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Catabolite-Insensitive Revertants of *Lac* Promoter Mutants*

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Abstract. The maximum rate of expression of the *lac* operon is severely reduced in *lac* promoter mutants. Revertants of these mutations which produce higher levels of enzyme were isolated. Some of these revertants had lost sensitivity to catabolite repression and transient repression. The mutations responsible for these losses took place at sites very close to the original promoter mutations. From these results we conclude that the promoter itself is the target site for both catabolite and transient repression of the *lac* operon.

We have previously reported that the target site for catabolite repression of the *lac* operon is in the promoter region (Fig. 1).[1] It was shown that partial

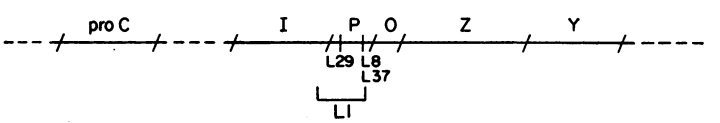

FIG. 1.—Genetic map of the *lac* region.[2] *Pro C*, gene essential for proline biosynthesis; *I, P, O, Z, Y: lac* repressor, promoter, operator, structural gene for β-D-galactosidase, and structural gene for β-D-galactoside permease, respectively; *L29, L8, L37*: *lac* promoter point mutations; L8 and L37 do not recombine with each other; L29 recombines with both. *L1*; *lac* promoter deletion also deleting part of *lac I* gene.

deletion of the promoter region (*L1*, Fig. 1) or fusion of the *lac* operon to the *trp* operon renders β-D-galactosidase synthesis insensitive to catabolite repression.[1]

In general, enzymes sensitive to *catabolite repression*[3,4] are also sensitive to the *transient repression* that ensues when glucose or any one of a number of other compounds is added to cultures exponentially growing on another carbon source such as glycerol.[5] A mutation mapping between *pyr C* and *pur B* confers insensitivity to catabolite repression caused by glucose but not to transient repression.[3] Conversely, a strain with a mutation in the phosphotransferase system[6] is insensitive to transient repression, but is still sensitive to catabolite repression.[7] It is thus possible to eliminate either effect separately by mutation. Yet transient repression, like catabolite repression, prevents the initiation of transcription of the *lac* operon,[8–10] and cyclic 3′:5′-adenosine monophosphate (AMP) counteracts both effects.[11] These results suggest a common target for

both transient and catabolite repression. In this paper we present genetic evidence that the common target is the promoter locus of the *lac* operon.

Materials and Methods. Bacterial growth is described in Silverstone *et al.*[1] Enzyme assays, protein synthesis, nitrogen starvation methods, and phage P1 transduction are described in Tyler *et al.*[3] Diploid construction with the *lac-pro A,B* episome is described in Silverstone *et al.*[1] Strains used in these experiments are described in Table 1. Mapping of closely linked promoter region markers was done as described by Arditti *et al.*,[12] except that 0.2% glycerol was used as an additional carbon source.

TABLE 1. *Bacterial strains.*

Strain	Pertinent characteristics*	Source or reference†
CA8.000	Hfr H *lac*$^+$, wild type	*a*
CA8.003	CA8.000 derivative with *lac* promoter mutation L8	*a*
CA8.005	CA8.000 derivative with *lac* promoter mutation L37	*a*
X8200	L8 promoter mutation in transposed position	*b*
AP64	"Full level" aminopurine revertant of CA8.005	*c*
UV5	"Full level" u.v. revertant of CA8.005	*c*
UV55	"Full level" u.v. revertant of CA8.005	*c*
N25	"Intermediate level" nitrosoguanidine revertant of CA8.003	*c*
UV89	"Intermediate level" u.v. revertant of CA8.005	
S-11	"Intermediate level" spontaneous revertant of CA8.003	*c*
X7165	F$^-$ *proC*$^-$ *lac*$^+$ *gal E*$^-$, Sm^r	*c*
X5075	F$^-$, *lac I*$^-$, Z$^-$, *pro C*$^-$, *trp*$^-$, Sm^r	*a*
M99	F$^-$, *pro A*$^-$, *lac* Z$^-$, Sm^r	*d*
E5014	Sm^s(*lac-pro A, B*)^{del}/F′ *lac*$^+$ *pro A,B*	*a*
E7089	Sm^s(*lac-pro A,B*)^{del}/F′ *lac* Z$^-$, *pro A,B*	*a*
M99 UV5	*lac*$^+$ transductant of M99, with UV5 promoter character	*e*

* The sex, *lac*, and streptomycin symbols are identical to those used by Tyler *et al.*[3] Promoter characters are explained in Fig. 1. *Pro A*, *B*, and *C* are specific mutations in genes involved in proline biosynthesis; *gal E*$^-$ is a specific mutation in the uridine diphosphogalactose-4-epimerase gene, involved in galactose metabolism.

† (*a*) From Jon Beckwith; (*b*) from William Reznikoff; (*c*) from R. Rita Arditti; (*d*) from Ethan Signer; (*e*) see Table 4.

Solid indicator medium for catabolite insensitivity (CR indicator medium) was a modification of that described in Tyler *et al.*:[3] to a minimal medium lacking $(NH_4)_2SO_4$ was added agar (1.5%), glucose (1.0%), D-glucosamine-HCl (0.2%, Calbiochem), lactose (0.1%), and the dye 5-bromo-4-chloro-3-indolyl-β-D-galactoside (40 μg/ml, Cyclo Chemical Corp.). In this medium catabolite repression is very strong due to the restriction of nitrogen metabolism. Under these conditions lactose can induce the production of β-D-galactosidase only if the strain is insensitive to catabolite repression. Induction of the enzyme results in formation of blue colonies. The test is not valid for constitutive strains or strains polyploid for the *lac* genes.

Results. Arditti, Scaife, and Beckwith[12] have described the isolation of revertants of *lac* promoter point mutants. Some of the revertants have levels of β-D-galactosidase approximating those of wild type and are called "full-level" revertants. Others have intermediate enzyme levels and are called "intermediate" revertants. We screened a number of these revertants on CR indicator medium to determine their sensitivity to catabolite repression. Of 15 "full level" revertants, 2 (UV5 and UV55) were found to be insensitive to catabolite repression, and of 20 "intermediate" level revertants, 3 (S11, N25, and UV89) were also found to be insensitive.

We examined these strains in liquid culture. Preliminary experiments showed that galactosidase was fully inducible in all strains; the basal enzyme levels

were 0.1–0.2% of the fully induced levels. We then compared the differential rate of synthesis of galactosidase in these strains in minimal medium containing either glucose-6-phosphate (repressing) or glycerol (nonrepressing). As controls, we used the wild strain, CA8.000; the promoter mutant parents (*lac* P^-), CA8.003 and CA8.005; and a "full level" catabolite-sensitive revertant, AP64. The results of these experiments are presented in columns 3 and 4 of Table 2.

TABLE 2. *Catabolite repression of β-D-galactosidase and tryptophanase.*

Strain	Color of colony on CR indicator plate	Differential Rate of Enzyme Synthesis* β-D-Galactosidase Glycerol	β-D-Galactosidase Glucose-6-PO_4	Tryptophanase Glycerol	Tryptophanase Glucose-6-PO_4
CA8.000	White	47	7.0	0.18	0.004
AP64	White	49	11	0.21	0.001
CA8.003	White	2.4	0.5	0.20	0.002
CA8.005	White	2.2	0.4	0.16	0.002
UV5	Blue	26	25	0.18	0.003
UV55	Blue	21	12	0.21	0.001
N25	Blue	11	8.8	0.18	0.002
UV89	Light blue	5.9	3.7	0.20	0.001
S-11	Light blue	9.8	2.2	0.17	0.001

* β-Galactosidase and tryptophanase units are expressed as enzyme units/ml of culture per increase in bacterial density expressed in Klett units. Enzyme units are given as mμmoles of substrate converted or product formed per minute. A Klett unit corresponds to about 4.2×10^6 bacteria.

In the controls, galactosidase synthesis was repressed five- to sevenfold by glucose-6-phosphate. Although the *lac* P^- strains make enzyme at only 5% of the wild type rate, they also experienced a fivefold repression by glucose-6-phosphate. The revertant UV5, however, was totally insensitive to repression by glucose-6-phosphate. The revertants N25, UV89, and UV55 were repressed by glucose-6-phosphate, although much less than the control strains. Finally, S11 was strongly repressed by glucose-6-phosphate although it had appeared to be insensitive to such repression on indicator plates. These revertants produce galactosidase at rates between 13% (strain UV89) and 60% (strain UV5) of the wild strain. The normal sensitivity of tryptophanase to catabolite repression in these strains (shown in columns 5 and 6 of Table 2) supports the conclusion that the effects are limited to the *lac* operon.

These strains were then examined for their susceptibility to transient repression by glucose. The results are shown in Figure 2. In sensitive strains, such as the wild type CA8.000 and the P^- parent CA8.003, the addition of glucose to cells growing on glycerol caused a strong repression of galactosidase synthesis for about one half of a generation. At the end of this period, synthesis resumed at a rate characteristic of catabolite repression, approximately one half the glycerol rate. In the P^- strain CA8.005 transient repression was also observed, but because the rate of enzyme synthesis during the subsequent period of catabolite repression is close to that of the transient repression rate, it is difficult to distinguish the two effects. Since Pastan and Perlman[13] failed to observe any transient repression in this strain, we examined this defective promoter after its transfer to another strain, X8200. In this strain, a distinct transient repression as well as catabolite repression were observed.

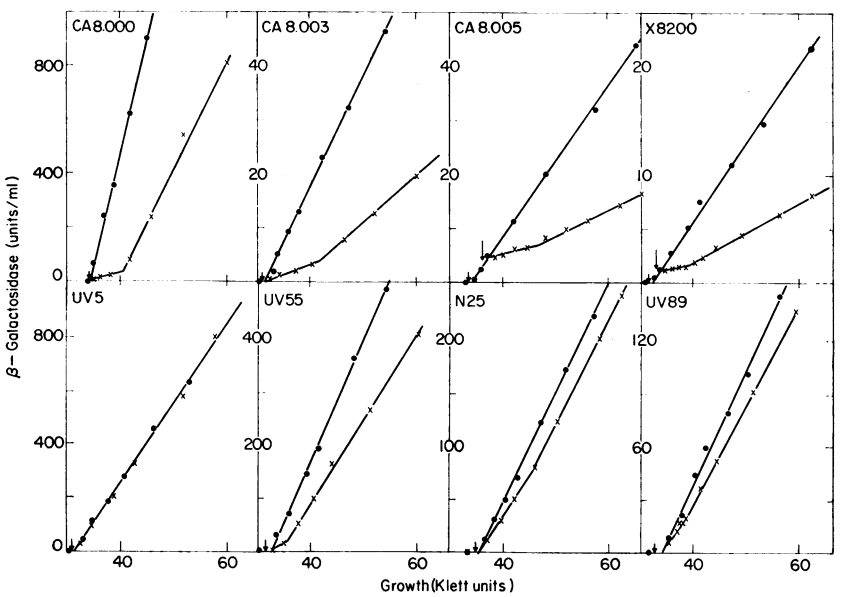

FIG. 2.—Transient and catabolite repression of β-D-galactosidase. Cultures of the indicated strains growing exponentially in glycerol minimal medium were split in two. To one part, IPTG (10^{-3} *M*) and glycerol (0.4%) were added (●). To the other, IPTG (10^{-3} M) and glucose (0.4%) were added (X). Single arrow indicates time of simultaneous addition of inducer and carbon source. Two arrows (CA8.005 and X8200) are used to indicate a slightly different procedure. Here, IPTG (10^{-3} M) is added at the first arrow. The cultures are then split and either glycerol (0.4%) or glucose (0.4%) is added at the second arrow. Samples were then taken for the measurement of enzyme and bacterial density.

In strain UV5 glucose caused neither transient nor catabolite repression. In strain N25 and UV89 glucose caused a slight catabolite and transient repression. Finally, UV55 was transiently and permanently repressed by glucose although not as much as the control strains. We therefore conclude that those strains that have altered sensitivity to catabolite repression have similarly altered sensitivity to transient repression.

Very strong repression arises in cells starved of nitrogen in the presence of a metabolizable carbon source.[4] This is shown in the experiment of Figure 3, in which cells of the strain CA8.000, pregrown in glycerol minimal medium, were starved of nitrogen and exposed to inducer. Addition of glucose enhanced the repression.[5] In strains UV5 and N25 neither glycerol alone nor glycerol plus glucose repress galactosidase synthesis during nitrogen starvation. Thus, UV5 and N25 are unaffected even under very strongly repressing conditions.

We mapped the mutation causing the insensitivity of the *lac* operon to catabolite repression in strain UV5. In a preliminary experiment we transduced strain X5075 (*pro C⁻lac I⁻Z⁻*) with a phage P1 lysate prepared on strain UV5. All of 50 independently isolated *pro C⁺lac⁺* recombinants proved to be fully inducible and insensitive to repression by glucose-6-phosphate. This result indicated close linkage of the site responsible for catabolite insensitivity to the *lac* region. In order to determine the relation of this new site to the original *P⁻*

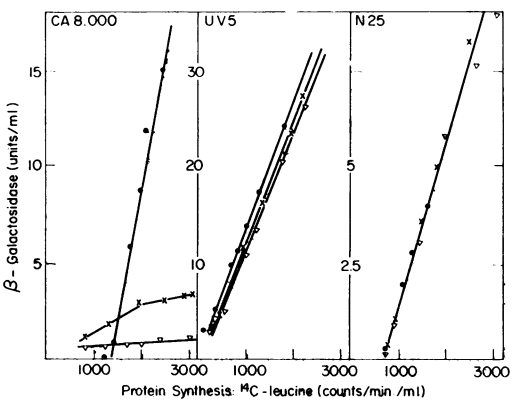

FIG. 3.—β-D-Galactosidase synthesis in nitrogen-starved cultures. The cells were harvested from exponential growth in a minimal medium lacking both a nitrogen source and a metabolizable carbon source. After 4 min, IPTG (10^{-3} M) and ^{14}C-leucine (0.1 μCi/-μg/ml) were added. The culture was divided into three parts. To one part, no carbon was added (●). To the second part, glycerol (0.4%) was added to create catabolite repression (X). To the third part, glycerol (0.2%) and glucose (0.2%) were added to create transient repression (▽). Samples were taken at intervals for the measurement of enzyme and incorporated radioactivity.

mutation, we selected for P^- recombinants in crosses between strains with revertant and wild type promoters.[12] As shown in Table 3, none of 220,000 Pro$^+$ recombinants was P^- when a phage P1 lysate of AP64 was used. In the case of the catabolite-insensitive strains UV5 and N25, 2 out of 40,000 Pro$^+$ transductants were P^- and catabolite-sensitive. We conclude that the mutations that restored the ability to form galactosidase at a higher rate and imparted insensitivity to catabolite and transient repression occurred at sites within the promoter locus, closely linked, though not identical to the site of the original P^- mutation. From recombination frequencies observed for other P1 transductions,[14] we can estimate roughly that the UV5 and N25 mutations are not more than 50 nucleotides from the original P^- mutation. We shall call the mutations in the promoter region that confer insensitivity to catabolite repression P^r.

TABLE 3. *Mapping of catabolite-insensitive revertants.*

Donor strain*	Catabolite sensitivity	Frequency of P$^-$ among *pro C*$^+$ transductants	Corrected recombination frequency (%)†
AP64	+	0/220,000	<0.002
UV5	−	2/41,300	0.02
N25	−	2/39,800	0.02

* P1 phage lysates grown on the donor strains were used to transduce strain ×7165 (*proC*$^-$*lac*$^+$ *gal E*$^-$). The procedure is that of Arditti *et al.*[12]

† The correct frequency is the observed frequency multiplied by 4, since *lac* is only 25% cotransduced with *pro C*.

We then constructed heterozygotes of the promoter characters to test the dominance relations of this region (Table 4). In a strain *lac* P^+Z^-/F′*lac* P^+Z^+ glucose-6-phosphate caused a fivefold repression of the episomal *lac Z*. In the strain *lac* P^rZ^+ glucose-6-phosphate caused no repression of the chromosomal *lac Z* gene. In the heterozygote *lac* P^rZ^+/F′*lac* P^+Z^-, the presence of an episomal P^+ did not restore the sensitivity of the chromosomal *Z* gene to glucose-6-phosphate repression. Finally, in the heterozygote *lac* P^rZ^+/F′*lac* P^+Z^+, glucose-6-phosphate caused a repression much weaker than that observed in *lac* P^+Z^-/F′*lac* P^+Z^+. This suggests that in this last heterozygote the episomal

TABLE 4. *Catabolite repression in heterozygotes of lac promoter mutants.*

Strain*	Pertinent characteristics	Relative differential rate of β-D-galactosidase synthesis†
M99/F′ *lac*$^+$	*lac* P^+Z^-/F′ *lac* P^+Z^+	20
M99UV5	*lac* P^rZ^+	102
M99UV5/F′ *lac*$^-$	*lac* P^rZ^+/F′ *lac* P^+Z^-	96
M99UV5/F′ *lac*$^+$	*lac* P^rZ^+/F′ *lac* P^+Z^+	70

* Strains are described in Table 1. Diploids were constructed by mating the indicated parent (which is *pro A*$^-$) with the appropriate streptomycin-sensitive donor (E5014 for *lac*$^+$ or E7089 for *lac*$^-$) and selecting for streptomycin-resistant proline prototrophs.

† Rates were determined as in Table 2. The values given are those in glucose-6-phosphate culture relative to the differential rate of synthesis in a control culture growing in glycerol, with the latter value taken as 100.

Z gene, but not the chromosomal *Z* gene, was repressed. We conclude, therefore, that the P^r effect is limited to the adjacent operon.

Discussion. We have shown that a single mutation can endow *lac* P^- mutants of *E. coli* with the ability to form galactosidase at a rapid rate even under conditions of severe catabolite and transient repression. The site of this mutation, *lac* P^r, is very close to the mutational site responsible for the reduced expression of the *lac* operon in the parent. The effects of both mutations are limited to the adjacent *lac* operon. These findings strongly support the idea that the promoter locus is the target of catabolite and transient repression. Furthermore, because *lac I* and *lac O* function normally in the *lac* P^r mutant we can rule out their participation in catabolite or transient repression.

The conversion of a catabolite-sensitive operon to a catabolite-insensitive one has been observed also in the case of the histidine degrading enzymes (*hut*) of *Bacillus subtilis*[15] and *Salmonella typhimurium*.[16] The synthesis of these enzymes can be prevented by mutations at a site closely linked to the structural genes for these enzymes. Just as in the *lac* system, some of the revertants of these putative promoter mutants are insensitive to catabolite repression.[15–17] Also, in the *hut* system, one can directly select for mutations to catabolite insensitivity; these are very closely linked to the promoter.[15,16]

The converse has been observed by Friedman and Margolin.[18] These authors studied a mutant unable to form any of the enzymes specific for leucine biosynthesis because of a defect at a site adjacent to the *leu* operon; a revertant, isolated from this mutant, could form these enzymes in the absence but not in the presence of glucose. Apparently an alteration of the leucine promoter made the normally catabolite-insensitive enzymes of the *leu* operon sensitive to catabolite repression.

From these findings on three distinct operons we argue that catabolite and transient repression are directed against the promoter function: the binding of RNA polymerase and the initiation of transcription. This conclusion is in good accord with the observation that catabolite and transient repression arrest the initiation of transcription of the *lac* operon.[8,9] Recent experiments have shown that cyclic AMP is required for transcription of the *lac* operon in a cell-free system.[19,20] Moreover, mutants lacking a protein that appears to be essential for the interaction of cyclic AMP with RNA polymerase are unable to form catabolite-sensitive enzymes.[21,22] The P^r promoter, however, allows galacto-

sidase to be formed by these mutants.[22] This suggests the nature of the difference between catabolite-sensitive and catabolite-insensitive promoters: attachment of the RNA polymerase to sensitive promoters requires cyclic 3′:5′ AMP; attachment to the insensitive promoters does not.

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