Proc. Nat. Acad. Sci. USA
Vol. 71, No. 2, pp. 507–511, February 1974

Multiple Regulation of the Galactose Operon—Genetic Evidence for a Distinct Site in the Galactose Operon that Responds to *capR* Gene Regulation in *Escherichia coli* K-12

(*lon*/operator/galactose enzymes/differentiation)

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Communicated by Albert Dorfman, October 4, 1973

ABSTRACT **Previous results demonstrated that the *capR* (*lon*) locus, which is not linked to the *gal* operon, independently controls the synthesis of the *gal* operon enzymes and *gal* mRNA, i.e., *galO*$^+$ *capR9* strains are derepressed 4- to 6-fold as compared to *galO*$^+$ *capR*$^+$ strains. A mutation has been isolated and localized in the galactose operator region that defines a new and distinct site of control. Mutation in this site, designated *gal-O*$^{capR+}$, causes a 4-fold increase in the galactose enzymes, galactokinase (EC 2.7.1.6) and UDP-galactose-4-epimerase (EC 5.1.3.2), in a *capR*$^+$ background. These mutants exhibit a reduced response to regulation by the unlinked regulator gene *capR* (*lon*). However, the *galO*$^{capR+}$ mutants are still subject to control by the *galR* repressor, since they can be further derepressed by growth in the presence of D-fucose. They also synthesize more galactokinase when grown in glycerol as compared to glucose. Thus there are now at least three, and probably four, sites for control of mRNA synthesis in the operator–promoter regions of the *gal* operon, making it one of the most complex control systems to date for a single operon in bacteria. The complexity is sufficient to accommodate models for differentiation in higher organisms that require more than one "switch" to control a single group of genes.**

A modified model (Fig. 1) for the structure of the galactose (*gal*) operon has been proposed (1). The operator region defined by response to the *galR*$^+$ repressor (designated O^{galR+} in Fig. 1) and the promoter region, responding to control by cyclic AMP (3′:5′) and cAMP receptor protein (CRP) (designated *prg* in Fig. 1), have been the subjects of intensive investigation in a number of laboratories and the reactions have been demonstrated *in vivo* and *in vitro* (for references, see refs. 1 and 2). The product of the *galR*$^+$ gene, the *galR* repressor, has been partially purified (2, 3). The *in vitro* system for transcription is subject to at least a 10-fold stimulation by the addition of cAMP and CRP (4). Two different λ *gal* DNA-dependent systems for coupled transcription and translation are also stimulated by the addition of cAMP and CRP (5, 6). One apparent dichotomy between the *in vivo* and *in vitro* situations is that *Escherichia coli* cells deficient in cAMP grow on D-galactose (7). We will return to this point in the *Discussion*.

The galactose operon is also derepressed by mutations in the *capR* (*lon*) (1, 8–10) or *capT* (1, 9) gene and the double mutant *capR9 capT* is no more derepressed than either of the single mutants (1). We proposed a site for a second repressor in the *gal* operon [designated O^{capR+} in Fig. 1 (1)] mainly on the basis of the following results. Control of the *gal* operon by *capR* (or *capT*) was shown to be independent of the *galR* repressor as follows: *galR*^{S} is a mutation in the *galR* gene that prevents induction of the *gal* operon by D-fucose or D-galactose. Such *galR*^{S} mutants could be derepressed 4- to 6-fold by introduction of a mutation in *capR* or *capT*. This was the same derepression produced by the *capR* mutation in a strain with a wild-type (*galR*$^+$) repressor (1). Control by *capR* and *capT* is at the level of transcription of *gal* mRNA (9, 10). Thus, the site in the *gal* operator region, O^{capR+}, was proposed to explain response of the *gal* operon to the product of the *capR* (or *capT*) gene that was independent of the *galR*$^+$ repressor. The assumption was made that such a target was in the *gal* operator region. In this paper we describe the isolation of mutants that map in the *gal* operator region that are still responsive to control by the *galR*$^+$ repressor, but are largely unresponsive to control by the product of the *capR*$^+$ and *capT*$^+$ genes. Such mutants establish the existence of the site O^{capR+} in the wild type.

Abbreviation: CRP, cyclic AMP(3′:5′) receptor protein.

MATERIALS AND METHODS

Bacteria. The bacteria utilized in this study are listed in Table 1. All strains of bacteria are derivatives of *E. coli* K-12.

Media and Chemicals. M9 minimal medium (11) was used to grow cells for enzymatic assay. Either 0.6% glucose, 1% glycerol (v/v), or 0.6% galactose was used as a carbon source. L broth (12) was used for F-duction and conjugation. L-broth supplemented with 2 mM $CaCl_2$ was used for P1 transduction. NAD, ATP, UDP-galactose, and D-fucose were purchased from Sigma Chemical Co.

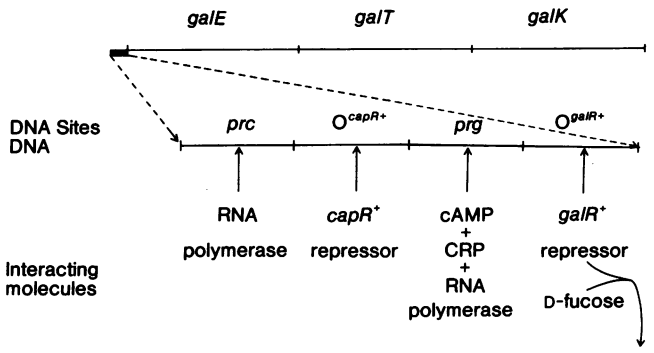

FIG. 1. A modified model for the structure of the galactose operon. Taken in part from Hua and Markovitz (1). The relative order of the controlling sites is discussed.

Genetic Methods. Transduction was performed as described by Lennox (13). Conjugation with Hfr strains was performed by the procedure of Taylor and Thoman. F-duction was carried out as described (14). Thymine-requiring mutants were selected on M9 minimal glucose plates containing 400 μg/ml of trimethoprim and 200 μg/ml of thymine, a modification of the method of Stacey and Simson (15).

Preparation of Cell-Free Extracts. Cells were grown in minimal medium at 23° in test tubes on a rotary drum. Late exponential phase cells were harvested for measurement of enzyme activity. Cell-free extracts were prepared by sonically disrupting the cells as described previously (1) except that 10 mM mercaptoethanol was used.

Enzyme Assays. Galactokinase (EC 2.7.1.6) was assayed according to the method of Sherman and Adler (16). Galactose and galactose-1-phosphate were separated by Whatman DE 81 cellulose paper according to the method of Wetekam *et al.* (6). Radioactive samples were counted as described previously (1). UDP-Gal-4-epimerase (EC 5.1.3.2) was assayed by the two-step method described by Kalckar *et al.* (17). Protein was determined by the method of Lowry *et al.* (18) with bovine-serum albumin as a standard.

RESULTS

Constitutive Levels of Galactokinase in galO Mutants. Strain HC2000 (F'*galR*^{s} *thyA*$^+$/*gal*$^+$ *thy*$^-$ *recA galR*^{s}) is phenotypically unable to grow on galactose minimal medium although the level of the enzymes of the *gal* operon are approximately equivalent to the level in *galR*$^+$ strains (ref. 1, and unpublished results). Presumably the *galR*^{s} mutation prevents growth on galactose by preventing entry of galactose and induction of the galactose enzymes. Spontaneous *galO* mutants were isolated by selecting phenotypically Gal$^+$ colonies from strain HC2000 on minimal galactose plates at 25°. The frequency of such mutants is 10^{-7}. A total of 107 independently isolated mutants were grown in minimal glucose medium and assayed for galactokinase activity. Fig. 2 presents the distribution of different levels of galactokinase in the mutants screened. The relative degree of derepression in galactokinase was calculated by arbitrarily setting the enzyme activity of the parent strain, HC2000, equal to 1. Seventy-five percent of the mutants tested exhibited a moderate degree of constitutitivity; 4- to 10-fold derepressed, while a small fraction of galactose-positive mutants contained almost as low activities as strain HC2000. Approximately 10% of the mutants were 18- to 20-fold derepressed, which corresponds to the levels observed in wild-type strains maximally induced by D-fucose or the classic *galO*^{c} strains (1).

TABLE 1. *Bacterial strains*

Strain	Derivative, source and/or genotype
MC100	R. Curtiss III (his strain X-156); F$^-$, *leu-6, proC34, purE38, trpE43, thi-1, ara-14, lacY1, galK2, xyl-5, mtl-1, tonA23, tsx-67, azi-6, str-109*, λ$^-$, P1^s, *capR*$^+$, *capS*$^+$, *capT*$^+$
MC129	P1(*gal*$^+$) × MC100, select *gal*$^+$
HC2000	F'*galR*^{s} *thyA*$^+$/*galR*^{s} *thy*$^-$ *recA;* derived from MC129
HC2101	*capR9 gal*$^+$ *galO*$^+$
HC2001	*capR*$^+$ *gal*$^+$ "
HC2106	*capR9 galO6* *galO* constitutive mutant
HC2006	*capR*$^+$ *galO6* "
HC2137	*capR9 galO37* "
HC2037	*capR*$^+$ *galO37* "
HC2142	*capR9 galO42* "
HC2042	*capR*$^+$ *galO42* "
HC2159	*capR9 galO59* "
HC2059	*capR*$^+$ *galO59* "
HC2170	*capR9 galO70* "
HC2070	*capR*$^+$ *galO70* "
HC2342	*capT galO42* Conjugation of M15 *non-2* × HC2042. Select Leu$^+$ and score for mucoid clones on eosin–methylene blue–glucose plates at 37° (1, 8).

Characterization of Derepressed Mutants as Operator Mutations by Linkage to galK. Bacteriophage P1 was grown on the presumptive operator mutants that were derepressed 4- to 10-fold in galactokinase. Strain MC100, a *galK*$^-$ *galR*$^+$ *capR*$^+$ strain, was used as a recipient for transduction. Since the *galO* region and *galK* belong to the same operon, and are separated only by *galE* and *galT*, we would expect approximately 95% cotransduction (19) of *galO* mutations with *galK*$^+$. For each transduction from a specific P1 (*galO* mutation) donor, four *galK*$^+$ transductants were selected for assay of specific activity of galactokinase. We observed different levels of galactokinase among the four transductants in some sets. Repeated measurements indicated that the differences were not due to errors in the method of assay. The different levels of galactokinase among the transductants may reflect the nature of the mutations (possibly deletions or insertions). It has been demonstrated that the frequency of point mutations is not increased by ultraviolet light in *recA* strains (20), while formation of deletions does not require the presence of *recA*$^+$ (21, 22). All of our operator mutations were isolated in a *recA* strain.

Our major concern was to isolate and identify some type of *galO* mutation which did not respond to *capR* regulation. After screening a large number of presumptive operator *galK*$^+$ transductants of strain MC100 by assaying galactokinase, mutants that had the level of galactokinase approaching that of a *galR*$^+$ *galO*$^+$*E*$^+$*T*$^+$*K*$^+$ *capR9* strain (i.e., 4- to 6-fold derepressed over wild type) were chosen for further investigation. Representative mutants which had different levels of constitutivity were also selected as controls. Each of the strains of interest was transduced to *proC*$^+$ with P1 (*proC*$^+$ *capR9*). *proC*$^+$ *capR*$^+$ and *proC*$^+$ *capR9* transductants were purified and studied.

Identification of Mutants in the galO Region that Exhibit Altered Responses to the capR$^+$ *and galR*$^+$ *Gene Products.* Three mutants were obtained that could be classified as altered in their response to *capR*$^+$ as compared to the *capR9* allele. Other mutants of the classic type, altered in their response to an active *galR* repressor, were also isolated. Representative mutants of each type classified by the basal level of galactokinase produced were chosen for presentation in this paper. The results are summarized in Tables 2 and 3. Strain HC2042 contains a mutation in the *galO* region that leads to partial derepression of the operon and an altered response to the

capR allele. The basal levels of galactokinase and UDP-galactose-4-epimerase in strain HC2042 are similar to the basal levels in strain HC2101 (*galO*$^+$ *capR9*) when grown in glucose (Table 2, lines 3 and 12). Strain HC2042 can be further induced by D-fucose (Table 3). Thus the site of action for the *galR*$^+$ repressor is functional and the promoter region is intact. The effect of *capR9* on the *gal* enzymes in combination with *galO42* was examined. The isogenic pair HC2142 (*capR9*) and HC2042 (*capR*$^+$) contained the same level of enzymes when grown in glycerol (Table 3, lines 1 and 3) and less than a 2-fold difference when grown in glucose (Table 2, lines 1 and 3). The *capT* gene mutation was similar to the *capR9* mutation in its interaction with *galO42*, although less work has been done using *capT* (Tables 2 and 3, line 2). In contrast, in a strain with a wild-type operator region (*galO*$^+$) *capR9* causes a 4-fold derepression in glycerol (Table 3, lines 12 and 13) and a 5-fold derepression in glucose (Table 2, lines 12 and 13). Two other mutations, *galO37* and *galO59*, do not respond to *capR* control in glycerol medium (Table 3) but are derepressed approximately two-fold when the *capR9* allele is introduced and they are grown in glucose (Table 2). Strains that contain two other mutations in the *gal* operator region similar to the classic *galO*^{c} mutation (designated *galO6* and *galO70*) have higher basal levels of *gal* enzymes than strain HC2042 (*galO42*) and introduction of the *capR9* allele causes a 3-fold derepression when cells are grown in either glucose or glycerol (Tables 2 and 3). The levels of galactokinase in all mutants in the *galO* region studied were further increased by D-fucose but the highest levels attained were in mutants containing the *galO42* or *galO59* mutation (Table 3). All this information indicates that *galO42* is a mutation in the site that normally responds to the *capR*$^+$ gene product. *galO37* and *galO59* are likely to be mutant in the same site as *galO42*.

Effect of Episomal capR$^+$ *Allele on the gal Enzyme Activity in the galO Constitutive Mutants.* We have constructed heterozygous partial diploids and homozygous partial diploids with the *capR*$^+$ allele on the episome, using an F′ 13 transferred from strain W3747. These strains, as well as the haploid *capR9* and *capR*$^+$ strains were grown at 23° (in glucose and in glycerol) and galactokinase was measured. The results are presented in Table 4. The results for the haploid strains are included in Tables 2 and 3. There is no greater repression of galactokinase in F′*capR*$^+$/*capR*$^+$ than in *capR*$^+$ haploid for the *galO* constitutive mutants or wild-type *galO*$^+$. The results with the heterogenotes, F′*capR*$^+$/*capR9*, demonstrate that the *capR*$^+$ allele is dominant when *capR*$^+$ is on the episome. In the *galO*$^+$ strain, the level of galactokinase was reduced 3-fold when the F′*capR*$^+$/*capR9* strain (Table 4, line 5) is compared to the *capR9* strain (Table 2, line 12; Table 3, line 12). Similarly, with the *galO6* mutation, the galactokinase was repressed to the same level in the F′*capR*$^+$/*capR9* heterogenote as in the *capR*$^+$ haploid or F′*capR*$^+$/*capR*$^+$ homogenote. The results with the *galO42* mutation are most in-

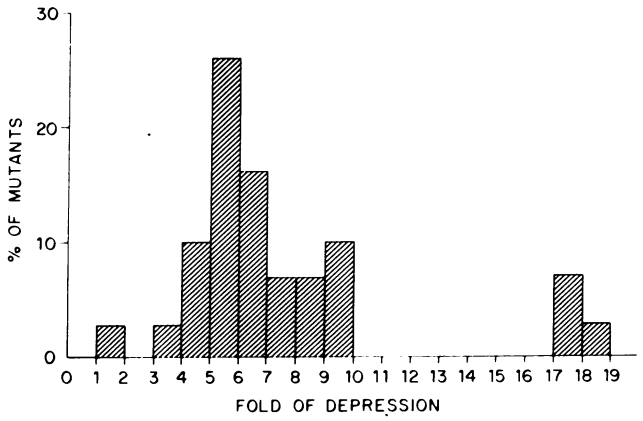

FIG. 2. Distribution of different levels of galactokinase in *galO* mutants. A total of 107 independently isolated mutants, obtained as Gal$^+$ revertants from strain HC2000 (F′*galR*^{s} *thyA*$^+$/*galR*^{s} *galE*$^+$*T*$^+$*K*$^+$ *thy*$^-$ *recA*), were grown in minimal glucose medium and assayed for galactokinase activity. The relative fold of derepression was calculated by setting the specific enzyme activity of the parent strain, HC2000, equal to 1.

TABLE 2. *Specific enzymatic activity of galactose enzymes in galO constitutive mutants grown in minimal glucose medium**

Strain	Relevant alleles	Galactokinase†	UDP-galactose-4-epimerase‡
HC2142	*capR9 galO42*	2260	54.8
HC2342§	*capT galO42*	2300	—
HC2042	*capR*$^+$ *galO42*	1420	30.8
HC2137	*capR9 galO37*	2690	—
HC2037	*capR*$^+$ *galO37*	1240	—
HC2159	*capR9 galO59*	2600	—
HC2059	*capR*$^+$ *galO59*	1220	—
HC2106	*capR9 galO6*	5140	129
HC2006	*capR*$^+$ *galO6*	1810	32
HC2170	*capR9 galO70*	7340	—
HC2070	*capR*$^+$ *galO70*	2250	—
HC2101	*capR9 galO*$^+$	1170	26.7
HC2001	*capR*$^+$ *galO*$^+$	224	7.9

* Results are the average of two or more separate experiments.

† Galactokinase was assayed at 37° and is expressed as nmol/hr per mg of protein.

‡ UDP-galactose-4-epimerase was assayed at 25° and is expressed as μmol/hr per mg of protein.

§ Results are the average of duplicate samples in a single experiment.

TABLE 3. *Specific enzymatic activity of galactokinase in galO constitutive mutants grown in minimal glycerol medium**

Strain	Relevant alleles	Galactokinase −D-Fucose	Galactokinase +D-Fucose†
HC2142	*capR9 galO42*	4060	16,400
HC2342	*capT galO42*	5400	—
HC2042	*capR*$^+$ *galO42*	4350	18,800
HC2137	*capR9 galO37*	4780	16,400
HC2037	*capR*$^+$ *galO37*	4480	16,800
HC2159	*capR9 galO59*	4010	18,800
HC2059	*capR*$^+$ *galO59*	3890	13,000
HC2160	*capR9 galO6*	7320	13,600
HC2006	*capR*$^+$ *galO6*	2890	13,500
HC2170	*capR9 galO70*	6620	12,400
HC2070	*capR*$^+$ *galO70*	2560	11,200
HC2101	*capR9 galO*$^+$	1240	12,300
HC2001	*capR*$^+$ *galO*$^+$	330	8180

* Footnotes in Table 2 apply.

† D-Fucose was added to a final concentration of 5 mM, and the cells were grown overnight at 23°.

TABLE 4. *Galactokinase activity* in heterozygous and homozygous partial diploids*

Genotype	Glucose as carbon source	Glycerol as carbon source
F'*capR*$^+$/*galO42 capR9*	1910	4290
F'*capR*$^+$/*galO42 capR*$^+$	1340	4380
F'*capR*$^+$/*galO6 capR9*	1810	2870
F'*capR*$^+$/*galO6 capR*$^+$	1810	2810
F'*capR*$^+$/*galO*$^+$ *capR9*	383	425
F'*capR*$^+$/*galO*$^+$ *capR*$^+$	247	361

* Specific enzymatic activity is expressed as in Table 2.

teresting. An episomal *capR*$^+$ allele fails to repress galactokinase in a F'*capR*$^+$/*capR9* heterogenote. These results clearly demonstrate that the *galO42* mutation is an alteration in the site where *capR*$^+$ gene product normally asserts its function from a *trans* position.

DISCUSSION

The first genetic evidence for a second independent operator site located in the *gal* operator region that is responsive to *capR*$^+$ gene-product control is presented in this paper. Transduction analysis indicates that all of the operator mutations studied are linked to *galK*. The following evidence indicates that the operator mutation designated *galO42* is at the site O^{capR^+} (Fig. 1). The *galO42* mutation, in the presence of *capR*$^+$, is 4- to 6-fold derepressed, compared to a *galO*$^+$ *capR*$^+$ strain, in both galactokinase and UDP-galactose-4-epimerase. Thus the *galO42* mutation affects the entire *gal* operon and not a rho-sensitive site at the end of the *galE* gene (23). When the *capR*$^+$ allele is replaced with the *capR9* allele in a strain containing *galO42* there is no further derepression of gal enzyme in glycerol medium and less than a 2-fold derepression in glucose (Tables 2 and 3, lines 1 and 3). Similar results were obtained in the interaction of *capT* with *galO42* although only galactokinase was assayed. *galO37* and *galO59* are similar to *galO42* in their interaction with *capR*. In contrast, when the *capR*$^+$ allele is replaced with the *capR9* allele in a strain containing gal*O*$^+$ there is a 4-fold derepression of *gal* enzymes in glycerol and a 5-fold derepression in glucose. Other *galO* mutations isolated in this study are still responsive to the *capR* allele (*galO6* and *galO70*) and are derepressed 3-fold more in a *capR9* background as compared to *capR*$^+$ in both glycerol and glucose media. *galO6* and *galO70* are therefore characterized as mutants at the O^{galR^+} site (Fig. 1).

All of the *galO* mutations isolated, including *galO42*, are further derepressed by D-fucose (Tables 3 and 4). Furthermore, all the *galO* constitutive mutants produced higher maximum levels of galactokinase when induced with D-fucose than the wild-type *galO*$^+$ strain (Table 4). The induction by D-fucose indicates that the *galR* repressor control is still exerted to some extent. Similar effects of operator mutations have been systematically studied in the *lac* operon (24). The *gal* operator mutations may alter the affinity of RNA polymerase or initiation by RNA polymerase, implying an overlap between operators and promoters (25, 26). However, there is no evidence for such an overlap in the *lac* operator and promoter regions (27), although there is evidence for two sites in the *lac* promoter region (28).

Before discussing our model of the *gal* operon we summarize as follows.

(*1*) There is now genetic evidence for two distinct *gal* operator sites; one that responds to the *galR* product and one that responds to the *capR* (or *capT*) product (refs. 1, 10, and present results).

(*2*) There is biochemical evidence for the *galR* repressor (2, 3).

(*3*) There is biochemical evidence for a cAMP–CRP-sensitive *gal* promoter (4–6). However, this promoter has not yet been defined by *gal* promoter mutations.

(*4*) Since there is considerable *gal* transcription in the absence of cAMP (7) there is presumably also a cAMP–CRP-independent *gal* promoter. There is no evidence as to whether these two *gal* promoters are physically separate.

(*5*) Both operator regions appear to regulate *gal* transcription (2, 9, 10).

At this point we focus our attention on the model presented in Fig. 1 and the proposed sequence of promoters and operators. We propose that the *gal* operon has two promoters, *prc* adjacent to the O^{capR^+} site and *prg* adjacent to the O^{galR^+} site. Data from the literature indicate that bacteria deficient in cAMP synthesis are able to grow on galactose (7). This is explained in the present model by having two separate promoters, *prg* being dependent and *prc* being independent of the cAMP–CRP system. The poor induction of galactokinase by D-fucose in the presence of glucose (in cells that are permeable to D-fucose in the presence of glucose) and the reversal of the glucose inhibition by cAMP is consistent with this model (29). When cells are grown in glucose medium *prc* is the favored promoter for initiation of mRNA synthesis. In glycerol medium both *prc* and *prg* function in binding RNA polymerase. We suggest the order of the sites as indicated in Fig. 1, supported by the following observations. Mutants in O^{capR^+} (*galO42*, *galO37*, and *galO59*) contain 2–3 times as much galactokinase when grown on glycerol as compared to glucose (Tables 2 and 3). This glucose effect was less evident in the *galO*$^+$ or the O^{galR^+} type strains observed in this study (Tables 2 and 3). These results are consistent with the site O^{capR^+} being adjacent to *prg* if one makes the following assumptions. Mutation in the O^{capR^+} site (*galO42*) will release functional overlapping between the sites and enhance transcription at *prg* in glycerol, but not in glucose. In glucose the cAMP–CRP-dependent promoter, *prg*, would be less functional. We also note that the *galO42* mutation still shows some response to *capR*$^+$ control, but this is only evident in glucose-grown cells. In glucose-grown cells we suggest the *prg* site is less functional and we do not observe the complication of extensive transcription from both promoters. The testing of this model will require the precise mapping of the various classes of *gal* operator mutations and the isolation and mapping of *gal* promoter mutations.

The mutations in *capR* have a number of effects on bacteria. They cause overproduction of capsular polysaccharide (mucoid clones) and derepressed synthesis of enzymes specified by at least four spatially separated operons involved in capsular polysaccharide synthesis. Some of these enzymes include GDP-mannose pyrophosphorylase (30, 31), UDP-glucose pyrophosphorylase (*galU*) (30, 32) and the *gal* operon (*galETK*) (1, 8, 10). The *capR*$^+$ allele, when present on the F'13 episome, was dominant to *capR9* in repressing capsular

polysaccharide synthesis (F'*capR*$^+$/*capR9* is nonmucoid), GDP-mannose pyrophosphorylase synthesis, and UDP-glucose pyrophosphorylase synthesis (30, 33). In the present study the F'13 *capR*$^+$/*capR9 galO*$^+$ strain also contained repressed levels of galactokinase. However, the *galO42 capR9* strain is not repressed by introduction of an F'13*capR*$^+$ episome (compare Tables 2, 3, and 4). These results also support the contention that *galO42* is a mutation in a site normally responding to *capR*$^+$ control.

We suggest that, on the basis of the complexity of the *gal* operon in *E. coli*, single operons controlled by multiple control sites could accommodate models for differentiation that require more than one "switch" to control a single group of genes.

We acknowledge the excellent technical assistance of Paula R. Butler. This investigation was supported by USPHS Grant AI 06966 and American Cancer Society Grant VC 116.

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