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lac Up-Promoter Mutants with Increased Homology to the Consensus Promoter Sequence†

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Four *lac* promoter mutants were constructed. The mutations increased the homology between the *lac* promoter and the consensus promoter sequences by introducing the consensus −10 and −35 regions and the consensus spacing of 17 residues between these two regions. The promoter mutants were cloned into a pBR322-derivatized vector upstream from the *lacZ* gene, and levels of β-galactosidase were an indication of promoter activity. All mutants exhibited higher activity than did the wild-type promoter.

There are several determinants of the promoter activity in *Escherichia coli*. The roles of the −10 and −35 regions and spacing between them have been well documented, and the consensus sequences for these regions have been proposed as follows: TATAAT for the −10 region and TTGACA for the −35 region, with a spacer of 17 base pairs (bp). It has also been noticed that promoter mutations which increase the homology between the two regions and the consensus sequence generally lead to an increase of activity of the mutant promoter (4, 15, 17). To elaborate on this observation, we introduced in this study the consensus −10 and −35 regions and consensus spacing into the *lac* promoter and evaluated the effects of these mutations on promoter activity in vivo.

The mutant promoter fragments were generated through enzymatic assembly of several synthetic oligonucleotides (Fig. 1). The mutations introduced were as follows: (i) T → A and G → A at positions −8 and −9, respectively, creating the *UV5* mutation and a consensus −10 region; (ii) deletion of 1 bp at position −23 (*D* mutation), generating a consensus length spacer; and (iii) T → G at position −34 (*G34* mutation), giving rise to a consensus −35 region. The *UV5* mutation was originally isolated as a reversion of the *L8* mutation that renders the *lac* promoter insensitive to catabolite activation (18) and was recently separated from *L8* and investigated in vivo (1). It was introduced into the set of mutants constructed to compare its activity in vivo with other mutants in a systematic way.

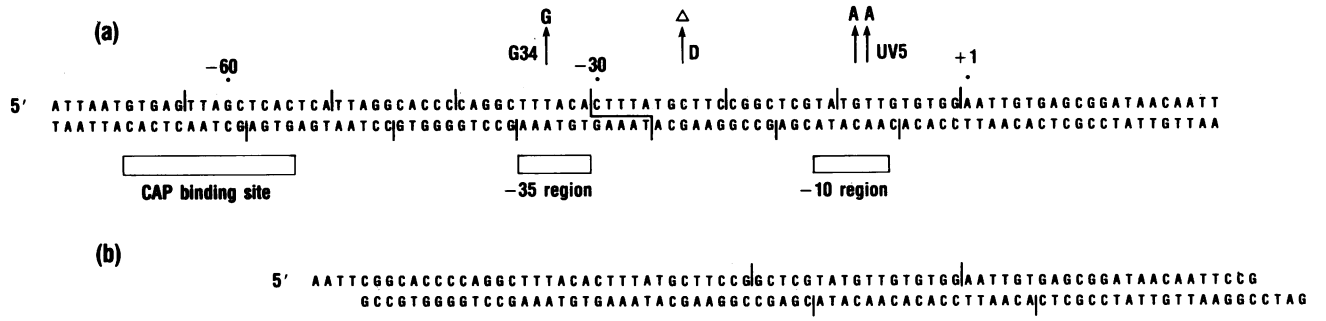

FIG. 1. Sequences of mutant *lac* promoters. DNA sequences of constructed synthetic promoter fragments are given. Oligonucleotides used to assemble the fragments are indicated by vertical bars. Two assembly patterns were used, i.e., for the wild-type, *UV5*, *D*, and *UV5-D* promoters (a) and for *UV5-D-G34* (b). A heavy line in the middle of panel a indicates halves of the promoter fragment assembled independently and subsequently ligated together. The phosphoramidite method (11, 12) was used in synthesis of oligonucleotides. Protocols for annealing of oligonucleotides, kinations, and ligations were as previously described (7). All cloned promoter fragments were sequenced by using the primer extension method (19) or the chemical cleavage method (13). Arrows indicate positions of mutations and residues introduced or deleted.

The following mutant promoter fragments were assembled: *UV5*, *D*, *UV5-D* double mutant, and *UV5-D-G34* triple mutant, as well as the wild-type *lac* promoter (Fig. 1). Since the *UV5-D-G35* mutant was constructed after the completion of work with other mutants, we used a different strategy to obtain the fragment, i.e., we did not include the catabolite

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TABLE 1. Activity of mutant *lac* promoters in vivo[a]

Plasmid	Promoter	β-Galactosidase activity (U) in[b]:					
		CSH26			X7940 (*cya*⁻)		
		Glucose	Glycerol	Stimulation	−cAMP	+cAMP	Stimulation
pWM29	*lacP* (wild type)	130	260	2.0	30	380	13
pWM32	*lacP-D*	240	480	2.0	50	620	12
pWM41	*lacP-UV5*	500	790	1.6	ND	ND	ND
pWM25	*lacP-UV5-D*	640	740	1.16	830	860	1.03
pWM142	*lacP-UV5-D-G34*	490	ND	ND	ND	ND	ND
pWM26	λp_r	860	ND	ND	ND	ND	ND
pRZ5202	No promoter	6	6	1.16	20	15	0.79

[a] β-Galactosidase assays were performed as described before (7, 10, 14). The λp_r promoter used in this study is a 67-bp synthetic fragment (3). Genotypes of the strains used were as follows: CSH26, F⁻ *ara* Δ(*lac pro*) *thi*; X7940, F⁻ Δ(*pro lac*) cya_{02} *metA* (courtesy of J. Beckwith).

[b] Units of β-galactosidase activity are defined by Miller (14). Abbreviations: −cAMP, without cyclic AMP; +cAMP, with cyclic AMP; ND, no data available.

gene activator protein (CAP) binding site into the fragment, since we knew that the *UV5-D* mutant does not respond to catabolite activation (Table 1).

The mutant promoter fragments were cloned into the *Sma*I site of plasmid pRZ5202 (7, 10) or, in case of the *UV5-D-G34* promoter, between the *Eco*RI and *Bam*HI sites of the same plasmid. The three sites constitute a multiple cloning site (16). Plasmid pRZ5202 carries the *lacZ* gene about 1,500 bp downstream from the multiple cloning site. Cloning of a promoter into that site renders the *lacZ* gene under the control of this promoter, and the β-galactosidase level is an indication of the promoter activity.

To evaluate the response of the mutant promoters, we compared the β-galactosidase expression in the cells grown under various physiological conditions that led to different cyclic AMP concentrations in the cell, which results in different levels of catabolite repression. We measured expression in the cells grown in medium supplemented with either glucose (partial repression) or glycerol (derepressed conditions). In addition, we assayed the promoter activity in the adenyl cyclase-deficient strain (*cya*⁻). Since *cya*⁻ cells do not produce cyclic AMP, one observed full catabolite repression in those cells. Repression can be relieved by supplying externally cyclic AMP.

The results of the assay are presented in Table 1. The comparison of β-galactosidase levels for the mutant promoters indicates that under derepressed conditions (glycerol; strain CSH26), the in vivo activity of the *lacP-D* and *lacP-UV5-D* promoters is 180 and 280%, respectively, of the wild-type *lac* promoter expression. This demonstrates that mutations which increase the homology between the *lac* promoter and the consensus promoter sequence can increase the promoter activity. Introduction of the *G34* mutation to the *UV5-D* background, however, decreased the promoter activity by 25%. Evidently, not every change towards the consensus promoter sequence results in an increase of promoter activity. A similar observation was made recently for the *lpp* promoter (6). An *lpp* promoter mutant with the consensus −10 and −35 regions (but 18-bp-long spacer) was twofold less active than mutants in which only one of the two regions had the consensus sequence.

The stimulatory effects of CAP on the wild-type and the *lacP-D* promoters are the same. For either promoter, the expression in the medium supplemented with glycerol is about twofold higher than on the medium with glucose (CSH26 strain), and cyclic AMP stimulation in a *cya*⁻ strain (X7940) is about 12- to 14-fold. The *UV5-D* promoter is different. Its activity is the same regardless of whether the medium is supplemented with glucose or glycerol (Table 1), and it is insensitive to stimulation by cyclic AMP. This characteristic of the *UV5-D* mutant can be explained in two ways. (i) *UV5* mutations in conjunction with the deletion render unnecessary the change in RNA polymerase or promoter structure or both, which is normally obtained as a result of the CAP action. (ii) CAP exerts the same structural changes as in the wild type; however, the unstimulated expression is so high (about three times more than the full wild-type expression) that it is limited by an interaction step which is not connected with the CAP action. The results presented in this paper are insufficient to distinguish between the two alternatives.

The CAP dependence observed appears to be, in all cases, less than what has been reported in the literature (up to 50-fold stimulation when the *lac* promoter is on the chromosome [5]). It seems likely that the system has been perturbed by the multicopy vector used for assay. Possibly, the CAP itself has been partially titrated; therefore, full stimulation by CAP cannot be realized. The use of a multicopy plasmid might also affect the absolute values of the β-galactosidase activity measured because of some uncertainity as to the plasmid copy number. Nevertheless, we expect that the promoter activities measured are at least qualitatively correct. Since the main conclusions of this paper are of qualitative type, they should not be affected.

The results show that the pattern of stimulation by CAP is not effected by the 1-bp spacer deletion at position −23. It was found previously that a 2-bp insertion into the spacer does not change the stimulation of expression by CAP (10). It seems, therefore, that the length of the spacer between the −35 and −10 regions is not a factor in the activation of the promoter by CAP, as was previously suggested (20). On the other hand, it was shown that the spacer between the CAP binding site and −35 region length is critical for stimulation by CAP (deletions or insertions decreased or abolished the stimulatory effect [7]). Earlier results showed that mutations which reduce the effect of catabolite activation on the *lac* promoter (*L305* and *L241* [5]) lay in the −35 region. This seems to indicate the crucial role of the −35 region and of its spacial alignment with the CAP binding site for the activation of the *lac* promoter by CAP.

The constructed promoters can be used to express genes in *E. coli* as alternatives to strong promoters, such as λp_r, *tac*

(2), or *trp*. One of them, *UV5-D*, was recently applied to obtain high levels of expression of the human complement factor C5a (8, 9).

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