MUTANTS THAT MAKE MORE LAC REPRESSOR

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The gene that makes the *lac* repressor functions at an extremely low rate. It synthesizes only a few thousandths of a per cent of the total protein of an *E. coli* cell: about 5–10 molecules each generation.[1] In order to study this molecule more easily, we have sought to enhance the amount present in a cell. Two approaches have been successful. The repressor gene, the *i* gene, itself can be mutated to a form that produces more repressor, or the number of copies of the gene can be drastically increased by incorporating the gene into a phage chromosome which will multiply in the cell. The combination of these two approaches yields a cell strain which can make 0.5 per cent of its protein *lac* repressor.

The i^q Mutant.—Several mutants that make about tenfold more repressor than the wild type have been found. These i^q mutants (*q* for quantity) were selected by forcing temperature-sensitive repressor mutants, strains constitutive at 43° and inducible at 30°, to revert to an inducible phenotype at 43°C. At the high temperature, the temperature-sensitive mutants make too little repressor, either because the protein itself is unstable (i^{TL}) or because the final assembly of active repressor is blocked (i^{TSS}).[2] Although the reversion could simply correct the original defect, the temperature effects can also be overcome by producing *more* of the protein (or a more active protein: so that the small amount left at the high temperature will suffice). However, the ability of the *lac* repressor to bind IPTG (isopropyl-1-thio-β-D-galactopyranoside) gives one a direct, quantitative measure of the number of molecules present.[1] By screening extracts from "revertant" strains, we could identify those mutants which, in contrast to the wild type, gave an easily detectable binding in the crude extract.

The revertants were isolated using TONPG (o-nitrophenyl-1-thio-β-D-galactopyranoside), which will inhibit the growth of *lac* constitutive cells that have a functioning *lac* permease. y^- (permease$^-$) mutants will arise and can be counterselected against by growth on lactose in the presence of IPTG. Both i^{TSS} and i^{TL} strains have yielded overproducing derivatives. Table 1 shows the IPTG-binding data for some of these strains; the data suggest that there is about ten times more repressor in the i^q strains. One of these mutants, a derivative of an i^{TSS} strain, has been studied in detail, and was used to produce pure repressor for physical characterization (studies that will be reported elsewhere). The affinity of this i^q repressor for IPTG has been checked and is the same as the wild type; therefore, the increase in the binding truly means an increase in amount of repressor.

Dominance and Complementation.—To study further the properties of this i^q mutant, we constructed heterozygous diploids with a variety of *i* and *o* (operator)

TABLE 1. *Amount of repressor present in various strains.*

Strain	Excess IPTG bound (%)	Mg protein/ml	Specific activity of repressor	Number of repressor molecules/cell
i^+	$\leqq 2$	46	$\leqq 0.05$	$\leqq 20$
$i^+/F'i^+$	$\leqq 2$	40	$\leqq 0.05$	$\leqq 20$
i^{qTSS}	12	49	0.25	100
i^{qTL}	8	40	0.20	80
$i^{qTSS}/F'i^{qTSS}$	22	42	0.50	200

Cells were grown in yeast tryptone medium. Repressor was determined as described in.[1] Specific activity of the *lac* repressor is defined as excess IPTG bound in %/mg protein/ml. The number of repressor molecules per cell is based on the specific activity of pure repressor:[5] 2000.

mutants. Although an *i* gene with an i^s mutation, producing a repressor that does not easily recognize the inducer, is dominant over the wild-type i^+, and the diploid $F'i^s/i^+$ is *lac*$^-$, an $F'i^s/i^q$ diploid is *lac*$^+$! Table 2 shows this dominance quantitatively. The *lac* genes are inducible, even in the presence of the i^s repressor. The dominance is not total: the induction is only to 25 per cent of the full diploid level.

The simplest explanation is intragenic complementation between subunits of an oligomeric repressor, as suggested by Sadler and Novick.[2] Purified *lac* repressor can indeed be shown to be a tetramer, of molecular weight 150,000 daltons.[5] In an i^q/i^s heteromerozygote most of the i^s subunits would occur in the mixed tetramer $i^q{}_3i^s$. These mixed oligomers must be sensitive to the inducer and driven off the DNA.

One would expect that in a diploid containing both the i^q and an o^c mutation, the o^c would synthesize β-galactosidase at a lower rate than in the presence of a wild-type i^+ gene, being sensitive to the greater amount of repressor. An $F'i^+/i^+o^c$ diploid generally functions at about half the level of the parental i^+o^c. However, when such diploids are constructed with the i^q, the i^q and i^+ genes behave comparably, as is shown in Table 3. The i^q, while making more repressor, must make a repressor with lessened affinity for the operator. This interpretation is supported by the i^q's mutation's not fully depressing the basal level to the normal value, even at low temperatures (also shown in Table 3). We infer that the affinity for the operator has decreased by a factor of 5–10. This can be confirmed *in vitro:* when radioactive repressor is prepared from the i^q, it binds to the operator region on a DNA molecule with an affinity that is about fivefold weaker than that of the wild type.[5] The i^q gene here is derived from an i^{TSS}, and the repressor involved contains two changes with respect to the wild type. Presumably, the defect that results in lessened affinity is in the original TSS repressor.

TABLE 2. *Dominance of i^q over i^s.*

Strain	Specific activity of β-galactosidase
i^sz^+	20
i^qz^+	10,000
$i^qz^+/F'i^sz^+$	5,500

Cells were grown in minimal medium M56[3] in the presence of $10^{-3}M$ IPTG with glycerol as carbon source at 37°. The β-galactosidase was determined as described previously.[4] The i^s strain used is AB 785 derivative Al-42 isolated by us.

TABLE 3. *The i^q repressor does not repress as well as wild-type i^+ repressor.*

Strain	Specific activity of β-galactosidase	Strain	Specific activity of β-galactosidase
$i^+o^+z^+$	15	$i^+o^cz^+/F'i^+o^+z^+$	750
$i^qo^+z^+$	140	$i^+o^cz^+/F'i^qo^+z^+$	1050
$i^+o^cz^+$	1300		

Cells were grown in the absence of inducer and assayed for β-galactosidase as described under Table 2. All strains were grown at 37° with exception of the $i^qo^+z^+$, which was grown at 20°. The o^c strain used is strain 2000 o^c obtained from C. Willson.

There is an anomalous class of *i*-gene mutants called originally "i^-o^c" and thought at one time to be deletions cutting into both the *i* gene and the operator. Davies[6] has mapped these and discovered that some of them are point mutants which map in the middle of the *i*-gene ("i^-o^c" nos. 24, 198, 522). When diploids are constructed with these mutants and the rates of β-galactosidase synthesis compared in these "i^-o^c" strains carrying either an $F'i^+$ or an $F'i^q$ episome, the galactosidase level is lowered from the wild-type level by a factor of nine in the presence of the i^q. These mutants clearly do not behave as operator constitutives. They are sensitive to the amount rather than to the quality of the repressor. The dominance of the constitutive character in diploids is thus a complementation phenomenon. These are trans-dominant i^-'s which we shall call i^{-d} (for dominant). The i^{-d} repressor is not only incapable itself of binding to the operator, but even the presence of one or more i^{-d} subunits in a mixed tetramer renders the whole structure incapable of functioning as a repressor. The i^q making an excess of subunits then dominates over the i^{-d}. That the i^{-d} is in fact trans-dominant has been confirmed by inserting an $i^{-d}z^-$ episome in an i^+z^+ rec^- cytoplasm. The chromosomal z^+ (β-galactosidase) gene then functions constitutively, as expected.

The Mechanism of the i^q Mutation.—Although the very low rate of synthesis of *lac* repressor is presumably only one extreme of a spectrum of rates that span four orders of magnitude, how in fact is this low rate achieved in the i^+ gene, and how was it altered in the i^q mutant? Some rate-limiting step in the over-all synthesis has been changed. One possibility is that the *i* gene has an inefficient promoter:[13, 14] an attachment and initiation site for the RNA polymerase with low affinity for the enzyme. The alteration of a base may have increased the affinity for the polymerase by 1 or 2 kc, and thus increased the rate by a factor of 10. Alternatively, some limiting step in the translation process might be altered. The rate at which ribosomes attach and initiate may be influenced by sequences near the chain-initiating codon; the messenger may contain sequences of codons that are inefficiently read, either because the sRNA's are in too low an amount or because they cannot sit down next to each other with any ease; or possibly, the final amino acid sequence itself might be unusually sensitive to proteolytic attack before the protein folds into its final form.

There is no definitive understanding of this problem at present, but we believe that the i^q mutation is most likely an enhanced promoter, a mutation leading to an increased rate of messenger RNA synthesis. The very low rate of normal synthesis would be due to an extremely inefficient promoter: the probability of

initiating the messenger RNA for the i gene being so low that only one or two mRNA molecules are synthesized per gene for each generation. Each messenger would then make five or ten molecules of repressor.

This assumption allows a simple explanation for the classical observation that if an i^+z^+ piece of DNA is introduced into an i^-z^- cytoplasm, the β-galactosidase gene functions at a high rate initially and appears to be only slowly turned off.[7] One can interpret this slow turn-off as being an exponential decrease in the number of β-galactosidase-producing cells: the cell population shuts off heterogeneously as each cell succeeds in making a messenger for the repressor and then a burst of repressor molecules. The same explanation applies to the behavior of the i^{TL} mutation.[2] The published curves for the i^{TL} can be plotted as exponential decays, yielding a time constant corresponding to 2.4 i-gene messengers per generation in haploid cells, i.e., about one from each nucleus for each generation.

If this interpretation is correct, a comparison of the i^+ and i^q genes in a mating experiment should show whether or not more i-gene messenger is made by the i^q. Only in that case would the period of constitutive synthesis be less extensive, for if the mutation increased the yield per messenger, i.e., if it had an effect on translation, the cells must still wait the same time before the synthesis of the first messenger. Figure 1 shows such a mating experiment: the i^q shuts off β-galactosidase synthesis about seven times more effectively than does the wild type.

Still More Repressor.—The production of *lac* repressor is subject to gene dosage, the rate increasing appropriately in diploid and triploid cells. Thus one expects the rate of synthesis to increase markedly if several hundred gene copies could be put in the same cell. This can be achieved by incorporating the i^q mutation into the genome of an inducible prophage. We have used a derivative of the ϕ80-λ hybrid, carrying the *lac* genes as a replacement of late functions. The phage used as a single defective lysogen is heat-inducible and carries also a specific defective mutation t_{68}. This mutation prevents lysis (although not the production of phage lysozyme) and furthermore prevents the shutoff of all functions that normally occurs about an hour after induction, whether or not the cell lyses.

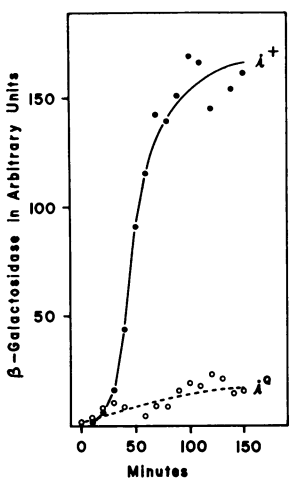

FIG. 1.—Onset of repression in a mating experiment. At time $t = 0$, Sm^s male cells growing exponentially at 37° and carrying either the F′i^+z^+ or the F′i^qz^+ episome were mixed with a 2 to 1 excess of Sm^r female cells carrying the *RV lac* deletion. After 10 min of further growth at 37°, in the casamino-acid-M56[3]-glycerol medium, mating was interrupted by vortexing and adding 30γ/ml of sodium dodecyl sulfate. Streptomycin (250γ/ml) was added to stop further growth of the male cells. At 10-min intervals, samples were withdrawn to measure β-galactosidase.[4] The efficiency of transfer of the lac^+ character was determined by plating. The β-galactosidase levels are given in arbitrary units normalized to equal transfer after subtraction of a male cell blank. ● ●● F′i^+z^+ males; ○○○ F′i^qz^+ males.

When cells carrying this *dlac* prophage are heat-induced, returned to low temperature, and harvested six hours after the induction, there is a 10- to 20-fold increase in the amount of repressor (Table 4). If the i^q mutation has been crossed into the phage genome, this increase, starting from the enhanced level of the i^q, now yields extracts in which 0.5 per cent of the protein is *lac* repressor.

TABLE 4. *Amount of repressor after phage induction.*

Strain	Specific activity of repressor	Strain	Specific activity of repressor
i^+	$\leqq 0.05$	i^q	0.25
ϕi^+	0.5	ϕi^q	10

Cells were grown in yeast tryptone medium at room temperature. The phage (λ *h80* C_{I857} *lac* t68) was induced by heating for 15 min to 43°. The cells were then cooled to 32° and grown for 6–7 hr. Repressor was determined as described under Table 1.

Summary—An *i*-gene mutant isolated from *E. coli* makes ten times the normal amount of *lac* repressor. After heat induction of a prophage carrying this mutated *i* gene, 0.5 per cent of the soluble protein of the cell is repressor.

Experimental Details.—Isolation of the repressor overproducing mutants: Bacteria, either a strain carrying an i^{TSS} mutation (isolated by us) or a strain carrying an i^{TL} mutation (strain E103 from Sadler and Novick[2]), were treated with N-methyl-N′-nitro-N-nitrosoguanidine as described previously.[8] After segregation in rich medium and adaptation to mineral medium at 44°, 5×10^8 bacteria were inoculated at 44° into 100 ml of mineral medium M56[3] containing 2×10^{-2} *M* glycerin and 3×10^{-3} *M* TONPG (o-nitrophenyl-1-thio-β-D-galactopyranoside). The TONPG inhibits lac^+ constitutive bacteria from growing but allows growth of lac^+ wild type. The selection was repeated with 10^7 bacteria in the same volume of fresh M56, glycerin, and TONPG. The bacteria were then plated on minimal plates containing 10^{-2} *M* lactose and 10^{-4} *M* IPTG at 44°. Large colonies were picked and their β-galactosidase level was determined after growth at 44°. These strains were tested *in vitro* for the presence of excess repressor. Three out of six in the case of the i^{TSS} gave better binding to IPTG than the wild type. One of them was examined in greater detail and was called i^q.

Construction of the strain carrying the phage λ *h80* c_{I857} *dlac* i^q t_{68}: A phage stock of λt_{68}[9] was obtained from E. Signer. This mutation prevents lysis (although not the production of phage lysozyme) and furthermore prevents the shut-off of all functions that normally occurs about an hour after induction, whether or not the cell lyses. A temperature-sensitive allele of this mutation, ts_{9b}, leads to phage overproduction: one infers that the totally defective form does so too. We constructed the single lysogen of the defective phage carrying both the *dlac* and the t_{68} character in the following way: the t_{68} mutation was introduced into a *dlac* prophage by the recombination act associated with the formation of a double lysogen upon infection of λt_{68} into the single lysogen, *K* 12 *RV* (λ *h80* c_{I857} *dlac*). The double lysogen was selected for by growth at 41°C. The single lysogen (λ *h80* c_{I857} t_{68} *dlac*) was isolated by transduction from an Hft obtained from this double lysogen. The λ *h80* c_{I857} *dlac* single lysogen was constructed from a double lysogen[10–12] obtained from E. Signer. The i^q marker was introduced by inserting an $F'lac\ i^q z^+ y^-$ into the single lysogen. Lac^- segregants were isolated and scored for the i^q/i^q property.

Growth and heat induction of phage carrying strains: If not indicated otherwise, bacteria were grown in rich medium containing 16 gm bactotryptone, 10 gm yeast extract, and 5 gm NaCl/l to saturation at room temperature. For phage induction, an exponential culture at an OD_{550} of 1 was heated to 43° for 15 min, then cooled to 32°. The bacteria were harvested after 6–7 hr further growth.

Assay of repressor and β-galactosidase: As described previously.[1, 4]

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