JOURNAL OF BACTERIOLOGY, Jan. 1997, p. 228–234 Vol. 179, No. 1
0021-9193/97/$04.00+0

Functional Characterization of Roles of GalR and GalS as Regulators of the *gal* Regulon

MARK GEANACOPOULOS AND SANKAR ADHYA*

Laboratory of Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892-4255

Received 29 August 1996/Accepted 22 October 1996

An isorepressor of the *gal* regulon in *Escherichia coli*, GalS, has been purified to homogeneity. In vitro DNase I protection experiments indicated that among operators of the *gal* regulon, GalS binds most strongly to the external operator of the *mgl* operon, which encodes the high-affinity β-methylgalactoside galactose transport system, and with less affinity to the operators controlling expression of the *gal* operon, which codes for enzymes of galactose metabolism. GalS has even less affinity for the external operator of *galP*, which codes for galactose permease, the major low-affinity galactose transporter in the cell. This order of affinities is the reverse of that of GalR, which binds most strongly to the operator of *galP* and most weakly to that of *mgl*. Our results also show that GalS, like its homolog, GalR, is a dimeric protein which in binding to the bipartite operators of the *gal* operon selectively represses its *P*1 promoter. Consistent with the fact that GalR is the exclusive regulator of the low-affinity galactose transporter, galactose permease, and that the major role of GalS is in regulating expression of the high-affinity galactose transporter encoded by the *mgl* operon, we found that the DNA binding of GalS is 15-fold more sensitive than that of GalR to galactose.

The genes and operons comprising the *gal* regulon in *Escherichia coli* enable the cell to use galactose both as a source of energy and as a source of intermediates necessary for biosynthetic glycosylation reactions (1). The polycistronic *gal* operon encodes enzymes participating in the Leloir pathway for galactose metabolism, while the *mgl* operon and the *galP* gene encode the high-affinity β-methylgalactoside transport system and the galactose permease, respectively, the two major galactose uptake systems in *E. coli* (18). The expression of the enzymes involved with the Leloir pathway and galactose transport activities is regulated by two highly homologous, galactose-sensitive repressors, GalR and GalS, encoded by the genes *galR* and *galS*, respectively. These repressors are members of the GalR-LacI family of transcriptional regulators in *E. coli* (17). The precise role played by each repressor in regulating expression of the genes of the regulon is unclear. GalR, the first of these proteins to be identified and which has been purified, functions as the major repressor of the *gal* operon and is sufficient for repression of its genes in the absence of galactose (1). GalS was identified because in strains in which *galR* was deleted, the *gal* operon could be further induced by galactose, a phenomenon called ultrainduction (9). Subsequent genetic studies indicated that GalS functions to control its own expression and that of the β-methylgalactoside transport system, the galactose transporter in *E. coli* (16). In order to obtain a better understanding of the roles of GalR and GalS in coordinating both galactose metabolism and uptake, we have purified GalS and studied the interaction of both of these repressors with the regulatory sequences in the regulon. Our results indicate that GalS, like GalR, is a dimeric protein and that it interacts most strongly with the *mgl* operator, less strongly with the bipartite *gal* operators (O_E and O_I), and most weakly with the *galP* operator. This order of affinities is the reverse of that which we found for GalR. Nevertheless, like GalR, GalS selectively represses the downstream promoter, *P*1, of the *gal* operon and slightly activates the upstream promoter, *P*2, in vitro. Its DNA binding is sensitive to galactose, which effects a 12-fold change in its affinity for the *mgl* operator, similar to the affinity change caused by galactose binding to GalR. Since GalR appeared in vitro to be the sole repressor of the expression of the low-affinity galactose uptake system and since the primary function of GalS is in regulating high-affinity galactose transport, we assessed the sensitivity of DNA-bound complexes of these repressors to galactose and found that GalS was 15-fold more sensitive to galactose than was GalR. The implications of these findings for control of the *gal* regulon are discussed.

* Corresponding author. Mailing address: Developmental Genetics Section, Laboratory of Molecular Biology, National Cancer Institute, National Institutes of Health, Building 37, Room 2E16, 37 Convent Dr. MSC 4255, Bethesda, MD 20892-4255. Phone: (301) 496-2495. Fax: (301) 496-2212. E-mail: sadhya@helix.nih.gov.

MATERIALS AND METHODS

Strains and plasmids. A list of the strains and plasmids used in this study is given in Table 1. GalS was overexpressed in BL21(λDE3) cells in which the bacteriophage T7 RNA polymerase gene is placed downstream of the *lac* promoter (14). In these cells *galR* was deleted by transduction with bacteriophage *P*1 which had been grown on strain AG701 in which a defective Tn*10* transposon had been inserted in *galR*. Tetracycline-resistant transductants were isolated and transformed with pVGS5a, which contains the *galS* coding region under the control of a T7 promoter (15). For DNase I footprinting and transcription assays of the *gal* promoter, we used pSA509, which was made by inserting a 291-bp fragment of the *gal* operon promoter containing O_E and O_I into pSA508 (7). Digestion with *Eco*RI and *Pst*I yields a fragment in which O_I is centered 29 bp from the 3′ end and O_E is located 147 bp from the 5′ end. *mglB* and *galP* regulatory sequences were cloned into pSA508 by PCR amplification from the chromosome of strain MC4100 with primers that contained unique and appropriate restriction sites in their sequences. Digestion of the amplified fragments with *Eco*RI and *Pst*I for *mglB* and with *Kpn*I and *Pst*I for *galP* was followed by ligation into similarly digested pSA508. Upon digestion the amplified *galP* segment yielded a 339-bp fragment in which the external operator, O_E, was centered 73 bp from the 5′ end and the putative internal operator was centered 66 bp from the 3′ end. The *mgl* promoter was contained on a 337-bp fragment in which the lone upstream operator was centered 182 bp from the 5′ end. pSA508 contains a transcription termination site 100 bp from the multiple cloning site for use in in vitro transcription assays (7). Media used in this study were prepared as described by Miller (12).

Purification of GalS. We observed that during logarithmic growth of MJW114 containing plasmid pVGS5a, GalS was overexpressed, even in the absence of IPTG (isopropyl-β-D-thiogalactopyranoside), and that continuous growth to high density was not possible under fermentation conditions. Therefore, cells were

TABLE 1. Bacterial strains and plasmids

Strain	Characteristics	Source or reference
Strains		
Mc4100	F⁻ *ara*Dl39Δ(*argF-lac*)*U169 rpsL150 deoC1 relA1 thiA ptsF25 flbB5310*	M. Casadaban
BL21	F⁻*hsdS gal* r_B m_B[λDE3(*Bam*HI)*int*::*lacUV5*-T7*gene1]*	9
MJW114	BL21 *galR*::Cm^r	4
Plasmids		
PVGS5a	pVEX11 *bla* Δ(*Nde*I-*Eco*RI)::*galS*	4
pSA508	pIB124 *rpoC* Δ(*Eco*RI-*Hin*dIII)::*attB′OB attP′OP*	7
pSA509	pSA508 Δ(*Eco*RI-*Pst*I)::$galO_E$-*P2P1*O_I	7
pSA520	pSA508 Δ(*Eco*RI-*Pst*I)::*mglB* promoter	This study
pSA521	pSA508 Δ(*Eco*RI-*Kpn*I)::$galPO_E$-O_I	This study

grown at 37°C in Luria-Bertani medium containing ampicillin (50 μg/ml) to an optical density of 4.0, harvested, and stored at −70°C. All subsequent steps were carried out on ice or at 4°C. Frozen cells (25 g) were suspended in 25 ml of lysis buffer (25 mM Tris-HCl [pH 8.0] containing 5 mM EDTA, 1 mM dithiothreitol, lysozyme [1 μg/ml], and the protease inhibitors phenylmethylsulfonyl fluoride [2 mM], leupeptin [1 μg/ml], tosyl-lysine chloromethylketone [20 μg/ml], and aprotinin [1 μg/ml]). After incubation for 30 min with occasional vortexing, the lysed suspension was adjusted to 1 M KCl, 1 M ammonium acetate, and 25 mM spermidine, incubated on ice for 1 h, and centrifuged for 1 h at 33,000 × *g*. The supernatant was dialyzed overnight against buffer A (25 mM Tris-HCl [pH 8.0] containing 200 mM KCl, 1 mM EDTA, and 1 mM dithiothreitol [DTT]). Nucleic acids were removed by the slow addition of protamine sulfate to a final concentration of 1 mg/ml followed by centrifugation at 33,000 × *g* for 1 h. After dialysis of the supernatant (fraction I) against buffer A containing 50 mM KCl, it was applied to a 30-ml Q-Sepharose column (5 by 1.9 cm [inner diameter]) that had been equilibrated with buffer A containing 50 mM KCl. The column was washed with three void volumes of buffer A and developed with a KCl gradient (50 to 300 mM) at a flow rate of 1.5 ml/min. Fractions that were enriched for GalS eluted in a peak centered at 150 mM KCl. These were identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and pooled as fraction II. After dialysis against 50 mM KCl, anion exchange was repeated by fast-performance liquid chromatography in a Mono-Q column (1-ml bed volume). The column was developed with the same KCl gradient at a flow rate of 1 ml/min. Fractions enriched in GalS, as revealed by SDS-PAGE, were pooled (fraction III), applied to a hydroxylapatite column (5-ml bed volume) which had been equilibrated to 10 mM KPO_4, and developed with a KPO_4 gradient (10 to 500 mM). GalS-containing fractions, which eluted at approximately 200 mM, were pooled (fraction IV) and dialyzed against buffer B (25 mM sodium citrate [pH 5.5] containing 50 mM KCl, 1 mM EDTA, and 1 mM DTT). GalS was precipitated under these conditions and redissolved in buffer A containing 600 mM KCl. After the addition of glycerol to 15% (fraction V), it was stored at −70°C.

To determine whether another method of purification and specifically whether omission of the low-salt precipitation step would increase the operator-binding activity of GalS, we developed an alternative purification protocol. Fraction II, prepared as described above, was dialyzed against 60 mM KCl and applied to a Hi-trap heparin sulfate column (Pharmacia) that had been previously equilibrated in buffer A containing 60 mM KCl. The column was developed with a KCl gradient (60 mM to 1 M KCl). GalS eluted at 100 mM KCl, with most impurities failing to bind to the column. The KCl concentration of the GalS-containing fractions was increased to 150 mM, and after centrifugation at 10,000 × *g*, the contents were applied to a hydroxylapatite column as described above. Enriched fractions were dialyzed against 50 mM Tris-HCl (pH 8.0) containing 350 mM KCl and 15% glycerol and stored at −70°C. GalS was more than 95% pure as evidenced by gel electrophoresis and Coomassie staining. The *mgl*-operator binding activity of GalS prepared in this way was identical to that of GalS prepared by the original method.

DNase I footprinting. DNase I footprinting titrations were performed as described by Brenowitz et al. (3). Restriction fragments containing *gal*, *mgl*, and *galP* promoters were radioactively end labeled with Klenow fragment in the presence of α-^{32}P-labeled deoxynucleoside triphosphates and then purified on a polyacrylamide gel. The fragments were incubated at a final concentration of 0.05 nM or less with GalR or GalS for 30 min in 200 μl of assay buffer (10 mM Tris-HCl [pH 8.0] containing 5 mM $MgCl_2$, 1 mM $CaCl_2$, 200 mM KCl, 2 mM DTT, 50 μg of bovine serum albumin per ml, and 2 μg of calf thymus DNA per ml), and then 2 μl of an appropriate dilution of DNase I was added such that approximately 80% of the labeled DNA was cut after 2 min. After the reaction was quenched with 0.7 ml of DNase I stop solution containing 645 μl of ethanol, 5 μl of tRNA solution (1 mg/ml), and 50 μl of 7 M ammonium acetate, the samples were placed on dry ice for 20 min and centrifuged at 15,000 × *g*. The precipitate was washed in 70% ethanol, vacuum dried, resuspended in loading buffer, and then applied to 8% polyacrylamide sequencing gels containing 8 M urea. Protection in each lane was normalized with reference to a standard block in the same lane containing unprotected bands. All gels were scanned with PhosphorImager:425 (Molecular Dynamics), which gives a linear response to radiation intensity in the range used in these experiments. Datum points were fit to the Langmuir isotherm by using the Kaleidagraph computer software package. Errors in the binding constants are derived from a common error based on the scatter of the datum points about the best curve. For titrations with inducer, D-galactose was added to the DNase I assay buffer immediately prior to addition of the repressor, and datum points were fitted to the equation for the fraction bound as a function of galactose concentration as described below.

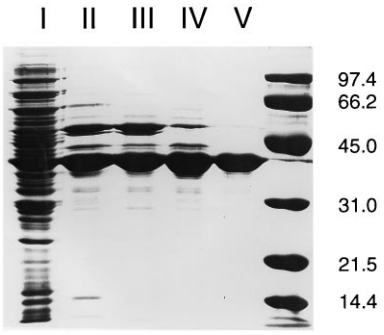

FIG. 1. SDS-PAGE of GalS purification pools. Lanes: I, crude lysate after protamine sulfate precipitation; II, Q-Sepharose; III, Mono-Q; IV, hydroxylapatite; V, low-salt precipitation. Molecular weights (in thousands) are indicated on the right.

Gel filtration chromatography. GalS was added in a volume of 200 μl to a Superose 12 (Pharmacia) gel filtration column (volume, 24 ml; height, 30 cm; inside diameter, 1.0 cm) that had been calibrated with a set of molecular weight standards ranging from 2,000 to 650,000 (Bio-Rad) and eluted in buffer A at a flow rate of 0.5 ml/min. The elution profile was continuously monitored by measuring absorbance at 280 nm. The concentration of GalS dimer was calculated by using a molar extinction coefficient of 41,000, derived from the protein sequence (11). This method of determining the extinction coefficient has been confirmed by amino acid analysis after acid hydrolysis (5).

In vitro transcription. Transcription reactions were carried out as previously described (7) in 20 mM Tris-acetate [pH 7.8] containing 10 mM magnesium acetate, 100 mM potassium glutamate, 2 nM DNA template, 1 mM ATP, 0.1 mM GTP, 0.1 mM CTP, 0.01 mM UTP, and 10 to 20 μCi of [α-^{32}P]UTP (3,000 Ci/mmol). After incubation for 5 min at 37°C, RNA polymerase was added to a concentration of 20 nM. Reactions were terminated after 10 min by addition of an equal volume (50 μl) of RNA loading buffer containing 80% (vol/vol) deionized formamide, 89 mM Tris borate, 2 mM EDTA, 0.025% bromophenol blue, and 0.025% xylene cyanol. After being heated at 90°C for 5 min, the samples were loaded onto 8% DNA sequencing gels containing 8 M urea. Quantification of transcription products was done with a phosphorimager. Bands were normalized for varying background by subtracting the total radioactivity of an equal area immediately above or below the band of interest. The RNA I transcript originating from the plasmid, which was not affected by repressor addition, served as an internal control between lanes. Transcription data were fitted to the function $1/(1 + kx)$, where x is the concentration of free repressor and k is an apparent association constant, except for *galP*2, for which repression was not observed.

RESULTS

Purification and molecular sizing of GalS. The SDS-PAGE profile of fractions from the GalS purification described in Materials and Methods is shown in Fig. 1. A series of dilutions of fraction V was electrophoresed, and the percentage of impurities was determined by Coomassie blue staining. GalS in fraction V was greater than 98% pure. The N-terminal 20 amino acids of GalS were determined by Edman degradation to be MITIRDVARQAGVSVATVSR; this is the order predicted by the gene sequence. Purification by an alternative method described in Materials and Methods, in which chromatography with a heparin sulfate column was added to the protocol and low-salt precipitation was omitted, gave a protein

TABLE 2. Molecular weight of GalS by gel filtration chromatography

Protein	Monomer concn (μM)	Mol wt
GalS	250	92,500
	10	84,600
	3	76,500
GalR	10	84,200
GalS/GalR	10/10	82,000

TABLE 3. Affinity of GalR and GalS for *gal* regulon operators

Operon	K_d (nM) for indicated operator			
	GalR		GalS	
	O_E	O_I	O_E	O_I
gal	1.3 ± 0.2	2.9 ± 0.2	31.0 ± 12.8	72.4 ± 27.8
galP	0.3 ± 0.06	ND[a]	>100	ND
mgl	11.1 ± 2.2		18.1 ± 2.6	

[a] ND, not detected.

preparation that was greater than 95% pure and had identical *mgl*-binding activity.

Gel filtration indicated that GalS eluted with an apparent molecular weight of 76,500 at a concentration of 3 μM (Table 2). This is the molecular weight of the dimeric protein, indicating that GalS, like previously analyzed GalR, is a dimer at micromolar concentrations. Mixing of GalR and GalS did not lead to formation of a higher-molecular-weight species. Some aggregation of GalS dimers was evident at higher concentration, with GalS eluting with an apparent molecular weight of 92,500 at a concentration of 250 μM.

Binding of GalS and GalR to galactose regulon operators. We measured the binding of GalR and GalS to the operators of the *gal* operon as well as those of the *mgl* operon and the *galP* gene. Only the interaction of GalR with the operators of the *gal* operon had been determined previously. The length of the sequences protected from DNase I cleavage was similar at all sites for both GalS and GalR, suggesting that they bound as dimers (Fig. 2). As expected from in vivo observations, both proteins bound to the upstream (O_E) and downstream (O_I) operators of the *gal* operon. However, in the *galP* gene, they bound only to the upstream (O_E) operator and not to the downstream (O_I) operator, which among *gal* regulon operators is the most divergent from the consensus sequence. Both proteins bound to the upstream operator of the *mgl* operon; there is no sequence homologous to the *gal* operators internal to *mgl*. DNase I footprint titrations were quantified to obtain binding isotherms and dissociation constants for the binding of the repressors to the individual operators (Table 3). GalR bound most strongly to the external operator, O_E, of the low-affinity galactose transporter gene, *galP*, with a dissociation constant of 0.3×10^{-9} M. At pH 8.0, the K_d values of GalR for O_E and O_I of *gal* were 1.3×10^{-9} and 2.9×10^{-9} M, respectively, similar to those measured previously (4). GalR bound more weakly to O_E of *mgl*, with a K_d of 11×10^{-9} M. GalS bound with similar affinity to O_E of *mgl*, with a K_d of 18×10^{-9} M; note that at a saturating concentration, GalS gave more complete protection of the operator site than GalR. The binding of GalS to the *gal* operators was considerably weaker, with a K_d values of 31×10^{-9} M for O_E and 72×10^{-9} M for O_I. GalS showed some specific protection of O_E of *galP*, but its affinity was too low to be measured quantitatively. Thus, the affinity of GalR appears to be higher than that of GalS for all operators tested, although

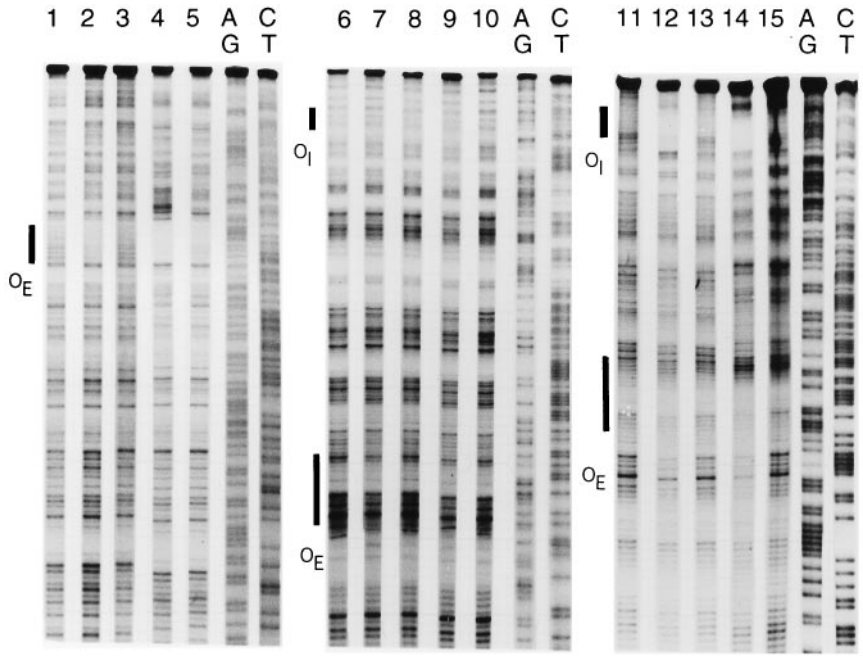

FIG. 2. Galactose-sensitive DNase protection by GalR and GalS. Lanes 1 to 5 show DNase I cutting of a 337-bp DNA fragment spanning from −235 to +102 of the *mgl* operon. Lanes: 1, no additions; 2 and 3, 50 nM galR in the absence and presence of 10 mM galactose, respectively; 4 and 5, 50 nM GalS in the absence and presence of 10 mM galactose, respectively. Lanes 6 to 10 show DNase cutting of a 337-bp fragment from −164 to +101 of the *galP* gene. Lanes: 6, no additions; 7 and 8, 1 nM GalR in the absence and presence of 10 mM galactose, respectively; 9 and 10, 200 nM GalS in the absence and presence of 10 mM galactose, respectively. Lanes 11 to 15 show DNase cutting of a 291-bp fragment from −207 to +84 of the *gal* operon. Lanes: 11, no additions; 12 and 13, 5 nM GalR in the absence and presence of 10 mM galactose, respectively; and 14 and 15, 100 nM GalS in the absence and presence of 10 mM galactose, respectively.

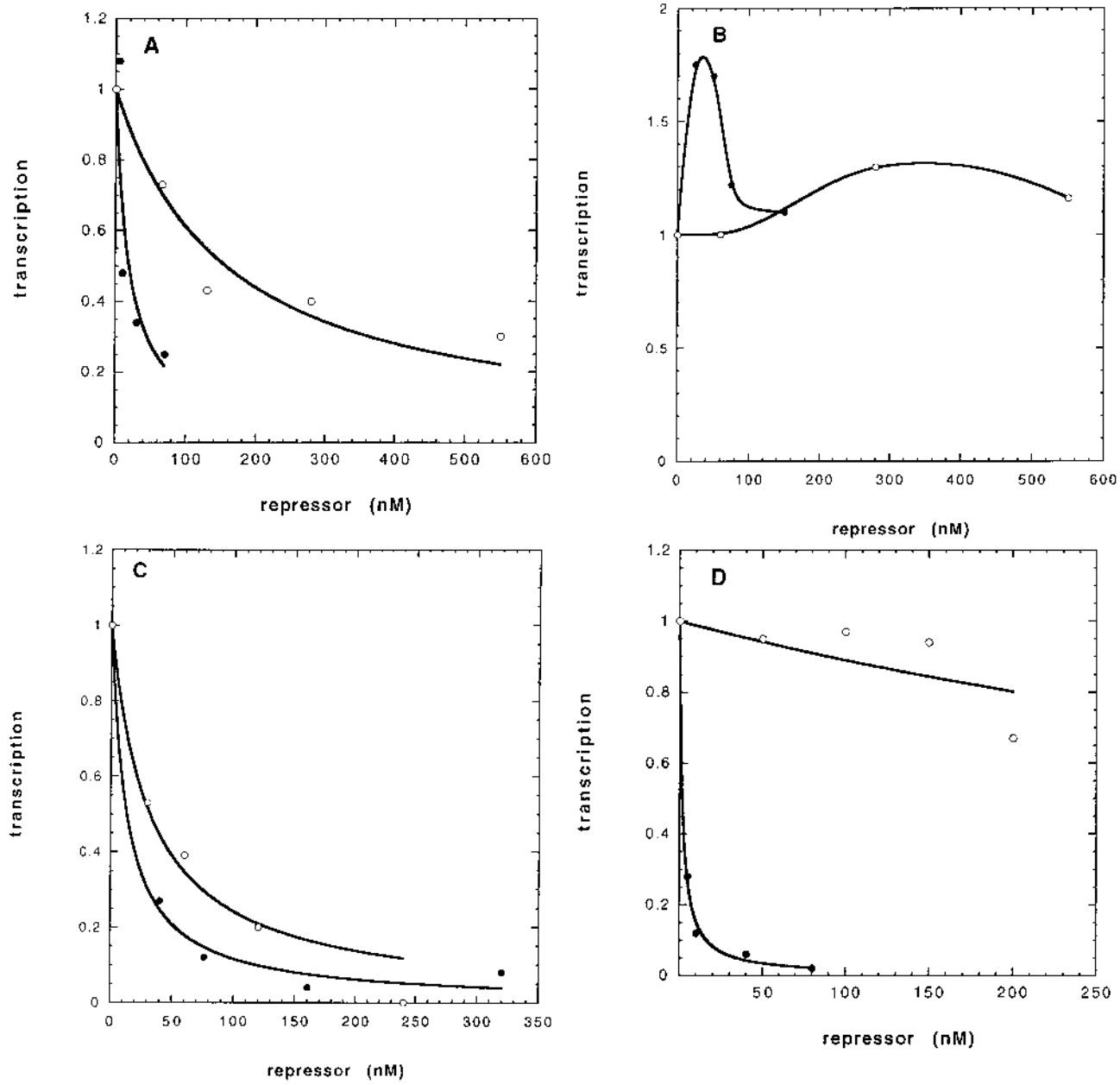

FIG. 3. Repression of in vitro transcription from *gal* regulon promoters by GalR (●) and GalS (○). Transcription was carried out as described in Materials and Methods. Bands were quantified with a phosphorimager, and the level of transcription was normalized to the plasmid RNA I transcript and then expressed as a percentage of the transcription that was obtained in the absence of repressor proteins. (A and B) Transcription from *galP*1 and *galP*2, respectively; (C) transcription from the *mgl* promoter; (D) transcription from the *galP* promoter.

for the single *mgl* operator the two proteins had comparable affinities. In summary, the order of affinities of the two proteins is reversed: GalR binds most strongly to *galP* and most weakly to *mgl*; the opposite is true for GalS. GalR and GalS binding to all sites was sensitive to the inducer galactose as indicated in Fig. 2.

Repression of transcription by GalR and GalS in vitro. The relative affinities of GalR and GalS for the operator sequences in *gal*, *mgl*, and *galP* were reflected in the effectiveness of the two repressors in inhibiting transcription of these genes in vitro (Fig. 3). The *gal* operon genes are transcribed from two promoters, *P*1 and *P*2, which are expressed with approximately equal strength in vitro in the absence of the cyclic AMP (cAMP) receptor protein. As previously shown (8), GalR selectively inhibited transcription from *P*1 of the *gal* operon in vitro and activated transcription from *P*2, although its effect on transcription from *P*2 is biphasic, with the blockage of elongating transcripts by repressor bound to O_I causing repression at higher GalR concentrations (8) (Fig. 3A and B). Like GalR, GalS selectively inhibited transcription from *P*1, albeit more weakly, and caused a slight activation of transcription from *P*2.

In contrast to that of *gal*, transcription of both *mgl* and *galP* originated from a single promoter and was highly dependent on the cAMP repressor protein, which in the presence of cAMP activated transcription from the *mgl* and *galP* promoters by 40- and 30-fold, respectively (data not shown). GalR was

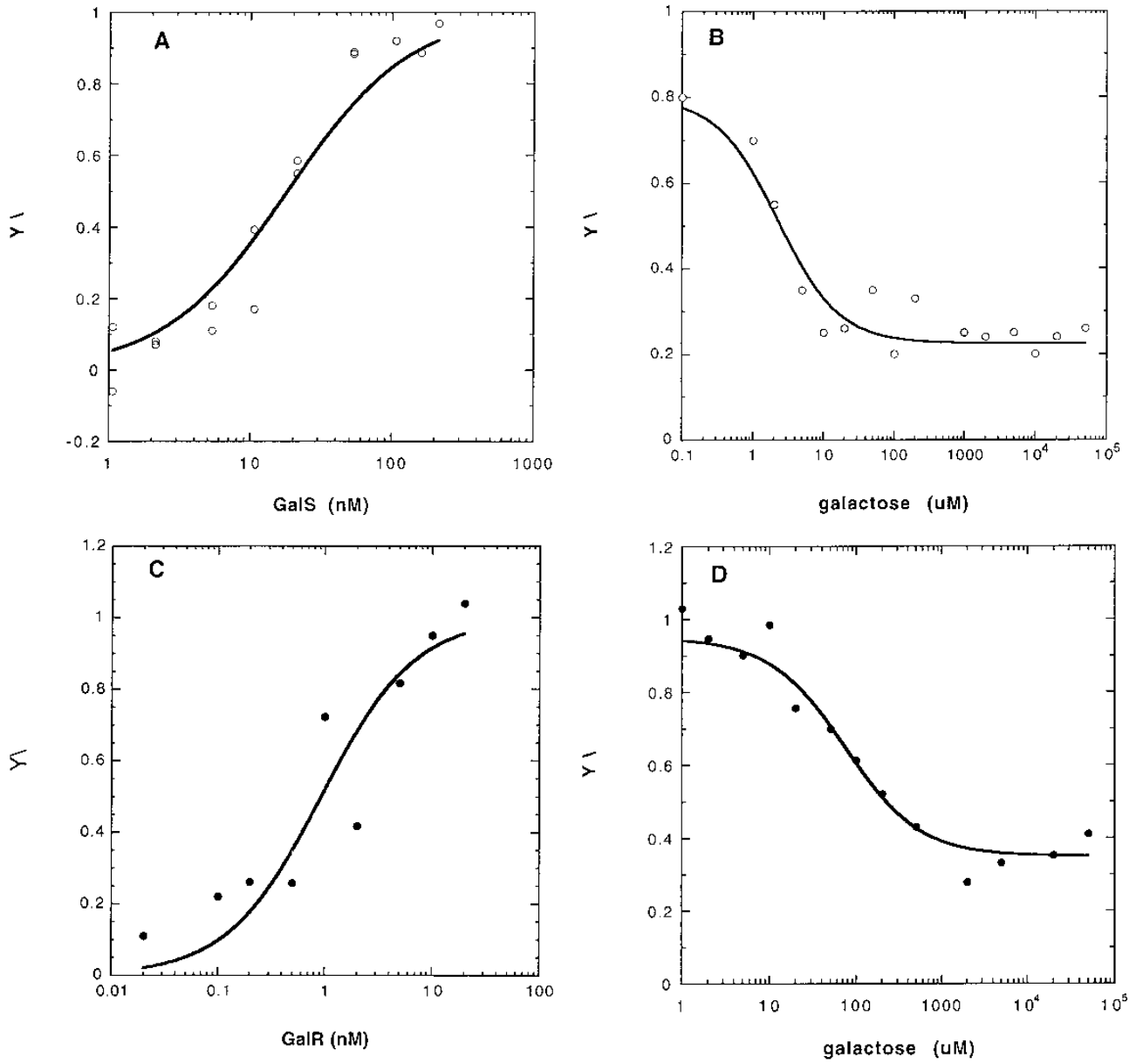

FIG. 4. Titration of repressor-operator complexes with galactose. (A and C) The binding of GalS to O_E of *mgl* and GalR to O_E of galE, respectively, is shown. The fraction of operators bound (Y\) at each repressor concentration was determined by quantitative DNase footprinting. Based on these titrations, GalS was added to O_E of *mgl* (B) and GalR was added to O_E of *galE* (D) at concentrations at which approximately 85% of the operators were bound in each case, and the repressor-operator complexes were titrated with increasing concentrations of D-galactose. *Y* was determined by DNase protection as described in the text.

more potent as an inhibitor of transcription of the *galP* gene and less effective as an inhibitor of transcription of the *mgl* operon compared to its potency as a repressor of *gal* operon transcription from *P*1 (Fig. 3C and D). As expected from the binding measurements, GalS was most potent as an inhibitor of *mgl* transcription (Fig. 3C). It did not inhibit transcription of the *galP* gene at concentrations up to 200 nM (Fig. 3D).

Sensitivity of GalR and GalS operator complexes to galactose. GalR contains a single tryptophan residue, and its interaction with galactose has been analyzed by tryptophan fluorescence; at pH 8.0, the dissociation constant of GalR for D-galactose is 16×10^{-5} M (6). In spite of the presence of several tryptophan residues, the tryptophan fluorescence of GalS is not sensitive to galactose, and we did not measure galactose binding to GalS directly. The sensitivities of the GalS and GalR DNA complexes to the inducer, galactose, can be compared if the proteins are bound to operators at similar loading free energies and if the bound complexes were titrated with galactose. The affinity of the proteins for the operators was first determined by quantitative DNase footprinting (Fig. 4A and C), and with this information, the proteins were then added such that 80 to 90% of DNA was protected by the bound repressor. Under these conditions, the dissociation of the repressors from the DNA in response to a given concentration of galactose is a function of the affinity of GalS or GalR for galactose and the change in affinity of the proteins for the operator which is caused by galactose binding. Neither GalR nor GalS completely dissociated from the operators (Fig. 4B

and D); both dissociation curves reached a plateau at the high galactose concentrations. This indicates that both proteins in what is very likely to be the doubly bound form (one galactose molecule per monomer) have measurable levels of affinity for the operators. Although the affinity change that occurs upon galactose binding is similar for the two proteins, the midpoints of the dissociation curves are different (5×10^{-6} M galactose for GalS and 100×10^{-6} M galactose for GalR). Thus, at a given degree of occupancy of an operator, GalS is more sensitive to galactose than GalR.

DISCUSSION

In *E. coli* two regulatory proteins, GalR and GalS, regulate the uptake and metabolism of galactose by binding to conserved operator sequences in the *gal* regulon. The two proteins are highly homologous; 55% of their amino acids are identical and 88% are similar, with only two substitutions occurring in the first helix of the DNA-binding domain and a single conservative substitution in the recognition helix (15). The gel filtration chromatography results indicate that, like GalR, GalS is dimeric at a 10×10^{-6} M concentration, which is 1,000-fold higher than the dissociation constant for the tetramer-dimer equilibrium of LacI (13). Both GalR and GalS lack the leucine-rich C-terminal domain involved in LacI tetramerization. In vivo genetic experiments have begun to clarify the functions of GalR and GalS in controlling expression of genes comprising the *gal* regulon. They indicate that the sets of genes controlled by these repressors overlap. In a *galR* deletion strain the *gal* operon can be further induced by galactose, and the ultimate level of expression is higher than that which can be obtained by adding galactose to wild-type cells (9). On the basis of this phenomenon, called ultrainduction, the existence of the *galS* gene was predicted and subsequently verified (9, 15). GalR appeared to be the major repressor of the *gal* operon, because in *galS* mutants the *gal* operon is repressed, while mutation of *galR* leads to a high level of expression. The *gal* expression is highest in a *galR galS* double mutant (9).

Our biochemical data confirm the genetic results in that at pH 8.0, GalR binds much more strongly than GalS to the *gal* operators O_E and O_I. The difference in affinity is reflected in their efficacy as repressors of in vitro transcription (Table 3 and Fig. 3). Both GalR and GalS selectively repress the *gal* operon transcription from the *P*1 promoter, while transcription from *P*2 is activated slightly. Failure to repress *P*2 could be explained by the failure of both proteins to tetramerize and form a DNA loop; when O_E and O_I are mutated to form *lacI* operators, addition of LacI causes simultaneous repression of both *P*1 and *P*2 (7). Recently, a protein that permitted *galR*-mediated inhibition of transcription from *P*2 in vitro was purified from cell extracts and identified as HU, a histone-like DNA-binding protein that imparts a sharp bend to sequences to which it binds (2). Although HU has no effect on transcription in the absence of GalR, it forms a cooperative complex with GalR bound to the tandem operators, O_E and O_I, which effectively represses *P*2. Genetic experiments also confirmed the involvement of HU in repression of *P*2. Interestingly, preliminary results indicate that HU does not mediate repression of transcription from *P*2 by operator-bound GalS (1a).

In vitro, GalS bound with highest affinity to O_E of *mgl*, the operon encoding the high-affinity galactose transport system. This agrees with the genetic evidence that repression of the *mgl* operon is the primary role of GalS in the cell (15, 16). Nevertheless, its affinity was slightly less than that of GalR for the *mgl* operator (Table 3). This result was unexpected because most in vivo experiments have indicated that GalR is not involved in regulation of *mgl*. For example, induction of high-affinity galactose transport was not affected by a $GalR^s$ mutation, which, while not affecting the DNA-binding activity of GalR, makes it insensitive to an inducer (10). Similarly, deletion of *galR* had no effect on *mgl* expression as measured by β-galactosidase levels in *mgl-lacZ* translational fusion strains; however, in a *galS* deletion strain, primer extension studies indicated that transcription of *mgl* was still inducible by galactose (15). We cannot rule out the possibility that the GalR and GalS protein preparations used in the in vitro studies reported here differ in their activity, although two methods of GalS purification produced protein preparations with the same *mgl* binding activity, and the DNA-binding activity of GalR in our preparations agrees closely with results reported previously (4). Alternatively, in vitro conditions could favor the binding of GalR over that of GalS. Thus, although our in vitro results support the genetic results suggesting that the major function of GalS is to regulate *mgl* expression, the issue of the extent to which *mgl* regulation is shared by the two proteins has not been resolved by these experiments.

The greatest difference in the DNA-binding affinity of GalR and GalS was for O_E of the *galP* gene coding for the low-affinity galactose transporter, galactose permease. GalR had an affinity for this sequence that was 5- to 10-fold higher than its affinity for the operators of the *gal* operon, although we could not precisely determine the affinity of GalS for this operator due to nonspecific binding at high protein concentrations. We conclude that the K_d value is greater than 100×10^{-9} M. Thus, GalR appears to be the major, if not the sole, regulator of the expression of the low-affinity galactose transporter in *E. coli*.

If the primary role of GalS is to control the expression of the high-affinity galactose transporter and GalR controls the low-affinity permease, then it might be predicted that GalS would respond to lower concentrations of galactose than would GalR. To test this hypothesis, GalR and GalS were added to operator sequences such that binding reached similar degrees of saturation and were then titrated with D-galactose. We found that GalS-operator complexes were more sensitive to the inducer than GalR-operator complexes (Fig. 4). If one assumes a highly simplified model for galactose-induced dissociation in which release of the repressor from DNA is mediated by binding of a single galactose molecule to one of two identical and noninteracting sites of a repressor dimer, R, and that binding of a second molecule of galactose has no further effect on the DNA affinity of a repressor dimer, then under conditions where galactose concentrations are much higher than the total repressor concentration and the repressor is in great excess of the operators, the fractional saturation ($Y\backslash$) of the operator as a function of galactose concentration (G) can be expressed as follows:

$$\frac{1}{Y\backslash} = 1 + \frac{K_4(G) + 1}{R_t[K_1 + K_3K_4(G)]}$$

where K_1 and K_3 refer to the association constants of free and galactose-bound repressor for the operator, respectively; K_4 is the apparent association constant for galactose for the free repressor; and R_t is the total concentration of repressor. By using the known values of R_t (50×10^{-9} for GalS and 5×10^{-9} for GalR) and K_1 (5.5×10^7 for GalS and 1.2×10^9 for GalR) and fitting the dissociation plots to the data, we obtained values for K_3 of 5.97×10^6 M^{-1} for GalS and 1.13×10^8 M^{-1} for GalR. The apparent association constant, K_4, of galactose for the free repressor was $(1.25 \pm 0.4) \times 10^6$ M^{-1} for GalS and $(9.0 \pm 1.1) \times 10^4$ M^{-1} for GalR, indicating that free GalS

binds galactose with 14-fold-higher affinity than GalR. Clearly, galactose-induced dissociation could occur by models more complex than that outlined above, but these would also predict that the binding event(s) coupled to the affinity change of the repressor for the operator occurs at lower galactose concentrations for GalS than for GalR. Thus, galactose binding to GalS could permit induction of the high-affinity galactose transporter at low levels of intracellular galactose before galactose concentrations become high enough to require an increase in galactose-metabolizing enzymes or the low-affinity galactose transporter, galactose permease. The reduction in DNA-binding affinity indicated in the dissociation curves shown in Fig. 4 (11-fold for GalR and 9-fold for GalS) would be misleading if a fraction of the DNA-bound repressor were inactive with respect to galactose binding. However, the observed change in the DNA-binding affinity of GalR is that which is predicted by the observation that GalR affinity for galactose changes 15-fold in the presence of saturating operator DNA (6a), indicating that at least for GalR, this is not the case. This relatively small change in affinity is consistent with the in vivo results where the levels of expression of the *gal* operon obtained in a ΔgalR ΔgalS strain are higher than those in wild-type strains induced with galactose. This finding suggests that inducer-bound GalR or GalS may still partly repress transcription under conditions where inducer concentrations are high enough to saturate the repressor.

The presence of two repressors responding to galactose with different levels of sensitivity would increase the possibility of regulatory control as the cell responds to the presence of external galactose. For instance, as internal levels of galactose begin to rise, genes under the control of GalS would be induced first. They would be the *mgl* operon involved in high-affinity galactose transport as well as chemotaxis in response to galactose and GalS itself, which, unlike *galR*, controls its own expression. As galactose levels increase further, genes under GalR control, the low-affinity galactose transporter, galactose permease, as well as galactose-metabolizing enzymes would be induced. It is possible that GalS levels could rise sufficiently high for it to function as a repressor in its galactose-bound form of the *mgl* operon, the expression of which in the presence of high galactose concentrations and galactose permease might be superfluous. Such a scheme for the coordinated control of galactose transport genes remains to be tested.

In conclusion, we have purified GalS, which functions as a dimeric repressor of the *gal* regulon. Our results indicate that its major role is to regulate transcription of the *mgl* operon. It has lower affinity for the bipartite operators controlling the expression of the *gal* operon and, like GalR, is incapable, in the absence of other factors, of repressing transcription of the upstream *gal* operon promoter. The increased sensitivity of GalS to galactose relative to that of GalR permits the induction of the *mgl* operon coding for the high-affinity galactose transporter at galactose concentrations lower than those that would significantly induce the *gal* operon and the low-affinity transporter, galactose permease, which our results indicate are largely under the control of GalR.

ACKNOWLEDGMENTS

M.G. is a recipient of a National Institutes of Health Intramural Research Training Award Fellowship.

The assistance of S. Roy in analysis of galactose-induced dissociation results and the advice and suggestions of S. Ryu concerning protein purification are gratefully acknowledged. We thank C. Klee for N-terminal sequencing of GalS and Michael Weickert for critical review of the manuscript.

REFERENCES

1. **Adhya, S.** 1987. The galactose operon, p. 1503–1512. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.

1a. **Aki, T.** Unpublished results.

2. **Aki, T., H. E. Choy, and S. Adhya.** 1996. Histone-like HU as a specific transcriptional regulator: cofactor role in the repression of *gal* transcription. Genes Cells **1:**179–188.

3. **Brenowitz, M., D. F. Senear, and R. E. Kingston.** 1987. DNase I footprint analysis of protein-DNA binding, p. 12.4.1–12.4.16. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), Current protocols in molecular biology. Greene Publishing Associates and Wiley Interscience, New York.

4. **Brenowitz, M., E. Jamison, A. Majumdar, and S. Adhya.** 1990. Interaction of the *Escherichia coli gal* repressor protein with its DNA operators *in vitro*. Biochemistry **29:**3374–3383.

5. **Boutelje, J., A. R. Karlstrom, M. G. N. Hartmanis, E. Holmgren, A. Sjolgren, and R. L. Levine.** 1990. Human immunodeficiency viral protease is catalytically active as a fusion protein: characterization of the fusion and native enzymes produced in *Escherichia coli*. Arch. Biochem. Biophys. **283:**141–149.

6. **Brown, M. P., N. Shaikh, M. Brenowitz, and L. Brand.** 1994. The allosteric interaction between D-galactose and the *Escherichia coli* galactose repressor protein. J. Biol. Chem. **269:**12600–12605.

6a. **Chatterjee, S., Y. Zhou, S. Roy, and S. Adhya.** Unpublished results.

7. **Choy, H. E., and S. Adhya.** 1992. Control of *gal* transcription through DNA looping: inhibition of the initial transcribing complex. Proc. Natl. Acad. Sci. USA **89:**11264–11268.

8. **Choy, H. E., S. W. Park, T. Aki, P. Parrack, N. Fujita, A. Ishihama, S., and S. Adhya.** 1995. Repression and activation of transcription by Gal and Lac repressors: involvement of alpha subunit of RNA polymerase. EMBO J. **14:**4523–4529.

9. **Golding, A., M. Weickert, J. Tokeson, S. Garges, and S. Adhya.** 1991. A mutation defining ultrainduction of the *Escherichia coli gal* operon. J. Bacteriol. **173:**6294–6296.

10. **Lengeler, J., K. Hermann, and H. J. Unsold.** 1971. The regulation of the B-methylgalactoside transport system and of the galactose binding protein of *Escherichia coli* K12. Eur. J. Biochem. **19:**457–470.

11. **Mihalyi, E.** 1968. J. Chem. Eng. Data **13:**179–182.

12. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

13. **Royer, C. A., A. E. Chakerian, and K. S. Mathews.** 1990. Macromolecular binding equilibria in the *lac* repressor system: studies using high-pressure fluorescence spectroscopy. Biochemistry **29:**4959–4966.

14. **Studier, F. W., and B. A. Moffat.** 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J. Mol. Biol. **189:**113–130.

15. **Weickert, M., and S. Adhya.** 1992. Isorepressor of the *gal* regulon in *Escherichia coli*. J. Mol. Biol. **232:**248–260.

16. **Weickert, M., and S. Adhya.** 1993. Control of transcription of *gal* repressor and isorepressor genes in *Escherichia coli*. J. Mol. Biol. **175:**251–258.

17. **Weickert, M. J., and S. Adhya.** 1992. A family of bacterial regulators homologous to *gal* and *lac* repressors. J. Biol. Chem. **267:**15869–15874.

18. **Weickert, M. J., and S. Adhya.** 1993. The galactose regulon of *Escherichia coli*. Mol. Microbiol. **10:**245–251.