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Multiple Regulator Gene Control of the Galactose Operon in *Escherichia coli* K-12

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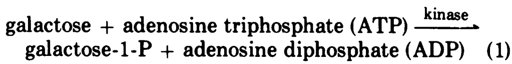
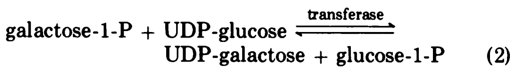
Previous studies showed that nonsense mutations in either of two genes (*capR* or *capS*) or an undefined mutation in a third gene (*capT*) led to pleiotropic effects: (i) increased capsular polysaccharide synthesis (mucoid phenotype); (ii) increased synthesis of enzymes specified by at least four spatially separated operons involved in synthesis of capsular polysaccharide including the product of the *galE* gene, UDP-galactose-4-epimerase (EC 5.1.3.2) in *capR* mutants. The present study demonstrated that the entire galactose (*gal*) operon (*galE*, *galT*, and *galK*) is derepressed by mutations in either the *capR* or the *capT* genes, but not by mutation in *capS*. Double mutants (*capR9 capT*) were no more derepressed than the *capR9* mutant, indicating that *capR9* and *capT* regulate the *gal* operon via a common pathway. Isogenic double mutants containing either $galR^+$, $galR^-$, $galR^s$, or $galO^c$ in combination with either $capR^+$ or *capR9* were prepared and analyzed for enzymes of the *gal* operon. The results demonstrated that *capR9* caused derepression as compared to $capR^+$ in all of the combinations. Strains with a $galR^s$ mutation are not induced, for the *gal* operon, by any galactose compound including D-fucose, and this was confirmed in the present study using D-fucose. Nevertheless, the derepression of $galR^s$ *capR9* compared to $galR^s$ $capR^+$ was four- to sixfold. The same derepression was observed when $galR^+$ *capR9* was compared to $galR^+$ $capR^+$. The data eliminate the explanation that internal induction of the *gal* operon by a galactose derivative was causing increased *gal* operon enzyme synthesis in *capR* or *capT* mutants. Furthermore, the same data suggest that the *galR* and *capR* genes are acting independently to derepress the *gal* operon. A modified model for the structure of the *gal* operon is proposed to explain these results. The new feature of the model is that two operator sites are suggested, one to combine with the *galR* repressor and one to combine with the *capR* repressor.

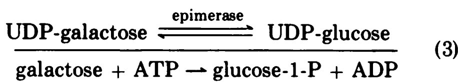
The synthesis of capsular polysaccharide in *Escherichia coli* K-12 is controlled by three regulator genes designated *capR*, *capS* and *capT* that map in different regions of the chromosome (29, 31, 32). Mutations in any one of the regulator genes results in overproduction of the same polysaccharide and leads to a mucoid phenotype (29, 31, 32). The capsular polysaccharide, called colanic acid by Goebel (13), contains D-galactose, L-fucose, D-glucose, D-glucuronic acid, acetate, and pyruvate in molar ratios of 2:2:1:1:1:1, and its structure has been largely elucidated (40, 46). Polysaccharide of the same composition, and probably the same basic structure, is also found in *Salmonella* and *Aerobacter* species (14). The biochemical pathway for the synthesis of the polysaccharide and the genetic map listing the locations, where known, of the relevant genes on the chromosome are presented in Fig. 1 and Table 1. The *capT* gene has not been mapped except that it is at a different location than either *capR* or *capS* (Markovitz and Shaparis, *unpublished data*). However, the phenotype of *capT* strains, originally described in strain M15 (29), is different than either *capR* or *capS*, the former being mucoid on complex medium at 37 C and below; all three are mucoid on minimal agar at 37 C and below. Among *cap* mutants, only *capR* (*lon*) mutants are sensitive to ultraviolet light (5, 17, 32; Markovitz, *unpublished data*).

Synthesis of many of the enzymes involved in capsular polysaccharide synthesis is derepressed in all mucoid strains, but there are important differences when mutants in *capR*, *capS*, and *capT* are compared. Data previously obtained are summarized in Table 1. Of par-

ticular interest for the present study is the fact that uridine diphosphate (UDP)-galactose-4-epimerase (epimerase; EC 5.1.3.2) is derepressed in mutant strains that contain either *capR* (29) or *capT* (this study). It is clear from Fig. 1 that epimerase is directly involved in *synthesis* of two of the capsular polysaccharide precursors. On the other hand, epimerase is also involved in catabolism of D-galactose when *E. coli* is growing on D-galactose via the Leloir pathway (20) as follows:







Extensive studies on the Leloir pathway enzymes established the following facts.

All three enzymes are coordinately induced from 10- to 25-fold by D-galactose or its nonmetabolizable analogue, D-fucose (9, 20), and appear in the sequence epimerase, transferase, and kinase (33).

The sequence of the structural genes for the galactose (*gal*) operon is *galE* (epimerase), *galT* [UTP: D-galactose-1-phosphate uridyl transferase (transferase) EC 2.7.10], *galK* [ATP: D-galactose phosphotransferase (kinase) EC 2.7.1.6] (7, 8), and mutations that cause derepression of all three are located near *galE* (O^c type; 4, 10) and are *cis*-dominant (10). Other mutations near but not in the *galE* gene cause decreased levels of all three enzymes, and these were designated O^o mutations (4, 42, 43); these mutations were also *cis*-dominant (4). Furthermore, nonsense mutations in the *galE* gene caused polar effects on *galT* and *galK*, and nonsense mutations in *galT* caused a polar effect on *galK* (4, 18). The frequencies of translation of the structural genes for epimerase and kinase are equivalent under a variety of conditions (53). Other mutations in a gene (designated *galR*) unlinked to the *gal* region cause either increased *gal* enzyme synthesis (*galR*$^-$, 10) or prevent induction of the *gal* enzymes by D-galactose or D-fucose (*galR*^{s}, 41). The messenger ribonucleic acid (mRNA) of the *gal* operon forms a hybrid with one of the two deoxyribonucleic acid (DNA) strands of the *gal* region (16). Recent work has resulted in the partial purification of the product of the *galR*$^+$ gene (37). It is a protein that binds to λ*pgal* DNA, and the binding is inhibited in vitro by D-galactose or D-fucose (37). Other studies revealed that both in vivo and in vitro transcription and translation of the *gal* operon requires cyclic adenosine 3',5'-monophosphate (c-AMP) and c-AMP receptor protein (CRP; 34–36, 52). All of these results have been considered as strong support for the initiation of transcription by DNA-dependent RNA polymerase at a single point (the promotor) near the DNA of the operator where the *galR* repressor would bind.

Mackie and Wilson demonstrated that one of the mucoid mutations (*capR6*) that caused derepression of epimerase (29) also caused derepression of the other enzymes of the *gal* operon and that derepression was at the level of transcription since *gal* mRNA was increased in the *capR6* strain as compared to the isogenic *capR*$^+$ strain (Fed. Proc. **30**:1262, 1971). The results of the present study, combined with those previously mentioned, suggest that the

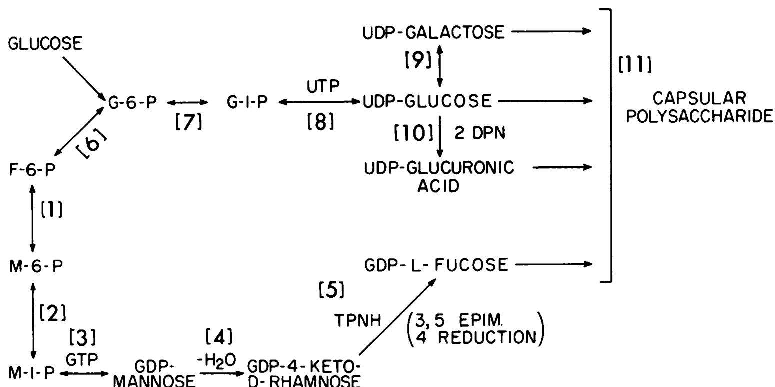


FIG. 1. *Postulated biosynthetic pathway for capsular polysaccharide in Escherichia coli K-12 (26). The precursors of acetate and pyruvate in the polysaccharide (46) are not included. The numbers in brackets refer to enzymes named in Table 1.*

TABLE 1. *Patterns of derepression in enzymes for capsular polysaccharide synthesis in mucoid mutants*[a]

No. on Fig. 1	Gene symbol	Map position (min)	Enzyme	Derepressed in mutant in[b]		
				capR9	*capS*	*capT*
1	*man*	33	Phosphomannose isomerase	+	−	ND[c]
2		ND	Phosphomannomutase	−[d]	−[d]	−[d]
3	*non-3*	ND	GDP-mannose pyrophosphorylase	+	+	+
4		ND	GDP-mannose hydrolyase	+	+	ND
5		ND	GDP-fucose synthetase	+	+	ND
6	*pgi*	79	Phosphoglucose isomerase	ND	ND	ND
7	*pgm*	16[e]	Phosphoglucomutase	−[d]	−[d]	−[d]
8	*galU*	25	UDP-glucose pyrophosphorylase	+	+[d]	+[d]
9	*galE*	17	UDP-galactose 4-epimerase	+	−	+[f]
10		ND	UDP-glucose dehydrogenase	+	+[g]	+[g]
11		ND	Polysaccharide polymerase(s)	ND	ND	ND

[a] Results are taken from previous publications (24-26, 29, 31) except where noted otherwise. Abbreviations: GDP = guanosine diphosphate; UDP = uridine diphosphate.

[b] *capR9* maps at 11.5 min (29), *capS* at 22.5 (31), and *capT* has not been mapped.

[c] Not done.

[d] C. E. Buchanan, *unpublished data.*

[e] Reference 3.

[f] Present study.

[g] A. Shaparis and A. Markovitz, *unpublished data.*

galactose operon must respond to several different systems of controls: (i) *galR* repressor; (ii) cyclic AMP plus cyclic AMP receptor protein; (iii) *capR* and *capT*.

The purpose of this communication is to investigate (i) to what extent the entire *gal* operon was affected by the *capR*, *capS*, and *capT* mutations, (ii) the interaction of mutations in *capR* and *capT* with *galR*$^-$, *galR*^{s}, and *galO*^{c} mutations, (iii) regulation of the *gal* operon in strains carrying mutations in both *capR* and *capT*, and (iv) the effect of glucose, as a measure of the c-AMP system, on derepression of the *gal* operon caused by *capR* and *capT* mutations.

MATERIALS AND METHODS

Bacteria. All strains of bacteria utilized in the experiments were derivatives of *E. coli* K-12. The properties of the basic strains employed are given in Table 2.

Media. M-9 minimal medium (1) was supplemented with 2.5×10^{-3} M $CaCl_2$ and 10 μg of thiamine-hydrochloride/ml. Amino acids and purine base were added at 50 μg/ml when required. Either 0.6% glucose, 1% glycerol (v/v) or 0.45% sodium succinate was used as a carbon and energy source. These media were solidified by adding 1.5% agar. Thymine and streptomycin were used at 100 and 200 μg/ml, respectively.

EMB plates were made using eosin methylene blue agar (Difco) with 1% sugar added after sterilization. L broth (28) was used to grow strains for transduction and conjugation.

Chemicals. Nicotinamide adenine dinucleotide (NAD), NAD phosphate, ATP, glucose-1,6-diphosphate, galactose-1-phosphate, L-fucose, and D-fucose were purchased from Sigma Chemical Co. ^{14}C-galactose was purchased from Amersham-Searle (Nuclear-Chicago Corp.), and Dowex 1 (Ag-2X) from Calbiochem. Omnifluor was obtained from New England Nuclear Corp.

Enzymes. UDP-glucose dehydrogenase, glucose-6-phosphate dehydrogenase and phosphoglucomutase were purchased from Sigma Chemical Co.

Genetic methods. Transduction was performed as described by Lennox using bacteriophage PlKc (23). Conjugation with Hfr strains was performed by the procedure of Taylor and Thoman (48). Thymine-negative mutants were selected on M9 minimal glucose plates with 400 μg of trimethoprim (TMP) per ml and 200 μg of thymine (45) per ml.

Growth of bacteria. Bacteria were maintained on minimal glucose plates. For measurement of enzyme activity, a stationary-phase culture was diluted 1:50 in fresh minimal medium, grown as indicated in each case at 23 C (except where noted otherwise) with reciprocal shaking, and harvested while still in exponential phase.

Preparation of cell-free extracts. The cells were harvested by centrifugation, washed once with 0.01 M NaH_2PO_4-Na_2HPO_4, pH 7.0, containing 10^{-3} mercaptoethanol and resuspended in the same buffer at approximately a 10-fold higher concentration of cells. The cells were disrupted by sonic oscillation with an MSE probe-type sonic oscillator for 45 sec. The extract was centrifuged at $37{,}000 \times g$ for 20 min, and the supernatant fluid was used as the crude soluble enzyme. The crude extract was kept at 4 C and assayed within 24 hr. The cells for some kinase assays were treated with toluene as follows: 0.2 ml of ethylenediaminetetraacetic acid (EDTA; 10^{-2} M), 0.05 ml of mercaptoethanol (1.4 M), and one drop of toluene were added to 1 ml of bacterial culture; the

TABLE 2. *Bacterial strains*

Strain	Mutant alleles important to this study	Phenotype[a]	Derivation, source, and/or genotype
MC100	None	N	R. Curtiss III (his strain χ-156)[b]; 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⁺*
MC116	None	N	N. S. Schwartz (his strain 156-2 derived from X-156)[b]; Same as MC100 except *galK⁺, lacG*, P1[r]
H81-2	*galO^c*	N	G. Buttin via S. Adhya (2); HfrH, *thi, galO^c*
H7	*galR^s*	N	S. Adhya; HfrH, *thi, galR^s*
X9001*galR⁻*	*galR⁻*	N	W. Epstein; F⁻, *thi, galR⁻*
MC129	None	M	P1 (*gal⁺*) × MC100. Select (sel.) *gal⁺*
MC102	*capR9 galK2*	M	P1 (*proC⁺ capR9*) × MC100; sel. Pro⁺; score for mucoid phenotype (*capR9*)
MC169	*capR9 galU galK2*	N	P1 (*trp⁺ galU*) × MC102; sel. Trp⁺; score for nonmucoid phenotype (*galU*)
MC120	*capS*	M	*capS* mutant of MC116 (31, 32)
MC158	*capT galK2*	M	Conjugation of M15 (an HfrH, ref. 32) × MC100; sel. Leu⁺ score for mucoid phenotype on complex medium (*capT*)
HC1002	*capR9*	M	P1 (*gal⁺*) × MC102; sel. Gal⁺
HC1003	*capT*	M	P1 (*gal⁺*) × MC158; sel. Gal⁺
HC1010	*galR^s*	N	P1 (*thy⁺ galR^s*) × MC129 *thy*; sel. *thy⁺*, score *galR^s*
HC1011	*capR9 galR^s*	M	P1 (*thy⁺ galR^s*) × HC1002 *thy*; sel. *thy⁺*, score *galR^s*
HC1012	*capT galR^s*	M	P1 (*thy⁺ galR^s*) × HC1003 *thy*; sel. *thy⁺*, score *galR^s*
HC1016	*galR⁻*	N	P1 (*thy⁺ galR⁻*) × MC129 *thy*; sel. *thy⁺*, score *galR⁻*
HC1017	*capR9 galR⁻*	M	P1 (*thy⁺ galR⁻*) × HC1002 *thy*; sel. *thy⁺*, score *galR⁻*
HC1018	*capT galR⁻*	M	P1 (*thy⁺ galR⁻*) × HC1003 *thy*; sel. *thy⁺*, score *galR⁻*
HC1022	*galO^c*	N	P1 (*galO^c gal⁺*) × MC100; sel. *gal⁺*, score *galO^c*
HC1023	*capR9 galO^c*	M	P1 (*galO^c gal⁺*) × MC102; sel. *gal⁺*, score *galO^c*
HC1024	*capT galO^c*	M	Conjugation of an M15 derivative (*capT non-1*) × HC1022; sel. Leu⁺, score for *galO^c* and *capT* (as in MC158)
HC1025	*capR9 capT galU*	N	P1 (*trp⁺ galU*) × HC1003; sel. *trp⁺*, score *galU* and then P1 (*proC⁺ capR9*) sel. *proC⁺*, score *capR9* for UV sensitivity
MC170	*capR9 capS galU*	N	P1 (*trp⁺ galU*) × MC120; sel. *trp⁺*, score nonmucoid phenotype and then P1 (*proC⁺ capR9*), sel. *proC⁺*, score *capR9* for UV sensitivity

[a] On M9 minimal medium at 37 C. M = mucoid, N = nonmucoid.

[b] Genealogy of the strains was provided by B. Bachman from files of the Coli Genetic Stock Center (CGSC). The allele designations supersede previous published designations from this laboratory (21, 24–26, 29–32, 40).

mixture was incubated at 37 C with mild shaking for 20 min and kept in an ice bath until it was assayed.

Enzyme assays. Kinase was assayed according to the method of Sherman and Adler (44) except the final volume of assay was adjusted to 0.5 ml. Galactose and galactose-1-phosphate were separated with the aid of a Dowex 1 formate column (55). Radioactive samples were counted in a Packard Tri-Carb model 3310 liquid scintillation counter using a mixture of Triton X-100-toluene (1:3) containing 4 g of Omnifluor/liter. A 0.5-ml amount of aqueous fraction was counted in 7.5 ml of the counting mixture. Epimerase and transferase were assayed according to the methods described by Kalckar, Kurahashi, and Jordan (20). The extracts disrupted by sonic oscillation were assayed at 25 C, and the cells treated with toluene were assayed for kinase at 37 C.

Chemical analysis. Protein was determined by the method of Lowry et al. (27) with bovine serum albumin as a standard. Capsular polysaccharide was estimated as nondialyzable methylpentose in supernatant fractions from boiled cultures by the method of Dische and Shettles (10-min boiling) (11).

RESULTS

Effect of capR, capS, and capT mutations on the levels of enzyme in the gal operon. The *capR9* mutation caused a four- to sixfold derepression of all three enzymes in the *gal* operon when cells were grown in minimal glucose medium at 23 C. When either glycerol or succinate was used as carbon source, a similar

derepression was observed. Kinase levels were highest in all strains grown in succinate (Table 3). The *capS* mutation did not cause much derepression. Repeated measurements indicated that the enzymes in *capS* were approximately 50% higher than in the wild type. The *capT* mutation caused a three- to fourfold derepression of all three enzymes.

The derepression of the *gal* operon observed with the *capR9* mutation is not a result of internal induction. According to the studies of Wu and Kalckar, *galK* strains are internally induced, but a strain that is *galU* [UDP-glucose pyrophosphorylase-negative (UTP: D-glucose-1-phosphate uridyltransferase, EC 2.7.7.9)] or *galE* in addition will not be subject to internal induction (54, 55). However, strain MC169 (*capR9 galK galU*) was derepressed in transferase and epimerase similar to the *capR9* gal^+ strain (Table 3). We will present more convincing evidence on this point when we consider interactions of *capR9* and $galR^s$.

As expected, the *capR9 capS* double mutant was derepressed to the same extent as *capR9*. Although *capR9* and *capT* individually caused derepression, the double mutant *capR9 capT* was no more derepressed than the *capR9* strain. Glucose-6-phosphate dehydrogenase was approximately 50% higher in *capR9* strains (data not shown), similar to results obtained previously (31). It is clear that bacteria that contain a mutation that blocks polysaccharide synthesis (*galU*) are nevertheless derepressed for the *galETK* enzymes (Table 3).

Induction of the gal operon by D-fucose. The results presented in Table 4 (compare also with Table 3) demonstrate that kinase and epimerase are derepressed by D-fucose when glycerol, but not glucose, is used as a carbon and energy source. This statement applies to mutants in either *capR*, *capS*, or *capT* and to the double mutants, *capR9 capT* and *capR9 capS*. In fully induced cultures, kinase is sixfold higher in *capR9* or *capT* strains compared to wild type. The results demonstrate that induction by D-fucose is largely independent of mutations in *cap* genes. However, D-glucose prevents induction by D-fucose in *cap* gene mutant strains as well as the wild type. Such results indicate that transport of D-fucose is probably inhibited by glucose in *cap* mutant strains as it is in the wild type (2, 22). Induction of kinase appears to be twofold higher than epimerase in most strains, but similar

TABLE 3. *Level of galactose enzymes in capR9, capS, and capT mutants*[a]

Strain	Mutant alleles	Carbon source	Galactokinase[b]	Galactose-1-P uridyle transferase[c]	UDP-galactose-4-epimerase[d]	Polysaccharide[e]
MC129	None	Glucose	15.6	0.42	4.0	6
HC1002	*capR9*	Glucose	107	2.6	17.2	716
HC1003	*capT*	Glucose	66	2.7	9.2	517
MC169	*galK, galU, capR9*	Glucose		2.4	18.2	8
MC116	None	Glucose	14.8	0.46	4.7	6
MC120	*capS*	Glucose	15.5	0.67	6.3	254
MC170	*galU, capR9, capS*	Glucose	70.9	3.9	14.6	8
HC1025	*galU, capR9, capT*	Glucose	96.8	2.2	17.1	9
MC129		Glycerol	32.8	0.85		6
HC1002		Glycerol	193.5	2.8		410
HC1003		Glycerol	124	2.3		300
MC169		Glycerol		3.2		7
MC116		Glycerol	29.6	0.79		7
MC120		Glycerol	45	1.4		184
MC170		Glycerol	143	3.2		5
HC1025		Glycerol	152	2.0		8
MC129		Succinate	136			6
HC1002		Succinate	446			320
HC1003		Succinate	413			272
MC116		Succinate	133			4
MC120		Succinate	196			62

[a] Results are the average of two separate experiments.
[b] Expressed as nanomoles of galactose-1-phosphate formed per hour per milligram of protein.
[c] Expressed as micromoles of substrate converted per hour per milligram of protein.
[d] Expressed as micromoles of UDP-glucose formed per hour per milligram of protein.
[e] Expressed as micrograms of nondialyzable methylpentose per milliliter per unit of cell turbidity (optical density at 600 μm).

TABLE 4. *Induction of galactose enzymes by* D-*fucose in mucoid mutants*[a]

Strain	Mutant alleles	Carbon source	Galactokinase	Galactose-1-P uridyle transferase	UDP-galactose-4-epimerase	Polysaccharide
MC129	None	Glucose	16.7	0.68	4.75	6
HC1002	*capR9*	Glucose	118	2.7	19.9	716
HC1003	*capT*	Glucose	96.8	2.4	10.1	495
MC116	None	Glucose	17.1	0.50	4.1	7
MC120	*capS*	Glucose	19.2	0.78	5.0	270
MC170	*galU, capS, capR9*	Glucose	75.5	3.0	13.4	10
HC1025	*galU, capT, capR9*	Glucose	100	3.6	18.1	12
MC129		Glycerol	327		60.2	7
HC1002		Glycerol	2070		136	390
HC1003		Glycerol	2160		117	327
MC116		Glycerol	261		55.3	7
MC120		Glycerol	350		61.2	178
MC170		Glycerol	1190		119	11
HC1025		Glycerol	1020		112	18

[a] All footnotes in Table 3 apply. D-Fucose was added to a final concentration of 5×10^{-3} M, and the cells were grown for five generations at 23 C.

result were noted with D-fucose as an inducer in the experiments of others (19). The synthesis of capsular polysaccharide was not altered by induction with D-fucose.

Transduction of galR^s into the mucoid strains. Bacteria that contain a *galR*^{s} mutation are not induced by D-fucose or D-galactose to form enzymes of the *gal* operon; the *galR*^{s} mutation is dominant to the wild-type *galR*$^+$ allele (41). The genes *galR* and *thyA* are cotransducible at a frequency of 50% (47). *thyA* mutants of all mucoid strains were prepared by selection on TMP plates and then transduced to *thy*$^+$ by P1 (*galR*^{s} *thy*$^+$). The *galR*^{s} transductants were identified by the observation that *galR*^{s} colonies are white on EMB-galactose plates and do not grow on minimal galactose plates after overnight incubation at 37 C. They do grow slowly on minimal galactose plates after 3 days of incubation at 37 C. Three identified *galR*^{s} transductants for each different strain were assayed for kinase activity and inducibility by D-fucose. Representative results are summarized in Table 5. It is clear that the *galR*^{s} derivatives of wild type, *capR9* and *capT* strains were not induced by D-fucose when grown in glycerol at 37 C, establishing that they were in fact *galR*^{s} derivatives, and further, they were not partly inducible by D-fucose. It should be noted that the derepression of kinase by *capR9* or *capT* was lower at 37 C than at 23 C in both *galR*^{s} and *galR*$^-$ derivatives (Table 5). This was also true of the entire *gal* operon in *cap* strains with *galR*$^+$ (data not shown). In the presence of *galR*^{s}, the *capT*, *capR9*, and *capS* mucoid mutants remained mucoid. The amount of kinase and

TABLE 5. *Activity of galactokinase in galR*^{s} *and galR*$^-$ *mutants*[a]

Strain	Genotype	D-Fucose	Galactokinase
MC129	*capR*$^+$ *galR*$^+$	−	1.15
HC1002	*capR9 galR*$^+$	−	3.6
HC1003	*capT galR*$^+$	−	2.8
HC1010	*capR*$^+$ *galR*^{s}	−	0.98
HC1011	*capR9 galR*^{s}	−	2.9
HC1012	*capT galR*^{s}	−	2.7
HC1010	*capR*$^+$ *galR*^{s}	+	0.94
HC1011	*capR9 galR*^{s}	+	3.0
HC1012	*capT galR*^{s}	+	2.6
HC1016	*capR*$^+$ *galR*$^-$	−	12.3
HC1017	*capR9 galR*$^-$	−	27.5
HC1018	*capT galR*$^-$	−	26.4

[a] Cells were grown in glycerol medium at 37 C. D-Fucose, where indicated, was added to a final concentration of 5×10^{-3} M. Galactokinase was assayed at 37 C on toluenized cells (Material and Methods) and is expressed as nanomoles/10^9 cells.

epimerase was four- to sixfold more in *capR9 galR*^{s} and *capT galR*^{s} strains compared to the *capR*$^+$ *capT*$^+$ *galR*^{s} strain (Table 6). Furthermore, the degree of derepression caused by the *capR9* mutation is the same in *galR*^{s} and *galR*$^+$ strains (compare Table 3, lines 9 and 10 with Table 6, lines 1 and 2). These data have important implications: the possibility that the derepression of *gal* enzymes by *capR* or *capT* mutations is due to internal induction via a galactose derivative is eliminated. We will pursue this topic further in the Discussion.

Transduction of galR$^-$ into the mucoid strains. By similar transduction procedures to

TABLE 6. *Derepression of galactokinase and UDP-galactose-4-epimerase in galR^s strains*[a]

Strain	Genotype	Galactokinase	UDP-galactose-4-epimerase	Polysaccharide
HC1010	*galR^s capR^+*	25.6	6.1	5
HC1011	*galR^s capR9*	148	26.5	558
HC1011	*galR^s capT*	109	20.1	475

[a] Specific enzymatic activity and polysaccharide are expressed as indicated in Table 3. Cells were grown in minimal glycerol medium at 23 C.

those described above the *cap* strains can be made *galR^-*. *galR^-* transductants form darker colonies than *galR^+* on EMB-galactose plates after overnight incubation at 37 C. Two identified colonies of each strain were assayed for kinase activity. Representative data, presented in Table 5, show that kinase is derepressed 10-fold beyond the basal level in the identified *galR^-* transductants. Genes *capR9* or *capT* cause another twofold increase.

Construction of a galO^c strain with capR9 or capT mutations. The basic strain in this study carries a *galK* mutation. *galO^c* can be transduced into the strains by P1 (*galO^c gal^+*), and selection for Gal-positive phenotype on minimal galactose plates. Strain MC102 (*galK capR9*) was transduced to *gal^+* with P1 (*galO^c gal^+*).

Direct transduction of *galO^c* into *capT* mutants was not successful. The strain was successfully constructed by transferring the *capT* from an M15 derivative (*capT* strain) into strain HC1022(*galO^c gal^+*) through conjugation and selection for *leu^+*. Among 376 nonmucoid recombinant colonies, there were six mucoid colonies. They were purified and streaked on EMB-glucose at 37 C and were mucoid under these conditions. This indicates the presence of a *capT* mutation. The *gal* enzyme levels in *galO^c* strains with either a *capR^+*, *capR9*, or *capT* mutation are summarized in Table 7. *capR9* and *capT* cause a twofold increase in kinase and epimerase in *galO^c* strains.

DISCUSSION

Nonsense mutations in either of two genes (*capR* or *capS*) or an undefined mutation in a third gene (*capT*) led to pleiotropic effects as follows: (i) increased capsular polysaccharide synthesis (29, 31); (ii) increased synthesis of enzymes specified by four and probably more spatially separated operons apparently involved in the synthesis of the polysaccharide (*capR*, *capT*) (15, 24–26, 29, 31) or several of the enzymes (*capS*) (Table 1, Fig. 1, 25); (iii) sensitivity to ultraviolet and ionizing radiation manifest as formation of nonseptate filaments and subsequent death (*capR*) which is identical with *lon* (5, 6, 12, 17, 29, 31). Other studies indicate that the *capR* gene product is a protein composed of subunits (21, 24, 30, 32, 49).

The simplest model to explain these results, implicit in a previous publication (29), is that the product of the *capR* locus is a repressor that binds to the DNA of the operators of the structural genes that are controlled. Nonsense mutations (*capR6* and *capR9*) would make an inactive repressor (30). This model requires either that several different operator regions have very similar recognition regions (base sequences) or that several different effector molecules interact with the *capR* repressor to permit it to recognize different base sequences of at least four separate operator regions.

One of the first enzymes that was found to be derepressed by the *capR6* mutation was UDP-galactose-4-epimerase (29). The present results demonstrate that the entire *gal* operon is derepressed by the *capR9* mutation. This supports similar findings of Mackie and Wilson that the *capR6* mutation also caused derepression of the entire *gal* operon. Furthermore, they demonstrated that the *capR6* mutation increased the amount of *gal* operon-specific mRNA, supporting the idea that *capR* control functions at the transcription level (Fed. Proc. **30**:1262, 1971). Their data and ours support a model in which the *gal* operon is controlled by two different repressors, the *galR* and *capR* gene products. The *capT* mutation also caused derepression of the *gal* operon enzymes, but the double mutant, *capR9 capT* was derepressed to the same extent as the *capR9* mutants. We therefore conclude that *capR* and *capT* function via a common pathway; perhaps the *capT^+* gene product is an enzyme that synthesizes a corepressor that

TABLE 7. *Enzyme levels of the galactose operon in galO^c strains*[a]

Strain	Genotype	Galactokinase	UDP-galactose-4-epimerase
MC129	*galO^+ capR^+*	10.1	3.5
HC1022	*galO^c capR^+*	302	124
HC1023	*galO^c capR9*	747	215
HC1024	*galO^c capT*	563	198

[a] Specific activity is expressed as in Table 3. Cells were grown in minimal glucose medium at 23 C.

combines with the *capR* protein to make holorepressor. (We have not excluded the possibility that the roles of the *capR* and *capT* gene products could be reversed.) Other functions might also be proposed for *capT*, but more experiments are required before further discussion is warranted. The *capS* mutation has little effect on the *gal* operon.

How does the *capR* repressor interact with the *gal* operon? One might suggest a very indirect action caused by internal induction; i.e., somehow *capR* repressor causes an increase in a galactoside within the cells and this galactoside interacts with the $galR^+$ repressor to cause derepression. This model was critically tested in this study by preparing strains that contained the $galR^s$ mutation in combination with *capR9* and *capT*. These strains were compared with $galR^+$ derivatives of *capR9* and *capT*. If internal induction via a galactoside occurred in the $galR^+$ derivative it would not be observed in a $galR^s$ strain, i.e., $galR^s$ is an altered form of the $galR^+$ gene and does not respond to galactosides (41; Table 5). The results demonstrate that either *capR* or *capT* caused a four- to sixfold derepression in a $galR^s$ strain (Table 6), and the degree of derepression caused by the *capR9* mutation is the same in $galR^s$ and $galR^+$ strains (compare Table 3, lines 9 and 10 with Table 6, lines 1 and 2). Other combinations of *capR9* or *capT* with $galR^-$ or $galO^c$ demonstrated that mutations in either *capR9* or *capT* caused derepression beyond that caused by the $galR^-$ or $galO^c$ mutation. This particular $galO^c$ was not induced further by D-fucose, indicating that it is truly insensitive to $galR^+$ repression (*unpublished data*). We conclude that *capR* and *capT* do not cause derepression by increasing the supply of a galactose derivative. Results in which possible internal induction was eliminated by introducing *galU* (UDP-glucose pyrophosphorylase deficiency; 54, 55) into a $galK^-$ *capR9* strain showed that *capR9* still caused derepression and support our conclusions.

We must now consider our data that relate to the effect of glucose on derepression in *capR* and *capT* strains. The data of Table 3 show that all strains (wild type or mutants in either *capR*, *capS*, or *capT*) had highest levels of *gal* enzymes when grown on succinate as compared to glycerol (next highest) or glucose. Thus the decrease of enzymes by growth in glucose is evident in all *cap* strains and may be taken as an indication that the c-AMP CRP system influences transcription in *cap* strains. There is considerable evidence from other laboratories that the c-AMP CRP system stimulates in vivo and in vitro transcription of the *gal* operon and that this effect is at a step prior to mRNA chain elongation (34, 35). This stimulation may be related to action of the c-AMP CRP system at a promotor site in the *gal* operon by analogy with cyclic AMP action in the *lac* system (50, 51, 56).

The implication of our experiments, taken together with the more extensive studies in other laboratories on *galR*, is that two negative control systems, the *galR* repressor and *capR* repressor, control the transcription of *gal* mRNA and, in addition, the c-AMP CRP system controls the same transcription in a positive fashion (34–36, 52). This is at present the most complex type of control proposed for a single operon, and it may be suggested that the reason for this complexity lies in the fact that one system is needed for catabolism of D-galactose and another for synthetic purposes related to cell wall and capsular polysaccharide synthesis.

Do the two repressors act cooperatively or independently? Mackie and Wilson (Fed. Proc. **30**:1262, 1971) found that the *capR6* mutation caused less derepression in a $galR^-$ and several $galO^c$ strains than in a $galR^+$ strain. We obtained similar results; the *capR9* (and *capT*) mutation caused less-fold derepression in a $galR^-$ and a $galO^c$ strain than in a $galR^+$ strain (Tables 5 and 7). The $galO^c$ $capR^+$ strain we used was fully constitutive since it could not be further induced with D-fucose. These results are inconclusive since one can propose that *galR* and *capR* act cooperatively *or* that the maximum rate of expression for the *gal* operon was reached under the conditions of growth. However, our results comparing $galR^+$ or $galR^s$ in combination with *cap* alleles are not subject to the contention that maximum rates of expression were reached. The results showed that either *capR9* or *capT* caused a four- to sixfold derepression of the *gal* operon in a $galR^s$ strain and the fold derepression caused by the *capR9* mutation is the same in the $galR^s$ and $galR^+$ strains (compare Table 3, lines 9 and 10 with Table 6, lines 1 and 2). Therefore, the two repressors appear to act independently. We consider this question to be of considerable importance and offer this as a tentative conclusion, one that will be subjected to more critical analysis with cell-free transcription systems when the $capR^+$ repressor is isolated.

At this time we would like to propose the following working model (Fig. 2). There are two operator sites at the *galE* end of the *gal* operon; one designated O^{galR^+} binds the $galR^+$

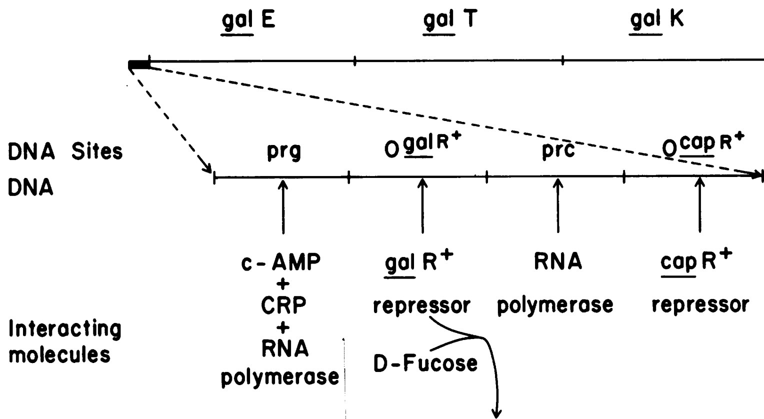


FIG. 2. *Proposed model for control of the galactose operon. Taken in part from Wilson and Hogness (53). The number of base pairs in the galE gene is approximately 1,900; the galE, galT, and galK genes are drawn in proportion (53) and the sequence of galE, galT, and galK are known (7, 8). The operator site (O^{galR^+}) responding to galactose and that binds the galR+ repressor (37) is to the left of galE (4, 7, 8, 10, 18, 42, 43), but the order of the remaining sites are unknown. O^{galR^+} binds the galR+ repressor, which may be removed by D-fucose or D-galactose (37) and, if c-AMP and c-AMP receptor protein are available, the galR promotor site (prg) will bind RNA polymerase. A second operator site (O^{capR^+}) binds capR+ repressor and contains an adjacent hypothetical capR promotor site (prc) where RNA polymerase also binds.*

repressor which may be removed by D-fucose (37) and, if c-AMP and CRP are available, the *galR* promotor site (*prg*) will bind DNA-dependent RNA polymerase; a second operator site (O^{capR^+}) binds *capR*+ repressor [and may be removed by an unknown inducer (*capT* controlled?) or inactivated by mutation as in *capR9* or *capR6*] and contains an adjacent *capR* promotor site (*prc*) where DNA-dependent RNA polymerase also binds. There is some precedent for part of our model. Reichardt and Kaiser recently presented evidence for two promotor sites for regulation of synthesis of the λ repressor, one that functions at a high rate during establishment of lysogeny and another that functions at a low rate for maintenance of lysogeny (38). There is no evidence for the existence of our postulated promotor (*prc*) adjacent to O^{capR^+}. The new feature of this model is related to the idea that two separate and independent repressors (*capR* and *galR* proteins) bind to different operator sites. There is no direct genetic evidence to support a separate operator site for *capR* binding near *galE*, i.e., there are no known mutants in the postulated O^{capR^+} site that are insensitive to *capR*+ (*capT*+) repression. In any model with two different repressor binding sites for control of synthesis of one mRNA molecule there is the problem of how DNA-dependent RNA polymerase will read through or compete with one of the repressors after only the repressor distal to the *galE* site has been removed. One solution to this problem would be a repressor (*capR*+) with low affinity for the DNA site compared with DNA-dependent RNA polymerase. In the context of our model we might expect to find two different mRNA species transcribed from the *gal* operon if mRNA synthesis is initiated at two promotor sites. However, it is also possible that DNA-dependent RNA polymerase might attach at either of two promotors and move to the second one before mRNA synthesis is initiated, in which case only one mRNA would be expected. Most aspects of this model are subject to experimental verification at the present time. Dual operator regulation has been produced artifically by fusing the *trp* and *lac* operons (39).

With regard to practical enzymology, it may be mentioned that the double mutants, *capR9 galO*^{c}, or a *capR9* strain induced with D-fucose produced the highest levels of galactose enzymes observed and, with a mutation in *galU* to prevent polysaccharide synthesis, would be the strains of choice for purifying the enzymes of the galactose operon. The results of Table 4 also show that a *capR9* strain induced with D-fucose in glycerol produced 125 times as much galactokinase as the wild type grown under repressing conditions, i.e., glucose. The derepression of the *gal* operon thus approaches that of the classic *lac* operon (values of 1,000-fold) when *all three* of the factors controlling *gal* operon expression, i.e., *galR* repressor, *capR* repressor, and c-AMP CRP system, are turned off or turned on.

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