Mutational Analysis of a C-dependent Late Promoter of Bacteriophage Mu

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ABSTRACT

Late transcription of bacteriophage Mu initiates at four promoters, P_{lys}, P_I, P_P and P_{mom}, and requires the Mu C protein and the host RNA polymerase. Promoter-containing DNA fragments extending ~200 bp upstream and downstream of the 5′ starts of the *lys*, *I* and *P* transcripts were cloned into a multicopy *lacZ*-expression plasmid. Promoter activity, assayed by β-galactosidase expression, was determined under two different conditions: (1) with C provided from a compatible plasmid in the absence of other Mu factors and (2) with C provided from an induced Mu prophage. β-galactosidase activities were greatest for P_{lys}, intermediate for P_I, and lowest for P_P. Similar analysis of plasmids containing nested sets of deletions removing 5′ or 3′ sequences of P_{lys} demonstrated that a 68-bp region was sufficient for full activity. Point mutations were generated within the 68-bp region by mutagenic oligonucleotide-directed PCR (Mod-PCR). Properties of the *lys* promoter mutants indicated that, in addition to the −10 region, a 19-bp region from −52 to −34 containing the C footprint is required for C-dependent promoter activity.

BACTERIOPHAGES have evolved complex regulatory mechanisms to ensure the ordered expression of phage genes and execution of developmental processes. For bacteriophage Mu, three phases of gene expression have been defined: early, middle and late (STODDARD and HOWE 1989; MARRS and HOWE 1990). Late gene expression is controlled by the Mu middle gene product C, which activates transcription by host RNA polymerase at four late promoters, P_{lys}, P_I, P_P and P_{mom} (HATTMAN *et al.* 1985; HEISIG and KAHMANN 1986; MARGOLIN and HOWE 1986; MARGOLIN, RAO and HOWE 1989; STODDARD and HOWE 1989; MARGOLIN and HOWE 1990).

Examination of DNA sequences upstream of the start sites of all four Mu late transcripts revealed a consensus sequence that resembles the consensus promoter for *Escherichia coli* RNA polymerase ($E\sigma^{70}$) in the −10 region but not in the −35 region (Figure 1; MARGOLIN, RAO, and HOWE 1989). Instead, the Mu late promoters contain a highly conserved 14-bp sequence upstream and overlapping the −35 region. In addition, a 10-bp sequence is repeated in P_{lys} and P_I and six contiguous T residues are present in P_{lys} and P_{mom}.

Previous studies of the mechanism of action of C protein demonstrated that C is not an alternate RNA polymerase, antiterminator or sigma factor (MARGOLIN and HOWE 1986; STODDARD and HOWE 1989; MARGOLIN and HOWE 1990). Furthermore, C binds to DNA sequences that overlap the upstream half of the Mu late consensus sequence in the *lys* and *mom* promoters (Figure 1; BÖLKER, WULCZYN and KAHMANN 1989). Therefore, the likely mechanism for C-dependent activation of late transcription is that C bound to the late promoters allows recognition and initiation by host RNA polymerase, either through protein-protein interactions or C-mediated changes in the DNA structure.

The goal of this study was to define the Mu late promoter sequences involved in transcription initiation. First, deletion mutations were made to define an approximate limit of DNA sequences important for the C-dependent activation of P_{lys}. Then Mod-PCR (CHIANG, KOVARI and HOWE 1993) was used to saturate the *lys* promoter with point mutations. The altered levels of activity of the promoter mutants defined the bases important to *lys* promoter function.

MATERIALS AND METHODS

Media, chemicals, enzymes and strains: LB agar, LB broth, SM and soft agar were described previously (HOWE 1973), as were TCMG plates (SCHUMM *et al.* 1980). MacConkey lactose plates contained 50 g of MacConkey agar (Difco) per liter and were supplemented with appropriate concentrations of isopropyl β-D-thiogalactopyranoside (IPTG; Sigma) from a 10 mg/ml stock prepared fresh in distilled H_20. Supplemented minimal medium for β-galactosidase assays contained M9 salts (MILLER 1972), 20% (vol/vol) LB broth, 0.2% glucose, 1 μg/ml thiamine, 40 μg/ml proline and all other amino acids at 20 μg/ml. Ampicillin (Ap), tetracycline (Tc) and chloramphenicol (Cm) (Sigma), were used as necessary at 40, 20 and 25 μg/ml, respectively. The indicator 5-bromo-4-chloro-3-indolyl-β-D-galactopyran-

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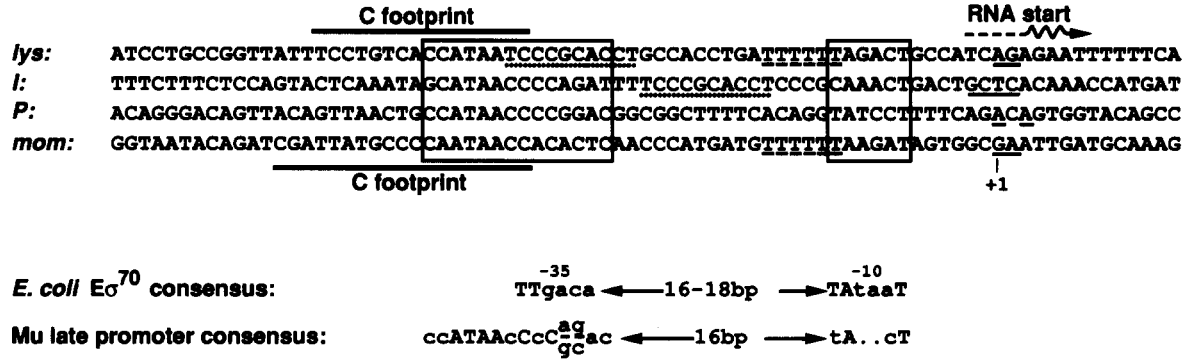


FIGURE 1.—Mu late promoter sequences. The Mu late promoters were aligned based on their similarity in the −10 and −35 regions (boxed) with respect to the transcription start sites (underlined) determined by S1 nuclease mapping (P_{lys}, P_I, P_P; MARGOLIN, RAO and HOWE 1989) and primer extension (P_{mom}; BÖLKER, WULCZYN and KAHMANN 1989). The position designated +1 was chosen to conform to the coordinates used by BÖLKER, WULCZYN and KAHMANN (1989) for P_{mom}. Heavy bars labeled "C footprint" indicate sequences in P_{lys} and P_{mom} protected by C in MPE·Fe(II) footprinting analysis (BÖLKER, WULCZYN and KAHMANN 1989). Dotted underlines show a 10-bp match between P_{lys} and P_I; dashed underlines show a 6-bp match between P_{lys} and P_{mom}. Shown below the alignment for comparison are the *E. coli* $E\sigma^{70}$ −10 and −35 and the Mu late promoter consensus sequences (MARGOLIN, RAO and HOWE 1989). Capital letters and lower-case letters indicate greater than 75% base conservation and less than or equal to 75% base conservation at the given position, respectively; dots indicate no apparent sequence conservation.

oside (Xgal; Sigma or Bachem) was added to LB agar from a 20 mg/ml stock solution in *N,N*-dimethylformamide to give a final concentration of 40 μg/ml.

Calf intestinal alkaline phosphatase was from Boehringer Mannheim Biochemicals. AmpliTaq polymerase was from Perkin Elmer Cetus. Klenow fragment, *Taq*I methylase, T4 DNA ligase, bovine serum albumin (10 mg/ml stocks) and restriction enzymes were from New England BioLabs. Exonuclease III and SI nuclease were from Promega Corporation. Enzymes were used as recommended by the supplier except where noted. SeaKem ME and NuSieve GTG agarose were from FMC Bioproducts; acrylamide and bis-acrylamide were from Bio-Rad. Deoxyadenosine 5′-(α-thio-) triphosphate, ^{35}S used for labeling sequence reactions was from NEN Research Products. β-Cyanoethyl phosphoramidites were from Applied Biosystems.

The bacterial strains used (Table 1) were derivatives of *E. coli* K-12. The Mu phage used was Mu *cts*62 (HOWE 1973). λpMu phages used were 423, 514, 724, 4M114–7, 4M121, and 4M145–5 (O'DAY *et al.* 1979).

Plasmid constructions and manipulations: Large-scale plasmid preparations for cloning were made essentially as described previously (MARRS and HOWE 1983). Plasmid minipreps used in screening clones were made according to the method of BIRNBOIM and DOLY (1979). Plasmid transformations were done by the RbCl-dimethyl sulfoxide method of KUSHNER (1978), or by $CaCl_2$ treatment (MANDEL and HIGA 1970). General cloning methods were as described by MANIATIS, FRITSCH and SAMBROOK 1982).

The promoter-expression plasmid pRS415 is a pBR322 derivative containing an *Eco*RI-*Sma*I-*Bam*HI polylinker upstream of the *lac* operon (SIMONS, HOUMAN and KLECKNER 1987); our isolate appears to contain five rather than four tandem repeats of the *rrn*B transcription terminator-containing sequence (Figure 2). Plasmid pLC1 is a *lacY* derivative of pRS415, generated by deletion of the *Sna*BI fragment in *lacY* (Figure 2).

Plasmid pLC3 is a $lacI^Q$ derivative of plasmid pWM13 (MARGOLIN, RAO and HOWE 1989) that expresses C under the control of the P_{lacUV5} promoter (Figure 2). The $lacI^Q$ gene was recovered from pMJR1560 (STARK 1987) by *Eco*RI + *Hin*dIII digestion and made blunt-ended by Klenow treatment before ligation into the *Taq*I methylase-insensitive *Hin*cII site of pWM13. The direction of transcription of the cloned insert, as determined by restriction analysis, is opposite to that of the P_{lacUV5} promoter.

TABLE 1

Bacterial strains

Strain	Genotype	Reference or derivation
MH429	F⁻ *thyA malT*::(Mu *cts*62)	GRUNDY and HOWE (1984)
MH3016	F⁻ *thyA malT*::(Mu *cts*62) (P1ʳ P2ʳ Muʳ)	GRUNDY and HOWE (1984)
MH6685	Hfr PO45 *thr-300 ilv-318 rpsE300 rel recA56 srlC300*::Tn*10* (P1 *clr100* Cm)	JC10240; CSONKA and CLARK (1980)
MH8501	F⁻ *gal lac rpsL*	WD5021; HOWE (1973)
MH8509	F⁻ *gal lac rpsL* (pRS415)	pRS415 transformant of MH8501
MH8565	F⁻ Δ*pro-lac trp*Δ*ED24*	MH290; Howe lab
MH8572	F⁻ Δ*pro-lac trp*Δ*ED24 recA srl*::Tn*10*	Rec⁻ Tet^R transductant of MH8565 by P1 from MH6685
MH8598	F⁻ *supE44 supF58 hsdR514 galK2 galT22 metB1 trpR55 lacY1*	LE392; MANIATIS, FRITSCH and SAMBROOK (1982)
MH8608	F⁻ Δ*pro-lac trp*Δ*ED24 recA srl*::Tn*10* (pLC2)	pLC2 transformant of MH8572
MH8624	F⁻ Δ*pro-lac trp*Δ*ED24 recA srl*::Tn*10* (pLC3)	pLC3 transformant of MH8572
MH8682	F⁻ Δ*pro-lac trp*Δ*ED24 recA srl*::Tn*10* (pLC2) (pLC13)	pLC13 transformant of MH8608
MH8726	F⁻ Δ*pro-lac trp*Δ*ED24 recA srl*::Tn*10* (Mu *cts*62)	Mu *cts*62 lysogen of MH8572

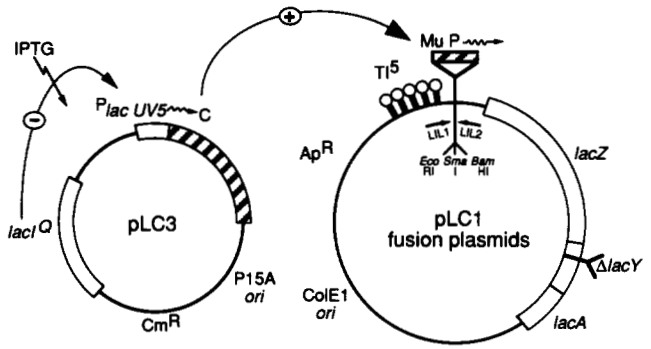


FIGURE 2.—The two plasmid Mu late promoter transactivation system. The approximate locations of relevant genes and sequences are shown on the plasmid maps: $T1^5$ refers to the tandemly repeated copies of the T1 terminator from the *rrn*B operon that reduce vector-specific transcription of *lacZ*. Wavy arrows indicate transcript origins and directions of synthesis. Primers LIL1 and LIL2 (short arrows) were used to sequence the cloned promoter fragments (Mu P). In the absence of IPTG, *lac* repressor made from $lacI^Q$ represses P_{lacUV5}-dependent transcription of the Mu *C* gene. Addition of IPTG leads to relief of repression and induction of C protein synthesis. The *lacY* deletion ensures diffusion-mediated, induction-independent transport of IPTG (NOVICK and WEINER 1957) and, consequently, IPTG concentration-dependent expression of C. Interaction of C with the Mu late promoter then leads to induction of β-galactosidase synthesis.

Plasmid pLC2 was constructed as a C^- control plasmid for pLC3 by cloning the same $lacI^Q$ cassette in the same orientation into the unique *Hin*cII site of the parent plasmid of pWM13, pNO2660 (BEDWELL and NOMURA 1986). The unique *Hin*dIII site in pLC2 allows cloning of any gene of interest to form an operon fusion with P_{lacUV5}. The $lacI^Q$ gene in pLC2 and pLC3 should supply *lac* repressor at a ratio of ~10 repressor molecules per P_{lacUV5}-promoter, thereby providing strong repression of the P_{lacUV5}-controlled gene in the absence of inducer and good induction in its presence (STARK 1987).

The general strategy for cloning Mu late promoters involved gel purification and ligation of a small promoter-containing DNA fragment to the linearized, gel-purified and phosphatase-treated *lacZ*-fusion vector, pLC1 (Figure 2). Ligation mixtures were transformed into MH8624; transformants were selected by Ap^R and checked by restriction digestion and sequence analysis. The 0.36-kb *Bgl*II-*Eco*RV P_{lys}-containing fragment from pKN50 and 0.41 kb *Mlu*I-*Xmn*I P_I-containing fragment from pKN35 (SCHUMANN *et al.* 1980) were made blunt-ended with Klenow polymerase and ligated into the *Sma*I site of pLC1 to form pLC10 (P_{lys}), and pLC11 and pLC12 (both orientations of P_I), respectively. The *Eco*RI-*Bam*HI promoter-containing fragments from pWM29 (P_{lys}) and pWM45 (P_P) (MARGOLIN, RAO and HOWE 1989) were ligated into *Eco*RI-*Bam*HI-digested pLC1 to form pLC27 and pLC29, respectively. For two other promoters that had been previously cloned into pRS415, clones isogenic to the pLC1 construct were made by deleting the *Sna*BI fragment on the pRS415-derived plasmids pWM55 (P_{lacUV5}) and pWM48 (P_P) (MARGOLIN, RAO and HOWE 1989) to form pLC13 and pLC15, respectively. Plasmid pLC13, containing a P_{lacUV5}-*lacZ* fusion, was constructed in MH8608 ($lacI^Q$) to avoid selection of mutations as a result of *lacZ* expression (SIMONS, HOUMAN and KLECKNER 1987) and then transformed into MH8624.

Plasmids pLC10Δ5 and pLC10Δ10 contain upstream deletions of P_{lys} generated by exonuclease III digestion of pLC10 linearized with *Eco*RI. Following SI-nuclease and Klenow-polymerase treatment to make the ends blunt, fragments containing the deletions were subcloned into pLC1 to restore the same context of vector sequences present in all of the promoter-fusions. Additional deletion and point mutations were made by cloning promoter DNA fragments synthesized by PCR amplification with primers whose sequences were chosen to generate the desired endpoints or mutations (described below).

All promoter-fusions isolated, regardless of the cloning steps involved, are present in pLC1 in the same context, *i.e.*, as if promoter sequences were directly cloned into the *Sma*I site (Figure 2).

Oligonucleotide synthesis: Oligomers were synthesized on an Applied Biosystems DNA synthesizer (Model 380B) using the phosphite triester method (MATTEUCCI and CARUTHERS 1981; BEAUCAGE and CARUTHERS 1981) and purified by ether extraction and ethanol precipitation (BAXA, CHIANG and HOWE 1992). Oligomers to be used as primers for PCR and DNA sequence analysis were designed to satisfy, as well as possible, the following criteria: 17–20 nucleotides (nt) homology to the template, a T_m of 52–56° (by an estimate of 2° for each A-T basepair and 4° for G-C), an overall G+C content of 40–50%, pyrimidine tracts ≤ 6 nt, G-tracts ≤ 4 nt, and purine tracts ≤ 4 nt. When PCR products were to be cloned, one-half *Sma*I-sites, *Eco*RI or *Bam*HI sites, and 2–4 extra 5′ nucleotides were designed into the primer 5′-ends to allow complete cutting and restoration of vector sequences at the cloning sites. Primer pairs for PCR had ≤ 2 bp complementarity at the 3′ ends to minimize primer-dimer formation. Specific primer sequences (CHIANG 1992) will be provided upon request.

Mutagenic primers for Mod-PCR mutagenesis were synthesized as described previously (CHIANG, KOVARI and HOWE 1993). Briefly, degeneracy was introduced to the positions targeted for mutagenesis by simultaneous delivery of equal volumes of nucleotide from two bottles, one with the wild-type (WT) nucleotide at 0.1 M and a second containing an equimolar mixture of all four nucleotides at a total phosphoramidite concentration of 0.025 M. Thus, the synthesis mixture contained 85% WT and 15% non-WT nucleotides, resulting in a misincorporation rate of 0.15 per nucleotide. The eight primers synthesized were as follows (a lower case letter indicates a position targeted for misincorporation): LIL8 (right-end WT), ACGGGATCCCCAATTCTCTGATGGCAGTCTA; LIL30 (left-end WT), ATGGAATTCCCGCCGGTTATTTCCTGTCAC; LIL31 (region I), ATGAATTCCCgcccggttattTCCTGTCACC; LIL32 (region II), CGGAATTCCCGCCGGTTATTtcctgtcaccatAATCC; LIL40 (region VI), ACGGGATCCCCaattctctgatgGCAGTCTA; LIL41 (region V), CGGGATCCCCAATTCTCTGATGgcagtctaaaaAATCAG;LIL42 (region III), GGTTATTT CCTGTCACCATaatcccgcacctGCCAC; and LIL43 (region IV), TCTCTGATGGCAGTCTAAAAaatcaggtggcAGGTG.

Primers synthesized for site-directed mutagenesis of specific bases between positions −54 and −29 in P_{lys} involved the same primer sequences used for Mod-PCR in regions I, II and III (LIL31, LIL32 and LIL42, respectively) except that only one position was mutagenized, by synthesis in the presence of one, two or three non-WT nucleotide precursors.

Polymerase chain reaction (PCR): A mini-prep procedure was used to isolate template DNA as total cellular DNA present 10–15 min before lysis following heat induction of the Mu *cts62* lysogen MH429 or MH3016 (BAXA, CHIANG and HOWE 1992). Then, 100 ng of the template was denatured in a 10-μl volume of 0.2 M NaOH for 15 min at room temperature, followed by 10-fold dilution into H_20; 1 ng of denatured template was used immediately in each amplification reaction.

PCR amplifications were carried out in 100-μl reactions containing *Taq* DNA polymerase reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl and 0.001% w/v gelatin), 1.5 mM $MgCl_2$, 200 μm of each dNTP, 1 μM each of the opposing primers, 2.5 U of AmpliTaq polymerase and 1 ng of denatured template. For Mod-PCR the $MgCl_2$ concentration varied from 1.5–10 mM, depending on the primer pair, to maximize for specificity and recovery of the amplified product. After being overlaid with 50 μl mineral oil, the reactions were subjected to 35 cycles of PCR: 1 min at 94° to denature, 30 sec at 45° to anneal and 2 min at 72° to extend. The amplification products were extracted once with 24:1 CH_3Cl:isoamyl alcohol to remove the mineral oil, and 5 μl were subjected to electrophoresis in a 4% 3:1 NuSieve:SeaKem agarose minigel to monitor the yield.

Recombinant PCR, involving two sequential PCR reactions, was used for mutagenesis of regions III and IV. In the first amplification, the opposing WT primer (LIL8 or LIL30) was used with the mutagenic primer (LIL42 for region III and LIL43 for region IV) to synthesize one end of the promoter fragment while incorporating the desired mutations. After gel purification in 4% NuSieve to remove excess primer and target DNA, 1 ng of product diluted from the molten agarose was added to the second PCR reaction along with LIL8 and LIL30 primers. In the second amplification, the entire promoter fragment was amplified because complementary base pairing between one strand of the short product and the missing-end primer generated 3′ ends for *Taq*-mediated DNA synthesis.

Cloning of PCR products: PCR products to be cloned were extracted once with an equal volume of phenol-CH_3Cl (saturated with Tris-HCl, pH 8.0) and ethanol precipitated. The pellet was resuspended in 10 μl of a modified *Eco*RI/*Bam*HI restriction buffer (0.15% Triton X-100, 100 mM NaCl, 50 mM Tris-HCl, pH 7.5, 10 mM β-mercaptoethanol and 5 mM $MgCl_2$) containing 5 U *Bam*HI, 2 U *Eco*RI and 0.1 mg/ml bovine serum albumin, and incubated for 2 hr at 37° or overnight at room temperature. The products were purified in a 4% NuSieve agarose gel, ligated directly in the melted NuSieve agarose to pLC1 DNA (prepared as described above) and transformed into MH8624.

Determination of plate phenotypes: MH8624 transformants with mutagenized plasmids were picked and patched on LB plates containing Cm and Ap. After 24 hr at 37° when patches were approximately 3 mm in diameter, these "master" plates were replica-plated onto MacConkey lactose plates containing 0, 10^{-4}, 10^{-3}, 10^{-2}, 0.1 and 1 mM IPTG. Patch color was scored relative to that of control strains after 12–15 hr incubation at 37°. Control strains contained pLC18 with the WT *lys* promoter, which generally induced (turned red) at 10^{-2} mM IPTG, and pLC13 with the three- to fourfold stronger P_{lacUV5} promoter, which induced at 10^{-3} mM IPTG.

Sequence analysis: Plasmid templates for sequencing were prepared by a modified HOLMES and QUIGLEY (1981) miniprep method involving lysis by boiling as described previously (BAXA, CHIANG and HOWE 1992). The minipreps were alkali denatured in 100 μl 0.2 M NaOH for 30 min at 37°, neutralized with 40 μl 3 M sodium acetate, pH 4.8, and ethanol precipitated before being subjected to standard US Biochemicals Sequenase sequencing methods with or without manganese buffer. The sequence was visualized by 6% PAGE in the presence of 8 M urea followed by autoradiography (MANIATIS, FRITSCH and SAMBROOK 1982). Promoter clones and deletion mutants were sequenced on both strands over the entire promoter-containing fragment using LIL1 and LIL2, primers homologous to sequences in the pLC1 vector (Figure 2); point mutants were sequenced on one strand using LIL1.

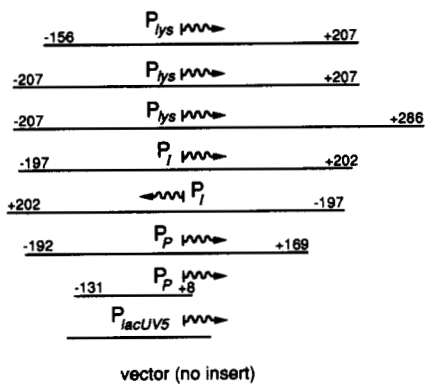
β-galactosidase assays: Strains grown overnight in LB broth with Cm and Ap were inoculated (0.02 ml) into 2 ml of supplemented minimal medium also containing Cm and Ap, grown with shaking at 37° to approximately 1×10^8 cfu/ml, and divided into 1-ml aliquots. One ml of the same medium containing IPTG was added to one aliquot to induce C production (final concentration, 2 mM IPTG). One ml of medium without IPTG was added to the remaining aliquot to serve as an uninduced control. Both aliquots were grown an additional 40–60 min with shaking at 37° followed by immersion into an ice-water bath for at least 20 min.

β-Galactosidase activity was assayed by the method of MILLER (1972) with the following modifications. Cell samples in 0.1-ml aliquots in microcentrifuge tubes were lysed by incubation on ice for 10–15 min following 10 sec of vigorous vortexing with 20 μl of 0.1% sodium dodecyl sulfate and 10 μl of CH_3Cl. Z buffer (MILLER 1972) containing 0.8 mg/ml *O*-nitrophenyl β-D-galactopyranoside (ONPG) substrate was made fresh and prechilled on ice; 0.5 ml were added to each cell sample on ice. Assay times of 20 min were started by immersing the chilled samples in a 28° water bath. The reactions were stopped by addition of 0.25-ml room temperature 2 M sodium carbonate. After 2-min centrifugation to pellet cell debris, the optical density was determined at a wavelength of 420 in a Gilford model 250 spectrophotometer. Miller units of activity were calculated as described (MILLER 1972) using optical densities determined within the linear response range of the assay. A single culture assayed 40 times in one day gave a range of 570–740 U with a standard deviation of 31 U (CHIANG 1992). Reproducibility was less between assays done on different days; however, essentially all activities on a given day were coordinately higher or lower. Therefore, to compare results from different days, we normalized the experimental activities to that of a control sample assayed in parallel (MH8624 containing pLC17 for the deletion mutants and pLC18 for the point mutants). Generally, normalized activities for the same mutant were within a twofold range.

In experiments in which C protein was provided by an induced Mu prophage, cells were grown at 32° with Ap (but not Cm) and induced by addition of 55° medium without IPTG. The cultures were then shaken at 42° for an additional 60 min (approximately 10 min past the expected time of cell lysis); uninduced control samples were taken at 0 min. The A_{600} values used for estimating Miller units of β-galactosidase activity were extrapolated, based on growth curves of isogenic heat-induced Mu lysogens, from each A_{600} measured at 35 min after induction to the expected maximum cell density just before lysis.

RESULTS

Design of promoter assay system: To facilitate alteration of the promoter sequences and determination of promoter activity by β-galactosidase expression, we cloned the Mu late promoters into a multicopy promoter-expression plasmid, pLC1 (Figure 2), a Δ*lacY* derivative of pRS415 (SIMONS, HOUMAN and KLECKNER 1987). Transcription terminators upstream of the promoter-cloning site in pLC1 ensured low basal β-galactosidase activity. The activator protein C needed for Mu late promoter activity was provided from the compatible plasmid pLC3 containing the *C* gene under the control of P_{lacUV5} and the $lacI^Q$ repressor (Figure 2). Strain MH8624 (F⁻Δ*pro-lac trp*Δ*ED24 recA srl*::Tn*10* pLC3) was used as the host, because (1) C induction by different concentrations of IPTG varied over a broad range; (2) the *recA* mutation allowed stable maintenance of both



		β-Galactosidase Activity			
		pLC3		Mu	
		-IPTG	+IPTG	0 min	60 min
P_{lys} -156 +207	pLC10	20	2520	61	1100
P_{lys} -207 +207	pLC17	14	1440	62	1000
P_{lys} -207 +286	pLC27	15	2560		
P_I -197 +202	pLC12	9	1820	26	570
P_I +202 -197	pLC11	69	65	99	56
P_P -192 +169	pLC15	45	770	92	430
P_P -131 +8	pLC29	58	660		
P_{lacUV5}	pLC13	66	7450		
vector (no insert)	pLC1	5	6	12	6

FIGURE 3.—C-Dependent β-galactosidase production by Mu late promoter-*lacZ* fusions. DNA fragments containing the *lys*, *I* and *P* promoters were cloned into pLC1; the fragment endpoints relative to the start of each transcript (+1) are indicated. Rightward and leftward arrows indicate the direction of transcription. β-galactosidase activities are presented for cultures with induction of C from pLC3 for 40 min (+IPTG) and without induction (−IPTG). Activities resulting from C provided by a heat-induced Mu lysogen are given for cultures before (0 min) and 60 min after (60 min) induction. Each activity shown is an average from at least two experiments and has been normalized to the average control pLC17 "WT" activity of 1440 U for pLC3 induction, and 1000 U for Mu induction.

plasmids; and (3) the relative copy number of the two plasmids (a ratio of 4:1, pLC1-derivative:pLC3) was constant under different states of induction (CHIANG 1992).

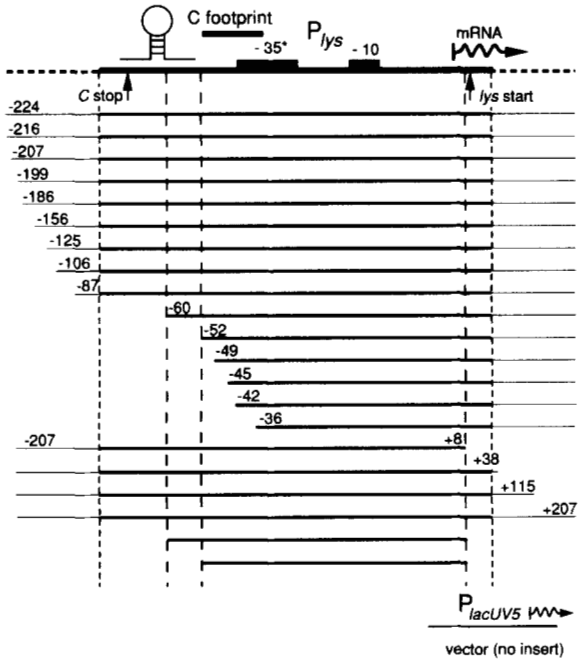
Comparison of Mu late promoter activities: DNA fragments containing at least 160 bp 5′ and 3′ of the late transcript start sites for P_{lys}, P_I and P_P were cloned into pLC1. The *mom* promoter was omitted because its regulation is affected by additional host factors (reviewed in BÖLKER and KAHMANN 1989; WULCZYN and KAHMANN 1991; HATTMAN *et al.* 1991). An *I* promoter fragment cloned in the opposite orientation was included as a negative control; it exhibited low C-independent β-galactosidase activity (69 U; pLC11, Figure 3) similar to that observed for many DNA fragments cloned into sensitive promoter-expression vectors (SIMONS, HOUMAN and KLECKNER 1987). In the absence of C (−IPTG), the Mu late promoters exhibited low β-galactosidase activity, even less than that of pLC11. In the presence of C provided by pLC3 (+IPTG), β-galactosidase levels increased to 770-2560 U with P_{lys} activity being the greatest, P_P the lowest and P_I intermediate (Figure 3).

These promoter-*lacZ* fusions were also assayed for promoter activity under conditions in which C was provided by heat induction of a Mu *cts* prophage. The Mu induction assays gave the same hierarchy of promoter strengths and roughly half the β-galactosidase levels obtained when C was provided from pLC3 (Figure 3). The *lys* promoter, which was the strongest in both assays, was chosen for mutational analysis.

Deletion mapping of the *lys* promoter: PCR (for review, see ERLICH 1989; INNIS *et al.* 1990) was used to generate nested sets of 5′ and 3′ deletions in the *lys* promoter by using the PCR primers to specify the deletion endpoints. Each amplified promoter fragment was then cloned into pLC1 using *Eco*RI and *Bam*HI restriction sites located at the 5′ ends of the primers. Transactivation assays with C provided from pLC3 were used to define the limit of sequences required for C-dependent activation (Figure 4). The deletions present in pLC10Δ5 (deleted to −60), pLC17Δ15 (deleted to −52), and pLC17Δ8 (deleted to +8) were the largest 5′ and 3′ deletions that still exhibited nearly full C-induced activity. When clones containing −60 to +8 (pLC18) and −52 to +8 (pLC20) were assayed, pLC18 gave nearly full activity which was twice that of pLC20. Therefore, we concluded that sequences −60 to +8, and possibly −52 to +8, were sufficient for promoter activity.

No additional phage factors (besides C) appeared to participate in *lys* promoter expression. The pattern of β-galactosidase expression for the deletions was essentially the same when C was provided from pLC3 or an induced Mu prophage (Figure 4). When C was provided by the phage, P_{lys} activity was approximately one-half that obtained when C was provided from pLC3. In addition, there was no detectable change in promoter activity when other phage factors were supplied by a set of λpMu phages (λ phages carrying various portions of Mu DNA; O'DAY *et al.* 1979) spotted on lawns of the deletion mutants on Xgal indicator plates (CHIANG 1992).

Strategy for saturation mutagenesis of the *lys* promoter: To define the specific bases required for C-dependent *lys* promoter activity, we proceeded to isolate point mutations in the −60 to +8 region present in pLC18 by using the strategy outlined in Figure 5. The 68-bp *lys* promoter was divided into six 10- to 12-bp regions for separate mutagenesis. The regions were chosen based on known or predicted features of the promoter: region I corresponded to sequences upstream of the C footprint; II contained most of the C footprint; III contained most of the −35 Mu late promoter consensus; IV corresponded to the region between −10 and −35; V encompassed the −10 region; and VI included the 5′ start of the transcript. For each region mutagenic oligonucleotide-directed PCR (Mod-PCR; CHIANG, KOVARI and HOWE 1993) was used to generate a population of *lys* promoter fragments with point mu-



β-Galactosidase Activity	pLC3		Mu	
	-IPTG	+IPTG	0 min	60 min
pLC24	17	1550		
pLC23	12	2060		
pLC17	14	1440	62	1000
pLC17Δ35	10	2000		
pLC17Δ12	13	2290	64	1130
pLC10	20	2520	61	1100
pLC17Δ9	15	2960	77	1530
pLC17Δ10	18	1870	78	910
pLC17Δ13	12	2190	87	1120
pLC10Δ5	15	1710	51	1100
pLC17Δ15	20	1030	52	1120
pLC17Δ16	11	310	36	160
pLC17Δ11	6	120	33	58
pLC17Δ29	13	15	43	31
pLC10Δ10	13	20	48	30
pLC17Δ8	10	1160	40	660
pLC17Δ6	6	1000	42	610
pLC17Δ5	8	1470	62	1130
pLC17	14	1440	62	1000
pLC18	6	1310		
pLC20	14	670		
pLC13	66	7450		
vector (no insert) pLC1	5	6	12	6

FIGURE 4.—Endpoints and β-galactosidase activities of the *lys* promoter deletion mutants. Relevant sequence features near P_{lys} are drawn approximately to scale (thick line); flanking sequence (dashed and thin lines) is represented at one-quarter scale. A stem-loop structure indicates the transcriptional terminator of the middle operon. The −10 region, −35 Mu consensus (−35*), and C footprint are indicated by bars. Small vertical arrows indicate a translational start or stop; a wavy arrow indicates the start of a transcript. The extent of Mu DNA present in each plasmid is represented by a line with the deletion endpoint coordinate given at its end. The columns to the right present the results of promoter activity assays which were performed and calculated as described in the legend to Figure 3.

tations confined to the target region. Each regional mutant population was then cloned into the *lac*Z-fusion vector and screened on indicator plates to assess the importance of that region to promoter activity. Further characterization by DNA sequence analysis and β-galactosidase assay was carried out for many mutants, regardless of phenotype, in phenotypically important regions; whereas, in less important regions, only mutants with altered phenotypes were studied.

Mod-PCR: A pool of oligonucleotide primers, containing an average of 1.5 random point mutations per primer within the region targeted for mutagenesis, was synthesized for each region. For regions I, II, V and VI, each such mutagenic primer (LIL31, LIL32, LIL41 and LIL40, respectively) was used in a separate PCR reaction with the WT primer of the opposing end (LIL8 or LIL30) to prime amplification of the entire 68-bp promoter. To avoid synthesis of a long mutagenic primer, we made the mutant populations for regions III and IV by recombinant PCR involving two sequential PCR reactions (see MATERIALS AND METHODS).

Previous analysis (CHIANG, KOVARI and HOWE 1993) demonstrated that Mod-PCR-generated mutations were randomly distributed within the targeted region, showing no bias toward insertion or replacement of specific bases, and that WT bases were accurately restored outside the targeted region. The recovery of mutants with multiple base substitutions was reduced, as expected, due to the requirement for primer hybridization to target DNA during PCR amplification. The resulting populations contained approximately 40% single mutants, making them suitable for phenotypic screening and mutant isolation.

To ensure that each population contained sufficient mutations, we determined the point mutant frequency for each region by sequencing a number of plasmid clones (Figure 6). For regions I, II, V and VI, the mutant frequency was high (55–66%); for regions III and IV (generated by recombinant PCR) it was only 20–25%. The high recovery of WT clones in regions III and IV can be explained by insufficient removal of WT template DNA prior to the second PCR; careful gel purification of the first PCR product in other recombinant PCR experiments reduced the recovery of WT clones. Due to the low mutant frequency and small number of clones scored (Figure 6), Region IV may not have been completely saturated with mutations.

Phenotypic properties of regions within P_{lys}: Transformants of MH8624 containing the Mod-PCR mutagenized promoter fragments cloned into pLC1 were picked at random and screened for their promoter phenotypes on MacConkey lactose plates containing different concentrations of IPTG. The phenotypic properties of the six mutant populations (Figure 6) indicated that region II, which includes the C footprint, had the greatest proportion of mutants with altered promoter activity, as reflected by 39% promoter-down mutants. Regions IV and VI were the least important with only 4% down mutants, and the others were intermediate with 12 to 20% down mutants.

Single point mutations in P_{lys}: Characterization of clones from the most important region, region II, included analysis of those with both WT and altered phenotypes. For the other regions, all clones with altered phenotypes were characterized, and a few with WT phenotypes were included to confirm that phenotypically silent changes were present.

Among 367 *lys* promoter mutants sequenced, 78 different single-base substitutions were obtained, with mutations in 44 of 68 positions. Promoter phenotypes

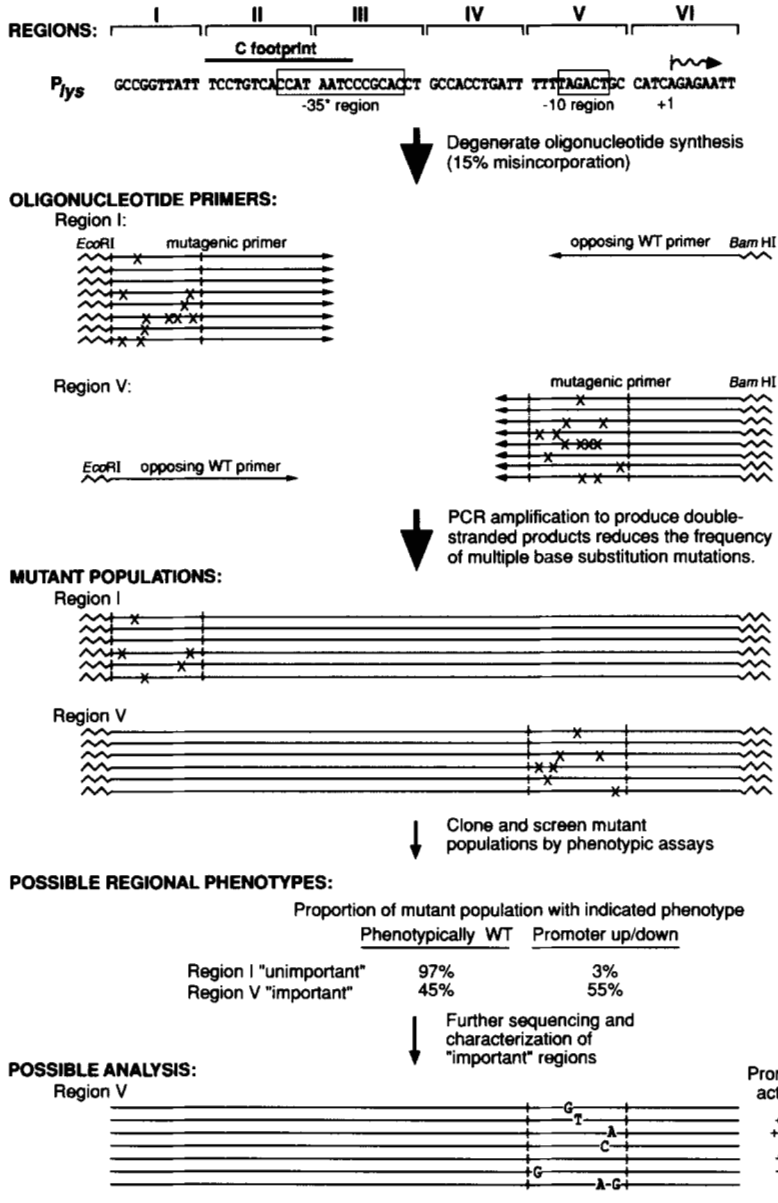
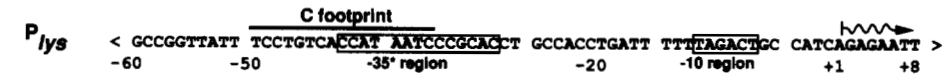


FIGURE 5.—Strategy for Mod-PCR mutagenesis of the *lys* promoter. The WT sequence of P_{lys} from −60 to +8 is shown, with breaks in the sequence delimiting the regions which were separately mutagenized with different mutagenic primers. The primers are drawn to correspond to the sequence; an X indicates a non-WT base incorporated into the primer during oligonucleotide synthesis; jagged lines indicate restriction sites on the 5′-end of each primer; an arrowhead designates the primer 3′-end.

were assigned based on β-galactosidase assays performed on liquid cultures of the mutants, with C provided by IPTG induction of pLC3 (Figure 7).

One group of promoter-down and noninducible mutations clustered in the region from −52 to −34, which includes the C-footprint and two adjacent 5′- and one adjacent 3′-bases (Figure 7); they probably delimit the extent of sequence information required for C binding. Surprisingly, no mutants with altered phenotypes were found in the region immediately downstream (−33 to −29), even though these five bases were originally included in the Mu late consensus (Figure 1; MARGOLIN, RAO and HOWE 1989). To construct specific desired mutations within and adjacent to the C-footprint, we isolated additional mutations by PCR with primers containing specific site-directed mutations (underlined in Figure 7). The phenotypic properties of all the single mutants are given in Figure 7; in addition, the fold increase or decrease in activity relative to WT for all the mutants in the extended C-footprint region (bases −54 to −29) is summarized in Figure 8. The results are consistent with positions −52 to −34 being required for C-dependent promoter activity; positions −33 to −29 are probably not required, although all possible base changes were not isolated. The location of these required sequences, just upstream of −35, is typical of proximal-site positive activators of σ^{70}-dependent promoters (COLLADO-VIDES, MAGASANIK and GRALLA 1991).

The only single mutant with constitutive β-galactosidase expression had a change at −41 from C to A, resulting in a canonical −10 TATAAT in this region. Upstream in the vector at the expected distance were sequences resembling the *E. coli* −35 consensus, suggesting that low level constitutive activity was due to creation of a new weak promoter (data not shown).



Region	I	II	III	IV	V	VI
Wildtype	73%	42%	88%	88% (59%)	78%	91%
Down	16%	39%	12%	4% (37%)	20%	4%
Up	11%	19%	1%	7% (4%)	2%	5%
Total Scored	106	256	200	91 (138)	200	199
Point Mutant Frequency	0.56	0.55	0.25	0.20	0.57	0.66

FIGURE 6.—Point mutant frequency and distribution of promoter phenotypes within regions of P_{lys} based on the indicator plate assay. Mutants that induced (turned red) at the same IPTG concentration as the WT control (pLC18) were scored as WT, those that required higher levels of IPTG to induce and those that never induced were scored as promoter-down mutants. Mutants that induced at lower IPTG concentrations were scored as promoter-up mutants. (Note: In subsequent β-galactosidase assays they exhibited wild type levels of activity.) Numbers for region IV represent a corrected distribution excluding mutants with a noninducible Lac⁻ phenotype; these mutants contained a 13-bp deletion arising by primer-dimer formation due to poor primer design (3-base complementarity at the 3′ ends of the LIL43 and LIL30 primers). Numbers in parentheses for region IV denote the original, uncorrected distribution. The point mutant frequency for each region was determined from the clones sequenced (84, 94, 47, 49, 83 and 17 total for regions I, II, III, IV, V and VI, respectively). Although the clones were chosen for sequencing based in part on their phenotypes, the overall point mutant frequency presented was calculated to reflect the rate for clones picked at random.

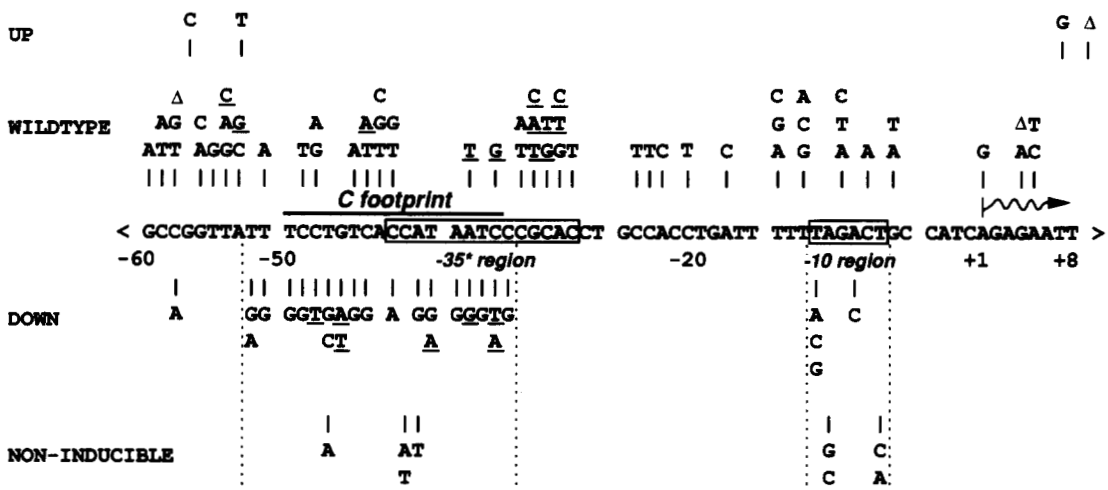


FIGURE 7.—Properties of single point mutations in the *lys* promoter. The *lys* promoter sequence and relevant features are indicated as in Figure 5. Nucleotides above and below the sequence indicate the specific base-changes isolated. Promoter activity for each mutant was determined at least twice by β-galactosidase assay after 50 min induction of C from pLC3; the percent WT activity was calculated based on the average activity of three WT (pLC18) control strains tested in the same experiment. Since 18 independently isolated WT clones exhibited a range of 50–160% WT activity (CHIANG 1992), only values outside this range were considered phenotypically mutant. Promoter-up (>230% pLC18 activity) and phenotypically WT (40–230% pLC18 activity) mutations appear above the sequence; promoter-down (<40% pLC18 activity) and noninducible mutations appear below the sequence. The majority of the mutations were isolated by random Mod-PCR mutagenesis; those isolated by site-directed mutagenesis are underlined.

Curiously, this mutant was not activated by C, suggesting that either the base change involved or RNA polymerase bound at the alternate promoter prevents C binding.

An additional cluster of promoter-down and noninducible mutations were located from −12 to −7 (Figure 7). These mutations reduced the match to the *E. coli* −10 consensus and exhibited the expected decrease in promoter activity.

One other promoter-down mutation, −58 C to A (Figure 7), reduced promoter activity to 27%; a small decrease in activity was also observed for pLC20 (analogous to pLC18 but deleted to −52; Figure 4), which also contains an A at position −58 due to vector sequences (GAATTCCC, −60 to −53). Since most of the base changes upstream of −52 did not significantly affect promoter activity, the mechanistic basis for this change is unclear, as is that of mutations −57 G to C and −53 A to T, which increased activity (to 248% and 235% WT, respectively). These bases may exert subtle effects on DNA conformation or the rate or strength of C binding. The only other promoter-up mutations, +7 T to G and +9 Δ (Figure 7), are located in the transcript and may affect promoter clearance or RNA stability.

DISCUSSION

The assay system used for this analysis of the Mu late promoters involved the determination of changes in β-

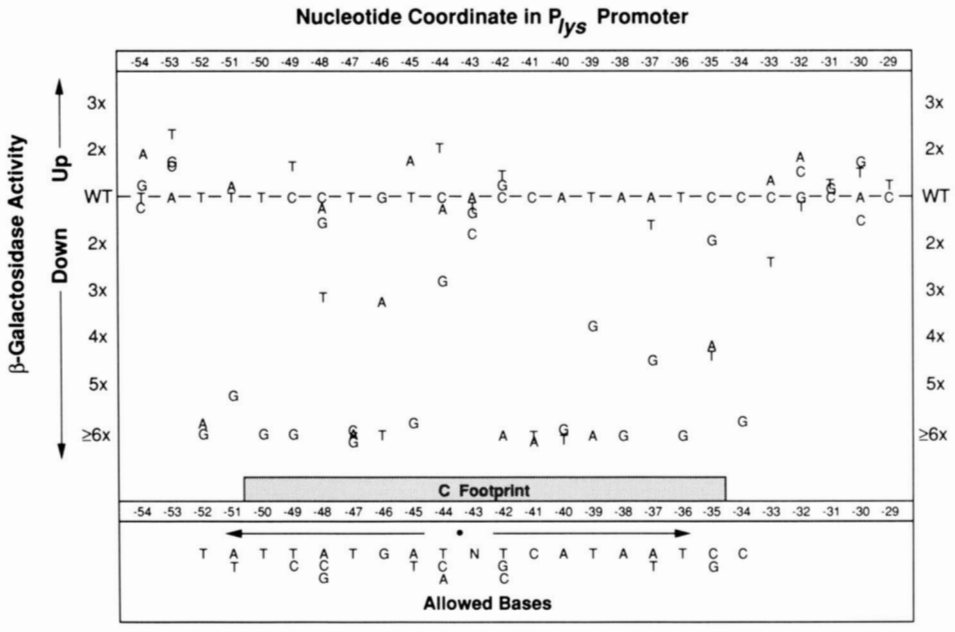


FIGURE 8.—β-galactosidase activity of single point mutants from −54 to −29 of P_{lys}. Nucleotides above and below the WT sequence indicate the specific base changes which are plotted along the y-ordinate to denote the fold increase (above WT) or decrease (below WT) in β-galactosidase activity relative to WT pLC18. Allowed bases are those within the important bases which exhibited >40% of WT activity. Horizontal arrows indicate a possible dyad symmetry element with the axis of symmetry at the dot.

galactosidase expression from promoter-*lacZ*-fusions present on multicopy plasmids. Theoretically, the β-galactosidase activity observed may be influenced by other factors such as copy number of the plasmid-borne fusions. Nevertheless, we believe that these results reflect differences in C-mediated promoter activity for the following reasons: (1) The relative copy number and stability of the two plasmids, pLC3 and the pLC1-derivative, were constant under different levels of induction and promoter activity (CHIANG 1992) as observed previously for pRS415 (SIMONS, HOUMAN and KLECKNER 1987). (2) The fusion-containing strains were grown in the absence of C and were assayed for promoter activity soon after induction; therefore, changes in plasmid copy number due to differential promoter activity should not be apparent within the time frame of the experiment. In addition, the intact *lac* operon terminator should prevent transcription from reaching the replication origin (SIMONS, HOUMAN and KLECKNER 1987). (3) All mutants were tested within the linear response range to C induction. (4) The effects observed were C-dependent and were exhibited by multiple deletion and point mutations in the regions identified. As with the results of any promoter-expression system, the conclusions must be verified by biochemical analysis and tests of their relevance *in situ*.

Mu late promoters: The Mu late promoters, as assayed by these *lacZ*-operon fusions, are relatively weak promoters; even the strongest promoter was three- to fourfold weaker than P_{lacUV5}. In addition, the promoters exhibit a hierarchy of promoter strengths with P_{lys} being the strongest, P_I intermediate and P_P the weakest (two- to fourfold weaker than P_{lys}).

Deletion mapping has now been carried out on three of the late promoters: P_{lys}, P_P and P_{mom}. In each case the results have identified a similar region, beginning approximately −60 to −45, as important to promoter activity. For P_{lys} the region −60 to +8 was sufficient for full C-dependent *lys* promoter activity; deletion to −52 resulted in a small drop in activity, and deletion to −49 and beyond severely reduced promoter function (Figure 4). For the P promoter a similar region (−59 to +4) was sufficient for full activity, and additional deletions to −45 had only a minor effect (CHIANG 1992). For P_{mom}, sequences to −51 sufficed for full C-regulated promoter activity (BÖLKER, WULCZYN and KAHMANN 1989). Since vector sequences adjacent to a deletion endpoint may influence promoter activity, more precise definition of the critical bases will require point mutant analysis as conducted for P_{lys}.

Lys promoter structure: Point mutant analysis identified an AT-rich 19-base region from −52 to −34, which encompasses the C footprint, as critical for C-dependent P_{lys} activity (Figure 7). Although dyad symmetry was previously detected within the C footprint of P_{mom} and P_{mod} (from Mu-like phage D108), such symmetry was not detectable in the WT sequence of P_{lys} (BÖLKER, WULCZYN and KAHMANN 1989). Substitution of certain allowed bases would increase the apparent dyad symmetry (Figure 8), raising the possibility that C might bind as a dimer. Several complementary changes within the halves of the dyad symmetry element caused similar qualitative changes in promoter activity (*e.g.*, −46T and −41A, −47C and −40G), lending support to this hypothesis. However, other complementary changes had substantially different effects (*e.g.*, −45G and −42C), calling into question the significance of the symmetry. Experiments defining whether C binds as a monomer or dimer should help to resolve this question.

Positions −35 and −34 are located in a region expected to be contacted by RNA polymerase; some mutations in these positions decreased promoter activity.

Since little or no basal activity was observed in the absence of C protein, and since the −35 sequence is so poor (CCCGCA in P_{lys} *vs. E. coli* −35 TTGACA), recognition of the −35 region by RNA polymerase may not occur in the absence of C. The absence of single-base changes in the −35 region that result in constitutive activity supports this hypothesis. In fact, one change, −35 C to T, which increased the similarity to an *E. coli* −35 consensus, significantly reduced C-induced promoter activity.

Single-base changes isolated in three conserved sequences (consensus positions −33 to −29, the 10-bp sequence found in P_{lys} and P_I, and the six T-residues found in P_{lys} and P_{mom}; Figure 1) had little effect on promoter activity, except where they overlapped the C-binding or −10 region. Their conservation may be due to random chance, evolution from a common ancestor or selective pressures unrelated to late transcription initiation.

Promoter-down and noninducible mutations outside the C-binding region were located in the −10 region from −12 to −7. These reduced the similarity to *E. coli* −10 consensus and would be expected to reduce promoter activity.

In summary, the properties of Mu *lys* promoter mutants suggest that the critical features for promoter activity include a recognition site for C binding, a −10 region that can be recognized by the host RNA polymerase, and specific bases in the −35 region different from those normally recognized by the host polymerase. These mutants should provide useful tools for future biochemical analyses to determine the molecular mechanism of C-activated transcription initiation.

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