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A Mutant of *Escherichia coli* with a New, Highly Efficient Promoter for the Lactose Operon

(transcription/*lac* mRNA/competition hybridization)

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ABSTRACT **We have isolated a mutant of *E. coli* whose maximal rate of synthesis of *lac* mRNA is 25-fold greater than that of its parental wild-type strain. The mutant also shows alterations in the kinetics of β-galactosidase synthesis, the functional half-life of β-galactosidase mRNA, and the properties of *lac* repressor.**

The lactose (*lac*) operon of *Escherichia coli* consists of three coordinately transcribed structural genes (*z*, *y*, and *a*) encoding, respectively, β-galactosidase, galactoside permease, and thiogalactoside transacetylase, an operator region (*o*), the site of action of *lac* repressor, and a promotor region (*p*), the site of initiation of transcription by RNA polymerase (1). The repressor (*i*) gene lies adjacent to the *lac* operon and is transcribed separately, though from the same DNA strand (2). The order of these genetic elements is *ipozya*.

The *lac* promoter consists of two RNA polymerase initiation sites: a catabolite-sensitive initiation site and a catabolite-insensitive initiation site (3). Initiation at the first but not at the second site requires cyclic AMP and CAP protein (cyclic AMP-binding protein; ref. 3). The second site is about 2% as efficient as the first (3, this paper). *lac* promoter mutants are known which yield less than the normal amounts of the *lac* enzymes after induction (4). There are no known promoter mutants which yield higher levels than the wild type, although some operator mutants synthesize as much as twice the normal amounts of the *lac* enzymes (5).

We recently undertook a search for mutants which would express *lac* operon functions for a long period after a brief exposure to the gratuitous inducer IPTG (isopropyl-β-D-thiogalactoside). Such a phenotype, we hoped, would be associated with *lac* mRNA of greater than normal stability. Among approximately 1000 isolates tested after nitrosoguanidine mutagenesis, one had the desired phenotype. We show here that this strain has *lac* mRNA of only slightly altered functional half-life but synthesizes about 25 times more *lac* mRNA than does the wild type. In another publication, we show that the mutation *lac*-741 lies in the *lac o*-distal portion of the *i* gene (Bruenn and De Lucia-Curtin, manuscript in preparation).

MATERIALS AND METHODS

Selection Procedure. Our selection procedure consisted of inducing mutagenized cells in the presence of amino acid analogs at 42°, then selecting for growth on the noninducing substrate phenyl-β-D-galactoside at 32°. We previously determined that wild-type cells preinduced with IPTG will grow on phenyl galactoside as carbon source in the absence of inducer, whereas uninduced cells will not. The amino-acid analogs used (azetidine-2-carboxylic acid and thienylalanine) are incorporated into proteins (6, 7) and yield inactive β-galactosidase and (or) *lac* permease. Of 1000 isolates tested, 15 had low constitutive levels of β-galactosidase in glucose minimal medium. Of these 15, only the isolate E741 showed as much as a 2-fold increase in the number of colonies growing on phenyl galactoside after a second selection as above.

Enzyme Assays. β-Galactosidase was assayed by the method of Zipser, as cited by Miller (8). The units we use are $^1/_{60}$ those of Miller. Thiogalactoside transacetylase was assayed as described by Michels and Zipser (9).

Pulse Inductions. Cells were grown in glucose minimal medium at 32° to an OD_{550} of about 0.1. IPTG was added to 0.5 mM for 10 min and removed by filtering the cells onto Millipore 25-mm 0.45 μm cellulose nitrate filters and washing with fresh medium at 32°. After resuspension of the cells, growth was continued at 32° and samples were taken at intervals for assay of β-galactosidase. Uninduced controls were similarly treated, except that IPTG was omitted.

RNA·DNA Filter Hybridizations. Denatured DNA was immobilized on Schleicher and Schuell B-6 filters by the procedure of Denhardt (10). DNA from F'*lac* strains, for determinations of *lac* DNA, was denatured by boiling for 15 min in 0.1 N NaOH. After an initial 6-hr incubation in Denhardt's medium at 37° (10), hybridizations were carried out at 37° for 3 days in 50% formamide, 0.30 M NaCl, 0.03 M citrate buffer (pH 7.0) and in the presence of 16 μg/ml of yeast tRNA. Reaction mixtures were then processed to isolate the RNase-stable hybrids (11), and counted in toluene scintillator fluid in an Intertechnique scintillation counter. Competition hybridizations were performed in two steps (11) with λ and λ*plac*5 DNA filters carrying 0.1 μg of denatured DNA. The initial hybridizations with nonradioactive RNA were performed as above. Any remaining pancreatic RNase was inactivated with iodoacetate as described by Zimmerman (12). The filters were washed and used for the second step hybridization with a molar excess of [^{32}P]RNA synthesized *in vitro*.

Total Cellular RNA and DNA. Unlabeled or [^{3}H]uridine-labeled *E. coli* RNA was purified and freed of DNA according to Bade (13). DNA was purified from Sarkosyl lysates (14) by

Abbreviations: *lac*, the lactose operon; CAP protein, cyclic AMP-binding protein; IPTG, isopropyl-β-D-thiogalactoside; *cap* and *cya*, the genes coding for CAP protein and adenylate cyclase, respectively; i^Q, a super-producing *i* gene mutation.

TABLE 1. *Properties of lac-741 strains and controls*

Strain	Genotype	Uninduced specific activities: Transacetylase (stationary phase in broth)	Uninduced specific activities: β-Galactosidase (log phase in minimal glucose)	Induced differential rate of synthesis of β-galactosidase: Average*	Induced differential rate of synthesis of β-galactosidase: Maximum†	*lac* DNA‡
E102	F'*lac*$^+$/Δ*lac*	0.010	0.021	38	38	1.0
7553	F'*lac*$^+$/*lac*$^+$ (parent of 741)	0.015	0.026	80	80	—
E101	F'*lac*-741/Δ*lac*	0.250	16	114	320	0.9
E741	F'*lac*-741/*lac*$^+$	0.193	0.61	180	320	—
X116	F$^-$*lac*-741	—	15	50	125	—
X7700	F$^-$*lac*$^+$	—	0.025	13.3	13.3	—
E129	F'*lac*-741/*lac* i^Qz^-	0.037	0.042	23.2	45	—
E150	F'*lac*$^+$/*lac* i^Qz^-	—	0.007	33	33	
E151	F'*lac*-741,z^-/*lac*$^+$	—	0.047	14.5	14.5	—
E154	F'*lac*-741,z^-/*lac* i^-	—	3.9	17	17	—
E121	F'*lac*-741/*lac* i^+p^+Δ(*ozya*)	—	0.051	22	—	

* Slope of the best straight line relating β-galactosidase activity to OD_{550} during continuous induction in glucose minimal medium with 0.5 mM IPTG.

† Maximum slope of the best smooth curve as above.

‡ Relative amounts of *lac* DNA were measured by hybridization of ^{32}P *in vitro lac* mRNA to total cellular DNA (see *Materials and Methods*).

phenol extraction, RNase and Pronase treatment, further phenol extraction, and extensive dialysis.

[^{32}P]*RNA Was Synthesized In Vitro* on template DNA consisting of *lac* DNA fragments of average length 1000 nucleotides (a generous gift of N. Maizels). The synthesis was catalyzed by *E. coli* RNA polymerase holoenzyme (a gift of B. Allet) as described by Gilbert *et al.* (15). RNA synthesis was for 25 min at 25°. The RNA was purified by 1:5 dilution into 10 mM Tris·HCl (pH 7.0)–10 mM $MgCl_2$, incubation with 1 μg/ml of Worthington electrophoretically pure DNase for 10 min at 37°, extraction with one volume of phenol saturated with 0.1 M Tris·HCl (pH 7.9) and Sephadex G-75 chromatography.

RESULTS

Some of the properties of the mutant E741 and derivative strains carrying the mutation *lac*-741 are summarized in Table 1. *lac*-741 confers partial constitutivity for β-galactosidase, thiogalactoside transacetylase, and lactose permease. This last is shown by the ability of the mutant to grow on raffinose in minimal medium (data not shown). Typically, *lac*-741 haploid strains in the logarithmic phase of growth have about 20% of their fully induced β-galactosidase specific activity, whereas full i^- strains have 100%. Various diploids were constructed in order to determine the effects of *lac*-741 in *cis* and in *trans*. The original mutation was transduced from the haploid E101 to a strain which was F'*lac* z^- *proA*$^+$, *B*$^+$/Δ(*lac pro*), selecting for Lac$^+$. *lac*-741 was 93% cotransducible with this *lac z* mutation (U118). One of the transductants was then crossed to (*lac pro*) deletion strains lysogenic for defective phages carrying portions of the *lac* operon. The result is that *lac*-741 is dominant in *cis* (E741) but not in *trans* (E151). Since *lac*-741 maps within the *i* gene (Bruenn and De Lucia-Curtin, in preparation), it would be expected that *lac*-741 strains would produce an altered *lac* repressor. In fact, *lac*-741 does confer partial loss of the operator binding activity of *lac* repressor (E154). *lac*-741 strains are repressible, since (in the diploid E129) *lac*-741 appears to be recessive to i^Q, a mutation which results in an increased rate of synthesis of *lac* repressor (16). *lac*-741 strains with an excess of normal *lac* repressor, e.g., E121 [F'*lac*-741/i^+p^+Δ(*ozya*)] and E129 (F'-*lac*-741/i^Qz^-) are not inducible even to the wild-type level of β-galactosidase. This property of *lac*-741 is not readily explained.

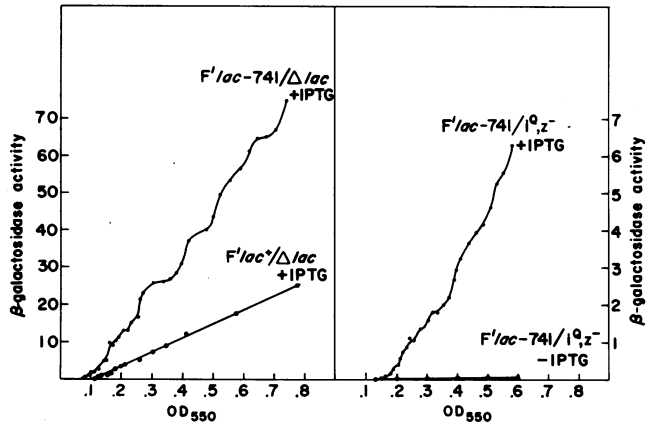

FIG. 1. Continuous inductions of *lac*-741 strains and controls.

The most striking effect of *lac*-741 is that the maximal differential rate of synthesis of β-galactosidase (the increase of β-galactosidase activity per OD_{550}) is 8–10 times higher in *lac*-741 haploids than in isogenic Lac$^+$ haploids, both in glucose minimal medium and in glucose-free broth (not shown). The differential rate of synthesis of each strain is maximal in broth and is about 2.5 times as great in broth as in glucose minimal medium. The F'*lac*$^+$ strains consistently have 2–3 times the differential rate of synthesis of β-galactosidase of F$^-$*lac*$^+$ strains, presumably because of multiple copies of the episome (17).

The differential rate of synthesis of β-galactosidase in *lac*$^+$ strains is constant in any given set of conditions (18), but this

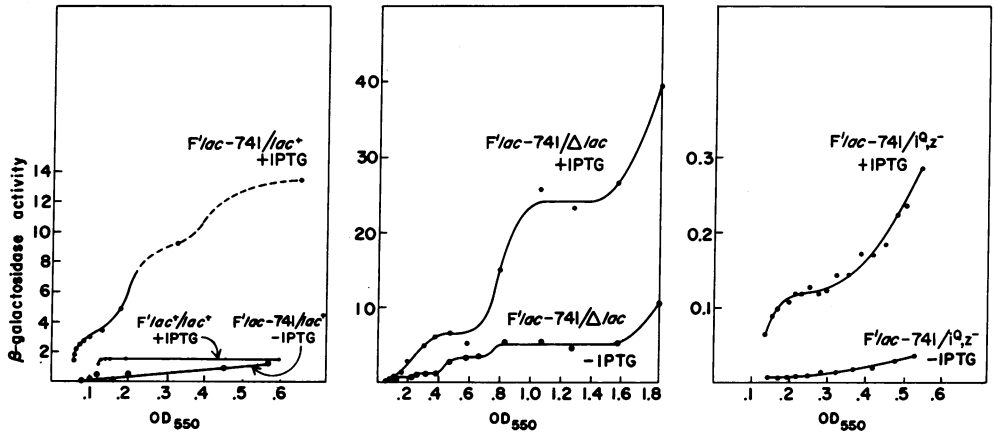

FIG. 2. Pulse inductions of *lac*-741 strains and controls.

is not the case with E741. Fig. 1 illustrates the behavior of E101 (F'*lac*-741), the isogenic haploid strain E102 (F'*lac*$^+$), and E129 (F'*lac*-741/i^Q) when exposed to a constant concentration of IPTG (0.5 mM). Clearly the rate of increase of β-galactosidase activity per OD_{550} is not constant in *lac*-741 strains. The log(OD_{550}) of these cultures is exactly linear with time under these conditions (not shown).

Pulse inductions of E101 (F'*lac*-741), E741 (F'*lac*-741/*lac*$^+$), E129 (F'*lac*-741/i^Q), and 7553 (F'*lac*$^+$/*lac*$^+$) are shown in Fig. 2. Cells were induced with IPTG for 10 min, then the IPTG was removed (*Materials and Methods*). The wild type entirely ceases synthesis of β-galactosidase within minutes after the removal of IPTG. The pulse-induced cultures of *lac*-741 strains continue to synthesize β-galactosidase at an average rate 3–15 times that of the uninduced culture. The rate of synthesis of β-galactosidase in the mutant cultures is not constant, but seems to be a periodic function of cell mass, increasing every cell generation. With respect to its behavior on pulse or continuous induction, *lac*-741 is dominant to i^Q, although E129 (F'*lac*-741/i^Q, z^-) is about 7-fold less inducible than E112 or E741 (F'*lac*-741/*lac*$^+$).

In the absence of an increased number of *lac* operons in *lac*-741 strains (Table 1), we sought in promoter abnormalities an explanation for the phenomenon of increased *lac* operon expression in *lac*-741 haploids. The relative efficiency of the *lac*-741 promoter was measured in three ways: first, by the amount of β-galactosidase synthesize in an induced culture after addition of rifampicin; second, by RNA · DNA hybridization of [^{3}H]uridine-labeled cellular RNA to *lac* DNA; and third, by competition hybridization of unlabeled cellular RNA with ^{32}P *lac* mRNA syntheized *in vitro*. Strain E741 (F'*lac*-741/*lac*$^+$) was induced for 20 min before the addition of rifampicin to 200 μg/ml. Under these conditions, E741 synthesizes 4.2 times as much β-galactosidase per unit mass after addition of rifampicin as does the diploid wild-type control (Fig. 3). An identical experiment with haploid strains gave similar results: 8 times as much β-galactosidase is synthesized by the *lac*-741 strain as by the *lac*$^+$ strain after a 20-min induction followed by rifampicin (results not shown). Similar experiments with one-minute inductions gave the same results (not shown). Since the same number of copies of *lac* DNA are present in *lac*-741 strains as in isogenic controls (Table 1), a greater frequency of initiation of *lac* mRNA chains is indicated in *lac*-741 strains.

When E101 (F'*lac*-741/Δ*lac*) was induced for 10 min in glucose minimal medium and then exposed to a [^{3}H]uridine pulse of 10 min duration, 4.4% of the input labeled RNA hybridized specifically to the *lac* DNA sequences of λ*plac*5. In an identical experiment done in parallel, the isogenic wild type (E102, F'*lac*$^+$/Δ*lac*) gave 0.15%. These experiments were performed with the same total input counts and with DNA in excess. Our value for the wild type is somewhat higher than that measured by Adesnik and Levinthal, who found 0.066% in a wild-type diploid (19). This discrepancy is most likely due to the fact that our labeling conditions were not steady-state conditions; mRNA was still labeled preferentially. Under these conditions, the *lac*-741 promoter appears to be about 29 times as efficient as the wild-type promoter.

In order to eliminate possible labeling artifacts, we measured the amount of *lac* mRNA in E101 and E102 by competition hybridization. E101 and E102 were grown in glucose medium and induced with IPTG. The synthesis of β-galactosidase was followed in each culture and samples for RNA

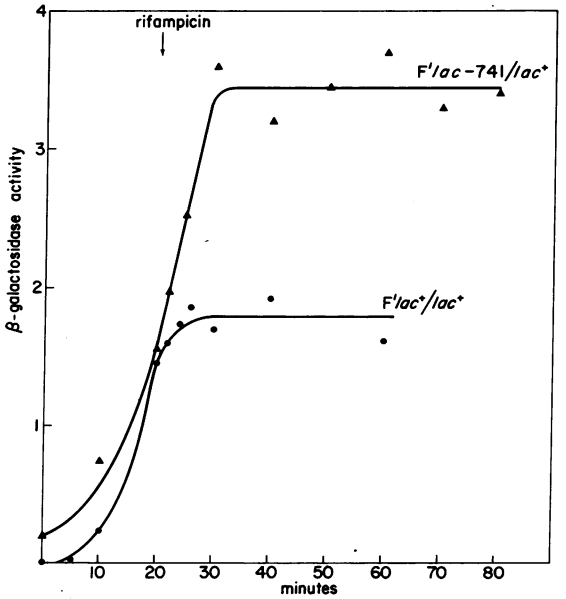

FIG. 3. Residual synthesis of β-galactosidase after addition of rifampicin. Curves are normalized to the same OD_{550}.

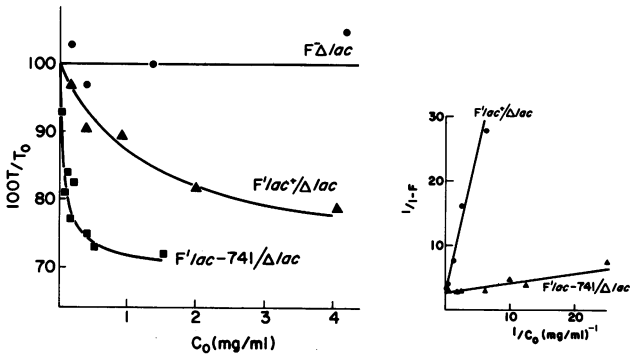

FIG. 4. Competition hybridization measurements of *lac* mRNA. T is the amount of radioactivity, in cpm, hybridized to λ*plac*5 in the second hybridization, T_0 the equivalent cpm without prehybridization, and C_0 the total concentration of nonradioactive RNA added in the first hybridization. In the *inset*, F is the fraction of ^{32}P *in vitro lac* mRNA hybridized to λ*plac*5 DNA in the second hybridization. $1/(1 - F) = a/(1 - T/T_0) = 1 + 1/c_0\, tk_2$, where a is the fraction of ^{32}P *in vitro* RNA that is *lac* mRNA (0.75), c_0 the concentration of unlabeled *lac* mRNA hybridizable to λ*plac*5, t the time of incubation, and k_2 a constant. T_0 was 750 cpm more than background (30 cpm).

analysis were taken at various differential rates of synthesis. The differential rate of synthesis in E102 was 35 and in E101 was 100 and 300 when RNA samples were taken. The two initial samples of wild-type and mutant RNA were both taken 1 hr after induction. Two-step competition hybridizations were performed as described in *Materials and Methods*. In this procedure, nonspecific competition is eliminated, since we measure only the amount of RNase-stable hybrids formed between unlabeled *lac* mRNA and *lac* DNA on the filters (11). Two separate types of control show this to be true. First, the amount of λ-specific RNA bound to the filters in the second hybridization is independent of the prehybridization. Second, λ*plac*5 filters treated in the same way as the prehybridized filters but without added nonradioactive RNA, or with RNA from the *lac* deletion strain 532 grown in the presence of IPTG, bind the same amount of ^{32}P *in vitro lac* mRNA as untreated filters.

The results of the competition hybridization experiments are shown in Fig. 4. If we assume that the RNAs from E101 and E102 are competing for the same *lac*-specific sequences of

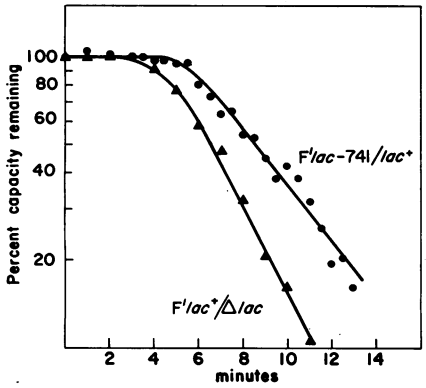

FIG. 5. Functional half-life of β-galactosidase mRNA. Cells were induced in glucose medium with 0.5 mM IPTG, followed 1 min later by 200 μg/ml of rifampicin. 100% corresponds to an increase in specific activity of 1.9 for E741 and 0.5 for E102.

TABLE 2. *Effect of cap⁻, cya⁻ background on lac-741**

Genotype	Average differential rate of synthesis of β-galactosidase (in minimal glucose) −IPTG	+IPTG
F'*lac*-741 *proA*+,*B*+/Δ(*lac pro*)*cap*⁻₀₀*cya*⁻₀₂	0.28	1.5
F'*lac*+ *proA*+,*B*+/Δ(*lac pro*)*cap*⁻₀₀ *cya*⁻₀₂	0.0025	1.0
F'*lac*-741 *proA*+,*B*+/Δ(*lac pro*)	15	92
F'*lac*+ *proA*+,*B*+/Δ(*lac pro*)	0.069	33

* *cap* and *cya* are the genes coding for CAP protein and adenylate cyclase, respectively.

λ*plac*5 DNA, we can calculate the relative proportion of each that is *lac* mRNA. This assumption appears justified by the fact that the double reciprocal plot of Fig. 4 extrapolates (at infinite nonradioactive input RNA) to the same amount of *in vitro* [^{32}P]RNA homologous to *in vivo lac* mRNA in each case. That this value is not the 75% of the [^{32}P]RNA that is *lac* specific, but rather 38%, is attributable to lack of strand specificity in the *in vitro* transcription.

The relative amount of *lac* mRNA in the mutant, estimated either from the 50% competition point or from the slope of the double reciprocal plot, is 25 times that of the wild type. This is true at both differential rates of synthesis of β-galactosidase, which were estimated, as described in Table 1, from measurements taken every 5 min on the same cultures harvested for determination of total RNA. A repetition of the competition hybridization experiment with another, independent, set of nonradioactive RNA samples gave identical results.

We examined the half-life of β-galactosidase mRNA to determine if the increased quantity of *lac* mRNA in E101 (F'-*lac*-741/Δ*lac*) was due to β-galactosidase mRNA of grossly altered stability. Strains were grown to mid-log phase in glucose minimal medium at 32° and induced with 0.5 mM IPTG followed, 1 min later, by 200 μg/ml of rifampicin. Fig. 5 presents the results in the form of decreasing capacity for the synthesis of β-galactosidase versus time. All four *lac*-741 strains tested are different from wild type in two respects: the functional half-life of β-galactosidase mRNA measured in this way is 2.8–3.0 min, in contrast to 1.8–1.9 min for the wild type; and the first detectable increment of β-galactosidase activity is at about 3.5–5 min, rather than at 2.5 min. The half-life measurements are reproducible to within ±5%, but the time of the first increment in β-galactosidase activity is difficult to estimate in the constitutive strain because of the high initial level of β-galactosidase. All four wild-type strains tested gave identical results, in agreement with published data for the first appearance of β-galactosidase (2.5 min) and the functional half-life of *z* gene mRNA (1.72–1.92 min) at 32° (20, 21). The chemical half-life of β-galactosidase mRNA (the half-life as measured by RNA·DNA hybridization) is the same in wild-type and *lac*-741 strains—about 3.0 min (Bruenn and Bade, unpublished), again in agreement with published data (21).

Since *lac*-741 *lac* mRNA has the same chemical half-life as wild-type *lac* mRNA, we conclude that the *lac*-741 promoter is 25 times as efficient as the wild-type *lac* promoter. A rough calculation based on the number of rRNA cistrons (22) shows

the *lac*-741 promoter to be about as efficient as a promoter for an rRNA cistron. Nevertheless, the *lac*-741 promoter, like the wild-type promoter, is dependent on CAP protein and cyclic AMP (Table 2). *lac*-741 haploid strains also respond to exogenous cyclic AMP as the wild type does (not shown).

DISCUSSION

In a fully induced wild-type haploid strain of *E. coli*, the steady-state level of *lac* mRNA is about 1% of the total mRNA (19). This means that in *lac*-741 haploid strains, which synthesize *lac* mRNA of normal chemical stability at 25 times the wild-type rate, *lac* mRNA should account for about 20% of the total mRNA. It is hard to predict what physiological consequences this imbalance would have, and a more detailed analysis is needed. However, the fluctuations in the differential rate of synthesis of β-galactosidase in the mutant, unaccompanied by fluctuations in the level of *lac* mRNA, already suggest complicated interactions. The lowered inducibility of *lac*-741 in the presence of excess normal *lac* repressor also requires further explication.

Since *lac*-741 haploids have a normal complement of *lac* DNA, their increased rate of synthesis of *lac* mRNA is most likely due to the presence of a new promoter for the *lac* operon with an unusually high affinity for RNA polymerase. The *cis* dominance of *lac*-741 is consistent with this interpretation.

If, as seems likely, the new promoter is within the *i* gene (Bruenn and De Lucia-Curtin, in preparation), then transcription of the rest of the *lac* operon in the mutant must result from rather efficient read-through beyond the end of the *i* gene into the operator and normal promoter, then into the *lac* structural genes. Normally, there is probably a signal for transcription termination at the end of the *i* gene (2). There could be read-through, however, if this termination site is not very effective or, alternatively, if in the original isolate the termination site is altered or deleted.

There is precedent for promoters of increased efficiency or abnormal location. The UV5 *lac* promoter is a highly efficient CAP- and cAMP-independent promoter with a location distinct from that of the wild-type *lac* promoter (3, 23). *lac*-741 could be an alteration of an existing internal promoter, as in the *trp* operon (24). The original promoter may not necessarily have been CAP- or cAMP-dependent, since in at least the case of the *leu* operon (25) a mutational alteration can make a promoter catabolite-sensitive. Alternatively the 741 mutation could be a modified nonpromoter nucleotide sequence, or an insertion of a "foreign" catabolite-sensitive promoter.

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