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Further Inducibility of a Constitutive System: Ultrainduction of the *gal* Operon

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In wild-type *Escherichia coli*, expression of the *gal* operon is negatively regulated by the Gal repressor and is induced 10- to 15-fold when the repressor is inactivated by an inducer. In strains completely deleted for *galR*, the gene which encodes the Gal repressor, the operon is derepressed by only 10-fold without an inducer. But this derepression is increased further by threefold during cell growth in the presence of an inducer, D-galactose or D-fucose. This phenomenon of extreme induction in the absence of Gal repressor is termed ultrainduction—a manifestation of further inducibility in a constitutive setup. Construction and characterization of gene and operon fusion strains between *galE* and *lacZ*, encoding β-galactosidase as a reporter gene, show that ultrainduction occurs at the level of transcription and not translation. Transcription of the operon, from both the cyclic AMP-dependent *P1* and the cyclic nucleotide-independent *P2* promoters, is subject to ultrainduction. The wild-type *galR*$^+$ gene has an epistatic effect on ultrainducibility: ultrainduction is observed only in cells devoid of Gal repressor protein. Titration experiments show the existence of an ultrainducibility factor that acts like a repressor and functions by binding to DNA segments (operators) to which Gal repressor also binds to repress the operon.

In wild-type *Escherichia coli*, the synthesis of three enzymes (galactokinase, galactose-1-phosphate uridyltransferase, and uridine-diphosphoglucose 4-epimerase) of the *gal* operon is induced 10- to 15-fold by growing the cells in the presence of D-galactose or its nonmetabolizable analog, D-fucose (7). It has been shown that the inducer acts by inactivating the Gal repressor, which is the product of an unlinked gene, *galR* (1a, 8, 21, 22, 30). In the absence of an inducer, the Gal repressor binds to two *gal* operators, O_E and O_I, resulting in the repression of the operon (12, 16, 17, 21). Consistently, the expression of the *gal* operon is also derepressed when an effective repressor interaction is destroyed by mutation in *galR* or in O_E or O_I.

It has been noted by several investigators that in *E. coli* strains with mutations in the *galR* gene the derepressed expression of the operon can be further induced in the presence of an inducer (1, 30, 31). Such findings were explained by assuming residual activity of the mutant *galR* gene products. This observation has been investigated in the current study by the construction of strains completely devoid of the *galR* gene and then studying the effect of inducer on *gal* expression in them. The results show that the *gal* operon is subject to another regulatory factor whose effect is revealed in the complete absence of Gal repressor. The activity of the new regulatory factor is also modulated by D-fucose and D-galactose but is epistatic to the Gal repressor.

MATERIALS AND METHODS

Bacteria, bacteriophage, and plasmids. The bacterial strains, plasmids, and bacteriophage used in this study are listed on Table 1.

Microbiological media and biochemical buffers. The compositions of the media used in this study are as follows: LB (Luria-Bertani) broth contained 10 g of tryptone (Difco), 5 g of yeast (Difco), and 5 g of NaCl per liter; LB agar plates contained 15 g of Bacto-Agar per liter of LB broth; MacConkey-galactose-agar plates contained 40 g of MacConkey agar base (Difco) and 10 g of galactose per liter; TB agar plates contained 10 g of tryptone, 11 g of Bacto-Agar, and 5 g of NaCl per liter; TB top agar contained 7 g of tryptone and 6 g of Bacto-Agar per liter; super broth contained 32 g of tryptone, 20 g of yeast extract, 5 g of NaCl, and 5 ml of 1 N NaOH per liter; TBMM broth contained 10 g of tryptone, 5 g of NaCl, 1 mg of thiamine, 2 g of maltose, and 2.4 g of $MgSO_4$ per liter; M56 buffer contained 13.6 g of KH_2PO_4, 2.6 g of $(NH_4)_2SO_4$, 0.01 g of $CaCl_2$, and 0.5 mg of $FeSO_4 \cdot 7H_2O$ per liter adjusted to pH 7.0 with KOH; EMBO agar plates contained 10 g of tryptone, 1 g of yeast extract, 5 g of NaCl, 2 g of K_2PO_4, 0.085 g of methylene blue, 0.4 g of eosin Y, and 15 g of agar per liter; M56 agar plates contained 15 g of Bacto-Agar per liter of M56 buffer; DOG plates contained 0.2% 2-deoxygalactose, 0.2% glycerol, 0.1% Casamino Acids, 1 μg of thiamine per ml, and 15 g of Bacto-Agar per liter of M56 buffer; XG-DOG-KAN plates contained 50 μg of 4-chloro-3-indolyl-β-D-galactoside and 50 μg of kanamycin per ml (final concentrations).

The E buffer used for DNA electrophoresis and TE buffer used for DNA were described previously (28).

Enzymes and other materials. Restriction endonucleases and polynucleotide ligase were obtained from Bethesda Research Laboratories. A 1,300-bp Cm^r gene cassette was purified by digesting plasmid pBR325 with restriction enzyme *Fnu*DII.

DE81 filters were from Whatman. D-[1-^{14}C]Galactose (61 μCi/mmol) was purchased from Amersham. All other chemicals were of reagent grade and bought from Sigma Chemical Co.

Methods. The microbial genetic techniques employed were from previously published procedures (3, 9, 33). Plasmid DNA preparation and DNA manipulation were as described by Perbal (28). Restriction endonuclease digestions and DNA ligations were carried out as recommended by the manufacturers.

pGR17 was partially digested with the restriction enzyme

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TABLE 1. Bacteria, bacteriophages, and plasmids

Strain, phage, or plasmid	Description	Source or reference
E. coli K-12		
C600	F⁻ *thi thr-1 leuB6 supE lacY1 tonA21*	A. Campbell
N720	F⁺ *supF mel*	NIH[a] Collection
SA15	F′ *gal*⁺/*galT*	1
SA321	Δ[*gal*-(*attL-int*)λ*c*Its857]	11
SA500	F⁻ Str^r *his relA1*	3
SA1638	SA500 $galO_E^c$	2
SA1796	SA500 *galR*^{s}	2
SA2211	HfrH *galT*::[λ*cry*]	S. Adhya
SA2345	SA500 Δ*cya ilv-2*::Tn*10*	2
SA2700	SA500 Δ*gal*P2P1::Cm^r	A. Majumdar
SA2718	SA500 $galO_I^c$	16
MC4100	F⁻ *araD139* Δ(*arg-F-lac*)U169 *rpsL150 relA deoC1 ptsF25 rbsR*	S. Gottesman
JT33	SA500 Δ*galR*(A-C)::Cm^r	This study
JT34	SA500 Δ*galR*(B-C)::Cm^r	This study
JT37	SA2345 Δ*galR*(B-C)::Cm^r	This study
JT38	SA500 Δ*cya-ilv2*::Tn*10*	This study
JT61	SA1630 Δ*galR*(B-C)::Cm^r	This study
JT62	SA2718 Δ*galR*(B-C)::Cm^r	This study
JT210	MC4100 φL(*galE*::*lac*)[b]	This study
JT214	MC4100 φR(*galE*::lac)[c]	This study
JT224	JT214 Δ*galR*(B-C)::Cm^r	This study
JT226	JT210 Δ*galR*(B-C)::Cm^r	This study
JT234	F′ *gal*⁺/JT210	This study
JT235	F′ *gal*⁺/JT214	This study
JT245	F′ *gal*⁺/JT214	This study
JT247	F′ *gal*⁺/JT226	This study
Phages		
B99	λ*imm*^{21}cIts *gal*⁺	24
B505	λ*imm*^{21}cIts *galT*	24
B511	λ*imm*^{21}cIts *galK*	24
B776	λ*imm*^{21}cIts *galE*	24
W30	λcI-*b*-2	NIH collection
W248	λ*h*80 Δ(*att-int*)cI⁻	NIH collection
Y12	λcI857 *J*am *int*	NIH collection
Y196	λcI857 *S*am *galR*⁺	27
Y2050	λcI857 *galR*⁺ *int*	This study
P1*vir*	P1*vir*	D. Chattoraj
P1clr100	P1*clr*100 Cm^r	J. L. Rosner
λp*lac*Mu9	λplac Mu1 Kn^r	N. Trun
λp*lac*Mu53	λplac Mu50 Kn^r	N. Trun
λMu507	λcI857 *S*am *A*⁺ *B*⁺	N. Trun
Plasmids		
pBR322	Ap^r Tc^r	5
pGR17	pBR322 (*Pst*I)::*galR*⁺	22
p291	pBR322 Δ(*Eco*RI-*Hin*dIII):: *gal* $O_E^+O_I^+$	17
pH101	p291 $galO_E^-$	14
pH103	p291 $galO_I^-$	14
pH108	p291 $galO_E^-$-$galO_I^-$	14
pMT5	pGR17 Δ*galR*(A-C)::Cm^r	This study
pMT6	pGR17 Δ*galR*(B-C)::Cm^r	This study
pBR325	Cm^{r}Ap^r	4

[a] NIH, National Institutes of Health.
[b] The symbol φL for *galE*::*lac* fusion strains denotes a translational (gene) fusion. See the text.
[c] The symbol φR for *galE*::*lac* fusion strains denotes a transcriptional (operon) fusion. See the text.

*Hin*cII. DNA fragments of 7 to 9 kb were purified and recircularized in the presence of a 1,300-bp Cm^r gene block by T4 DNA ligase. pGR17 carries four *Hin*cII sites, three in or around bacterial *galR* DNA (marked A, B, and C in Fig. 1) and the fourth in the Tet^r gene of pBR322. The ligation products were used to transform strain C600 and select for Tet^r. The plasmid DNA of the Tet^r transformants was analyzed by restriction enzyme digestion; two of them, pMT5 and pMT6, were used further.

Cells were grown for assay of enzymes as described by Adhya and Miller (2). Galactokinase assays in toluene-treated cells were performed as described by Adhya and Miller (2), and β-galactosidase assays in sodium dodecyl sulfate-chloroform-treated cells were done by the procedure of Miller (25).

Y196 is a λ specialized transducing phage carrying the bacterial *galR* gene (27). The phage carries a temperature-sensitive repressor gene (*c*I857) and a lysis-defective amber mutation (*S*am7). The phage was crossed with λ strain Y12, which contains an integration-defective gene (*int*6), an amber mutation in the phage tail gene (*J*am6), and the *c*I857 mutation. A recombinant phage strain (Y2050) with the genotype *galR*⁺*int*6*S*⁺*J*⁺ was isolated and used as a vector to transfer *galR* mutations from plasmid to chromosome. Red plaque strain SA2211 was used to verify the Int phenotype (10). The GalR phenotypes were verified by spotting the recombinant phages on a *galR*s host strain on MacConkey-galactose-agar indicator plates (30, 32). Lysogens of λ were isolated by infecting appropriate hosts at 32°C and then selecting for immune colonies at 32°C on EMBO plates after challenging with *c*I*b*2 phage (W30). Cells cured of the prophage were isolated as colonies that survived on EMBO plates at 42°C. The frequency of such curing was about 10^{-3}.

Chromosomal fusion of *lac* structural genes to the *gal* promoters was performed by the phage transposition mechanism with two vector phage, λp*lac*Mu9 and λp*lac*Mu59, as described by Silhavy et al. (33). Insertions of the two phages into the chromosomal *gal* operon were selected on minimal XG-DOG plates (data not shown).

The location of the λp*lac*Mu insertions within the *gal* operon was determined by complementation spot tests with λ*gal*⁺, λ*galE*, λ*galT*, and λ*galK* tester phages on MacConkey-galactose-agar indicator plates (3, 9). When complementation occurred, there was bacterial growth of a solid red color at the spotted area after overnight incubation at 32°C when complementation did not occur, there was cell lysis on the first day and then red papillation due to recombination on the second day.

Since the *lac* operon transposed into the chromosome carries with it a Kn^r marker, the *gal*::*lac*-Kn^r fusions were transferred into a new background by phage P1 transduction with Kn^r as a marker and verified by a Gal⁻ phenotype.

The F′ *gal*⁺ episome was introduced into *gal*::*lac* fusion strains by F′-duction (1a, 9) with SA15 as donor and selection for Gal⁺ Str^r recombinants (9). A Δ*cya* marker was introduced into *gal*::*lac* fusion strains by phage P1 transduction with a neighboring Tet^r marker from an *ilv-2*::Tn*10* Δ*cya* strain (SA2345).

RESULTS

Bacterial strains carrying deletion-substitution mutations in the *galR* gene. *E. coli* strains were constructed in which the *galR* gene sequence was replaced by a chloramphenicol resistance (Cm^r) gene. The drug marker provided a positive selection for the deletion-replacement event. The constructions were made in plasmids (Fig. 1); then the engineered *galR* genes, by homologous recombination, were transferred first from the plasmid to a specialized λ transducing phage vector containing the *galR* gene and flanking regions and

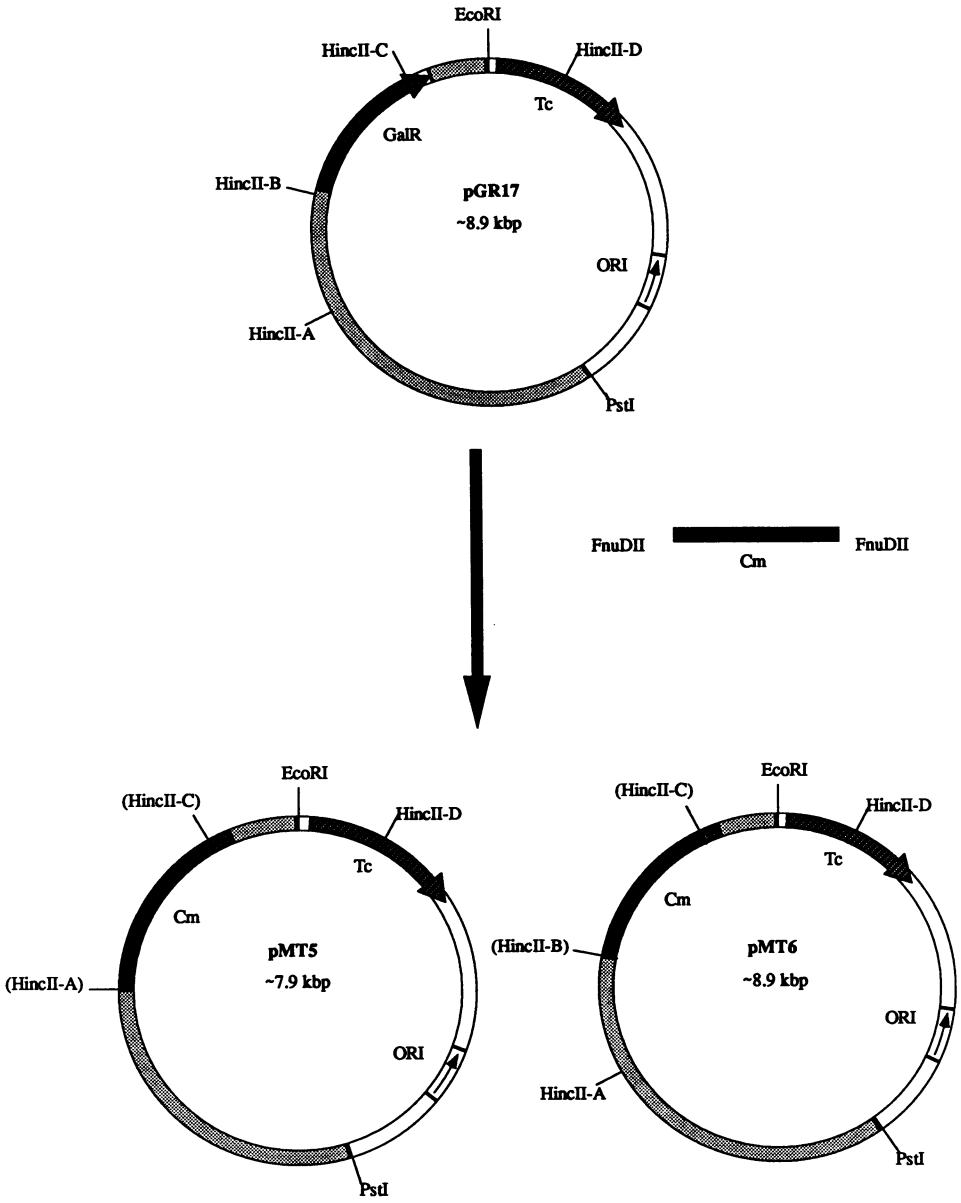

FIG. 1. Structure of mutants with deletions of the *galR* gene in plasmid vector. The construction details of pMT5 and pMT6 are described in text. *E. coli* DNA in pGR17 extends clockwise from the *Pst*I site to the *Eco*RI site. The structures of the plasmids were confirmed by restriction digestion analysis with the markers *Pst*I, *Hin*dIII and *Eco*RI. The approximate size of each plasmid is indicated in the center. The deletion-substitution isolates were at the three *Hin*cII sites (A, B, and C) in the combinations shown. pMT5 carries a Cm^r substitution from sites A to C, and pMT6 carries a Cm^r substitution from sites B to C. The fourth *Hin*cII site (D) is within the selected Tet^r gene.

finally from the phage to the *E. coli* chromosome as follows (Fig. 2) (23). Plasmid pGR17 contains the $galR^+$ gene subcloned between the *Pst*I and *Eco*RI restriction sites of pBR322 (22). Two different plasmids, pMT5 and pMT6, were made in which the amino-terminal 80% of the *galR* gene, including its promoter, is replaced by the Cm^r marker (Fig. 1). The substitution nature of pMT5 and pMT6 was verified by mapping restriction sites predicted from the known DNA sequences of the *galR* gene and Cm^r cassette. The substitutions in these plasmids are termed $\Delta galR$ (A-C)::Cm^r and $\Delta galR$ (B-C)::Cm^r, indicating that the *galR* gene from either the *Hin*cII-A site to the *Hin*cII-C site or the *Hin*cII-B site to the *Hin*cII-C site has been deleted and replaced by the Cm^r gene. λ phages carrying the Cm^r substitution-deletion mutation of *galR*, λ$\Delta galR$(A-C)::Cm^r and λ$\Delta galR$(B-C)::Cm^r were isolated by growing a λ$galR^+$ phage (Y2050) on hosts carrying plasmids pMT5 and pMT6, respectively, and then selecting for Cm^r lysogens in a $galR^+$ (SA500) host (Fig. 2A through C). The lysogens were formed by homologous recombinations between phage and bacterial *galR* regions and are diploid ($galR^+/\Delta galR$) for the *galR* regions. The bacterial chromosome was made haploid for the $\Delta galR$ (A-C)::Cm^r and $\Delta galR$(B-C)::Cm^r markers by isolating bacteria cured of the prophage, which have left behind the substitution mutations in the chromosome, as temperature-resistant survivors of the lysogens, which are also Cm^r (Fig. 2C through E).

The two $\Delta galR$ bacterial strains thus constructed (JT33

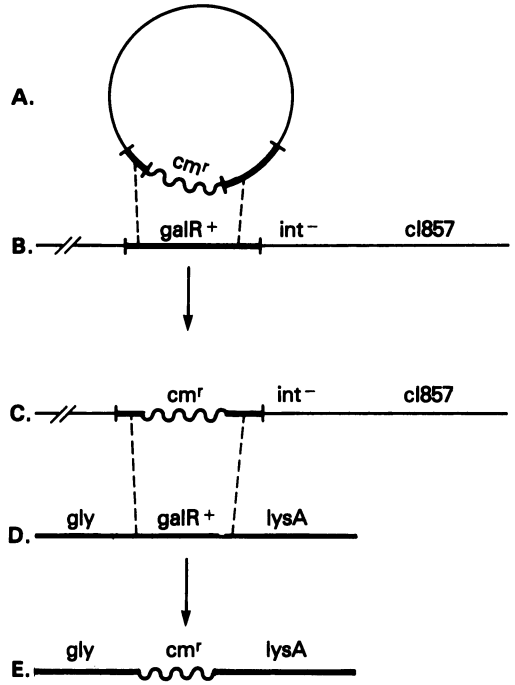

FIG. 2. Principle of transfer of the plasmid carrying the drug resistance marker Cm^r in the *galR* gene to the chromosome of *E. coli* (16). Thin lines represent vector plasmid or phage DNA, thick lines represent bacterial DNA, and wavy lines represent the Cm^r gene. (A) Plasmid in which a Cm^r marker was engineered into *galR*, replacing a large part of the latter. (B) λ phage vector (Y2050) carrying the $galR^+$ gene. A double crossover between the phage and the plasmid in the homology region, as shown, would generate a phage in which the drug resistance marker is transferred from the plasmid-carried *galR* gene to the phage (C). (D) The *galR* segment of the *E. coli* chromosome. *gly* and *lysA* are two neighboring chromosomal markers of *galR*. Two separate single crossover events between the phage, carrying the drug marker in the *galR* gene, and the bacterial chromosome in the regions indicated would generate an *E. coli* strain in which the *galR* gene carries an engineered deletion-substitution (E). The two-step transfer of a marker from the phage (C) into chromosome (D) is achieved first by lysogenizing the phage into the chromosome by a homologous recombination event as indicated (one of the two crossovers) and then by curing the prophage by the other crossover (C). The details are described in text.

and JT34) were tested for the absence of the Gal repressor by immunoblot assay of their extracts with anti-Gal repressor antibody. Neither JT33 or JT34 showed the presence of the Gal repressor (data not shown).

Galactokinase synthesis in mutant strains devoid of Gal repressor. Strains in which the *galR* gene was substituted by a drug resistance marker were tested for the expression of the *gal* operon by measuring the level of galactokinase after the cells were grown in the absence and presence of D-fucose. The results of enzyme assays in the wild type (SA500) and Δ*galR*(B-C)::Cm^r (JT34) are shown in Table 2. Wild-type $galR^+$ cells showed a low basal level of galactokinase when grown without an inducer and about 12-fold higher level of the enzyme when grown with D-fucose. In the absence of an inducer the level of galactokinase in the Δ*galR* strain was 10-fold higher than that of $galR^+$ strain, as expected. However, D-fucose addition caused a 1.5-fold further increase in the specific activity of galactokinase in

TABLE 2. Induction of galactokinase in wild-type and mutant strains of *E. coli*

Strain	Description	Sp act of galactokinase[a]	
		− Inducer	+ Inducer
SA500	Wild type	6.0	75
JT34	Δ*galR*(B-C)::Cm^r	58	85
SA1638	$galO_E^c$	103	118
JT61	Δ*galR* $galO_E^c$	130	130
SA2718	$galO_I^c$	72	109
JT62	Δ*galR* $galO_I^c$	148	145
JT38	Δ*cya*	<1.0	22.9
JT37	Δ*galR*(B-C)::Cm^r Δ*cya*	13.0	43.0

[a] Galactokinase was assayed in lysed cells after 4 h of growth at 37°C in minimal M56 medium containing 0.3% fructose as the carbon source, 0.1% Casamino Acids, and either 5 mM D-fucose (strains SA500, JT34, SA1638, JT61, SA2718, and JT62) or 0.3% D-galactose (strains JT38 and JT37) as an inducer when present. The specific activities of galactokinase are given as nanomoles of galactose 1-phosphate generated per minute per 10^9 cells. Assays of separate extracts from cells of the same strains grown under the same conditions generally showed approximately 15 to 20% variability for levels of enzymes.

JT34. The measurement of differential rate of galactokinase synthesis in SA500 and JT34 showed a threefold higher rate in the Δ*galR* strain than in the wild type (Fig. 3). Similar results were obtained with the Δ*galR*(A-C)::Cm^r strain (JT33) and when D-galactose, instead of D-fucose, was used as an inducer (data not shown).

Galactokinase synthesis in cells with mutant *gal* operators. Strains SA1630 ($galO_E^c$) and SA2718 ($galO_I^c$) were made Δ*galR*(B-C)::Cm^r and used to investigate the induction of the *gal* operon. When galactokinase synthesis was assayed in $galO_E^c$ and Δ*galR* $galO_E^c$ strains (SA1638 and JT61), no appreciable difference was observed in galactokinase specific activity in the two cultures grown with or without D-fucose in the medium (Table 2). In other words, the

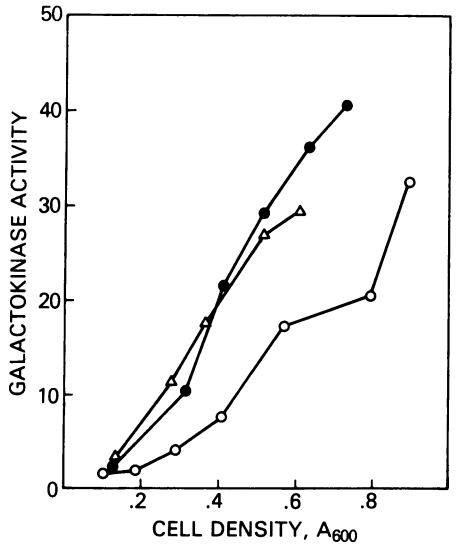

FIG. 3. Differential rate of β-galactosidase synthesis in a Δ*galR* strain. Cell growth and enzyme assays are as described in footnote *a* of Table 2. Symbols: ○, JT34; ●, JT34 grown in the presence of D-fucose; △, JT34 grown in the presence of D-galactose. Total galactokinase activity is plotted against cell growth as measured by A_{600}.

expression of the *gal* operon was constitutive and equally high in both strains, showing no further induction in the presence of inducer. The strain with the $galO_I^c$ mutation (SA2718) did show induction by D-fucose, but the strain with $galO_I^c$ Δ*galR* double mutations (JT62), like the strain with the double $galO_E^c$ Δ*galR* mutations, showed a very high galactokinase level with no further increase when the inducer was present (Table 2). These findings that the expression of the *gal* operon has reached the highest level in the $galO^c$ Δ*galR* strains in the absence of D-fucose indicate that the DNA sequences defined by the *gal* operator mutations are contributing elements of the induction effect seen in the absence of the Gal repressor.

Induction of the expression from the *P2* promoter. The experiments discussed above established further inducibility of the *gal* operon in bacterial strains with normal levels of cyclic AMP and its receptor protein, i.e., when the *gal* operon is expressed from the *P1* promoter (2, 26). Under these conditions, the activity of the *P2* promoter is inhibited. To determine whether the *P2* promoter is also subject to similar induction, the Δ*galR*(B-C)::Cm^r strain JT34 was transduced with a Δ*cya* (adenyl cyclase deleted mutation) marker by phage P1. The absence of cyclic AMP causes inactivation of *P1* and activation of *P2*. The Δ*galR*(B-C):: Cm^r Δ*cya* strain JT37 was then assayed for galactokinase activity after it was grown in the absence or presence of inducer. When the Δ*cya* mutation made the *P2* promoter active, as reported previously (2, 15), the Δ*galR* Δ*cya* derivative showed threefold induction in the presence of inducer, demonstrating that additional inducibility also occurs when the *gal* operon is expressed from the *P2* promoter (Table 1). It should be noted that for these experiments induction was performed with D-galactose only, because D-fucose is not taken up by Δ*cya* cells (1).

Study of *gal* expression in transcriptional and translational fusion strains. To investigate whether the additional induction of the *gal* operon in the absence of Gal repressor occurs at the level of transcription or translation, the structural gene of β-galactosidase (*lacZ*) was fused to the *gal* promoters in a strain completely deleted for the *lac* operon (MC4100) by the procedure of Silhavy and his colleagues with two λ phage strains, λp*lac*Mu9 and λp*lac*Mu53 (6, 33). The two phage create fusions by inserting the *lacZ* gene by the DNA transposition mechanism of phage Mu. λp*lac*Mu9 mediated the construction of translational (gene) *gal*::*lac* fusions. λp*lac*Mu53, on the other hand, created transcriptional (operon) *gal*::*lac* fusions. The transposition-mediated fusion events inactivated one or more of the *gal* cistrons as revealed by complementation tests performed with infecting λ*gal* specialized transducing phages carrying nonpolar mutations in the individual *gal* cistrons (24). Of the many independent fusions characterized this way, two—one gene fusion and one operon fusion—in the *galE* gene were used in this study. Because of a likelihood of multiple insertions of the λp*lac*Mu phages, the *galE*::*lac* fusions were transferred into the original MC4100 strain by transducing phage P1, and these transductants were used further.

That the expression of β-galactosidase in the fusion strains was under the control of *gal* promoters was confirmed by deletion analysis. A deletion mutation of the *gal* promoter region from strain SA2700 was transduced into the *galE*::*lac* fusions by phage P1 transduction. The promoterless strains were all Lac$^-$, and no β-galactosidase synthesis was detected in them either in the absence or presence of D-fucose.

In the two *galE*::*lac* fusions, the expression of the *gal* operon was monitored in the presence or absence of D-fucose by determining the levels of β-galactosidase (Table 3). Although the absolute levels of β-galactosidase activity vary, both strains show some induction by D-fucose, confirming that the *lacZ* gene in these strains is indeed regulated by the *gal* promoter (in this case, *P1*). The inducibility, unlike that of the wild-type *gal* operon, ranges from three- to fivefold. The basal level of expression in the two strains is high because an insertion mutation in *galE* abolishes the expression of the *galK* gene because of a polar effect (3, 18, 32). A galactokinase deficiency causes accumulation of D-galactose in cells by anabolic reactions and induces the *gal* operon. This is known as internal induction (19). This kinaseless constitutivity of the *gal* operon is recessive to the presence of an F′ *gal*$^+$ episome, under which condition the internal D-galactose is metabolized, thus preventing accumulation. Therefore, further studies of the fusion strains were carried out only after converting them to Gal$^+$ by introduction of an F′ *gal*$^+$ episome (9). β-Galactosidase synthesis was measured after F′ duction, i.e., in F′ gal$^+$/*galE*::*lac* strains in which internal induction should be eliminated, after the cells were grown in the absence and presence of D-fucose. As expected, a Gal$^+$ phenotype decreased the basal level and consequently restored a normal degree of *gal* induction by D-fucose (Table 3). To study the additional inducibility of the *gal* operon in the absence of Gal repressor, the F′ *gal*$^+$/*galE*::*lac* fusion strains were made Δ*galR* by P1 transduction with JT34 as the donor. Removal of the Gal repressor by deletion of the *galR* gene brought 10-fold derepression of the *gal* operon in all strains grown in the absence of D-fucose, as expected (Table 3). Most importantly, in both gene and operon fusions, cell growth in the presence of D-fucose caused an additional threefold or higher level of β-galactosidase synthesis, as observed by measuring the level of galactokinase. The subsequent studies of inducibility in a Δ*galR* strain were carried out in the F′ *gal*$^+$/Δ*galR* *galE*::*lacZ* operon fusion strain JT245.

TABLE 3. β-Galactosidase expression in *gal*::*lac* fusion strains[a]

Strain	Description	Fusion type	Level of β-galactosidase		Fold induction
			− D-fucose	+ D-fucose	
JT210	*galE*::*lacZ*	Gene	601	1,780	3.0
JT214	*galE*::*lacZ*	Operon	682	3,180	4.7
JT234	F′ *gal*$^+$/*galE*::*lacZ*	Gene	76	1,010	13.3
JT235	F′ *gal*$^+$/*galE*::*lacZ*	Operon	93	1,180	12.7
JT247	F′ *gal*$^+$/*galE*::*lacZ* Δ*galR*	Gene	950	3,860	4.0
JT245	F′ *gal*$^+$/*galE*::*lacZ* Δ*galR*	Operon	1,021	2,720	2.7

[a] β-Galactosidase is expressed in Miller units. Cell cultures were grown as described in footnote *a* of Table 1. The measured specific activity of β-galactosidase in a given strain varied from day to day depending upon inoculum size, age of culture, and medium, but the ratios of the specific activities of induced and uninduced cultures were always about the same. The data from a typical experiment are shown.

Induction in a Δ*galR* strain transformed with a plasmid carrying the *gal* operon control DNA sequences. The rationale of this experiment is based on the previous observations (13, 17) that a multicopy plasmid bearing the *gal* operator region, not necessarily the structural genes, titrates out the Gal repressor, leading to derepression of the single-copy chromosomal *gal* operon in the absence of an inducer. The same strategy was adopted to determine whether a potential repressorlike factor additionally regulates the *gal* operon and

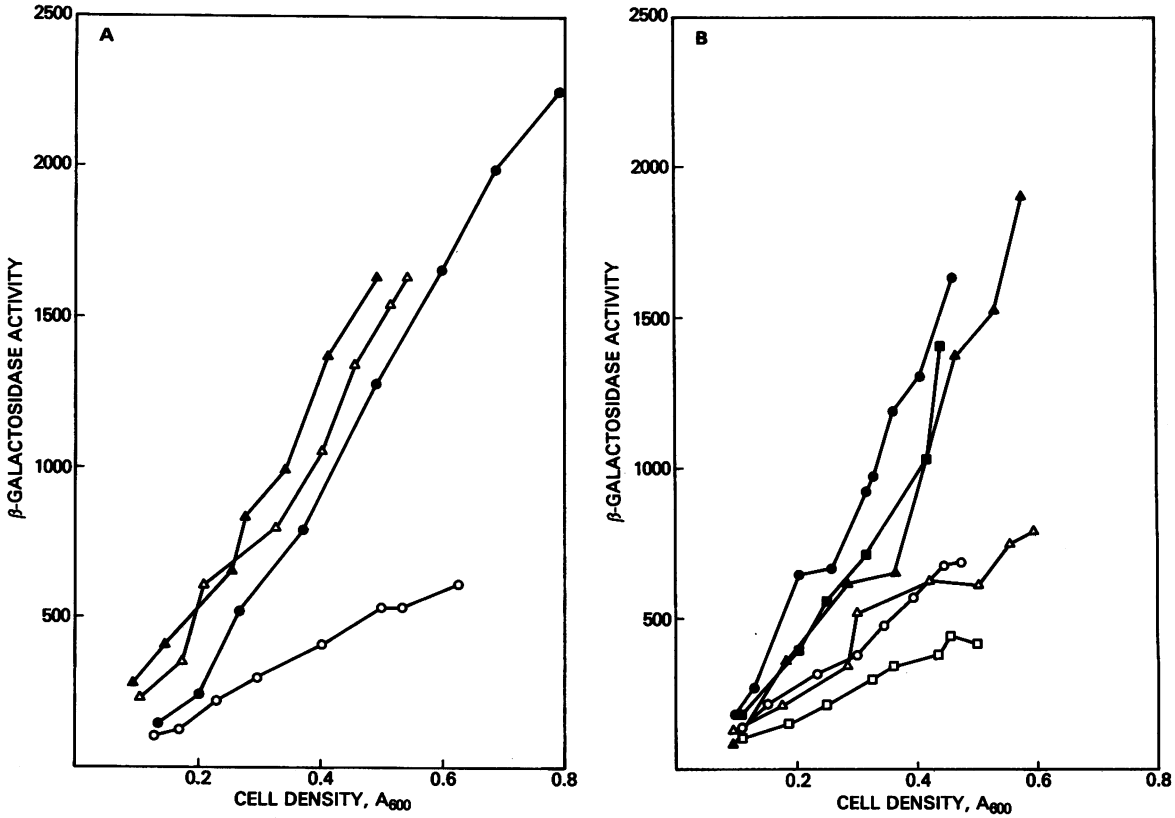

FIG. 4. Differential rate of β-galactosidase synthesis in a *galE::lacZ* operon fusion strain (JT245). Cell growth and enzyme assays are as described in footnote *a* of Table 2. (A) Symbols: ○, cells carrying no plasmids grown without an inducer; △, cells carrying plasmid p291 (O_E^+ O_I^+) grown without an inducer; ●, strain carrying plasmid pBR322 grown in the presence of D-fucose; ▲, cells carrying plasmid p291 grown in the presence of D-fucose. (B) Symbols: ○, △, □, cells carrying plasmids pH101 (O_E^- O_I^+), pH103 (O_E^+ O_I^-), and pH108 (O_E^- O_I^-), respectively, grown without an inducer; ●, ▲, ■, corresponding set of strains grown in the presence of D-fucose.

is responsible for its further induction in the Δ*galR* background and could be similarly titrated out by introduction of multiple copies of the *gal* control DNA sequences in the *galE::lac* fusion strain. For this experiment, the F′ *gal*$^+$/Δ*galR galE::lacZ* operon fusion strain (JT245) was individually transformed with several plasmids: pBR322; p291, carrying the wild-type *gal* operator segment (O_E^+ O_I^+); pH103, carrying an O_I mutation (O_E^+ O_I^-); pH101, carrying an O_E mutation (O_E^- O_I^+); and pH108, carrying both O_E and O_I mutations (O_E^- O_I^-). β-Galactosidase synthesis was measured in each transformant grown in the presence and absence of D-fucose (Fig. 4). The differential rate of β-galactosidase synthesis as calculated from the results of Fig. 4 are reported in Table 4. Whereas a plasmid-borne O_E^+ O_I^+ sequence causes the additional induction of the chromosomal *gal* operon to the full extent (threefold) irrespective of the absence or presence of an inducer, mutation in one of the two *gal* operators (O_E^- O_I^+ or O_E^+ O_I^- shows only partial (about 1.5-fold) induction. In the latter case, the addition of D-fucose restores further inducibility to the maximum level. A plasmid carrying either no *gal* DNA or *gal* control DNA sequences, in which both the *gal* operators were mutated (O_E^- O_I^-), does not show the additional induction unless D-fucose is added.

DISCUSSION

Cells grown in the presence of D-galactose or D-fucose show induction of the enzymes of the *gal* operon by 10- to 15-fold from promoter *P1* or *P2*, depending on the presence or absence of cyclic AMP receptor protein. It has been shown that induction of the *gal* operon occurs by inactivation of Gal repressor by inducer binding (21, 27). Thus, we would expect the same amount of, if not more, derepression of the *gal* operon if the *galR* gene were destroyed by mutation; the presence of an inducer should not have any additional effect on the already derepressed level of the *gal* enzymes. This was tested in a strain in which the repressor

TABLE 4. Repressor titration by plasmid-borne *gal* operators[a]

Plasmid	Plasmid-borne operators	Differential rate of β-galactosidase synthesis		Fold induction
		− D-fucose	+ D-fucose	
None		1,200	3,490	2.9
p291	O_E^+ O_I^+	4,000	4,240	1.1
pH101	O_E^- O_I^+	1,690	3,920	2.3
pH103	O_E^+ O_I^-	1,360	3,910	2.9
pH108	O_E^- O_I^-	993	3,780	3.8
pBR322		991	ND	

[a] The host strain was F′ *gal*$^+$/*galE::lac* Δ*galR* (JT245). The plasmids are shown in Table 1. Cell growth and β-galactosidase assays were as described in footnote *a* of Table 3, except 50 μg of ampicillin per ml was present in the growth medium for plasmid-carrying cells. The differential rates of β-galactosidase synthesis were calculated from the results of Fig. 4, representing (changes in β-galactosidase activity)/(change in optical density at 600 nm). ND, Not done.

gene was deleted. In plasmid clones harboring the *galR* gene, the latter was made completely inactive by deleting approximately the amino-terminal 80% of the structural gene as well as its promoter and substituting a gene cassette that confers chloramphenicol resistance to the cell. The Δ*galR*::Cm^r alleles in such plasmids were transferred to the *E. coli* chromosome by homologous recombination with a λ specialized transducing phage vector. The complete absence of Gal repressor in the Δ*galR* cells was confirmed by immunoblotting. The antibody to Gal repressor did not detect any Gal repressor protein in two Δ*galR* strains used in this study [Δ*galR*(A-C)::Cm^r and Δ*galR*(B-C)::Cm^r] (data not shown). Galactokinase, a gene product of the *gal* operon, was derepressed 10-fold in these Δ*galR* cells. However, these cells showed further induction of the operon by threefold when grown in the presence of an inducer as measured by the differential rate of galactokinase synthesis (Fig. 3). Thus, the *gal* operon can be induced to a higher level even when there is no Gal repressor to exert a negative regulatory effect. This phenomenon of additional induction by an inducer in the complete absence of Gal repressor has been termed ultrainduction—a manifestation of further inducibility. In this study, the phenomenon of ultrainduction was further investigated to answer the following questions. (i) At what level of gene expression does ultrainduction occur, transcription or translation? (ii) Is ultrainduction a manifestation of positive or negative control? (iii) Is any DNA element(s) involved in ultrainduction?

Ultrainduction occurs at the level of transcription. Specific genetic fusions have been employed to determine whether ultrainduction occurs at the transcriptional or translational level. λp*lac*Mu phage strains developed by Silhavy and co-workers (6, 33) are simple and easily applicable tools for isolating and studying genetic fusions in which the expression of the *lacZ* structural gene is brought under the control of another promoter in a single step after phage infection. By using the bacteriophage Mu transposition system, the *lac* structural genes were inserted into *galE*, creating translational (gene) and transcriptional (operon) fusions. In the former case, the *lacZ* gene is fused to a *gal* gene such that the expression of the *lacZ* gene from the *gal* promoters depends upon its translation from the translation initiation site of the particular *gal* cistron to which the *lacZ* gene is fused. If the phenomenon of ultrainduction occurs at the level of either translation or transcription of the *gal* genes, then ultrainduction of *gal* would be manifested at the level of β-galactosidase synthesis in the translational fusions. In the case of a transcriptional fusion, a complete *lacZ* gene (including its own translation initiation signal) placed anywhere along the transcription path would be expressed. If ultrainduction occurs at the level of transcription, then it would be reflected at the level of β-galactosidase synthesis in such a fusion. A translation control of ultrainduction would not be manifested in a transcriptional fusion. Measurement of specific activity of β-galactosidase in the F′ *gal*$^+$/*galE*::*lac* fusion strain under a variety of conditions has clearly shown that cell growth in the presence of D-fucose causes 10- to 12-fold induction of the operon, whereas cells completely devoid of Gal repressor showed 10-fold derepression (Table 3). However, the presence of an inducer causes an additional threefold induction beyond the normal constitutivity seen in the latter cells demonstrating ultrainduction. Ultrainduction was observed in both the gene fusion strain (which reports both transcription and translation) and the operon fusion strain (which reports only transcription). These results demonstrate that ultrainduction is regulated at the level of transcription.

Ultrainduction is due to a negative control. The phenomenon of ultrainduction could originate from a positive or negative control at the level of transcription. D-Fucose could activate an activation factor that effects *gal* transcription or inactivate a repressorlike factor that is inhibitory to *gal* transcription. These two control mechanisms may be distinguished by introducing an excess number of control DNA molecules, which interact with the positive or negative factor(s) to effect the pertinent control provided the regulatory proteins are present in limiting amounts in the cell. If a positive regulatory protein were involved, one would expect the induced level of gene expression to be lower, with increasing copy numbers of the controlling DNA elements, because the latter would bind and soak up the activated regulatory protein, whereas the level of expression observed without the inducer would remain lower and unaffected by an increase in the copy number of the DNA controlling elements. The expectation would be the opposite for a negative control. Excess controlling elements would bind and titrate out the repressor protein and enhance gene expression in the absence of an inducer, whereas in the presence of an inducer, i.e., when the repressor molecules are inactivated, the higher level of gene expression would be independent of the DNA controlling element copy number. When a multicopy plasmid bearing the entire control DNA segment of the *gal* operon was introduced into a Δ*galR* strain completely devoid of Gal repressor, the results, as measured by the differential rate of β-galactosidase synthesis in *galE*::*lac* fusion operon (Fig. 4, Table 4), clearly show a threefold higher level of *gal* expression in the presence of multicopy *gal* control region in the absence of inducer. Similar plasmid-mediated derepression was observed by measuring differential rate of galactokinase synthesis from the wild-type *gal* operon in a Δ*galR* background (data not shown). This threefold level of derepression is characteristic of ultrainduction. No further induction was observed when the inducer was present. These results strongly suggest that ultrainduction is due to the presence of a negative regulatory protein, termed the ultrainducibility factor (UIF), which limits the *gal* transcription in the complete absence of the known Gal repressor.

Site of action of the UIF. The titration assay discussed above also points out that the UIF acts by binding to the control DNA sequence present in the multicopy plasmid used in the assay. The DNA sequence used in this experiment encompasses the two *gal* promoters, *P1* and *P2*, the cyclic AMP receptor protein binding site, and the two *gal* operators, O_E and O_I (from −187 to +453 bp). The Gal repressor inhibits *gal* transcription by binding to O_E and O_I (8, 12, 17, 21, 27). If the DNA sequence cloned on the plasmid carried a mutation that would eliminate the affinity of the DNA toward UIF, then the plasmid would fail to elicit ultrainduction. When the differential rate of β-galactosidase synthesis was measured in strains carrying the plasmids bearing control DNA sequences with inactivated *gal* operators in various combinations, the strains showed reduced or no plasmid-elicited ultrainduction (Fig. 4B). Inactivation of one operator resulted in partial ultrainduction, whereas inactivation of both allowed no ultrainduction. The latter strain revealed ultrainduction only when D-fucose was present. These results suggest that the UIF binds to DNA sequences that are identical to or overlap with O_E and O_I, the binding sites of the Gal repressor.

The suggestion that the UIF acts to repress the *gal* operon

by binding to O_E and O_I DNA sequences was totally consistent with the direct effect of mutations in O_E and O_I on ultrainducibility. The results of galactokinase assays in *galO*^{c} strains (Table 1) clearly show the involvement of the wild-type operator alleles in ultrainducibility. Mutations in O_E or O_I cause derepression of the operon, which can be induced only slightly more by D-fucose. The operator mutations in the absence of Gal repressor cause the highest operon expression. The addition of an inducer does not cause any further induction.

The model. The results presented here strongly argue in favor of the idea that UIF is a negative regulatory factor, presumably a protein, that elicits operon control by interacting with the previously defined operators. Interaction of the regulatory factor with both operators is needed, because inactivation of either operator shows the highest expression in the Δ*galR* strain without an inducer being present. The UIF, like the Gal repressor, acts at the level of transcription. The question of how transcription is inhibited by the binding of the second repressor is not answered in the current study. Whatever the biochemical mechanism by which repression due to the UIF ensues, the same inducers, i.e., metabolizable D-galactose and its nonmetabolizable analog D-fucose, are capable of inactivating the action of the UIF. Note that, unlike the Gal repressor, the UIF does not bring about strong repression of *gal*, very likely because its affinity for the operators is lower than that of the Gal repressor.

Epistatic effect of the Gal repressor on ultrainduction. The Gal repressor-mediated repression of the *gal* operon can be removed by inactivation of the Gal repressor either by binding to an inducer or by mutation of the *galR* gene. One interesting feature of the phenomenon of ultrainduction is that it is mostly observed only when the Gal repressor is eliminated by mutation (deletion in this study). D-Fucose induces transcription of the *gal* operon in wild-type cells by 12-fold over the basal level by inactivating perhaps mostly the Gal repressor and 3-fold more than that by inactivating the UIF when the Gal repressor is genetically absent in the cell (Tables 2 and 3). The transcription of the *gal* operon can be induced by about 30-fold. Although D-fucose or D-galactose can inactivate both the Gal repressor and the UIF, the presence of high concentrations of inducer molecules during cell growth in itself does not inactivate both repressors at the same time to generate the extreme level of *gal* operon transcription characteristic of ultrainduction. The presence of the Gal repressor largely prevents D-fucose inactivation of the UIF. Thus, the Gal repressor has an epistatic effect on ultrainduction. The reason for this epistatic suppression needs further investigation.

Why is the *gal* operon subject to a second negative control? The results presented here show that the Gal repressor and the UIF, the predicted second repressor, act by binding through the same DNA elements in the *gal* operon, O_E and O_I. Although there are many examples of a positive regulatory factor and a negative regulatory factor mediating differential expression of genes by interacting through common DNA targets (20), the control of a promoter by the binding of two negative regulatory proteins to the same DNA sites is rare and is only known in lambdoid phages (29). In the phage systems, such dual negative control has profound biological consequences. The enzymes of the *gal* operon constitute an amphibolic pathway. They are needed for degradation of D-galactose as a carbon and energy source as well as for biosynthesis of complex carbohydrates in cell walls and cell membranes. Physiologically, these two processes are unrelated. The dual negative control of *gal* very likely provides *E. coli* with the ability to adapt to both catabolic and anabolic requirements of the galactose pathway, perhaps in a temporal fashion. In a strain defective for both the Gal repressor and the UIF, the *gal* operon should be derepressed to the ultimate level in the absence of an inducer. Using transposon Tn*10* insertion mutagenesis, we isolated *E. coli* mutants in a Δ*galR* background that show *gal* expression at the ultimate level in the absence of D-fucose, as expected for mutants defective in UIF (12a). One such mutation has been mapped at 46 min on the *E. coli* chromosome. Genetic analysis of the UIF mutants and biochemical investigation of the product of the wild-type allele of the mutant gene are now being carried out to answer several questions. Do the repressors act simultaneously or temporally? Why is the Gal repressor epistatic to ultrainduction? Is the UIF specific for the *gal* operon, or does it regulate the expression of other genes?

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