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The −45 Region of the *Escherichia coli lac* Promoter: CAP-Dependent and CAP-Independent Transcription

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The lactose (*lac*) operon promoter is positively regulated by the catabolite gene activator-cyclic AMP complex (CAP) that binds to the DNA located 61.5 bp upstream of the transcription start site. Between the CAP binding site and the core promoter sequence is a 13-bp sequence (from −38 to −50 [the −45 region]). The possible roles of the −45 region in determining the CAP-independent level of *lac* expression and in the CAP activation process were studied by isolating and characterizing random multisite mutations. Only a small percentage of mutants have dramatic effects on *lac* promoter activity. Among the mutations that did affect expression, a 26-fold range in *lac* promoter activity in vivo was observed in the CAP-independent activity. The highest level of CAP-independent *lac* expression (13-fold the level of the wild-type *lac* promoter) correlated with changes in the −40 to −45 sequence and required an intact RNA polymerase α subunit for in vitro expression, as expected for an upstream DNA recognition element. Mutant promoters varied in their ability to be stimulated by CAP in vivo, with levels ranging from 2-fold to the wild-type level of 22-fold. Only a change of twofold in responsiveness to CAP could be attributed to direct DNA sequence effects. The −40 to −45 sequence-dependent enhancement of promoter activity and CAP stimulation of promoter activity did not act additively. The mutant promoters also displayed other characteristics, such as the activation of nascent promoter-like activities overlapping *lac* P1 and, in one case, replicon-dependent changes in promoter activity.

Transcription is a multistep process that begins with the binding of RNA polymerase (RNAP) to specific DNA sites termed promoters. A majority of *Escherichia coli* promoters (those recognized by the σ^{70} holoenzyme) exhibit a general structural pattern consisting of two conserved hexameric sequences separated by approximately 17 bp, termed the core promoter. The strength of RNAP-core promoter binding, often correlated with the level of transcription, is determined by a number of factors. The major factors include the match of the promoter hexamers and DNA spacer to the established consensus sequence (9), the presence of activator binding sites (1) and functional activator proteins, and the presence of an upstream DNA recognition element (called an UP element) in some strong promoters, such as *rrnB* P1 and *rrnB* P2 (25; for a review, see reference 23). The UP element is recognized by the carboxyl-terminal domain of the RNAP α subunit (α-CTD).

E. coli lac P1 is a well-studied σ^{70} promoter containing a relatively weak core promoter. In addition, the sequence immediately upstream of the core promoter (−38 to −50, hereinafter called the −45 region) is a poor match to the sequences of UP elements frequently associated with highly active promoters (25). *lac* P1 is highly regulated, being subject to both repression and activation. Repression is maintained by a functional *lacI* gene product, coincident with the absence of a suitable inducer. Activation is achieved through the catabolite gene activator protein when it is bound by cyclic AMP (cAMP). Regulation of the *lac* operon provides paradigms for both repression and activation of bacterial operons (24).

We were interested in studying the mechanism by which the catabolite gene activator protein-cAMP (CAP) stimulates *lac* expression. A great deal of information is available concerning the role of CAP at the *lac* operon, derived from biochemical and structural studies dating back 30 years (15). Nevertheless, the precise molecular mechanism by which CAP operates at the *lac* operon remains unclear.

Extensive evidence supports the proposal that DNA-bound CAP acts by contacting the RNAP α subunit, in particular, the α-CTD (2, 4, 16, 29, 31, 33). The RNAP α-CTD is essential for CAP-dependent transcription of the *lac* operon. Several RNAP α-CTD deletion studies have shown that α-CTD subunit deletions reconstitute into a functional RNAP holoenzyme and initiate transcription from some promoters; however, the mutant RNAP will no longer respond to activation by CAP at the *lac* promoter (11).

Some studies have suggested that the DNA between the CAP binding site and the core promoter may also play a role in CAP stimulation of *lac* expression. For instance, introduction of two to four base gaps in the region between −46 and −49 depressed CAP activation of *lac* transcription in vitro (26). In addition, footprinting and studies of laser-induced cross-linking indicated a close proximity of protein to the −45 region when ternary complexes of RNAP-CAP-*lac* DNA but not binary complexes of CAP-DNA or RNAP-DNA were analyzed (16). There are two hypothetical roles for the −45 region DNA in the CAP activation process. The −45 region sequence might provide sequence-specific contacts to RNAP that are facilitated by α-CTD–CAP contacts. In this case, the α-CTD–CAP interaction would stabilize RNAP binding to the *lac* DNA complex. This stabilization might compensate for the fact that the *lac* −45 sequence cannot on its own act as an UP element sequence. An alternative role is that the −45 region sequence might contribute no sequence-specific contacts for RNAP. In this case, the −45 region might contribute nonspecific RNAP contacts (e.g., by virtue of the phosphate backbone) or play a passive role by providing the necessary spacing and orientation. These hypotheses might be distinguished by analyzing the effects of mutations in the −45 region sequence. If there are sequence-specific contacts, some mutations should change ratios of CAP stimulation.

In an effort to investigate these possibilities, we have made

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TABLE 1. Strains used in this study

Strain	Genotype	Source or reference
CJ236	*dut ung thi relA* pCJ105	Bio-Rad Muta-gene phagemid in vitro mutagenesis kit
MV1190	Δ(*lac-proAB*) *thi supE* Δ(*srt-recA*)*306*::Tn*10* (F′ *traD36 proAB lacI*^{q}*Z* ΔM15)	Bio-Rad Muta-gene phagemid in vitro mutagenesis kit
RZ6525	CSH26 [Δ(*lac-proAB*)$_{X111}$ *ara thi*] *rpsL* Δ*cya* (F′ *lac* UV5 ΔM15)	This study
RZ201	F$^-$ Δ(*lac-proAB*)$_{X111}$ *ara thi rpsL*	13
RZ203	F$^-$ Δ(*lac-proAB*)$_{X111}$ *ara thi rpsL* Δ*cya*	13
ER1458	F$^-$ Δ(*lac*)*U169* Δ(*lon*) *araD139 rpsL supF mcrA mcrB1 hsdR2 trpC22*::Tn*10 serB28*	New England Biolabs
JM101	*supE thi* Δ(*lac-proAB*) (F′ *traD36 proAB*$^+$ *lacI*^{q}*Z* ΔM15)	17

randomized mutations of the DNA between positions −37 and −50 of the *lac* promoter. We report that certain DNA sequences in the −45 region can significantly affect the activity of the *lac* promoter both in the absence of CAP and, to a lesser extent, in its activation by CAP (when the *lac* promoter is activated by CAP). These studies, focusing on mutations that increase *lac* expression, complement those recently reported by Flatow et al. (6), which analyzed −45 region mutations that decrease *lac* expression. Their results suggested that only a small percentage of changes in the −45 region significantly reduce CAP stimulation of *lac* (6).

MATERIALS AND METHODS

Bacterial strains, phagemid, and phage. The bacterial strains used in this study are presented in Table 1. pUC119 was used as the starting phagemid for site-directed mutagenesis (18). λ*plac*5-T743 was used for recombination procedures to generate single-copy λ lysogens (14). RZ6525 was generated by conjugal transfer of the F′ *lac* UV5 ΔM15 factor into RZ203 (generated by Russell Karls).

Media. Glycerol-M63 minimal medium plates (containing 1× M63 minimal medium [19], 4 μg of B1/ml, 1 mM $MgSO_4$, 0.2% glycerol, and 3 μg of Bacto vitamin assay Casamino Acids/ml and supplemented with 40 μg of X-Gal [5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside]/ml, 1 mM IPTG [isopropyl-β-D-thiogalactopyranoside], and 100 μg of ampicillin/ml) were used for the screening of mutagenesis products. Luria broth (LB) medium was used for the growth of liquid cultures.

Generation of −45 region mutations. pUC119 was subjected to oligonucleotide site-directed mutagenesis according to the Bio-Rad Muta-gene phagemid in vitro mutagenesis kit protocol, except for single-stranded DNA isolation. Single-stranded uracil-containing pUC119 DNA was isolated from CJ236 cells by a CTAB isolation protocol (3).

All mutant plasmids, except for mutants UP, p.cons, and 424, were generated from one mutagenic oligonucleotide, dAGCATAAAGTGTAAA **NNNNNNNNNNNNN**ATGAGTGAGCTAA, where **N** denotes equal probabilities of insertion of all four bases. The UP mutant was created with the specific oligonucleotide dCATAAAGTGTAAA**GAGGAAATTTAAAA**ATGAGTGAGC TAA. The bases in boldface type reflect the alteration of the *lac* wild-type promoter sequence from positions −38 to −50 to match the *rmB* P1 DNA sequence of the same region. Both oligonucleotides were obtained from the University of Wisconsin Biotechnology Center. The specific oligonucleotide dAA AGTGTAAAGCCT**TTTC**TGCCTAATGAGTG was used to generate mutant 424. The bases in boldface type reflect the DNA sequence alterations of mutant 524 between positions −40 and −45. The 424 oligonucleotide was obtained from Research Genetics.

The p.cons mutant was generated with the specific oligonucleotide dAAAGT GTAAAGCCT**TATT**TGCCTAATGAGTG, obtained from Research Genetics. The sequence for the consensus mutant p.cons was generated from analysis of 11 mutants with increased CAP-independent activities as judged by β-galactosidase assays (see Table 2) (please note that mutant 424 was not included in the analysis). Each mutant was weighted on the basis of its fold increase in CAP-independent activity (versus the level of the wild type), and individual bases for each mutant were counted on the basis of this weight. The base generating the highest score was considered the consensus base. Preliminary β-galactosidase assay values were used in the calculation. For example, to calculate the consensus base for position −41 (with the current data in Table 2) mutant 519 would contribute 4.4 A's; mutant 520 would contribute 3.5 T's; mutant 504 would contribute 6.4 T's, and so on. An additional criterion used to select the consensus bases was a score of at least 50% in the above-described analysis. Only positions −40 to −45 scored 50% or greater. The particular importance of positions −40 to −45 in upstream DNA regions is consistent with results from work done on the *rmB* P1 UP element, including a hydroxyl-radical footprint demonstration of an α-dependent interaction (5, 21, 25).

Mutagenesis products were transformed into either MV1190 or RZ6525 and grown overnight on glycerol-M63 minimal medium plates containing X-Gal, IPTG, and ampicillin (see "Media") at 37°C for 1 to 2 days. Colonies were screened based on color compared to that of a wild-type control.

In the MV1190 (wild-type) background, colonies were selected randomly for color variety, from dark blue to white. Eighty-one colonies were randomly selected from approximately 1,500 total colonies and characterized. Thirty-four of these candidates contained changes only in the targeted region, i.e., from −37 to −50. Of the remaining candidates, 39 contained changes outside of the targeted region and 8 had the wild-type sequence. Twenty-five of the characterized mutants had an altered position −37. Due to a 10-fold inhibitory effect of altering this position (data not shown), these mutants and this position were removed from analysis. The remaining nine mutants were further characterized. One mutant from this analysis, mutant 10, is presented. The remaining eight do not exhibit increased CAP-independent β-galactosidase activity in vivo and have not been further analyzed.

In the RZ6525 (*cya*-defective) background, colonies were screened in a biased manner on the basis of a dark-blue phenotype. Ninety candidates, found to be a darker blue than wild-type colonies, were chosen from approximately 7,000 colonies. Twenty of these candidates, randomly chosen, were sequenced, resulting in 16 mutants containing mutations exclusively in the targeted region. The first nine, chosen randomly, were further characterized and are presented in this paper.

The UP, p.cons, and 424 mutants were isolated as blue transformants in MV1190. For each, approximately six blue colonies were selected and the DNAs were sequenced to confirm that the corresponding mutation had been incorporated.

Sequence analysis. Mutant plasmid DNA was isolated by the Promega Wizard miniprep protocol. The plasmid DNA was sequenced by standard dideoxynucleotide DNA sequencing procedures according to the United States Biochemical Sequenase version 2.0 protocol.

Recombination onto λ*plac*5-T743. The pUC119 plasmid mutations were crossed onto λ*plac*5-T743. λ*plac*5-T743 contains the entire *lacZ* gene and regulatory region; however, it is Lac$^-$ due to a −11G mutation in the promoter region. Recombinant phages were isolated as very-light-blue to dark-blue plaques on ER1458 cells (14). All recombinants subsequently studied maintained the pUC119 polylinker. The presence of the polylinker region in the *lacZ* gene reduces the overall β-galactosidase activity by 3.5-fold (data not shown). However, since all constructs studied contained the polylinker region, the presence of the polylinker should not have affected the relative levels of β-galactosidase expression.

Lysogen formation. RZ201 (*cya*$^+$) and RZ203 (Δ*cya*) strains were lysogenized with λ recombinant phages as described by Karls et al. (14). At least four lysogens were picked and tested for monolysogeny according to the procedure of Powell et al. (22). Recombinant λ monolysogens were sequenced by the Promega *fmol* DNA sequencing system protocol to confirm their DNA sequences and the presence of the pUC119 polylinker.

β-Galactosidase assays. β-Galactosidase assays were performed on monolysogens in both wild-type (*cya*$^+$) and CAP-defective (Δ*cya*) backgrounds (19). Lysogens were grown in LB containing 100 μg of streptomycin/ml and 1 mM IPTG at 32°C. All assays were carried out in duplicate with three to four isolates. β-Galactosidase values were normalized to wild-type values, and then values for two or four assays for each isolate were averaged. Standard deviations of the mean values were typically ≤20%.

RNA isolation. Plasmid-encoded or chromosomal mRNAs were isolated by the protocol described by Karls et al. (14) with the following modifications. RZ203 cells containing the mutant, pUC119-derived plasmids were grown in 4 ml of LB containing 100 μg of ampicillin/ml and 1 mM IPTG at 37°C to an optical density at 600 nm (OD_{600}) of 0.5 to 0.8. For the wild-type background (*cya*$^+$), 50 mM cAMP was added to early mid-log-phase (OD_{600}, ~0.3) subcultures and incubated for an additional 30 min at 37°C. For the chromosomal constructs, RZ201 or RZ203 λ lysogens were grown in 4 ml of LB containing 100 μg of streptomycin/ml and 1 mM IPTG at 32°C to an OD_{600} of 0.5 to 0.8. Dried mRNA pellets were stored at −70°C.

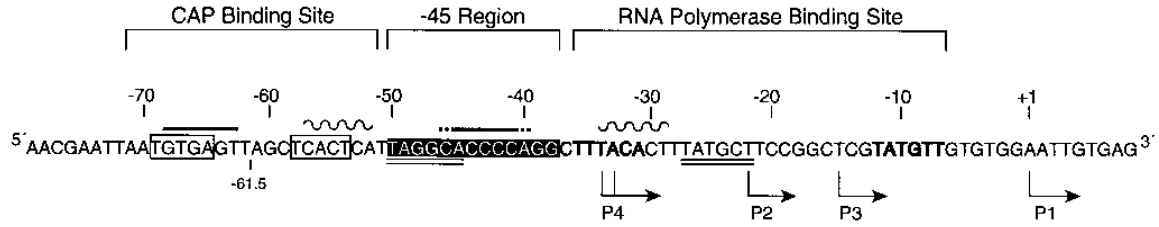

FIG. 1. Structure and DNA sequence of the *lac* promoter and start sites. The promoter region consists of the RNAP binding site, i.e., the core promoter, which includes the −35 and −10 hexamers (boldface letters), the −45 region (filled box), and the CAP binding sites (open boxes). −37C (lightly shaded box) is differentiated to denote that this position has been removed from the original definition of the −45 region. The +1 position corresponds to the P1 transcription start site. Transcription of P2 initiates at −22, transcription of P3 initiates at −15, and transcription of P4 initiates at −33 or −34. The −10 and −35 hexamers of P1, P2, P3, and P4 are marked by boldface letters, wavy lines, double lines, and boldface lines, respectively. The dashes at the ends of the P4 −10 hexamer denote alternative mutations which occur at different positions in these regions.

Primer extension analysis. RNA primer extensions were performed as described by Inoue and Cech (12) with the following exceptions. Two 5′-end-labeled oligonucleotides were used as primers in the primer extension analyses: 1212 primer (dGTTTTCCCAGTCACGAC), binding 40 bp upstream of the pUC119 polylinker region in the *lacZ′* gene, and Ap400 primer (dTCATTGG AAAACGTTCT), complementary to the β-lactamase mRNA. The 1212 primer was purchased from New England Biolabs (M13/pUC sequencing primer [position −40], catalog number 1212). The Ap400 primer was purchased from Research Genetics. Dried RNA pellets were resuspended in 1 pmol of Ap400 primer and/or 1 pmol of 1212 primer plus 10 μl of hybrid buffer. Hybridizations were carried out at 45°C for 15 min, and primer extension incubations were done at 45°C for 30 min in the presence of actinomycin D (100 μg/ml). Samples were run on 6% polyacrylamide–8 M urea sequencing gels. Gels were dried and exposed to X-ray film and Molecular Dynamics storage phosphor screens. Radioactive mRNA transcripts were quantitated with ImageQuant software and a Molecular Dynamics PhosphorImager. Quantitated data from three to four experiments were averaged, and mean values were normalized to wild-type values for each background. Standard deviations for mean values, with the exception of that for mutant 520 in the RZ203 background, were twofold or less. Mutant 520 in the RZ203 background had a standard deviation of 2.4-fold.

In vitro transcription reactions. DNA templates used for in vitro transcription were prepared by PCR to generate test templates (from −157 to +150 of the various mutants or wild-type pUC119) and a control template (from −157 to +200 of a pUC119 mutant bearing the UV5 promoter) serving as an internal standard. Single-round in vitro transcription reactions were performed in 30 mM Tris-HCl (pH 8.0) at 25°C, 10 mM $MgCl_2$, 100 mM KCl, 100 μM EDTA, 500 μg of bovine serum albumin per ml, 0.1 mM dithiothreitol, 0.2 mM cAMP, and 7% glycerol in a final volume of 20 μl. CAP (0.2 pmol) was added to the wild-type pUC119 control reaction mixtures. Each reaction mixture contained 5 nM test template DNA, 5 nM control template DNA, and 20 nM RNAP (wild-type or α-235). The reconstituted α-235 (deletion of amino acids 236 to 329 of the RNAP α subunit) and wild-type RNAPs were gifts from R. L. Gourse and M. S. Kainz. Reaction mixtures were preincubated with RNAP for 10 min at 37°C, and heparin was added to 40 μg/ml for 1 min at 37°C, followed by the addition of ribonucleotide triphosphates (200 μM GTP, ATP, and CTP and UTP at 5 μM plus 10 μCi of [α-^{32}P]UTP [3,000 Ci/mmol]). The reactions were stopped after 20 min at 37°C by placing the tubes on ice. An equal volume of 8 M urea–0.25% bromophenol blue–0.25% xylene cyanol–0.5× Tris-borate-EDTA was added, and samples were boiled (45 s to 1 min) and run on an 8% polyacrylamide–8 M urea gel.

RESULTS AND DISCUSSION

Construction of −45 region mutations. Recent studies have demonstrated that DNA sequences upstream of the core promoter can have profound effects on the transcription initiation process. For instance, the UP element is a 20-bp AT-rich sequence found upstream of the core *rrnB* P1 sequence that stimulates transcription initiation approximately 30-fold in vivo (25). Furthermore, evidence suggests that DNA upstream of the core promoter can affect CAP activation of the *lac* operon. The introduction of two to four base gaps in the region between −46 and −49 depresses the ability of CAP to activate *lac* transcription in vitro (26). To directly analyze the role of upstream DNA in both CAP-dependent and CAP-independent *lac* transcription initiation, we generated a collection of mutations in the −45 region of the *lac* promoter (Fig. 1) by oligonucleotide site-directed mutagenesis.

pUC119 was used as the starting phagemid in the site-directed mutagenesis protocol. For all of the mutants (except for UP, p.cons, and 424), the mutagenic oligonucleotide used contained a randomization of the −45 region. Phagemid double-stranded DNAs were synthesized and transformed. Mutant transformants were isolated on X-Gal–agar plates as described in Materials and Methods. Mutants with an altered position −37 were removed from the analysis due to a 10-fold inhibitory effect (data not shown). Presumably, modification of this position affects core promoter recognition (7, 10).

Approximately 1 to 2% of the total mutant population increased CAP-independent activity. Nine of these mutants are presented in this analysis. In addition, we generated three specific mutants. The UP mutant was created to contain the *rrnB* P1 promoter DNA sequence from positions −38 to −50. The p.cons mutant was generated by a weighted sequence analysis of 11 mutants listed in Table 2 (see below and Materials and Methods). Mutant 424 was designed to contain the DNA changes of mutant 524, which shows the highest CAP-independent activity, between −40 and −45.

To avoid complications due to the multicopy nature of the pUC119 phagemid and the requirement for α complementation in the β-galactosidase assays, the −45 region mutants were crossed onto a λ phage containing the entire *lacZ* gene. A UV5 derivative of pUC119 (mutations in the −10 hexamer to consensus TATAAT) was included as a CAP-independent control. β-Galactosidase assays were performed on single-copy λ lysogens in the presence and absence of CAP (strains RZ201 [*cya*$^+$] and RZ203 [Δ*cya*]). The β-galactosidase activities are given in Table 2. As will be discussed in more detail subsequently, the level of β-galactosidase activity was found to reflect the level of P1 transcript (Table 2), with the possible exception of that of mutant 512.

CAP-independent activity in vivo. All mutants presented in Table 2 demonstrate increased CAP-independent β-galactosidase activity in vivo. We isolated and characterized additional mutants from the initial mutant collection which did not exhibit this increase in CAP-independent β-galactosidase activity but rather had activities ranging from the wild-type level to 50% of the wild-type level. The latter type of mutant has been described in a recent communication by Flatow et al. (6). Our analysis is primarily restricted to mutants exhibiting increased CAP-independent β-galactosidase activity.

In the Δ*cya* background, RZ203, the activities of the −45 region mutants (including those of the additional mutants not presented) span a 26-fold range. In examining the β-galactosidase activities in comparison to the DNA sequence changes in the −45 region, an A/T preference between positions −40 and −45 emerges as a requirement for increased CAP-independent expression.

TABLE 2. Mutant DNA sequences, β-galactosidase activities, and quantitations of P1 mRNA transcripts

DNA	Sequence				β-Galactosidase activity in Miller units[c]			*lacZ* mRNA level[f]	
	−50	−45	−40	−37	*cya*$^+$	Δ*cya*	*cya*$^+$/Δ*cya*	*cya*$^+$[d]	Δ*cya*[e]
Wild type	t a g g c a c c c c a g g c				1.0[a] (236)[b]	1.0 (11)	22	1.0	1.0
UV5	t a g g c a c c c c a g g c				2.6 (620)	67 (718)	0.9	4.5	157
519	A G A · · T G A A A · A C ·				0.8 (184)	4.4 (53)	3	0.6	4.9
520	C G · T · G A T T T C C · ·				0.9 (212)	3.5 (37)	6	1.2	5.2
504	C G C A · G A A T A T A C ·				0.9 (217)	6.4 (68)	3	0.9	9.8
10	· · A T G T A T A A T C · ·				1.1 (258)	3.3 (32)	8	1.0	2.8
514	A T T T A T T T T T C T · ·				1.2 (296)	7.2 (77)	4	0.9	9.2
517	A T A · T G A A A A · · · ·				1.4 (326)	10.6 (117)	3	1.5	17.5
524	G C · · · · G A A A · · T ·				1.4 (342)	12.9 (139)	2	1.5	23.0
503	· T · A T · A A · T · · A ·				1.8 (491)	2.6 (32)	15	1.3	4.4
512	A T · C T · A A A A · T A ·				1.9 (458)	3.3 (35)	13	1.8	28.5
506	· · · A · · · T T T T C · ·				2.1 (507)	6.3 (76)	7	1.5	5.0
p.cons	· · · · · · · A A T A · · · ·				1.7 (438)	8.1 (89)	5	2.0	8.5
424	· · · · · · · G A A A · · · ·				1.8 (401)	11.5 (113)	4	ND[g]	ND
UP	· T T T A · A T T T C C T ·				2.2 (484)	12.8 (145)	3	1.8	21.9

[a] Values were normalized to wild-type values for each background prior to averaging.
[b] Average β-galactosidase activity.
[c] Standard deviations were typically ≤20%.
[d] RZ203 plus 50 mM cAMP was used for 5′ mRNA analyses (RZ201 for β-galactosidase assays).
[e] RZ203 was used for 5′ mRNA analyses and β-galactosidase assays.
[f] Standard deviations were ≤2.4-fold.
[g] ND, not determined.

The importance of the −40 to −45 sequence in effecting a high level of CAP-independent expression was examined by the generation of the p.cons and 424 mutants. The p.cons mutant sequence was derived, as explained in Materials and Methods, to represent a consensus for the entire −45 region for increased CAP-independent *lacZ* expression. The consensus sequence focused our interest on positions −40 to −45. Mutant p.cons yielded an eightfold increase in expression over that of the wild-type −45 sequence (Table 2). However, mutants 424, 517, 524, and UP all surpassed this effect, indicating that the p.cons DNA sequence is not the optimal sequence for the −45 region. Further data is needed before a −45 region consensus DNA sequence can be established.

Mutant 424, a derivative of mutant 524, was designed to test the particular importance of positions −40 to −45 relative to the other positions of the −45 region on CAP-independent *lacZ* expression. We expected that if only the composition of positions −40 to −45 was critical for high-level CAP-independent activity, then mutant 424 should exhibit the same activity as mutant 524. The minimal difference in β-galactosidase activities for these two mutants (Table 2) indicates that positions outside of −40 to −45, in particular, positions −50, −49, and/or −38, which differ between 524 and 424, are minor contributors to CAP-independent expression at *lac* P1. The DNA sequence of positions −40 to −45 has the major potential to yield increased CAP-independent *lacZ* expression.

The phenotypes of several mutants resemble that of the UP element described for the *rrnB* P1 promoter, in particular, the A/T richness of the −40 to −45 sequence. At the *lac* operon, the −45 region is located in a position analogous to that of half of an UP element. Thus, we generated a *lac* −45 region mutant (termed UP) with the downstream half of the *rrnB* P1 UP element sequence and examined its CAP-independent activation of *lacZ* expression (Table 2). As expected, the UP mutant enhanced *lacZ* expression substantially (13-fold). This observation is in agreement with the finding that the UP element is a separable transcriptional stimulatory element (25). This mutant offers approximately half the 30-fold stimulation of a complete *rrnB* P1 UP element, which would be expected for half of an UP element.

CAP-independent activity requires the RNA polymerase α-CTD in vitro. A hallmark of an UP element is a dependence on the α-CTD of RNAP for transcriptional stimulation (8, 25). We tested various mutants from Table 2, namely, 514, 517, 524 and UP, which show increased CAP-independent expression of β-galactosidase in vivo for their α-CTD dependence in single-round, in vitro transcription reactions as described in Materials and Methods. Each mutant was compared to the wild-type (CAP-stimulated) and UV5 controls.

Analysis of Fig. 2 shows a dramatic effect of the presence or absence of the α-CTD (an α-235 mutant RNAP was compared to wild-type RNAP) on the extent of transcription seen for the various mutants. In accordance with the in vivo data, the most significant stimulation is seen with mutants 524 and UP, while mutants 514 and 517 are more modestly stimulated. It appears that CAP-independent *lac* P1 expression can be stimulated to various extents by different sequences between the polymerase and CAP binding sites and that this stimulation is dependent on the α-CTD. Thus, the −45 region mutants described in this report behave as genuine UP elements.

CAP-dependent activity in vivo. Levels of CAP stimulation of our mutants (as manifested in the *cya*$^+$ host) demonstrate definite twofold differences resulting exclusively from changes in the −45 region sequence (Table 2, *cya*$^+$/Δ*cya* values). For example, comparison of two mutants with similar CAP-independent activities (520 at 3.5 and 512 at 3.3) reveals that CAP stimulation can vary for promoters with similar activities (520 at 6-fold and 512 at 13-fold). These modest differences in CAP activation are fairly prevalent among the mutants in Table 2 (compare 10, 519, and 520 with 503 and 512; also 504 and 514 with 506 and p.cons). The significance of this twofold effect of the −45 region on CAP-dependent activity is not clear. However, possible roles will be discussed below.

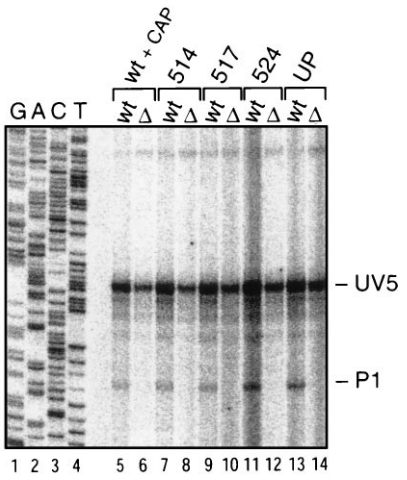

FIG. 2. Single-round in vitro transcription with wild-type RNAP and α-235 RNAP of the *lac* promoter. Several mutants were tested for their dependence on the α-CTD. Lanes 1 to 4 contain G, A, T, and C DNA sequencing markers. Five nanomolar DNA template (−157 to +150) and 5 nM UV5 internal standard (−157 to +200) were used. Each reaction mixture contained 20 nM wild-type (wt) or α-235 (△) RNAP, as indicated. The wild-type pUC119 control contained, in addition, 0.2 pmol of CAP. The UV5 internal standard (UV5) and the *lac* P1 transcript (P1) are marked.

CAP-dependent stimulation and UP element activity are not additive at *lac* P1. In analyzing the effect of the −45 region on CAP-dependent promoter activity, we were interested in whether CAP stimulation and UP element activity of transcription initiation can act additively. If the two phenomena act additively, then the wild-type 20-fold stimulation by CAP should be manifested in our mutants. Examination of the data in Table 2 demonstrates that CAP stimulation and UP element activation, in our mutants, do not appear to act additively. For instance, mutants with high levels of CAP-independent activity (more than sevenfold the level of the wild type) manifest sixfold or less CAP stimulation.

Currently we are considering three models to explain this apparent lack of additivity. (i) Both CAP and the UP element may affect the same kinetic step of transcription initiation. In this case, mutations that generate UP element-type activity will result in a mutant promoter with a new rate-limiting step unaffected by CAP activation. (ii) The region of the RNAP α-CTD which contacts CAP may be close to or overlap the region of the α-CTD which contacts UP element DNA. Thus, the two stimulatory mechanisms cannot function simultaneously. (iii) DNA sequences which positively affect UP elements are coincident with DNA sequences which have a negative impact on CAP-dependent stimulation of *lac* transcription.

Current published research supports the second model, i.e., that CAP and UP element stimulation cannot function simultaneously in our *lac* constructs due to the close proximity of the critical residues on the RNAP α-CTD required for either stimulatory component. The critical residues of the α-CTD required for UP element-dependent transcription and DNA binding have been localized to two regions of the α-CTD: amino acids 262 to 269 and 296 to 300 (8). Additionally, the α-CTD residues necessary for CAP-dependent transcription of class I promoters have been proposed. Zou et al. have reported that positions 265 to 270 of the α-CTD are important residues, with arginine at position 265 being the most critical (33). Tang et al., on the other hand, maintain that amino acids 258 to 265 are essential, with glutamic acid at position 261 being the most important (30). These analyses were extended in a recently published study of the α-CTD residues involved in the contact of the *rrnB* P1 UP element and CAP at the *lac* P1 promoter in vitro (20). These experiments demonstrated that most mutations in the α-CTD which affected CAP stimulation also influenced UP element activation (20). Thus, if the residues necessary for CAP activation and UP element usage overlap, both stimulatory elements cannot function concurrently at *lac* P1. Alternatively, concurrent activation would necessitate a very precise −45 region architecture that our mutants apparently do not possess.

It might be proposed that one α-CTD interacts with the half-UP element-like DNA sequences of some of our −45 region mutants and that the other α-CTD is available for CAP-dependent stimulation; however, due to the location of the CAP binding site, it is unlikely that sufficient space exists for both α-CTDs to function within our mutant constructs. It is important that although we favor the second model, the models proposed are not mutually exclusive. Further investigation is required to determine which components of these three models are responsible for the failure of CAP to stimulate mutants containing UP element-like sequences in the −45 region.

The failure of CAP and the upstream DNA to act additively at *lac* P1 appears to contradict previously described results. Savery et al. (27) reported that when an UP element was introduced into a class I CAP-activated promoter construct, the UP element increased CAP-activated transcription initiation but did not relieve CAP dependence. That is, CAP-independent activity was not increased by the UP element (27). We have observed an increase in transcription activation when CAP is not a player. However, these two results may not necessarily be in opposition, for the promoter system presented by Savery et al. is fundamentally different than that which we have investigated in this study. For instance, the construct studied by Savery et al. lacked a discernible −35 region and possessed an UP element containing two α-CTD contact sequences, and the CAP binding site was displaced to −71.5. When taking these differences into consideration, the results of Savery et al. (27) in part support our findings that CAP and the upstream DNA do not act additively, possibly due to insufficient space. When the CAP site is displaced upstream by an additional 10 bp and a full UP element is present, CAP and the UP element are able to work in conjunction (27). Thus, by providing the additional space needed for both α-CTDs, it is possible to observe an additive effect of CAP and an upstream UP element DNA.

In vivo analysis of *lac* transcripts. To determine a correlation of increased *lacZ* expression with changes in the expression of the *lac*P1 (+1 start site), we analyzed the 5′ end of the plasmid-encoded *lacZ′* mRNAs by primer extension methods. Representative primer extension analyses are presented in Fig. 3, and quantitation results are given in Table 2.

Examination of the wild-type and UV5 mRNA 5′ ends showed transcription initiation from the expected start site, i.e., P1 located at +1. Nevertheless, several mutants initiated transcription at additional start sites, discussed below. Upon quantitation of the primer extension products, compared to an internal β-lactamase standard, in vivo β-galactosidase activities appear to parallel that of the P1 transcript (Table 2), except for that of mutant 512. We expected this result, since previous studies have suggested that transcripts from start sites other than P1 would be inefficient at programming the synthesis of β-galactosidase. These transcripts (but not the P1 transcript) would contain a 37-base-long secondary structure that would occlude the *lacZ* Shine-Delgarno sequence (28).

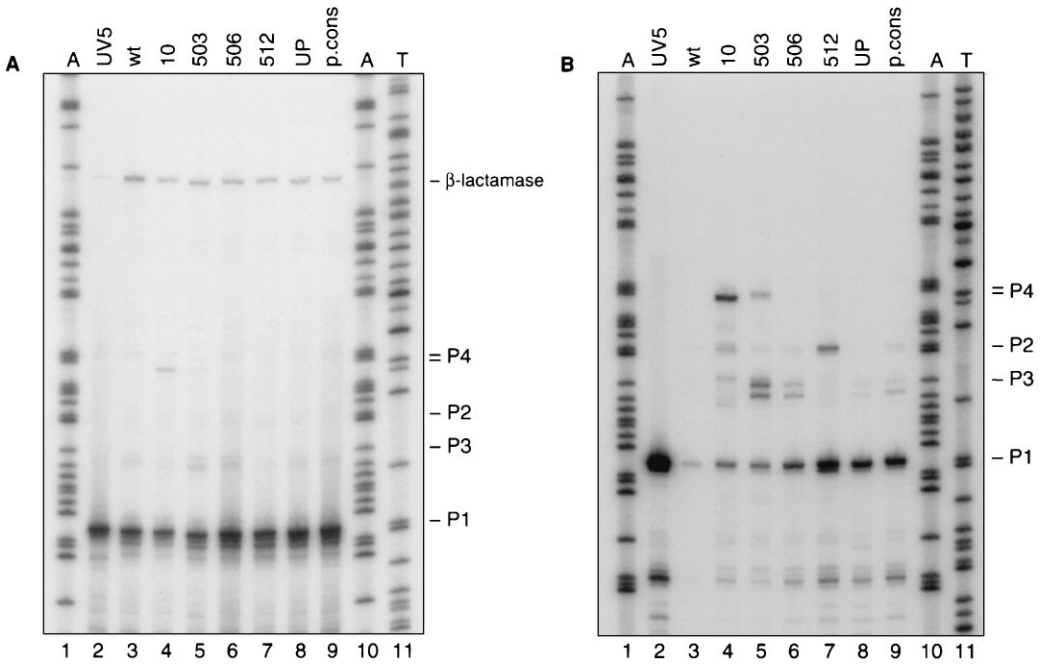

FIG. 3. Primer extension analysis of the *lac* promoter −45 region. In vivo plasmid-encoded mRNA transcripts were examined for start sites programmed by the *lac* −45 promoter mutations. Lanes 1, 10, and 11 contain A and T DNA sequencing markers. The four promoter start sites (P1, P2, P3, and P4) are marked. +1 corresponds to the P1 transcript start site. P2 initiates at −22, P3 initiates at −15, and P4 initiates at −33 or −34. (A) Reaction mixtures containing active CAP (*cya*$^+$). The internal β-lactamase standard is marked. (B) Reaction mixtures in the absence of CAP (Δ*cya*). The internal β-lactamase standard is not shown to emphasize the alternative promoter start sites present in the absence of CAP. All quantitations performed for Table 2 (*lacZ* mRNA levels) were normalized to the β-lactamase internal standard. wt, wild type.

Mutant 512 presents an interesting exception to the correlation between chromosomally programmed *lacZ* expression and plasmid-based P1 transcription. This mutant demonstrates greatly enhanced CAP-independent expression when located on a pUC-based replicon (as determined by primer extension analysis) (Fig. 3B, lane 7, and Table 2) relative to that seen for a chromosomal location (β-galactosidase activity) (Table 2). A possible model to explain this difference is that the run of nine A/T base pairs upstream of the −35 region in mutant 512 results in this promoter being activated by DNA topology found in plasmids but not on the chromosome. Additional evidence was provided by primer extension analyses performed on the chromosomal constructs. The results from the chromosome-based transcripts (data not shown) did not demonstrate a discordance of *lac* mRNA levels for mutant 512 relative to its β-galactosidase activity. Instead, localized sequence changes can have dramatic, replicon-dependent effects on transcription initiation frequencies. We note that mutant 514 also contains a run of A/T base pairs (12 from −52 to −41); however, this mutant does not exhibit the same effect on replicon dependence.

Previous reports indicated that alternative start sites for *lac* transcription initiation could be produced when the promoter region sequence was altered. These alternative start sites for the *lac* promoter include P2 at −22 and P3 at −15 (24, 32). In the absence of CAP (Δ*cya*), multiple start sites were, in fact, found for a majority of the mutants (Fig. 3B). Many mutants initiated at P2, as well as at P1. The −45 region is located in the spacer sequence between the −35 and −10 hexamers for P2 (Fig. 1). These results suggest that the DNA sequence composition of the −35 and −10 hexamer spacer region can affect promoter activity. Several mutants, namely, 503, 506, and p.cons, showed additional transcription initiation at P3. The P3 −35 hexamer is located at positions −45 to −50 (Fig. 1). Mutants 503 and 506 contain sequences in the −45 to −50 region which resemble the canonical −35 region TTGACA. p.cons, on the other hand, contains alterations only between −41 and −44, which fall in the spacer region between the −10 and −35 hexamers for P3. Two mutants, 10 and 503, also initiated at a novel start site, named P4. Alterations in positions −40 to −46 that create a strong P4 −10 hexamer show initiation at this previously undetected start site located at position −33 for mutant 10 and at position −34 for mutant 503 (Fig. 1). Mutant 10 has a perfect consensus −10 hexamer located between positions −40 and −45. Mutant 503 shows a −10 hexamer between −41 and −46 that is two bases from consensus. A suitable −35 hexamer, GTGAGT, is located 16 to 17 bp upstream.

In the presence of CAP, all the constructs initiated transcription at the preferred start site, P1 (Fig. 3A). No other start site was detectable, except that for mutant 10. Mutant 10, which contains a perfect consensus P4 −10 hexamer, also initiated at the new start site, P4. Our results confirm that CAP activation represses most alternative start site activities, as has been found previously (32).

Conclusions. A very small percentage of changes in the *lac* P1 −45 region DNA affects CAP-independent transcription and/or CAP-dependent transcription within our mutant constructs. The changes that affected CAP-independent transcription did so in a very specific fashion, behaving in a manner similar to that of an UP element, with AAA as the most frequent DNA sequence between −41 and −43 as opposed to the *rrnB* P1 UP element TTT.

Among the mutants we have analyzed, we have not detected an additive effect of CAP and upstream DNA. Our current hypothesis proposes that the α-CTD cannot simultaneously

provide stimulatory contacts with both CAP and the −45 region, at least for our mutants. It is possible that other −45 region mutants may have a unique structure that would allow both contacts simultaneously.

The actual role of the −45 region in CAP-dependent transcription remains elusive; however, we feel that the DNA most likely plays a sequence-nonspecific role in the stimulation of RNAP by CAP at *lac* P1, a role mainly in spacing and orientations of the DNA-bound CAP and RNAP and/or in sequence-independent contacts. Although the integrity of this region is critical (26), the precise DNA sequence can be changed dramatically without altering CAP stimulation significantly (reference 6 and our work where less than 2% of the randomized mutants showed phenotypic changes on plates). However, certain DNA sequence alterations in the −45 region demonstrated a twofold effect on CAP stimulation.

An interesting, unexpected result from this study of the *lac* promoter −45 region was the emergence of a novel promoter start site, P4, and the increase in activity of existing alternative start sites, P2 and P3 (32), for several mutants. By altering the DNA sequence of the −45 region, we unsuspectingly altered the activities of various *lac* promoter start sites. In retrospect, the increase in alternative promoter activities within the *lac* promoter region should be expected when altering the sequence composition. It is typical for a promoter region to contain several overlapping nascent promoters. Thus, it is likely that sequence changes would affect these otherwise silent promoters.

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