** Schematic representation of the *M. smegmatis-E. coli* promoter probe shuttle vector, pLG. The plasmid was constructed as described in Materials and Methods. The direction of the transcription of the *lacZ, galK*, and *Kan* (kanamycin resistance) genes are shown. Abbreviations: *Bm*, *Bam*HI; *Xb*, *Xba*I; *Eori*, *E. coli* specific origin of replication; *Mori*, *M. smegmatis* specific origin of replication; *lacZ*, *β-galactosidase*; *galK*, *galactokinase*; and *kan*, kanamycin resistance gene.

**Assay of β-galactosidase** The level of the β-galactosidase reporter enzyme in the *E. coli* transformants was measured according to the procedure of Miller (1992). To measure the β-galactosidase level in the *M. smegmatis* transformants, the cells were grown in a Luria broth (containing 0.3 % Tween 80 and desired antibiotic) upto 0.3-0.4 $OD_{590}$. One milliliter of this cell culture was centrifuged, and the cell pellet was suspended in a Z buffer and sonicated at 0-4°C. The sonicated extract (100-500 ml) was mixed with an equal volume of a fresh Z buffer. The assay was then performed according to the method of Miller (1992).

***In vitro* transcription assay** The *in vitro* transcription assay was carried out by the method of Levin and Hatfull (1993) with some modifications. Briefly, 250 µl of the reaction mixture was incubated at room temperature for 10 min. The mixture contained 40 mM Tris · HCl (pH 7.9), 0.15 M KCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.5 mg/ml BSA, 1.5 µg of denatured L1 850 bp DNA fragment of pCM8a, 0.15 mM each of NTPs (except UTP), [$\alpha^{32}$P]-UTP, and 1 µl of *M. smegmatis* RNA polymerase (isolated in our laboratory). The reaction was stopped by a buffer that contained 150 mM NaCl, 1% SDS, 50 mg/ml yeast tRNA, and 1 M LiCl. It was extracted once with 1 : 1 phenol-chloroform. The RNA in the aqueous phase was precipitated with 2 volumes of ethanol. The RNA pellet was collected by centrifugation at $12,000 \times g$ for 20 min, washed, and air dried. After dissolving the pellet in $1 \times$ gel loading buffer, it was loaded onto 6% polyacrylamide gel containing 8 M urea, followed by electrophoresis at 250 V for 2 h. After fixing the gel with 10% acetic acid, it was dried and set for autoradiography.

**DNA Sequencing** DNA sequencing of the L1 insert was carried out by an ABI automated sequencer according to the manufacturers protocol.

**Table 1.** Estimation of β-galactosidase activities in L1 promoter clones

| Plasmids | Approx. insert size (in bp) | β-galactosidase activity[a] (units/$OD_{590}$) in | |
|---|---|---|---|
| | | *E.coli* | *M. smegmatis* |
| pTAC3734 | nil | 0.94 | ND |
| pLP1 | 1000 | 176 | ND |
| pLP2 | 3700 | 320 | ND |
| pLP3 | 2300 | 2816 | ND |
| pLP4 | 2800 | 3120 | ND |
| pLG | nil | 0.60 | 0.40 |
| pLG1 | 1000 | 248 | 41 |
| pLG2 | 3700 | 510 | 78 |
| pLG3 | 2300 | 2726 | 2822 |
| pLG4 | 2800 | 3015 | NA |
| pCM8 | 850 | 2940 | ND |
| pCM8a | 850 | ND | 3000 |
| pCM8c | 430 | 1078 | ND |
| pCM8d | 430 | ND | 1160 |

[a]To estimate the enzyme level, the *E. coli* or *M. smegmatis* carrying the pTAC3734 or pLG derivatives were separately grown in Luria broth (containing ampicillin or Kanamycin) up to 0.3-0.4 OD, centrifuged, and the cell pellets were suspended in a Z buffer. The cell suspensions were sonicated and the β-galactosidase levels in the extracts were determined according to the method of Miller (1992). The β-galactosidase activity was expressed in Miller units. Each assay result is the average of three independent experiments. ND, not done; NA, no transformant available.

**DNA accession number** Upon deposition of the cloned 430 bp L1 DNA sequence of pCM8d, the accession number, AY266345, was obtained from GenBank.

## Results and Discussion

**Cloning of the promoters of phage L1 in *E. coli*** For cloning the promoters of the temperate L1 mycobacteriophage, we constructed a *Bam*HI-*Bgl*II genomic library of L1 DNA in an *E. coli*-specific promoter probe vector pTAC3734, because some promoters of the mycobacterial systems also functioned well in *E. coli* (Barletta *et al*., 1992; Das Gupta *et al*., 1993; Nesbit *et al*., 1995; Ramesh and Gopinathan, 1995). According to the procedure described previously, a total of twenty transformants, which produced blue colonies on Luria agar containing X-gal, were selected after screening about 5,000 transformants. The plasmid DNAs, isolated from the twenty individual transformants, were digested with both *Sma*I and *Hind*III (they lack the recognition site in L1 DNA but flank the cloning site, *Bgl*II, in the pTAC3734 vector). They were analyzed by agarose gel electrophoresis (picture not shown). The L1 DNA inserts that were present in these twenty plasmids were of four group sizes - 1,000, 3,700, 2,300, and 2,800 bp. Therefore, one representative plasmid from each of these four groups was used for further study. They were designated pLP1 to pLP4 (Table 1).

The β-galactosidase levels in the bacteria that harbored each of the four plasmids were measured (Table 1). The results suggest that the strength of the putative L1 promoters in the four clones is different. The L1 promoters in pLP3 and pLP4 are about 8 to 16-fold stronger than those in pLP1 and pLP2.

**Expression of L1 promoters in *M. smegmatis*** To test whether these promoters are of real L1 origin and functional in *M. smegmatis*, an *E. coli-M. smegmatis* promoter probe shuttle vector, designated pLG, was constructed (see Materials and Method). The 11.5 kb pLG vector carries two unique restriction enzyme sites, *Bam*HI and *Xba*I, at the upstream of the *β-galactosidase* reporter gene (Fig. 1). Since the L1 DNA inserts in the pLP vectors contain no *Xba*I and *Bam*HI sites (data not shown), the *Bam*HI-*Xba*I inserts from pLP1 to pLP4 that carry L1 promoters were subcloned between the same sites in pLG; the resulting constructs were designated pLG1-pLG4 (Table 1). The orientations of these promoters in pLG1 -pLG4 remain the same as those in their respective pLP parent derivatives. However, the estimation of the β-galactosidase levels in these clones showed that the subcloning of the L1 inserts from the pLP clones into the pLG vector did not significantly affect the L1 promoter activities in *E. coli* (Table 1). All of the four pLG derivatives were then transformed into both *M. smegmatis* and its L1*c*I*ts*391 lysogen. Surprisingly, pLG4 yielded no transformant with *M. smegmatis*, but did with its L1*cIts* 391 lysogen. Possibly the L1 DNA insert of the pLG4 plasmid carries a gene that is lethal to its *M. smegmatis* host but not to *E. coli*, and its expression is negatively

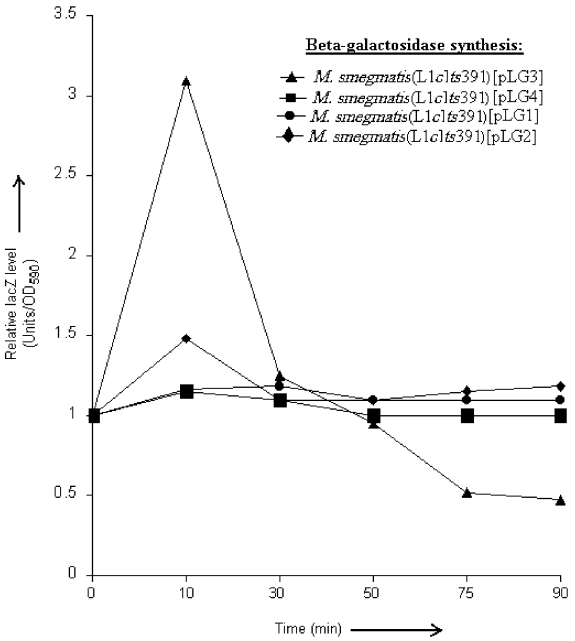


**Fig. 2.** Estimation of β-galactosidase in the induced culture of *M. smegmatis* (L1*cIts*391) carrying pLG derivatives. *M. smegmatis* (L1*cIts*391) cells carrying the pLG derivatives were grown at 32°C to around 0.4 $OD_{590}$, and induced separately at 42°C for 20 min, followed by their growth at 37°C. One-ml aliquots from each induced culture were taken at 0, 10, 30, 50, 75, and 90 min. The β-galactosidase levels in all of the aliquots were measured. The amount of enzyme synthesized was plotted as a function of the time at which the aliquot was taken out after induction. The level of the β-galactosidase reporter enzyme at 0 min was taken as 1 unit/ $OD_{590}$. Absolute values of the enzyme levels in the pLG1, pLG2, pLG3, and pLG4 clones are 180, 50, 573, and 40 units per $OD_{590}$, respectively.

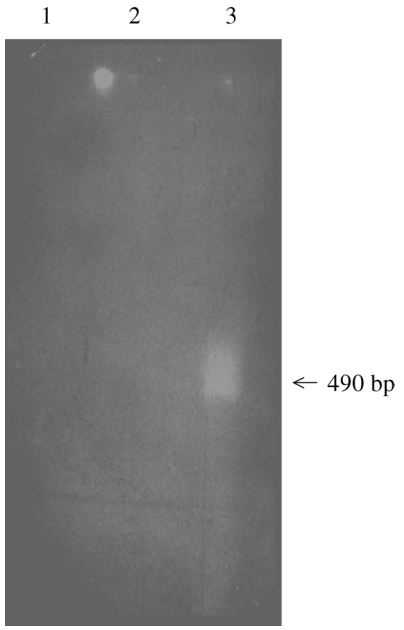


**Fig. 3.** Analysis of *in vitro* transcription products. The *in vitro* transcription assay was performed with an 850 bp L1 DNA fragment of pCM8 (as template), according to a modified Method (see in Material and Methods). The reaction products were run through a 6% polyacrylamide-urea gel, and the [$^{32}$P]-labeled RNA band was visualized by autoradiography. The size of the RNA band was determined by co-electrophoresis with a standard RNA size marker. Lanes: 1, *M. smegmatis* RNA polymerase; 2, 850 bp L1 DNA insert of pCM8; 3, reaction product from the 850 bp L1 insert and *M. smegmatis* RNA polymerase.

regulated by the L1 repressor in *M. smegmatis*.

Data on the measurement of the β-galactosidase levels in *M. smegmatis* that carry these pLG derivatives suggest that the promoters in pLG1 and pLG2 are about 40-60 fold less active than those in pLG3 in *M. smegmatis*. This implies that the L1 promoters in pLG1 and pLG2 are very weak in comparison to those in pLG3. These types of relatively weak promoters were also reported in the L5 phage (Nesbit *et al*., 1995). Interestingly, the putative L1 promoter in pLG3 showed almost an identical strength in both *M. smegmatis* and *E. coli* (Table 1), which suggests that the expression from this promoter possibly needs no phage-encoded activator for the optimal expression.

**Classification of the cloned L1 promoters into the early, delayed early, and late groups** The promoters of the most well characterized bacteriophages (such as lambda and T4) have been classified into the early, delayed early, and late types on the basis of the timing of their temporally-regulated expression during their lytic development. In order to classify the four cloned L1 promoters into these groups, the β-galactosidase levels were measured in *M. smegmatis* (L1*cIts*391) harboring these pLG derivatives during their post-induction growth (Fig. 2). During the post-induction growth of the lysogen carrying pLG3, the level of the enzymes increased about 3 fold over the zero time value, then decreased rapidly with a further increase in the growth time. The β-galactosidase level in the same lysogen carrying pLG2 (growing under identical conditions) showed almost the same kinetics as those shown by pLG3, except that the latter plasmid showed a 1.5 fold higher enzyme expression than the former at 10 min. These data suggest that the promoters that are present in both pLG2 and pLG3 are possibly of the early types.

The L1 promoters that are present in pLG1 and pLG4 showed no significant change in the expression of the reporter enzyme activity after heat induction.

**Identification of the minimal region of the insert pLG3 that has promoter activity** As the L1 promoter in pLG3 belongs to the early class and is nearly equally active in both *E. coli* and *M. smegmatis*, it could be utilized in developing a suitable *mycobacteria*-specific expression vector(s). A prerequisite to the construction of these vectors is to identify

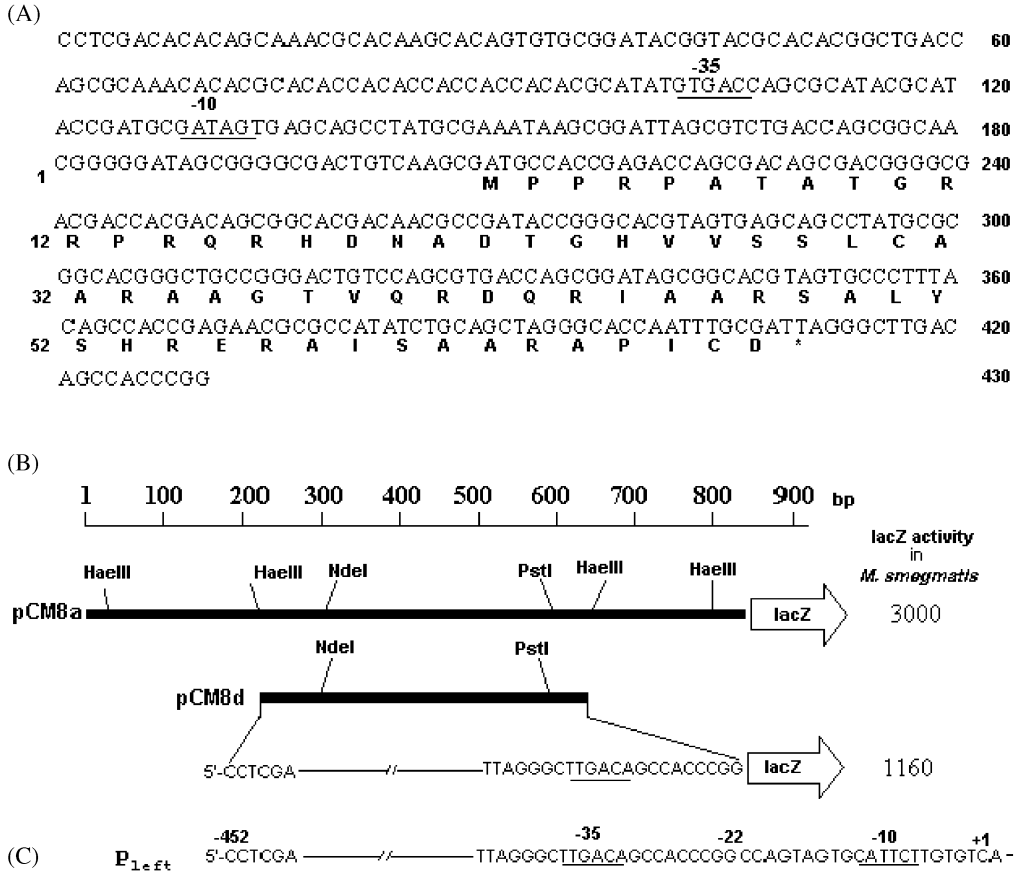


**Fig. 4.** Sequence analysis of the L1 DNA insert in pCM8d. (A) Single-strand DNA sequence of 430 bp L1 insert of pCM8d is shown. The −10 and −35 hexamers of the putative promoter *P4* are underlined. The 67 amino acid long putative protein is also shown below the DNA sequence. The stop codon is marked by asterisk (*). The numbers on the left and right sides indicate the numbers of the end amino acids and deoxynucleotides, respectively. (B) Maps of the L1 DNA inserts in both pCM8a and pCM8d (represented by solid bars) with several restriction enzymes are shown. A scale representing the size of the L1 insert is given at the top of the physical map. The β-galactosidase levels (lacZ) in *M. smegmatis*, carrying pCM8a and pCM8d, are presented separately. The single-strand sequence at the two ends of the L1 DNA in the pCM8d insert is presented. The end of the L1 DNA in pCM8d, which joins *lacZ*, carries the homologous −35 hexamer (underlined) of $P_{left}$ of the L5 phage. See the Results and Discussion section for details. (C) The single-strand DNA sequence at the two ends of the mycobacteriophage L5 DNA, encompassing 51673 to 52123 bp coordinates, is also shown for comparison. In fact, the region carries +2 to −452 regions of its early $P_{left}$ promoter. The transcription start site (+1) of $P_{left}$ is shown, and its −35 and −10 hexamers of $P_{left}$ are underlined (Nesbit *et al*., 1995).

the minimal region of the 2.3 kb insert in pLG3 that could show the promoter activity. To do this, the gel-purified 2.3 kb DNA insert of pLG3 was partially digested with *Sau*3AI. The resulting 0.3-1 Kb L1 DNA fragments were ligated with the *Bgl*II-digested pTAC3734, and ten clones, which appeared as blue colonies on LA-amp-X-gal, were selected. The β-galactosidase levels that were produced by these subclones revealed that only two retained identical promoter activity as that of pLP3 in *E.coli*. The sizes of the L1 DNA inserts in the latter two plasmids were about 0.85 and 1.2 kb, respectively (data not shown). The former clone was designated pCM8 (Table 1). Subcloning of the 850 bp *Bam*HI-*Xba*I fragment from pCM8 into pLG yielded pCM8a. The β-galactosidase levels that were produced by pCM8a and pLG3 in *M. smegmatis* were the same (Table 1).

To determine the possible location of the promoter within this 850 bp DNA fragment, an *in vitro* transcription assay was carried out according to the procedure mentioned in Methods. It was discovered that only a single transcript with an approximate size of 490 bp was produced from 850 bp DNA template (Fig. 3). This result suggests that transcription initiation occurs almost in the middle of the 850 bp insert. The 850 bp *Bam*HI-*Hind*III fragment of pCM8a was then subcloned into identical sites of pUC19 to obtain pCM8b. Using the latter plasmid, this 850 bp was sequenced, and a possible restriction map of the DNA segment was constructed by a free computer program (available at BCM Search Launcher) (Fig. 4). The two *Hae*III sites are located at the 212 and 641 bp coordinates of the 850 bp DNA. The region encompassing the 212 to 641 bp coordinates of the L1 insert

**Table 2.** Alignment and analysis of the $\sigma^{70}$ like promoter sequences in mycobacteriophages

| Phage/promoter | −35 hexamer | −10 hexamer | References |
|---|---|---|---|
| L5/$P_{left}$ | TTGACA | CATTCT | Nesbit *et al*., 1995. |
| L5/*P1* | TCCGCA | TATCCT | Nesbit *et al*., 1995. |
| L5/*P2* | TTGCTA | TACATT | Nesbit *et al*., 1995. |
| Bxb1/$P_{left}$ | TTGACA | CATACT | Jain and Hatfull, 2000. |
| Bxb1/$P_{right}$ | TTGATC | TAACCT | Jain and Hatfull, 2000. |
| Ms6/*P1* | CCGACC | TATCCT | Garcia *et al*., 2002. |
| Ms6/*P2* | CTGATG | TACACT | Garcia *et al*., 2002. |
| L1/*P4* | GTGACC | GATAGT | This work. |
| Consensus[a] | $T_{63}T_{75}G_{88}A_{75}C_{63}A_{50}$ | $T_{63}A_{100}T_{63}A_{50}C_{75}T_{100}$ | This work. |
| *E. coli* consensus | $T_{82}T_{84}G_{78}A_{65}C_{54}A_{45}$ | $T_{80}A_{95}T_{45}A_{60}A_{50}T_{96}$ | Lewin, B., 1990. |
| *M. smegmatis* consensus | | $T_{100}A_{93}T_{50}A_{57}A_{43}T_{71}$ | Bashyam *et al*., 1996. |
| *M. tuberculosis* consensus | | $T_{80}A_{90}C/T_{60}G_{40}A_{60}T_{100}$ | Bashyam *et al*., 1996. |

[a]To determine the consensus −35 and −10 hexamers of mycobacteriophage-specific promoters, the promoter sequences were aligned and the % frequency of occurrence of each base was calculated.

in pCM8a should carry the putative L1 promoter if the *in vitro* transcription data is correct. To test this possibility, the gel-purified 430 bp *Hae*III fragment of pCM8a was subcloned into the modified *Bgl*II site of pTAC3734. The resulting plasmid, designated pCM8c, yielded blue colonies with *E. coli* DH5α on a LA-X-gal plate.

Measurement of the β-galactosidase level in *E. coli* DH5α that carried pCM8c show that the promoter in the pCM8c clone is about 2.5-fold less active than that present in the pCM8 or pLP3 clone (Table 1). To test the activity of the promoter in the 430 bp DNA in *M. smegmatis*, a plasmid (designated pCM8d) was constructed by subcloning the 430 bp *Bam*HI-*Xba*I fragment of pCM8c between the same sites of pLG. It was discovered that the activity of the promoter in both pCM8d and pCM8c was nearly the same (Table 1). This result suggests that the reduction of the promoter activity in both pCM8c and pCM8d may be due to the deletion of an upstream region that positively regulates the activity. Alternatively, the 850 bp DNA fragment carries two convergent promoters, and one of them was deleted by the *Hae*III digestion.

**DNA Sequence analysis and identification of an *E. coli* $\sigma^{70}$ -like L1 promoter** To more precisely determine why the L1 promoter activity in pCM8d was reduced, the 430 bp DNA sequence, which is known to be a part of the earlier 850 bp DNA sequence, was analyzed by several computer programs. By analysis with the nucleotide-nucleotide BLAST program of NCBI, the DNA sequence of the 430 bp insert showed a 100% homology with the DNA segment that is bounded by the 51,694 to 52,123 bp coordinates of the L5 mycobacteriophage. This 430 bp DNA carries an open-reading frame corresponding to a protein that has 67 amino acid residues. This L5 region in fact corresponds to the –22 to –452 regions of its early promoter $P_{left}$ (Fig. 4C, Nesbit *et al*., 1995). The –22 equivalent end of the 430 bp L1 DNA was ligated to the upstream region of the *lacZ* reporter gene in pCM8d. It did not recreate the −10 hexamer of $P_{left,}$ whose homolog is present within the 850 bp L1 insert in pCM8, the mother clone of pCM8d (data not shown). In the mycobacterial system, the deletion of the −10 hexamer affects the promoter activity much more severely than does the deletion of the −35 hexamer (Bashyam *et al*., 1996; Kenney and Churchward, 1996). Therefore, these results suggest that an additional promoter is possibly present upstream of the $P_{left}$ equivalent in the 850 bp L1 DNA insert. This second promoter may be responsible for the relatively high lacZ expression in *M. smegmatis* carrying pCM8d. By a free promoter prediction program (available at BCM Search Launcher), we detected a putative *E. coli* $\sigma^{70}$ promoter like element, designated *P4*, between the 100 to 140 coordinates of the 430 bp insert of pCM8d. This supports our *in vitro* transcription data as the predicted promoter *P4* generates only 9 nucleotides longer mRNA than what we obtained experimentally (Fig. 3 versus Fig. 4). It is unclear why the 850 bp L1 DNA template yielded no 188 bp long $P_{left}$ homolog-specific mRNA. It is possible that it left the polyacrylamide gel at our condition of the electrophoresis of the *in vitro* transcription products.

The predicted −10 and −35 hexamers of the putative L1 promoter *P4* are 5'-GATAGT- and 5'-GTGACC-, respectively (Table 2). No conserved operator like sequence of L5 was found in and around this predicted promoter *P4* ( Brown *et al*., 1997). The immediate upstream region of the −10 element of *P4* also carries a *TGN* motif which is prevalent in bacterial promoters, including mycobacteria (Bashyam and Tyagi, 1998). However, by aligning and analyzing some of the best characterized mycobacteriophage promoters, along with the putative promoter *P4* of L1, we found that both the −10 and −35 elements of the mycobacteriophage promoters are quite

similar to the same elements of the *E. coli* $\sigma^{70}$ promoters (Table 2). The presence of base 'C' in the 5th position of the consensus −10 elements of the mycobacteriophage promoters may be due to the high GC content in the genomes of the mycobacteriophages. This type of GC bias was also observed in the −10 hexamers of the promoters of *M. tuberculosis* as well as other GC rich bacteria (Strohl, W. R., 1992; Bashyam *et al*., 1996). Finally, the expression from *P4* is probably not constitutive since the β-galactosidase expression in induced L1 lysogen carrying pLG3 was found to reduce gradually after 10 min (Fig. 2). Work is currently in progress to elucidate the biological role of *P4*.

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