JOURNAL OF BACTERIOLOGY, June 1973, p. 1040-1044

Vol. 114, No. 3
Printed in U.S.A.

Role of Cyclic Adenosine 3',5'-Monophosphate in the In Vivo Expression of the Galactose Operon of *Escherichia coli*

LUCIA B. ROTHMAN-DENES, JOANNE E. HESSE, AND WOLFGANG EPSTEIN

Departments of Biophysics and Biochemistry, The University of Chicago, Chicago, Illinois 60637

Received for publication 22 December 1972

Studies of levels of galactokinase in *Escherichia coli* with mutations affecting synthesis of, or response to, cyclic adenosine 3',5'-monophosphate show that this nucleotide does not play a major role in expression of the galactose operon, causing at most a twofold stimulation. The discrepancy between our in vivo results and the marked stimulation by cyclic adenosine 3',5'-monophosphate in in vitro systems indicates that current cell-free systems lack a factor which allows efficient expression of the galactose operon even in the absence of cyclic adenosine 3',5'-monophosphate or of the binding protein for this nucleotide.

Genetic (7, 19, 20) and biochemical (2, 4, 6, 7) evidence has accumulated in recent years, showing that the catabolite repression of inducible enzymes is mediated by cyclic adenosine 3',5'-monophosphate (c-AMP) and a protein factor (CRP) that specifically binds c-AMP. Recently, two groups of investigators reported that expression of the galactose operon in vitro, measured either as synthesis of galactose-specific messenger RNA (mRNA) or by coupled mRNA and enzyme synthesis, is markedly stimulated by c-AMP and CRP (17, 18, 23). In this communication we show that the expression of the galactose operon in vivo is not greatly influenced by c-AMP and CRP.

MATERIALS AND METHODS

Strains. Most of the strains used in this work were constructed by P1*kc* transduction from a TrkA/D mutant of *Escherichia coli* K-12 (9). These strains, prefixed LU, are all constitutive for the glycerol regulon (*glpR*$^-$) and, except as noted, constitutive for the lactose operon (*lac*i_s) and for the galactose operon (*galR*$^-$). The strains are either defective in adenyl cyclase, carrying the *cya* mutation from strain 5336 (19), defective in the c-AMP binding protein due to the *crp* mutation from strain 5333 (19), defective in both, or neither. In strain LU94 the normal host galactose region is replaced by deletion 507 (21) which extends from *nadA* to *att*λ, and all of the galactose operon is from the transducing phage λp*gal*8 (17). LU94 also carries λ helper which, like the transducing phage, carries the temperature-sensitive CI_{857} mutation. LU107 carries the *cap*R9 mutation of Markovitz (10). CAE134 and CAE135 were prepared by transduction of a *pdxA* Hfr Hayes strain; these two are isogenic except for the presence of the *araC*$^+$ and *araC*^{c}67 mutations, respectively. L104 is a strain constitutive for the β-methylgalactosidase transport system (13).

Chemicals. [1-^{14}*C*]galactose was obtained from Calbiochem, and [*U*-^{14}*C*]glycerol was obtained from International Chemical and Nuclear Corp.

Bacterial growth and preparation of extracts. Cells were grown at 37 C in 0.2% glucose K115 medium (9) with or without 3 mM c-AMP as indicated, harvested when the cultures had reached approximately 6 × 10^8 cells/ml, washed by centrifugation with 50 mM tris(hydroxymethyl)aminomethane-chloride (Tris-Cl) buffer (pH 7.0) containing 1 mM $MgCl_2$, and disrupted by sonic irradiation. After centrifugation for 12 min at 26,000 × *g*, the supernatant fluids were assayed for the enzymes. LU94 was grown at 34 C to prevent prophage induction.

Enzyme assays. β-Galactosidase was assayed by the rate of *o*-nitrophenyl-β-D-galactopyranoside hydrolysis (8). One unit of β-galactosidase produces 1 μmol of product per min at 37 C.

Galactokinase and glycerol kinase were assayed by the technique of Sherman (22) in which the conversion of radioactive substrate to a form retained on diethylaminoethyl-paper (Whatman DE81) was measured. Galactokinase assays contained: 100 mM Tris-Cl (pH 7.5), 6 mM $MgCl_2$, 4 mM NaF, 16 mM mercaptoethanol, 5 mM adenosine triphosphate, 1 mM [1-^{14}*C*]galactose, and cell extract. One unit of galactokinase produces 1 nmol of product per min at 37 C.

Glycerol kinase assays contained: 80 mM Tris-Cl (pH 7.5), 1.6 mM ethylenediaminetetraacetic acid, 22 mM NaF, 5 mM $MgCl_2$, 33 mM mercaptoethanol, 5 mM adenosine triphosphate, 4 mM [*U*-^{14}*C*]glycerol, and extract. All assays were performed at 37 C.

In the experiments presented in Fig. 1 and Table 3, β-galactosidase activity was measured in toluene-

treated suspensions as described elsewhere (8). For galactokinase measurements 1-ml samples of culture were brought to 2 mM in ethylenediaminetetraacetic acid and 70 mM in mercaptoethanol and shaken with 30 μliters of toluene for 30 min at 37 C. This suspension was assayed as described above except that the galactose concentration was 0.2 mM. Galactokinase values for the samples containing glucose are corrected for the 16% inhibition of activity by glucose under these assay conditions. Protein was determined by the method of Lowry et al. (14), using bovine albumin as standard.

RESULTS

Effect of cya and crp mutations on enzyme levels. Our initial experiments used a series of strains, constructed by P1*kc*-mediated transduction, which are fully constitutive for the lactose and galactose operons and for the glycerol regulon. The synthesis of enzymes of each of these three systems is markedly reduced in the presence of glucose, and for all three this glucose effect has been interpreted in terms of catabolite repression (5, 11, 16).

Table 1 shows that β-galactosidase and glycerol kinase levels are low in LU63 (*crp*), LU73 (*cya*), and LU83 (*cya crp*). The addition of 3 mM c-AMP to the growth medium of LU73 results in a 20-fold and 40-fold stimulation of glycerol kinase and β-galactosidase levels, respectively. In contrast, galactokinase levels are only slightly affected by these mutations or the presence of c-AMP.

Role of regulatory mutations on the effect of c-AMP. The dependence on c-AMP might be affected by the presence of protein components which are known, or suspected, to act at or near the galactose promoter. Two such factors are the galactose repressor produced by the *galR* gene, and the product of the *capR* gene. In strains defective in the *capR* product there is an increase in the levels of a number of enzymes of polysaccharide synthesis and approximately a doubling of the levels of the galactose enzymes (10, 15). Neither the presence of the galactose repressor (Table 2, strains LU55 and LU75) nor the absence of the *capR* product (Table 2, strain LU107) result in a marked dependence on c-AMP. In strain LU107 there is the paradoxical result of a slight reduction of galactokinase by c-AMP. In all of these strains c-AMP greatly stimulated β-galactosidase synthesis.

Behavior of the galactose operon from λpgal8. In one of the in vitro studies, deoxyribonucleic acid from a plaque-forming galactose transducing bacteriophage, λp*gal*8 was used as template, and a 15-fold stimulation of galactose operon transcription by c-AMP and CRP was observed (17). We examined the dependence of the in vivo expression of the galactose operon from this template in strain LU94, which carries a deletion from *att*λ to *nadA* and is lysogenic for λp*gal*8 and λ helper

TABLE 2. *c-AMP effects in strains with mutations affecting galactose operon regulation*[a]

Strain	Pertinent genotype	c-AMP (mM)	Galactokinase (μmole/min/mg of protein)	β-Galactosidase (μmole/min/mg of protein)
LU55	*galR*$^+$	0	0.0084	20.5
		3	0.0158	35
LU75	*cya galR*$^+$	0	0.0106	0.63
		3	0.0162	27
LU85	*cya crp galR*$^+$	0	0.0116	—
LU107	*cya galR capR9*	0	0.187	0.47
		3	0.152	20.8
LU94	*cya galR* λp*gal*8	0	0.242	0.61
		3	0.348	27.8

[a] Values are assays of a single culture except for LU75 where the average of assays of two cultures is given.

TABLE 1. *Effects of c-AMP on enzyme synthesis in wild-type and mutant E. coli*[a]

Strain[b]	Defect	c-AMP (mM)	Galactokinase (μmole/min/mg of protein)	β-Galactosidase (μmole/min/mg of protein)	Glycerol kinase (μmole/min/mg of protein)
LU53	—	0	0.083	15.3	0.34
		3	0.168	32.0	0.88
LU63	*crp*	0	0.130	0.61	0.042
		3	0.110	0.55	0.038
LU73	*cya*	0	0.141	0.62	0.041
		3	0.177	27.2	0.84
LU83	*crp cya*	0	0.123	0.51	0.038
		3	0.124	0.54	0.042

[a] Values are the averages of determinations on two separate cultures. Cultures of the mutants contained less than 1% revertants at the time the cultures were harvested.

[b] Strains listed are constitutive for the three enzymes assayed (see Results).

phage. The strain appears to carry an intact λ*pgal*8 since it gives rise to high-frequency transducing lysates for galactose utilization. There is no large dependence of galactokinase on c-AMP in this strain (Table 2).

Effect of inducer transport and c-AMP on the galactose operon. We examine the induction of galactokinase in strains with mutations altering the transport of fucose, the gratuitous inducer of the galactose operon used in our experiments. We find that the effects of glucose and c-AMP on galactokinase synthesis depend on the strain examined. Figure 1 shows data for CAE134, a strain constitutive for the arabinose transport systems which have affinity for fucose and are not inhibited by glucose (3). In this strain there is marked transient repression of β-galactosidase synthesis which is fully reversed by c-AMP (Fig. 1A), whereas galactokinase synthesis is less severely reduced and c-AMP does not fully reverse the inhibition (Fig. 1B). In the other strains examined (Table 3), galactokinase synthesis is more severely reduced by glucose, and c-AMP never fully reverses the reduction. The synthesis of β-galactosidase, on the other hand, was restored to control levels by c-AMP in all cases except for strain L104, for which 3 mM c-AMP seems to be insufficient to fully overcome transient repression.

DISCUSSION

The data of Tables 1 and 2, showing at most a twofold stimulation of galactokinase synthesis by c-AMP, lead us to conclude that this nucleotide plays only a minor role in the in vivo expression of the galactose operon, in contrast to its major role in the expression of the lactose operon and the glycerol regulon. The behavior of glycerol kinase and β-galactosidase show that the mutations we used are not very leaky. This point is also demonstrated by the data for LU83 which show the same levels of the enzymes as the strains which are defective in only *cya* or *crp*, i.e., LU63 and LU73.

In surveying the literature, we found only one experiment which appears to be inconsistent with this conclusion. That experiment showed that the glucose inhibition of galactokinase induction is fully reversed by c-AMP (5). Glucose can inhibit enzyme synthesis by at least two different mechanisms: interference with inducer uptake and catabolite repression (16). Interference with inducer uptake is an important factor in the glucose inhibition of galactokinase synthesis as shown by two facts: (i) glucose severely inhibits uptake of inducers of the galactose operon (1), and (ii) constitutive synthesis of the lactose transport system largely overcomes glucose inhibition (1, 12).

In interpreting the data of Table 3 we assume that, if the sole effect of glucose is to alter cell c-AMP levels, the reduction by glucose will not be greater than the stimulation by c-AMP in mutants defective in adenyl cyclase. By this criterion, changes in c-AMP alone can account

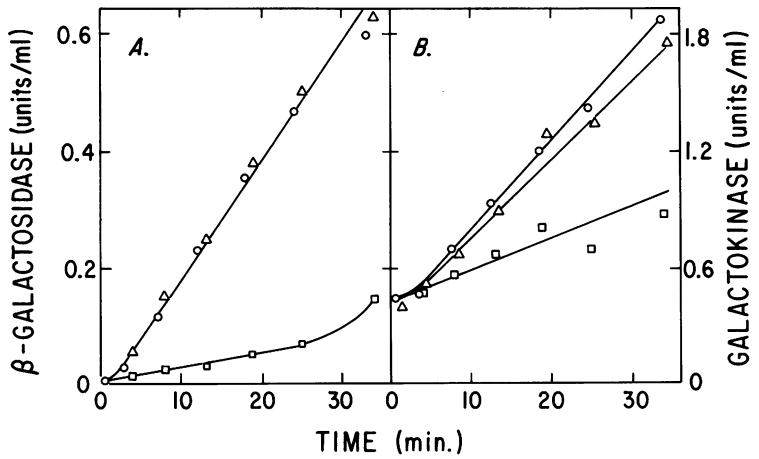

FIG. 1. *Effects of glucose and c-AMP on the induction of β-galactosidase and galactokinase. At t = 0 an exponential-phase culture of strain CAE134 at 5 × 10⁸ cells/ml in 0.2% glycerol minimal medium was induced and then divided into three portions. One served as control (○), a second received glucose to a final concentration of 11 mM (□), whereas a third received both 11 mM glucose and c-AMP to a concentration of 3 mM (△). Protein synthesis in the samples taken at the times indicated was arrested with 50 μg of chloramphenicol per ml. (A) Induction of β-galactosidase by 1 mM isopropyl-β-d-1-thiogalactopyranoside. (B) Induction of galactokinase by 5 mM fucose.*

TABLE 3. *Inhibition of enzyme synthesis by glucose and its reversal by c-AMP*[a]

Strain	Pertinent genotype	Galactokinase			β-Galactosidase		
		Control (units/ml)	Glucose (% of control)	Glucose + c-AMP (% of control)	Control (units/ml)	Glucose (% of control	Glucose + c-AMP (% of control)
CAE134	*araC*^{c}67	1.03	37	90	0.42	10	101
CAE135		0.97	18	67	0.52	15	100
L104	*mg/R*	0.83	18	33	0.20	5	72
LU57		1.05	9	36	0.53	13	99

[a] All strains are inducible for the lactose and galactose operons. The rates of enzyme synthesis were measured over the interval from 5 to 25 min after addition of inducer as described in Materials and Methods. Rates of enzyme synthesis in the presence of glucose are expressed as a percent of the rate in the control which received only inducer.

for inhibition of β-galactosidase synthesis but not for the inhibition of galactokinase synthesis, and thus imply that effects of glucose besides those on c-AMP are also present. Inducer exclusion is suggested by the greater glucose inhibition in strain CAE135 than in CAE134, the former having low basal levels and the latter having high constitutive levels of the arabinose transport systems. Strain L104 has high constitutive levels of the methylgalactosidase system which can transport fucose but is severely inhibited by glucose (13). The mechanism whereby c-AMP stimulates galactokinase synthesis induced in the presence of glucose is not clear, but since the stimulation can be as large as fourfold (Table 3, LU57) we suspect that c-AMP may have slight effects on inducer transport.

Our failure to detect a large dependence of galactokinase synthesis in vivo is not due to the presence of a mutant galactose promoter since the strains tested represent a total of four galactose operons of different origin. We, therefore, conclude that in vivo c-AMP and CRP are not essential for transcription of the galactose operon. The fact that they are needed in vitro implies that the cell-free systems currently in use are not a faithful reflection of in vivo conditions. We suggest that there are additional factors absent from current in vitro preparations which allow for efficient transcription of the galactose operon in the absence of c-AMP and CRP.

ACKNOWLEDGMENTS

We thank Winifred Boos, Max Gottesman, Robert Perlman, and Alvin Markovitz for providing bacterial strains and bacteriophages used in this work.

This work was supported by Public Health Service grant GM 15766, special postdoctoral fellowship GM 48803 to L. B. R-D., and Research Career Development Award GM 10725 to W.E., all from the National Institute of General Medical Sciences.

LITERATURE CITED

1. Adhya, S., and H. Echols. 1966. Glucose effect and the galactose enzymes of *Escherichia coli*: correlation between glucose inhibition of induction and inducer transport. J. Bacteriol. **92**:601-608.
2. Arditti, R. R., L. Eron, G. Zubay, G. Tocchini-Valentini, S. Connaway, and J. R. Beckwith. 1970. *In vitro* transcription of the lac operon genes. Cold Spring Harbor Symp. Quant. Biol. **35**:437-442.
3. Brown, C. E., and R. W. Hogg. 1972. A second transport system for L-arabinose in *Escherichia coli* B/r controlled by the *araC* gene. J. Bacteriol. **111**:606-613.
4. de Crombrugghe, B. B. Chen, M. Gottesman, and I. Pastan. 1971. Regulation of lac mRNA synthesis in a soluble cell-free system. Nature N. Biol. **230**:37-40.
5. de Crombrugghe, B. R. L. Perlman, H. E. Varmus, and L. Pastan. 1969. Regulation of inducible enzyme synthesis in Escherichia coli by cyclic adenosine 3',5'-monophosphate. J. Biol. Chem. **244**:5828-5835.
6. de Crombrugghe, B., H. E. Varmus, R. L. Perlman, and I. Pastan. 1970. Stimulation of lac mRNA synthesis by cyclic AMP in cell free extracts of *Escherichia coli*. Biochem. Biophys. Res. Commun. **38**:894-901.
7. Emmer, M., B. de Crombrugghe, I. Pastan, and R. L. Perlman. 1970. The cyclic AMP receptor protein of *E. coli*: its role in the synthesis of inducible enzymes. Proc. Nat. Acad. Sci. U.S.A. **66**:480-487.
8. Epstein, W. 1967. Transposition of the lac region of *E. coli* IV. Escape from repression in bacteriophage-carried lac genes. J. Mol. Biol. **30**:539-543.
9. Epstein, W., and B. S. Kim, 1971. Potassium transport loci *Escherichia coli* K-12, J. Bacteriol. **108**:639-644.
10. Hua, S., and A. Markovitz. 1972. Multiple regulator gene control of the galactose operon in *Escherichia coli* K-12. J. Bacteriol. **110**:1089-1099.
11. Koch, J. P., S. Hayashi, and E. C. C. Lin. 1964. The control of dissimilation of glycerol and L-α glycerophosphate in *E. coli*. J. Biol. Chem. **239**:3106-3108.
12. Lengeler, J. 1966. Untersuchungen zum glucose-effekt bei der synthese der galaktose-enzyme von *E. coli*. Z. Vererbungsl. **98**:203-229.
13. Lengeler, J., K. D. Hermann, H. J. Unsöld, and W. Boos. 1971. The regulation of the β-methylgalactoside transport system and of the galactose binding protein of *E. coli* K-12. Eur. J. Biochem. **19**:457-470.
14. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.
15. Mackie, G., and D. B. Wilson. 1972. Regulation of

transcription of the galactose operon of *E. coli* by the *cap*R gene. J. Biol. Chem. **247**:2973–2978.
16. Magasanik, B. 1970. Glucose effects: inducer exclusion and repression, p. 189–219. *In* J. R. Beckwith and D. Zipser (ed.), The lactose operon. Cold Spring Harbor Laboratory.
17. Nissley, S. P., W. B. Anderson, M. E. Gottesman, R. L. Perlman, and I. Pastan. 1971. *In vitro* transcription of the gal operon requires cyclic adenosine monophosphate and cyclic adenosine monophosphate receptor protein. J. Biol. Chem. **246**:4671–4678.
18. Parks, J. S., M. Gottesman, R. L. Perlman, and I. Pastan. 1971. Regulation of galactokinase synthesis by cyclic adenosine 3′,5′-monophosphate in cell-free extracts of *E. coli*. J. Biol. Chem. **246**:2419–2424.
19. Perlman, R. L., and I. Pastan. 1969. Pleiotropic deficiency of carbohydrate utilization in an adenyl cyclase deficient mutant of *E. coli*. Biochem. Biophys. Res. Commun. **37**:151–157.
20. Schwartz, D., and J. R. Beckwith. 1970. Mutants missing a factor necessary for the expression of catabolite-sensitive operons in *E. coli*, p. 417–422. *In* J. R. Beckwith and D. Zipser (ed.), The lactose operon. Cold Spring Harbor Laboratory.
21. Shapiro, J. A., and S. L. Adhya. 1969. The galactose operon of *E. coli* K-12 II. A deletion analysis of operon structure and polarity. Genetics **62**:249–264.
22. Sherman, J. R. 1963. Rapid enzyme assay technique utilizing radioactive substrate, ion-exchange paper and liquid scintillation counting. Anal. Biochem. **5**:548–554.
23. Wetekam, W., K. Staack, and R. Ehring. 1971. DNA dependent *in vitro* synthesis of enzymes of the galactose operon of *E. coli*. Mol. Gen. Genet. **112**:14–27.