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In Vitro Repression of the Transcription of *gal* Operon by Purified *gal* Repressor

(*E. coli*/λ phage/fucose/galactose/affinity chromatography)

SHIGETADA NAKANISHI, SANKAR ADHYA, M. E. GOTTESMAN, AND IRA PASTAN

Laboratory of Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20014

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ABSTRACT **We have studied the *in vitro* repression of *gal* mRNA synthesis by the *gal* repressor from *Escherichia coli*. By use of a four-step purification procedure involving chromatography on phosphocellulose, DEAE-cellulose, and an affinity resin, the *gal* repressor has been purified about 1600-fold from a crude cell extract. The purification was aided by use of a cell extract made after prophage induction of cells lysogenic for bacteriophage λ that carries the *gal* repressor gene (*galR*). The highly purified *gal* repressor is an effective and specific repressor of *in vitro* synthesis of *gal* mRNA with λ *gal* DNA as template. Both D-fucose and D-galactose overcome the action of *gal* repressor; the half-maximal concentrations of D-fucose and D-galactose for overcoming the action of repressor are 1 mM and 0.5 mM, respectively. The repressor fails to repress *gal*-specific transcription when the *gal* DNA contains a cis-dominant operator constitutive (O^c) mutation. We conclude that the *gal* repressor recognizes the *gal* operator site and acts by preventing *gal* transcription.**

The expression of the *gal* operon of *Escherichia coli* is known to be regulated by two small molecules. The first is the inducer, D-galactose, which relieves repression by the *gal* repressor (1–5). The second is cyclic AMP, which in concert with cylic AMP receptor protein (CRP), allows RNA polymerase to initiate *gal* transcription (6–9). Parks *et al.* (10) have partially purified the *gal* repressor from extracts of *E. coli* lysogenic for a λ transducing phage that carries the gene coding for *gal* repressor. After induction, prophage replication produced large quantities of *gal* repressor, presumably because of a gene dosage effect. With the partially purified protein obtained by affinity chromatography on *p*-aminophenyl-β-D-thiogalactoside-substituted agarose, they showed that *gal* repressor binds to the *gal* DNA containing a wild-type operator, and that the binding is prevented by the addition of either of the two inducers, D-fucose and D-galactose. However, the preparation of repressor used by Parks *et al.* (10) was not sufficiently pure to allow studies on its ability to repress *gal* transcription *in vitro*.

In this paper we describe a method for preparing *gal* repressor of sufficient purity to investigate how the *gal* repressor and inducers of the *gal* operon work. We find that *gal* repressor represses *gal* transcription *in vitro* and that the repression is lifted by the inducers, D-galactose or D-fucose.

MATERIALS AND METHODS

Chemicals. Cyclic AMP, optical grade cesium chloride, [5-^{3}H]CTP (20.4 Ci/mmol), D-galactose, and D-fucose (6-deoxy-D-galactose) were purchased from Schwarz-Mann; UTP, ATP, CTP, and GTP from P-L Biochemicals; ribonuclease-free deoxyribonuclease, bovine-pancreatic ribonuclease, and rabbit-muscle lactate dehyrogenase from Worthington; *E. coli* alkaline phosphatase from Sigma; *E. coli* tRNA from General Biochemicals; phosphocellulose (Whatman P1) and DEAE-cellulose (DE52) were from Reeve Angel. *p*-Aminophenyl-β-D-thiogalactoside linked to agarose was prepared as described by Parks *et al.* (10).

Cyclic AMP Receptor Protein, RNA Polymerase, and Phage DNA. CRP was purified as described by Anderson *et al.* (11). *E. coli* RNA polymerase was prepared according to the method of Berg *et al.* (12). The phage DNA was extracted as described by Nissley *et al.* (9).

Bacterial and Bacteriophage Strains Used. N1812, a strain of *E. coli* K-12, was used as the source of *gal* repressor for purification. Its genotype is: HfrH*thi*$^-$ *galE*$^-$ (*galR-lys*)$_\Delta$λ*cryJ*$^+$ lysogenic for λ*cI*857*Sam*7p*galR*$^+$*lys*$^+$. *gal* repressor was purified from cells grown in a medium containing 10 g of tryptone, 5 g of NaCl, and 5 g of yeast extract per liter. The temperature was raised to 41° for 20 min in the early-logarithmic phase of growth to induce the lysogen, then lowered to 34°. The cells were harvested 4 hr later and stored frozen. Bacteriophage λ*cI*857*Sam*7p*gal*25 (λp*galO*$^+$), carrying the entire *gal* operon except for a small distal segment of the *k* cistron, has been isolated by Feiss *et al.* (13). λ*cI*857 *Sam*7p*gal*$^+$ 8-O^c1038, (λp*galO*^{c}), which is isogenic with λp*gal*8 except for the O^c mutation, was isolated by S. Adhya (manuscript in preparation). The phages were purified by banding in cesium chloride density gradients (9).

Assay of gal Repressor. The DNA-binding activity of *gal* repressor was measured as described by Parks *et al.* (10). The preparation of λp*gal*[^{32}P]DNA has also been described (10).

In Vitro Transcription of gal Operon and Hybridization Procedures. Standard reaction mixtures contained 20 mM Tris·HCl (pH 7.9), 3 mM $MgCl_2$, 100 mM KCl, 0.1 mM dithiothreitol, 0.1 mM EDTA, 0.15 mM (each) ATP, UTP and GTP, 75 μM [^{3}H]CTP (20.4 Ci/mmol), 5% (v/v) glycerol, 16.5 μg/ml of λp*gal* DNA, 20 μg/ml of CRP, 0.1 mM cyclic AMP, and 24 μg/ml of RNA polymerase. The reaction mixture (0.1 ml) was first incubated without RNA polymerase at 37° for 3 min: the reaction was started by the addition of RNA polymerase and terminated after 20 min by the addition of 1 ml

Abbreviation: CRP, cyclic AMP receptor protein.

of a cold solution containing 0.1 M Tris·HCl (pH 7.0), 3.3 mM magnesium acetate, 0.33 mg/ml of tRNA, and 28 μg/ml of deoxyribonuclease. The RNA was extracted and hybridized to measure the amount of *gal* messenger RNA as described (9). Nissley *et al.* (9) observed that the addition of CRP and cyclic AMP to a transcription system containing λp*gal*8 DNA as template increased total RNA synthesis 2.5-fold, due to the read-through of RNA polymerase initiating at the *gal* promoter into λ genes. The increase in total RNA made provides a simple means of measuring the stimulation of *gal* transcription by CRP and cyclic AMP. We used this assay to study the effect of inducer concentration according to the procedures described by Anderson *et al.* (11), except that the reaction mixture was used as described above.

Sucrose Density Gradient Centrifugation. 0.2 ml of *gal* repressor preparation was layered on a 5.2-ml, linear 5–20% sucrose gradient containing 0.05 M potassium phosphate (pH 6.5)–0.1 mM EDTA–0.2 mM dithiothreitol. The gradients were centrifuged for 16 hr in an SW50.1 rotor at 48,000 rpm at 3°, and were collected by drops in 42 fractions. Rabbit-muscle lactate dehydrogenase and *E. coli* alkaline phosphatase were used as internal velocity markers. The activity of each enzyme was assayed according to the Worthington enzyme manual.

Protein Determinations. Protein was routinely measured by the method of Lowry *et al.* (14), with crystalline bovine-serum albumin used as a reference standard.

RESULTS

Purification of *gal* repressor

Step 1: Preparation of Cell Extracts and Precipitation with 70% Ammonium Sulfate. All steps were performed at 0–4°. All buffers contained 0.1 mM EDTA and 0.2 mM dithiothreitol. Frozen cells (200 g) were suspended in 800 ml of Buffer A [10 mM Tris·HCl (pH 7.5)–10 mM $MgCl_2$–0.1 mM EDTA–0.2 mM dithiothreitol] and disrupted at 10,000 lb/in² in an Aminco French pressure cell. 2 μg of DNase was added to each ml of cell extract. When the extremely viscous solution became fluid, the cell extracts were centrifuged for 2 hr at 19,000 rpm in a Spinco Type 19 rotor. To this supernatant fluid, solid ammonium sulfate (472 mg for each ml of solution) was added slowly; a pH of 6.5 was maintained by the addition of 5 N KOH. After 30 min the precipitate was removed by centrifugation at 10,000 × *g* for 30 min and dissolved in 100 ml of 0.1 M potassium phosphate buffer (pH 6.5). This solution was dialyzed against 20 volumes of the same buffer for 4 hr, and the insoluble material was removed by centrifugation at 10,000 × *g* for 20 min.

Step 2: Phosphocellulose Chromatography. The supernatant from *step 1* was applied to a phosphocellulose column (2.6 × 25 cm) equilibrated with 0.1 M potassium phosphate buffer (pH 6.5). The column was washed with 400–500 ml of the same buffer until the absorbance at 280 nm of the eluate was reduced to 0.10–0.15. The *gal* repressor was eluted with a linear gradient starting with 300 ml of 0.1 M potassium phosphate buffer (pH 6.5) in the mixing chamber and 300 ml of 0.5 M potassium phosphate buffer (pH 6.5) in the reservoir. Fractions of 10 ml (each) were collected, and *gal* repressor was eluted at a salt concentration of 0.15 M–0.2 M potassium phosphate (pH 6.5).

Step 3: Precipitation with 70% Ammonium Sulfate and DEAE-Cellulose Chromatography. Solid ammonium sulfate (472 mg for each ml of solution) was added slowly to the pooled phosphocellulose fractions from *step 2*. After 30 min the precipitate was removed by centrifugation at 10,000 × *g* for 30 min and was dissolved in 10 ml of 0.05 M potassium phosphate (pH 6.5). This solution was dialyzed against 100 volumes of the same buffer for 4 hr. The dialyzed solution was applied to a column (1.6 × 5 cm) of DEAE-cellulose equilibrated with 0.05 M potassium phosphate (pH 6.5). The column was washed with the same buffer until the absorbance at 280 nm of the eluate was reduced to 0.05. The *gal* repressor was then eluted with 0.25 M potassium phosphate (pH 6.5). The eluates were collected and dialyzed against 100 volumes of Buffer A containing 15% (v/v) glycerol.

Step 4: Chromatography on p-Aminophenyl-β-D-thiogalactoside Substituted Agarose. The dialyzed solution from *step 3* was applied to a column (1.6 × 5 cm) containing *p*-aminophenyl-β-D-thiogalactoside linked to agarose that was previously equilibrated with Buffer A containing 15% (v/v) glycerol. The column was washed with 20 ml of Buffer A containing 0.05 M KCl and 15% (v/v) glycerol, then eluted with a linear gradient starting with 25 ml of Buffer A containing 0.05 M KCl and 15% (v/v) glycerol in the mixing chamber and 25 ml of Buffer A containing 0.50 M KCl and 15% (v/v) glycerol in the reservoir. 1-ml Fractions were collected; *gal* repressor was eluted at a concentration of 0.3 M–0.4 M KCl. Fractions exhibiting high specific activity were pooled, dialyzed against Buffer A containing 25% (v/v) glycerol, and stored at −70° in 1-ml aliquots. Table 1 summarizes the results from a typical purification. This procedure gives about a 1600-fold purification. The loss of activity during purification is retarded by the addition of 15% glycerol. Assay of *gal* repressor for its ability to repress *gal* transcription *in vitro* is not feasible until *step 4*, because some factor present in the cell extract interferes with the *gal* transcription. The

TABLE 1. *Purification of the gal repressor*

Fraction	Total volume (ml)	Total protein (mg)	Total activity (units)*	Specific activity (units*/mg of protein)	Yield (%)	Fold purification
Crude extract	820	114,000	150	0.013	100	1
Phosphocellulose	90	50	103	2.1	69	160
DEAE-cellulose	20	7.3	46	6.7	31	515
Sepharose-*p*-aminophenyl-thiogalactoside chromatography	5	0.55	12	21	8	1600

* One unit of binding activity is defined as that required to bind 1.0 μg of λp*gal* of [^{32}P]DNA in a total volume of 0.3 ml.

TABLE 2. *Resistance of GalO^c DNA to repression by gal repressor*

Additions	λp*galO*$^+$	λp*galO*^{c}
	gal mRNA(cpm × 10^{-3})	
None	1.5	2.2
cAMP	9.4	7.0
Repressor	2.3	2.0
Repressor + cAMP	2.7	8.0
Repressor + cAMP + fucose	7.3	9.0

The experimental conditions are given in Fig. 2. λp*galO*$^+$ DNA and λp*galO*^{c} DNA were used as template and 33 μg/ml of *gal* repressor was added. All the values of cpm are for 10-μl aliquots of the total [^{3}H]RNA product (0.5 ml).

protein obtained from *step 4* is stable for at least 1 month stored frozen at −70°, but is frequently inactivated when kept at 4°.

Molecular weight

The molecular weight of *gal* repressor was derived from the sedimentation velocity of the protein in sucrose density centrifugation experiments with *E. coli* alkaline phosphatase (6.3 S) and rabbit muscle lactate dehydrogenase (7.6 S) as standard markers. As shown in Fig. 1, repressor activity moved as a single symmetrical peak, with a sedimentation coefficient of 4.6 S. This corresponds to a molecular weight of about 70,000.

Repression of *gal* transcription by purified *gal* repressor

The binding assay of *gal* repressor, which was used to purify this repressor, provides a simple means for the detection and quantitation of this protein. However, this property does not ensure that the repressor preparation would work in an *in vitro gal* transcription system. The effect of different concentrations of repressor on *gal*-specific mRNA synthesis is shown in Fig. 2. In the absence of *gal* repressor, addition of cyclic AMP produced about a 5-fold increase in *gal* mRNA. However, this stimulation was progressively inhibited by increasing concentrations of *gal* repressor, and completely inhibited by the addition of more than 30 μg/ml of repressor. The figure also shows that D-fucose overcomes the action of the *gal* repressor on *gal* transcription. These results clearly demonstrate that the purified *gal* repressor works as an effective repressor of *gal* mRNA synthesis, just as predicted from *in vivo* studies. The small amount of *gal* mRNA made in the absence of cyclic AMP was not subject to repressor action. It is not clear if this RNA is *gal* RNA; its nature is under investigation.

Comparison of λp*galO*$^+$ DNA and λp*galO*^{c} DNA as templates

In order to understand the mechanism of action of the *gal* repressor in inhibiting *gal*-specific transcription, we compared λp*galO*$^+$ DNA and λp*galO*^{c} DNA as templates (Table 2). The addition of cyclic AMP stimulated *gal* mRNA synthesis in the transcription system containing λp*galO*^{c} DNA as well as λp*galO*$^+$ DNA as template. These results show that *O*^{c} mutant DNA has an intact cyclic AMP–CRP sensitive site. However, there are marked differences in the response to inhibition by *gal* repressor between λp*galO*$^+$DNA and λp*galO*^{c} DNA. The lack of effect of *gal* repressor in a system containing λp*galO*^{c} is in marked contrast to the almost complete inhibition of *gal* transcription by *gal* repressor with λp*galO*$^+$ DNA. This result clearly shows that *gal* repressor interacts with the *gal* operator site to repress the synthesis of *gal* mRNA.

Effect of fucose and galactose on *gal* repressor

Two inducers of the *gal* operon, D-fucose and D-galactose, overcome the action of the *gal* repressor on *gal* transcription. The concentration dependency of the action of these two sugars is shown in Fig. 3. The apparent K_m values for D-fucose and D-galactose are 1 mM and 0.5 mM, respectively. The concentration curves are slightly displaced to a higher molarity as compared with those obtained by the binding assay (10). The displacement is probably due to the fact that repressor can act only on those DNA molecules in which RNA polymerase is bound to the *gal* promoter. In the *in vitro* transcription system, RNA polymerase is limiting and, therefore, not all the *gal* promoters are occupied. *In vivo*, D-fucose is a more potent inducer than galactose; but, in the transcription system, D-galactose is more effective. This difference is probably due to the rapid metabolism of D-galactose *in vivo* (2). Indeed, the endogenous threshold value for induction by D-galactose *in vivo* is 0.1–0.2 mM (15). This value is close to the threshold value for derepression by D-galactose found *in vitro* (Fig. 3).

DISCUSSION

The galactose operon in *E. coli* has been extensively studied both biochemically and genetically. The existence of the *gal* operon very close to a gene causing sensitivity to chlorate (*chlD*) and to prophage λ on the chromosome of *E. coli* has permitted genetic analysis of the system (16, 17) and isolation of nondefective λ*gal* transducing phages that carry various segments of the *gal* region (13). Therefore, the *gal* operon was our choice for study of the regulation of gene expression *in vitro*. The isolation of an unlinked recessive mutation (*galR*$^-$) (3, 4) and a linked *cis*-dominant mutation (*O*^{c}) (3) causing constitutive expression of the *gal* operon, together with the noninducible dominant mutation (*galR*^{s}) (5) suggested the repressor-mediated negative regulation of the *gal* operon. *In vivo* biochemical studies suggested that the

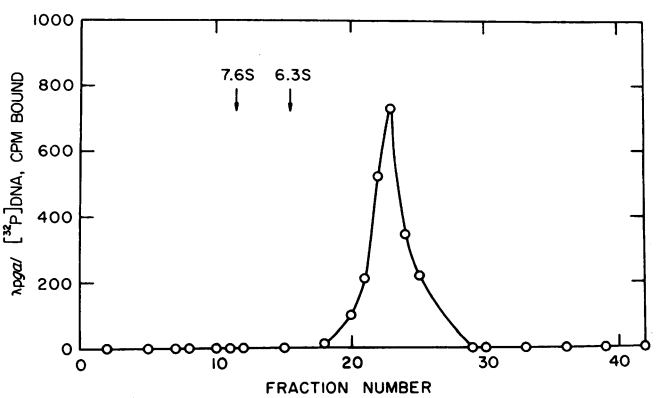

FIG. 1. Sucrose density gradient centrifugation of *gal* repressor. The experimental conditions are given under *Methods*. As the *gal* repressor preparation, the pooled fraction from *step 3* was used after dialysis against 50 mM potassium phosphate buffer (pH 6.5)–0.1 mM EDTA –0.2 mM dithiothreitol.

regulation occurred through a specific inhibition of *gal* transcription (7, 18). The enzymes of the *gal* operon have also been synthesized in a crude *in vitro* system in which transcription and translation are coupled (8, 19). Working at low DNA concentrations, Wetekam and Ehring demonstrated induction by fucose with *galO*$^+$ DNA, but not with *galO*^{c} DNA (19). However, the possibility remained that the inhibition of transcription was secondary to inhibition of translation. Using a purified transcription system, we have now shown that the *gal* repressor indeed works as an effective and specific inhibitor of transcription of *gal* operon, and inducer molecules act by relieving this inhibition. The experiments with λp*galO*^{c}DNA as template clearly show that *gal* repressor interacts with *gal* operator locus to repress the *gal* transcription of *E. coli*. This control system of the *gal* operon is so far in complete agreement with the original proposal of Jacob and Monod (20) about gene regulation. Recently, effective repression of RNA synthesis of the *lac* operon (21, 22) and bacteriophage λ (23, 24) has also been achieved *in vitro* with purified components. Effective repression of specific λ transcription requires a higher concentration of λ repressor when a template with an operator mutation is used (23). *lac* repressor has been shown to have a reduced affinity for *lacO*^{c}DNA (25, 26), but the effect of *lac* repressor on transcription of *lacO*^{c}DNA has not been reported.

There is another regulatory locus that controls the production of the enzymes of the *gal* operon. This locus, named *capR* or *lon*, also controls the synthesis of various other enzymes that synthesize sugars that are subsequently incorporated into cell-wall polysaccharide (27, 28). Since mutations in the *capR* locus result in overproduction of these enzymes, and the *capR*$^+$ is dominant over the *capR*$^-$ phenotype, Markowitz and coworkers (27, 28) proposed that the *capR* product was a repressor. Mackie and Wilson (29) have recently demonstrated that derepression caused by the *capR* mutation is independent of induction by fucose, and probably occurs at the level of transcription. Their data support the idea that the product of the *capR* locus is a repressor protein that binds to the operator region of the *gal* operon. Therefore, it seems possible that the *capR* product binds to *gal* DNA. It is very unlikely

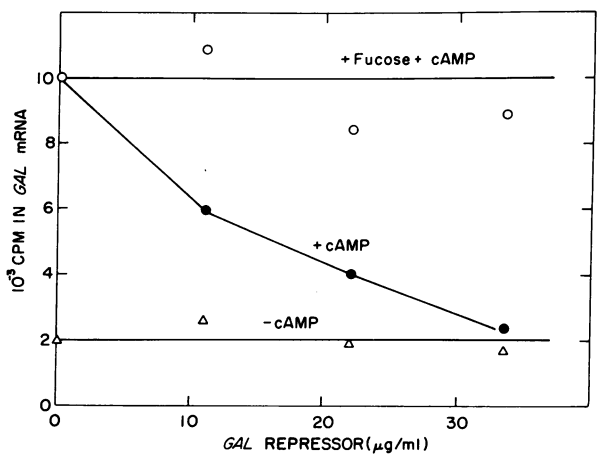

FIG. 2. Effect of *gal* repressor and fucose on *gal* mRNA synthesis. The details of the transcription system and hybridization procedures are given under *Methods*. As template λp*galO*$^+$ was used. The concentration of fucose was 30 mM. The *gal*-specific cpm are for a 10-μl aliquot of the total [^{3}H]RNA (0.5 ml) made in the *in vitro* transcription system.

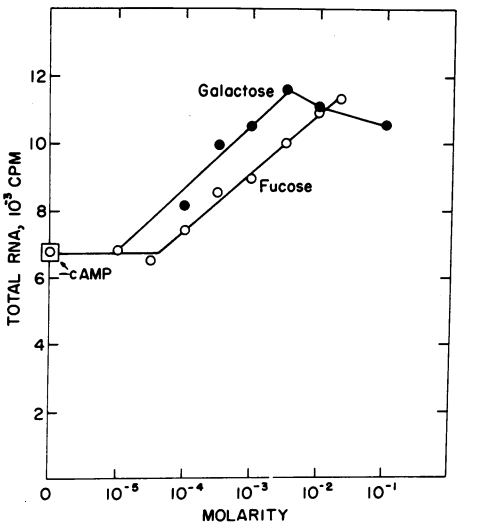

FIG. 3. Derepression by galactose and fucose of *gal*-promoted RNA synthesis. The concentration of λp*galO*$^+$ DNA and *gal* repressor were 8.2 μg/ml and 22 μg/ml, respectively. The specific activity of [^{3}H]CTP is 40 Ci/mol. Activity is reported as total cpm of Cl_3COOH-precipitable [^{3}H]RNA product.

that our purified *gal* repressor contains the *capR* repressor. Parks *et al.* (10) were unable to detect the presence of a specific *gal* DNA-binding protein in *galR*$^-$ extracts; they detected low binding in *galR*$^+$ extracts and high activity only after induction of the λ*galR*$^+$ lysogen. Further, we have been unable to detect in *lon*$^+$ and *galR*$^+$ extracts a protein that binds *gal* DNA in the presence of D-fucose.

The procedures for purification of the *E. coli gal* repressor reported here resulted in about a 1600-fold purification. However, the yield of activity is not high, and our preparation of *gal* repressor still remains impure. It would be interesting to investigate, with homogeneous *gal* repressor, the molecular mechanism of the repressor action. In particular, we should like to know if the repressor acts by competing with RNA polymerase for a common site on the DNA, or like the *lac* repressor (30) by preventing the initiation of polymerization.

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