The Role of the Regulator-Gene Product (Repressor) in Catabolite Repression of β -Galactosidase Synthesis in *Escherichia coli*

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1. The specific role of the *lac* repressor (*i*-gene product) in transient catabolite repression evoked by the introduction of glucose into the medium has been investigated in *Escherichia coli* by using mutants of the *i*-gene. 2. A temperature-sensitive mutant (*i*^{TL}) is normally inducible and demonstrates transient repression when grown at 32°. At 42° it is about 20% constitutive and transient catabolite repression is abolished. 3. A strain carrying an amber suppressor-sensitive mutation in the *i*-gene is phenotypically constitutive and also fails to show transient catabolite repression. 4. Insertion of *Flac*⁺ into this strain restores both inducibility and transient repression. 5. It is concluded that the *i*-gene product interacts with the catabolite co-repressor in such a way that its affinity for the operator is increased.

It has previously been shown (Palmer & Moses, 1967) that acute transient catabolite repression of β -galactosidase synthesis, observed when glucose is added to glycerol-grown cells of *Escherichia coli* (Moses & Prevost, 1966), requires the presence of a functional operator gene (*o*). The specific involvement of the *i*-gene product (repressor) was not then tested, since a regulator-gene deletion mutant (*i*^{del}) was not available to us. Though such a deletion mutant has still not been isolated to the best of our knowledge, the isolation of certain temperature-sensitive control mutants by Sadler & Novick (1965) and of amber suppressor-sensitive mutants by Müller-Hill (1966) has made possible an investigation of the role of the repressor in acute transient repression.

Two types of temperature-sensitive regulatory mutants have been obtained (Sadler & Novick, 1965). In one series the repressor itself appeared to be increasingly unstable as the temperature was raised, so that growth at 42° produced a cell largely constitutive in character, whereas the same strain cultured at 32° was typically inducible. This mutation has been designated *i*^{TL}. In another series (*i*^{TSS}) the synthesis of the repressor appeared to be prevented at high temperature although the repressor, once formed, was not destroyed by heat. There is some doubt, however, about the status of the repressor in the *i*^{TSS} mutants. Sadler & Novick (1965) observed that such strains acquired active repressor unusually rapidly when transferred from a high growth temperature, believed to prevent repressor synthesis, to a temperature at which

repressor synthesis was possible. Further, the constitutive synthesis of β -galactosidase at high temperatures is susceptible to inhibition by *o*-nitrophenyl β -fucoside (J. Palmer, unpublished work), a substance regarded as an activator of the repressor rather than an antagonist of the inducer (Jayaraman, Müller-Hill & Rickenberg, 1966). It is thus probable that some repressor functions in *i*^{TSS} strains are reversibly inactivated at high temperature, and it is possible that other repressor functions might persist under these conditions. Lowering the ambient temperature of the cells from 42° to 32° might permit spontaneous reactivation of a heat-inactivated repressor, accounting for the rapid onset of inducibility reported by Sadler & Novick (1965). We have therefore confined our studies of transient repression in heat-sensitive strains to the *i*^{TL} mutation. Investigation of the catabolite-repression behaviour of the *i*^{TL} mutant at 42° and 32°, and comparison with its wild-type parent, has allowed a more definite correlation than previously possible between the functional state of the repressor and the transient repression effect.

A further test has been made with an amber suppressor-sensitive mutant of the *i*-gene (*i*^{-sus}), comparing this with the behaviour of a diploid strain also carrying a wild-type *i*-gene (*i*^{-sus}/*i*⁺). The *i*^{-sus} mutant differs from *i*⁻ strains in that it carries a chain-terminating codon that leads in effect to a functional deletion of the *i*-gene rather than to a point mutation. Chain-terminating mutations of the *i*-gene may be suppressed by appropriate suppressor genes (Bourgeois, Cohn & Orgel, 1965).

Thus in the $i^{-\text{sus}}$ mutant all functions of the repressor are eliminated simultaneously, and the role of the i -gene may then be studied by introducing i^{+} .

MATERIALS AND METHODS

Organisms. The strains of *E. coli* used are listed with their relevant genetic characters. Strains E103 ($\text{Hfr}^{i^{\text{TL}} o^{+} z^{+}}$) and E102 (i^{+} parent of E103) were obtained from A. Novick, and their properties are described by Sadler & Novick (1965) and by Horiuchi, Horiuchi & Novick (1963); the i^{TL} mutant differs from its parent only by lesions in the i -gene of the *lac* operon, and selection procedures during its isolation would not be expected to select other genotypic changes. Strains 112-12-A-84 ($\text{F}^{-} i^{-\text{sus}} o^{+} z^{+} \text{his}^{-} \text{cys}^{-} \text{gal}^{-} \text{su}^{-}$) and JC2637 ($\text{thr}^{-} \text{leu}^{-} \text{pro}^{-} \text{met}^{-} \text{B}_1^{-} \text{Sm}^{\text{R}}/\text{F} \text{lac } i^{+} o^{+} z^{+} y^{+}$) were gifts from B. Müller-Hill and A. J. Clark respectively.

Growth conditions. The cells were grown at 32°, 37° or 42° in medium 63 (Pardee & Prestidge, 1961) containing 22 mM-glycerol and supplemented with the necessary specific nutrients. Temperatures were controlled to $\pm 0.5^{\circ}$ in a water bath. Aeration was provided by shaking or by magnetic stirring. Growth was measured as described by Moses & Prevost (1966); at an extinction of 1.0 at 650 m μ the cultures contained 225 μg . of bacterial protein/ml. Glucose was introduced to the cultures where indicated by adding M-glucose solution to a final concentration of 10 mM.

Enzyme induction and assay. β -Galactosidase synthesis was induced, where indicated, with 0.5 mM-isopropyl thio- β -D-galactopyranoside. Since many measurements

were made of constitutive enzyme synthesis, particular care was exercised to minimize errors in sampling volume. Samples (0.05–0.20 ml.) of culture were added to clean weighed 10 ml. shell vials containing 0.2 ml. of chloroamphenicol solution (1 mg./ml.), mixed and reweighed to correct for volume errors. Before assay, 1 drop (about 6 μl .) of toluene was added to each of the samples, and these were stoppered and shaken for 30 min. at 37°. The stoppers were then removed and the toluene was allowed to evaporate for 30 min. at 37°. Enzyme activity was measured by the rate of hydrolysis of *o*-nitrophenyl β -D-galactoside, as described by Kepes (1963), with 0.8 ml. of 3.3 mM-substrate solution, and 0.8 ml. of 0.75 M- Na_2CO_3 to stop the reaction. About 100 mg. of solid BaCO_3 was added to each vial, and after the suspension had been mixed the vials were centrifuged for 1 min. at 1400 g in a bench-top centrifuge to clarify the solution and remove turbidity due to the bacteria. The amount of enzyme catalysing the hydrolysis of 1 μmole of substrate/min. at 37° is defined as 1 unit.

Mating experiments. Strain JC2637 was used to insert i^{+} into the $i^{-\text{sus}}$ strain 112-12-A-84. The requirement of strain JC2637 for proline was confirmed; colonies of this strain failed to grow for at least 7 days in the absence of added proline. About 2×10^8 cells of the donor/ml. were incubated with 1×10^8 cells of the recipient strain/ml. for 2 hr. at 37° without agitation in glycerol-minimal medium containing all the growth requirements for both strains. The culture was then diluted with buffer and plated on glycerol-minimal plates containing histidine and cysteine to select for the recipient strain and against the donor. When visible colonies were obtained the plates were inverted for 1–2 min. over filter paper soaked with toluene and the colonies were

Fig. 1. Repression by glucose of β -galactosidase synthesis in strain E102 at (a) 32° and (b) 42°. Isopropyl thio- β -D-galactopyranoside (0.5 mM) was added at $\downarrow$ and glucose (10 mM) at $\uparrow$. Mass-doubling times (min.) before and after glucose addition and length of transient repression (min.) were respectively: at 32°: 78, 77, 25; at 42°: 70, 66, 29.

Fig. 2. Repression by glucose of β -galactosidase synthesis in strain E103 at (a) 32° and (b) 42°. Isopropyl thio- β -D-galactopyranoside (0.5 mM) was added at $\downarrow$ and glucose (10 mM) at $\uparrow$. Mass-doubling times (min.) before and after glucose addition and length of transient repression (min.) were respectively: at 32°: 70, 72, 23; at 42°: 118, 65, no transient repression.

then tested for β -galactosidase activity with sterile *o*-nitrophenyl β -D-galactoside solution. Colonies showing no nitrophenol colour for at least 5 min. were replated and further colonies selected that showed no hydrolysis of *o*-nitrophenyl β -D-galactoside for 1 hr. These were found to be *lac*⁺ on eosin-methylene blue-lactose plates. One isolate was designated PM1 and was used for further studies. It showed the same growth requirements as the recipient parent and not those of the donor. Isolate PM1 is therefore *i*^{-sus}*o*⁺*z*⁺*y*⁺/*Fi*⁺*o*⁺*z*⁺*y*⁺.

RESULTS

Effect of temperature on transient repression in i^{TL} *and i*⁺ *strains.* The effect on β -galactosidase synthesis of adding glucose to cells growing on glycerol at 32° and 42° is shown for the *i*⁺ strain E102 (Fig. 1) and the *i*^{TL} strain E103 (Fig. 2). Both strains had similar growth responses to the addition of glucose; they showed little change in doubling time when glucose was added at 32°, but a 10–25% increase in the growth rate when glucose was introduced at 42°. For strain E102 a transient repression of enzyme synthesis was observed at both temperatures, lasting about the same time in each case. Similar experiments performed with strain E103 showed that transient repression, though present at 32°, was no longer evident at 42° (Fig. 2). The mutation from *i*⁺ to *i*^{TL} thus abolished acute

transient repression and simultaneously permitted a partially constitutive synthesis of β -galactosidase when the cells were grown at 42°.

Effect of glucose on i^{-sus} *and i*^{-sus}/*i*⁺ *strains.* Addition of glucose to *i*^{-sus} cells growing on glycerol increased the growth rate by 11%. No transient repression of β -galactosidase synthesis was observed, and the steady differential rate of enzyme synthesis fell immediately to 61% of the rate in the absence of glucose (Fig. 3). When glucose was added to the *i*^{-sus}/*i*⁺ diploid cells, there was almost no effect on the growth rate, but transient repression was obtained (Fig. 3). Immediately after the addition of glucose the differential rate of enzyme synthesis fell by 79%; about 55 min. later (0.9 generation time) it had recovered to about 65% of the original rate on glycerol.

DISCUSSION

It was pointed out by Palmer & Moses (1967) that arguments excluding repressor from a role in transient repression are not acceptable when based on the fact that *i*⁻ constitutive mutants exhibit such repression. It is possible that *i*⁻ point mutants possess an altered repressor that, though it has lost its normal capacity to bind to the operator, may be activated to do so under metabolic conditions giving

Fig. 3. Repression by glucose of β -galactosidase synthesis (a) in strain 112-12-A-84 (i^{-sus}) and (b) in strain PM1 (i^{-sus}/i^{+}) at 37° . Isopropyl thio- β -D-galactopyranoside (0.5 mM) was added at $\downarrow$ and glucose (10 mM) at $\uparrow$. Mass-doubling times (min.) before and after glucose addition and length of transient repression (min.) were respectively: strain 112-12-A-84: 59, 53, 0; strain PM1: 60, 60, 55.

rise to catabolite repression. Similarly, the observation of repression by glucose of β -galactosidase synthesis during the 1 hr. period of constitutive synthesis of the enzyme after transfer by conjugation of $i^{+}z^{+}$ into lac -deletion recipients (Loomis & Magasanik, 1964) also does not eliminate repression from the mechanism of catabolite repression. One might argue that repressor is effective at lower concentrations in the presence of glucose metabolites. Barbour & Pardee (1966) have indeed obtained evidence that the repressor or its precursor is present in low concentration from a very early period during mating.

The observation that transient repression depends on a functional operator gene (Palmer & Moses, 1967) strongly suggests the likelihood of the repressor also being involved. A formal test of this possibility requires a comparison of the glucose effect in related strains, one of which contains the repressor and the other of which does not. Such a comparison cannot yet be made with a deletion mutant, but the i^{TL} temperature-sensitive regulator strain and the i^{-sus} mutant offer opportunities of testing repressor involvement.

In comparing strain E103 with its parent strain E102 we note that the change from i^{+} to i^{TL} simultaneously rendered the synthesis of β -galactosidase about 20% constitutive at 42° and at that tempera-

ture eliminated the transient repression due to glucose. At 32° the i^{TL} strain was inducible and showed transient repression. We attempted to insert $Flac^{+}$ from strain JC2637 into an F^{-} phenotype (Lederberg, Cavalli & Lederberg, 1952) of strain E103, but we failed to isolate an i^{TL}/i^{+} diploid. A successful isolation would have permitted a more rigorous test of these changes arising entirely as a result of mutation in the i -gene.

The absence of transient repression in the i^{-sus} strain supports the idea of a role for the repressor in this phenomenon. The repressor in this strain has presumably lost affinity for the operator, giving rise to the constitutive phenotype. Gilbert & Müller-Hill (1966) have shown that this repressor has also lost affinity for the inducer. This argues strongly for a large functional deletion in the i -gene and is thus consistent with transient catabolite repression being mediated by the i -gene product. The simultaneous restoration of both inducibility and transient repression by the insertion of i^{+} into i^{-sus} confirms this conclusion.

The observed relation of transient catabolite repression to various mutations in the i -gene may be explained by a model in which the repressor is a trivalent molecule, rather than the bivalent entity originally suggested by Jacob & Monod (1961). This concept has previously been briefly

discussed by Clarke & Brammar (1964), and was also considered by Loomis & Magasanik (1964) but was rejected by them for reasons that were subsequently criticized (Clarke & Brammar, 1964; Palmer & Moses, 1967). The three postulated sites on the repressor would be an inducer-interacting site (I), an operator-interacting site (O), and a catabolite co-repressor-interacting site (CR). According to this model, the O-site interacts with the operator in the absence of inducer and prevents transcription. In the presence of inducer the I-site binds to the inducer, and the O-site is allosterically modified in such a way that it no longer has affinity for the operator and transcription proceeds (Monod, Changeux & Jacob, 1963). The CR-site interacts with the catabolite co-repressor and allosterically increases the affinity of the O-site for the operator. Thus the CR- and I-sites have opposite effects on the degree of binding of the repressor to the operator and can be viewed as being competitive. Evidence has been published indicating that induction and catabolite repression may be competitive phenomena (Clark & Marr, 1964).

Each of the proposed three repressor sites may be conceived as existing in several allelic forms, giving rise to the following genotypes: I^+ , repressor is capable of interacting reversibly with inducer and thus losing affinity for the operator; I^- , repressor is incapable of interacting with inducer; I^{++} , repressor binds to inducer more tightly than normal; CR^+ , repressor is capable of interacting reversibly with the catabolite co-repressor and thus increasing affinity for the operator; CR^- , repressor is incapable of interacting with the catabolite co-repressor; CR^{++} , repressor binds to the catabolite co-repressor more tightly than normal; O^+ , repressor is capable of interacting reversibly with the operator; O^- , repressor is incapable of interacting with the operator; O^{++} , repressor binds to operator more tightly than normal.

To account for the presence of transient catabolite repression in i^- constitutive mutants, it is also necessary to postulate a further allele of the O-site: O^{CR} , repressor interacts with the operator only when the CR-site is occupied by the catabolite co-repressor.

By combining the appropriate alleles for the three sites into one gene all the known phenotypic variations of transient catabolite repression behaviour can be accounted for by this model.

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REFERENCES

- Barbour, S. D. & Pardee, A. B. (1966). *J. molec. Biol.* **20**, 505.
- Bourgeois, S., Cohn, M. & Orgel, L. E. (1965). *J. molec. Biol.* **14**, 300.
- Clark, D. J. & Marr, A. G. (1964). *Biochim. biophys. Acta*, **93**, 85.
- Clarke, P. H. & Brammar, W. J. (1964). *Nature, Lond.*, **203**, 1153.
- Gilbert, W. & Müller-Hill, B. (1966). *Proc. nat. Acad. Sci., Wash.*, **56**, 1891.
- Horiuchi, T., Horiuchi, S. & Novick, A. (1963). *Genetics*, **48**, 157.
- Jacob, F. & Monod, J. (1961). *J. molec. Biol.* **3**, 318.
- Jayaraman, K., Müller-Hill, B. & Rickenberg, H. V. (1966). *J. molec. Biol.* **18**, 339.
- Kepes, A. (1963). *Biochim. biophys. Acta*, **76**, 293.
- Lederberg, J., Cavalli, L. L. & Lederberg, E. M. (1952). *Genetics*, **37**, 720.
- Loomis, W. F., jun. & Magasanik, B. (1964). *J. molec. Biol.* **8**, 417.
- Monod, J., Changeux, J.-P. & Jacob, F. (1963). *J. molec. Biol.* **6**, 306.
- Moses, V. & Prevost, C. (1966). *Biochem. J.* **100**, 336.
- Müller-Hill, B. (1966). *J. molec. Biol.* **15**, 374.
- Palmer, J. & Moses, V. (1967). *Biochem. J.* **103**, 358.
- Pardee, A. B. & Prestidge, L. S. (1961). *Biochim. biophys. Acta*, **49**, 77.
- Sadler, J. R. & Novick, A. (1965). *J. molec. Biol.* **12**, 305.