Interaction of Gal repressor with inducer and operator: Induction of *gal* transcription from repressor-bound DNA

(tryptophan fluorescence/derepression/galactose/circular dichroism)

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ABSTRACT Gal repressor inhibits transcription from the *gal* promoter (*P*1) when it binds to the cognate operator (O_E). The repression is relieved by the presence of the inducer D-galactose. Compared with its interaction with free repressor, D-galactose binds to the repressor–operator complex with 10-fold reduced affinity as determined by fluorescence enhancement measurements. Thermodynamic analysis and fluorescence anisotropy showed that the stability of the repressor–operator complex is reduced by only 7-fold by the presence of the inducer in the complex. The formation of the inducer–repressor–operator ternary complex has been confirmed by CD spectral analysis. Fluorescence spectroscopy and energy transfer experiments suggest that individual allosteric effects of the two ligands, inducer and operator, on Gal repressor are responsible for the slightly weakened stability of the ternary complex compared with the stability of the inducer–repressor and repressor–operator complexes. *In vitro* transcription results demonstrated full derepression of transcription of the *P*1 promoter under conditions in which the concentrations of the inducer–repressor binary complex are severalfold higher than the dissociation constant of the inducer–repressor–operator ternary complex into inducer–repressor and free DNA. These results strongly suggest that the inducer binding to the repressor–operator complex does not lead to dissociation of the repressor from the operator during transcription induction. Because Gal repressor inhibits transcription by modulating the α subunit of the *P*1-bound RNA polymerase, we conclude that the inducer binding to the operator-bound repressor only allosterically relieves the inhibitory effect of repressor on RNA polymerase without dissociating the repressor from DNA.

In the classical negative control of gene expression, a repressor protein binds to cognate operator element(s) in DNA at or near the promoter and inhibits transcription initiation (1). Induction of transcription can occur in the presence of an inducer molecule that interacts with and causes an allosteric change in the repressor, which then loses its inhibitory power on transcription. It was originally proposed and commonly believed that repressor sterically prevents RNA polymerase from binding to the promoter site. According to this idea, because of inducer-generated allosteric change(s), the repressor loses its affinity for the operator and dissociates from DNA, thus allowing RNA polymerase to bind. We investigated the dissociation of repressor from the operator DNA by inducer under conditions of transcription derepression in the *gal* operon of *Escherichia coli* and found that this long-held

view of the mechanism of induction does not apply to the *P*1 promoter of the operon.

The *gal* operon is transcribed by two promoters, *P*1 and *P*2 (2). Repression of the two *gal* promoters involves Gal repressor (GalR) binding to two operators, O_E and O_I (3–6). Derepression occurs by the interaction of GalR with inducer D-galactose or D-fucose (7). Whereas full repression of both *P*1 and *P*2 requires the histone-like protein HU (8), interaction of repressor with O_E alone represses *P*1, but not *P*2. GalR·O_E-mediated repression of *P*1 observed in the absence of HU also is relieved by the presence of D-galactose or D-fucose (6). We report here the results of an analysis of (*i*) formation and stability of various binary and ternary complexes of operator, repressor, and inducer by fluorescence and CD spectroscopy, (*ii*) the antagonistic effect of inducer or operator on the binding of the other to repressor, and (*iii*) DNA-induced conformational changes in GalR. These results lead us to propose that inducer derepresses transcription in this system without dissociating the repressor from the operator.

MATERIALS AND METHODS

Materials. D-Galactose, D-fucose, adenosine 5′-[γ-thio]triphosphate (ATP[γ-S]), and acrylamide were obtained from Sigma. Fluorescein isothiocyanate and 5-({[(2-iodoacetyl)amino]ethyl}amino)naphthalene-1-sulfonic acid (IAEDANS) were purchased from Molecular Probes. Oligonucleotides purified by HPLC were from Midland Certified Reagent (Midland, TX). The sequence of the 20-mer wild-type O_E was 5′-TTGTGTAAACGATTCCACTA-3′, whereas that of the mutant O_E was 5′-TTGTGTAAATAATTCCACTA-3′. The sequence of the 29-mer wild-type O_E was 5′-xACTTCT-TGTGTAAACGATTCCACTAATTT-3′, x being a hexyl-amino group (2). Polynucleotide kinase was obtained from BRL. GalR was purified from a plasmid clone in which the *galR* gene was expressed from a late promoter of bacteriophage T7 as described previously (9). The concentration of GalR dimer was obtained from its extinction coefficient at 280 nm of 39,600 M^{-1}·cm^{-1} (10) and is expressed in terms of monomer. Plasmid pSA509 used as the source of DNA in transcription assays has been described before (6).

IAEDANS Labeling of O_E. Twenty nanomoles of O_E DNA duplex, 250 μmol of ATP[γ-S], and 10 units of T4 polynucleotide kinase were incubated at 37°C for 2 h in 0.1 M potassium phosphate buffer, pH 8.0. The mixture was extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). The aqueous layer was loaded onto a Sephadex G-25

Abbreviations: IAEDANS, 5-({[(2-iodoacetyl)amino]ethyl}amino)-naphthalene-1-sulfonic acid; AEDANS, 5-({[(acetyl)amino]ethyl}-amino)naphthalene-1-sulfonic acid; ATP[γ-S], adenosine 5′-[γ-thio]triphosphate.
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column (1.5 × 12.5 cm), equilibrated with 0.1 M potassium phosphate (pH 8.0) and eluted. Appropriate fractions were pooled, and IAEDANS was added at a final concentration of 0.5 mM. The mixture was incubated for 90 min in the dark and extensively dialyzed. The calculated incorporation ratio obtained from A_{260} and A_{340} was approximately 1.2 mol per mol of oligonucleotide duplex.

Synthesis and Purification of Fluorescein-Labeled O_E. The 29-mer complementary oligonucleotides with a hexylamino group (x) attached at their 5′ ends were separately synthesized in an Applied Biosystems 381A DNA synthesizer using amino link supplied by Applied Biosystems. The oligonucleotides were deprotected by incubating them at 55°C for 24 h in 30% ammonium hydroxide, precipitated by 1-butanol, then dried under vacuum and dissolved in water.

Ten milligrams of fluorescein 5-isothiocyanate was dissolved in 200 μl of 1.0 M NaHCO$_3$/Na$_2$CO$_3$ buffer (pH 9.0)/N,N'-dimethylformamide/water in the ratio of 5:2:3. The dye solution, the oligonucleotide solution, and the carbonate buffer were mixed in the ratio of 2:5:3, vortexed briefly, and incubated at 25°C for 20 h. The fluorescein-labeled oligonucleotides were further purified by C_{18} reverse-phase HPLC using a linear gradient of 0–60% acetonitrile in 0.1 M triethylammonium acetate buffer (pH 7.0). The appropriate fractions of each labeled oligonucleotide were mixed in equimolar proportion, and then annealed.

Fluorescence. Fluorescence spectra were recorded either in a Hitachi F3010 or SLM-Aminco (Urbana, IL) spectrofluorometer equipped with computers for spectra addition and subtraction facility. Experiments were conducted in a water-circulated thermostat chamber maintained at 25°C. Excitation band pass was 1.5 nm, and emission band pass was 20 nm. Samples were incubated at 25°C for 30 min before conducting the experiment. This incubation was necessary, because initial dilution of protein from stock solution caused the appearance of slight turbidity, which disappeared within several minutes upon standing at room temperature. The excitation wavelength was 295 nm; the emission wavelength was 360 nm. In preliminary experiments, the tryptophan residue in GalR was found to be somewhat photosensitive. The excitation slit was opened only during the fluorescence measurements and only at a single point to reduce exposure time. Repeated measurements under such conditions yielded constant fluorescence values. The experiments were performed in 12.5 mM Tris·HCl buffer (pH 8.0) containing 300 mM KCl and 0.5 mM EDTA. D-Galactose titrations of GalR were done at 2×10^{-6} M GalR at 25°C. For titration of GalR-O_E complex by D-galactose, GalR, and O_E concentrations were 2.4×10^{-6} M and 1.2×10^{-6} M, respectively. O_E titrations were performed at 2×10^{-6} M GalR. Increasing concentrations of DNA were added to GalR before measuring the emission spectra. The normalized integrated intensity was plotted as a function of O_E concentration. The fluorescence values were corrected for dilution and inner filter effect. The excitation and emission band passes were 2 nm each.

The direct binding of O_E to GalR and to D-galactose·GalR complex was measured fluorometrically by using fluorescein-labeled operator DNA in the buffer described above. The excitation and emission wavelengths were 490 nm and 525 nm, respectively. Excitation and emission band passes were 10 nm.

Fluorescence anisotropy was measured after each addition of GalR as described in *Results*.

Fluorescence energy transfer efficiency (E) was calculated from excitation spectra using the following equation (11):

$$F_{D+A}/F_A = 1 + (\varepsilon_D C_D/\varepsilon_A C_A)E,$$

where F_{D+A} is the fluorescence intensity of the donor–acceptor pair (dansylated O_E and GalR); F_A is the fluorescence of the acceptor (dansylated O_E) at the same wavelengths;

ε_D and ε_A are the molar extinction coefficients of the donor and acceptor, respectively, at the excitation wavelength, and C_D and C_A are the concentrations of the donor and acceptor, respectively. The distance between the dansyl group and the tryptophan in GalR was determined by the following equation:

$$R = R_o(E^{-1} - 1)^{1/6},$$

where R is the distance between the donor and the acceptor, R_o is the distance for which the energy transfer efficiency is 50%, and E is the experimentally observed energy transfer efficiency.

$$R_o = (J\kappa^2 Q\eta^{-4})^{1/6}(9.79 \times 10^3)\text{cm},$$

where J is the overlap integral (a measure of the spectral overlap), Q is the quantum yield, η is the refraction index, and κ is the orientation factor. The overlap integral was calculated according to the method of Wu and Stryer (12). The value of κ^2 was taken as 2/3, which is completely spatially averaged. Such complete spatial averaging is justified here because of aliphatic linkers separating the 5-({[(acetyl)amino]-ethyl}amino)naphthalene-1-sulfonic acid (AEDANS) molecule from the DNA as well as the experimentally obtained low polarization value. The energy transfer experiments were done in the buffer described above at 25°C. The excitation and emission band passes were 4 nm and 16 nm, respectively. The emission wavelength was set at 500 nm.

Circular Dichroism. CD spectra were obtained using a Jasco J-600 spectropolarimeter at 25°C. DNA concentration was 2.0 μM. Ten scans were averaged to produce a CD spectum. The bandwidth was 1 nm, and the time constant was 1 s. The experiments were conducted in the buffer described above. The buffer spectrum was subtracted from the O_E spectrum, whereas the buffer-plus-GalR spectrum was subtracted from O_E-plus-GalR spectrum. The protein did not contribute significantly to the intensities at the wavelengths used.

Data Processing. For all titration experiments, control experiments were carried out in which identical volumes of buffer were added to the protein solution without the ligand. Values from the blank titration were used to correct the volumes for ligand titrations. The corrected fluorescence values then were fitted to an equation of a single class of ligand binding by the nonlinear least-squares method for determining dissociation constants. The three variable parameters chosen (initial fluorescence value, fluorescence value at infinite ligand concentration, and the dissociation constant) were systematically varied within a given range. The values that gave the lowest χ^2 were chosen as the best-fit parameters. A similar least-squares fit procedure was used to obtain operator–repressor dissociation constants from anisotropy data.

***In Vitro* Transcription.** *In vitro* transcription assays were done as described previously (9) with slight modifications. The 348-bp *Eco*RI–*Bam*HI fragment of pSA509 containing the *gal* promoter region was used as the template. The transcription reaction mixture containing 12.5 mM Tris·HCl (pH 8.0), 200 mM potassium chloride, 5 mM magnesium chloride, 2 nM DNA, 20 nM RNA polymerase, 160 or 800 nM GalR, and different concentrations of D-galactose as indicated in Fig. 7 was preincubated for 5 min at 37°C. The reaction was started by the addition of 0.2 mM ATP, GTP, CTP, and 0.02 mM ^{32}P-labeled UTP. After incubation for 15 min at 37°C, the reaction was stopped by the addition of 0.2 vol of 0.2 M EDTA in 40% glycerol with dye. D-Galactose concentrations were 2.0 and 30 mM. The RNA was quantified by PhosphorImager 425 (Molecular Dynamics).

RESULTS

Inducer Binding to GalR and GalR·O_E Complex. Binding of ligands to proteins often causes quenching or enhancement of fluorescence of tryptophan, depending on the location of the amino acid(s) in the protein. Such enhancement or quenching can be used to derive binding isotherms. GalR is a dimer containing only one tryptophan residue per subunit. The intrinsic fluorescence of this tryptophan was used to monitor ligand binding. Fig. 1 shows the results of D-galactose, D-fucose, and D-glucose titration of GalR at 1.0×10^{-6} M as monitored by the intrinsic tryptophan fluorescence. Binding of D-galactose resulted in a 23% enhancement of tryptophan fluorescence, which quickly saturated. No significant fluorescence increase occurred at D-galactose concentrations greater than 2.0×10^{-4} M. This enhancement also was accompanied by a small (≈ 2 nm) red shift of the emission maximum and a change in acrylamide quenching pattern (data not shown). The data were used to fit a binding equation for a single class of binding site using a nonlinear least squares fit procedure. It yielded a dissociation constant (K_{D1}) of $2.2 \pm 0.3 \times 10^{-5}$ M. A similar dissociation constant for the interaction of D-galactose and GalR has been reported previously (13). D-Fucose is a nonmetabolizable analog of D-galactose and is a slightly weaker inducer (14). Binding of D-fucose also led to an enhancement of tryptophan fluorescence, although the extent of enhancement (12%) was less, resulting in a dissociation constant of $6.0 \pm 0.3 \times 10^{-5}$ M. D-Glucose, which is not an inducer, caused relatively minor changes in tryptophan fluorescence.

The binding of D-galactose to operator–repressor complex was studied by monitoring tryptophan fluorescence changes that occurred upon D-galactose binding. The concentration of GalR was 2.4×10^{-6} M, and that of O_E DNA was 1.2×10^{-6} M. As will be shown later, Gal repressor bound to O_E DNA with a dissociation constant (K_{D4}) of 4×10^{-9} M under these conditions. Thus, GalR and O_E should exist as a 1:1 complex under these conditions. A significant increase of tryptophan fluorescence was achieved by the addition of increasing concentrations of D-galactose, with little enhancement occurring beyond 1.0×10^{-3} M (Fig. 1). At the high D-galactose concentration, tryptophan fluorescence increased by 23% of the initial value. The degree of fluorescence enhancement was
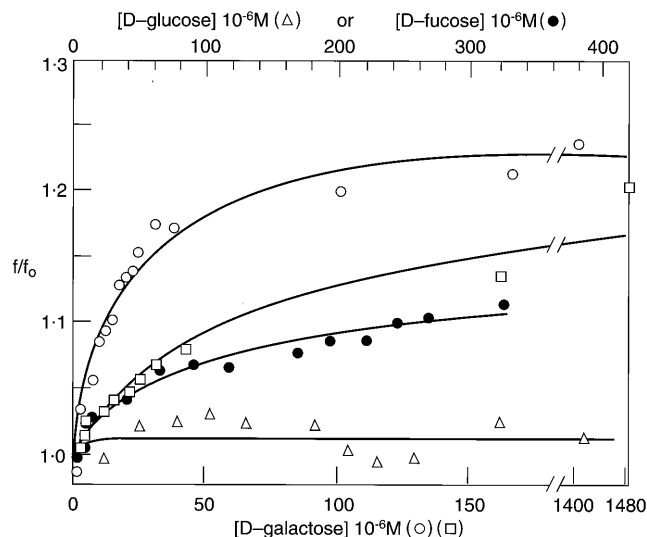
very similar to that observed on binding of D-galactose to free GalR. However, D-galactose bound to the GalR·O_E complex with a dissociation constant (K_{D3}) of 2.2×10^{-4} M, which is approximately 10-fold higher than K_{D1}, the dissociation constant of D-galactose binding to free GalR. This suggests that although binding of repressor to the operator reduced the affinity of inducer to repressor, because of the sufficient residual affinity of the inducer-bound repressor toward the operator, the inducer bound to the operator-bound GalR without dissociating the complex.

The inability of D-galactose to dissociate repressor from the DNA·protein complex was further tested by fluorescence anisotropy experiments. The O_E DNA was thiophosphorylated at the 5′ end by ATP γS and T4 polynucleotide kinase and subsequently allowed to react with IAEDANS as described in *Materials and Methods*. The AEDANS-labeled operator at 0.5×10^{-6} M gave an anisotropy value of 0.036 (data not shown), indicating significant internal motion of the IAEDANS probe. The presence of 1.0×10^{-6} M GalR monomer increased the anisotropy value to 0.064. This value is still low compared with that expected for a fully rigid probe, again indicating significant internal motion. Such low anisotropy values are not uncommon in 5′-end-labeled DNAs because of flexibility of the linker arm and lack of steric constraints at the end of the oligonucleotides (15). Further addition of 1.0×10^{-3} M D-galactose resulted in a very small (less than 5%) decrease in the anisotropy value obtained for GalR plus DNA. The value in the presence of D-galactose was still much higher than that of the free operator, indicating that binding of D-galactose to the repressor–operator complex does not lead to significant dissociation of the repressor from the operator.

Determination of the affinity of D-galactose to free repressor and repressor–operator complex allowed an estimation of the relative affinities of O_E for the free GalR and the D-galactose–GalR complex based on the binding cycle described in Fig. 2. Two pathways lead from the repressor to the end product, the inducer–repressor–operator ternary complex. Thus,

$$K_{D1} \cdot K_{D2} = K_{D3} \cdot K_{D4}$$

or

$$K_{D1}/K_{D3} = K_{D4}/K_{D2},$$

where the K_Ds are the dissociation constants of the respective binding equations. Thus, the predicted ratio of K_{D4}/K_{D2} is 0.1 as determined from K_{D1} and K_{D3} values of 2.2×10^{-5} M and 2.2×10^{-4} M, respectively.

FIG. 1. Relative tryptophan fluorescence change (f/f_o) of GalR as a function of hexose concentration. D-Galactose (○), D-fucose (●), D-glucose (△), and D-galactose plus 20-mer O_E-DNA (□). The GalR concentration was 2×10^{-6} M in all of the experiments, except repressor concentration was 2.4×10^{-6} M and O_E DNA was 1.2×10^{-6} M in the titration of GalR·O_E complex.
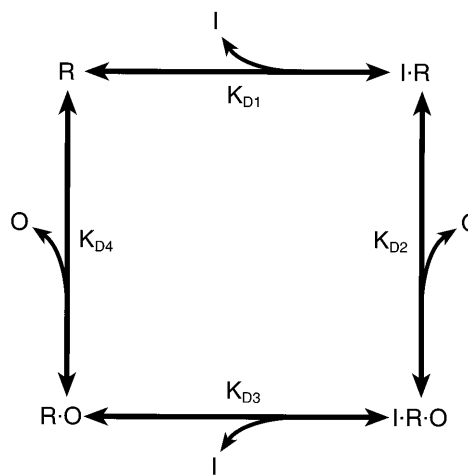

FIG. 2. The binding cycle of GalR (R) to ligands D-galactose (I) and operator (O).

GalR Binding to O_E in the Absence and Presence of Inducer.
To determine values of K_{D4} and K_{D2} experimentally, the
affinity of the O_E for GalR and D-galactose–GalR complex was
determined by fluorescence anisotropy measurements. A
29-bp O_E DNA with a hexylamino group at the 5′ end was
labeled with fluorescein isothiocyanate. Fluorescein has a high
molar extinction coefficient and quantum yield. The purified
duplex was titrated with GalR and anisotropy was measured at
each point. A plot of fluorescence anisotropy of the 2.0×10^{-9}
M O_E DNA at different GalR concentrations is shown in the
absence (Fig. 3A) and in the presence (Fig. 3B) of 1×10^{-3} M
D-galactose. In both cases, fluorescence anisotropy increased
by about 40–50%. The observed values of K_{D4} and K_{D2} were
4.2×10^{-9} M and 27.8×10^{-9} M, respectively. The measured
dissociation constant (K_{D4}) of the operator–repressor complex
under the present conditions was at least an order of magni-
tude higher than those obtained by gel electrophoresis and
DNase footprinting assays (9, 16). This was expected, as the
latter measurements were done in the presence of potassium
glutamate instead of KCl and at a lower salt concentration,
conditions that increase the affinity of DNA-binding proteins
for the target sequences (17). Nevertheless, the binding of
D-galactose to GalR reduced the affinity of repressor for the
operator by approximately 7-fold. The K_{D4}/K_{D2} ratio of 0.15,
measured by fluorescence anisotropy, is very similar to the
value (0.1) obtained from the indirect binding cycle analysis.

The binding of repressor to the operator was further inves-
tigated in the presence and absence of inducer by CD spec-
troscopy. Binding of the GalR to O_E leads to a significant
change of CD spectra of the operator (10). Fig. 4A shows the
CD spectra of the operator, the repressor–operator complex,
and the repressor–operator complex in the presence of D-
galactose. Binding of GalR to O_E at micromolar concentra-
tions, as expected, resulted in a change of the operator CD
spectrum. The direction and magnitude of the spectral change
was the same as that observed previously (10). The CD spectral
change indicated a DNA distortion that very likely is the DNA
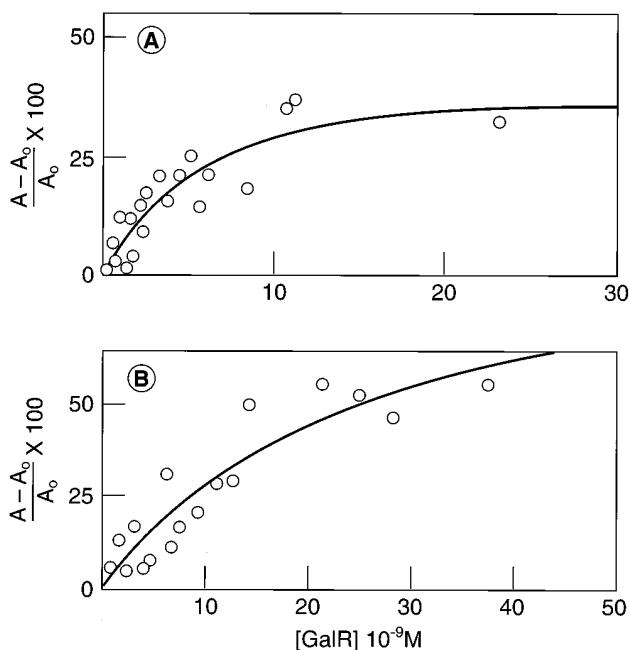bending caused by the binding of GalR to O_E (18). The
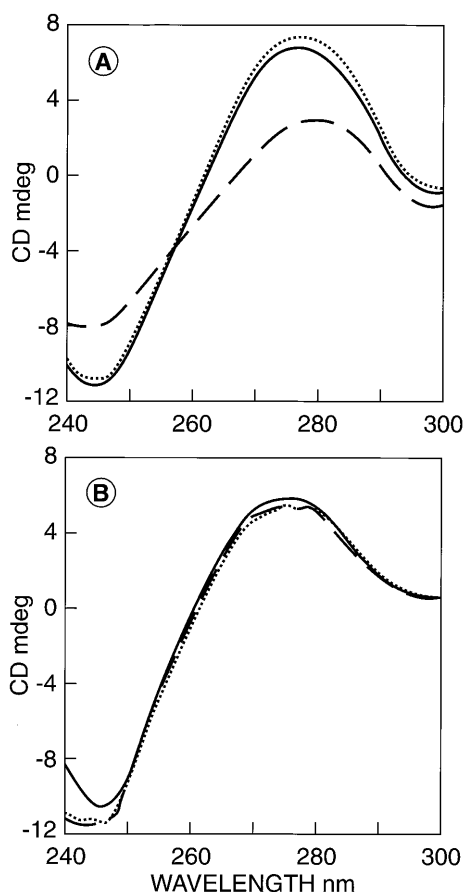
FIG. 4. (*A*) CD spectrum of the operator O_E (20-mer) at 2×10^{-6}
M ($-\,-\,-\,-$), in the presence of GalR at 4.0×10^{-6} M (⋯⋯), and in the
presence of GalR at 4.0×10^{-6} M and D-galactose at 2.0×10^{-3} M
(——). (*B*) CD spectrum of the mutant O_E^C operator (20 mer) at $2.0 \times$
10^{-6} M ($-\,-\,-\,-$), in the presence of GalR at 4.0×10^{-6} M (——) and
in the presence of GalR at 4.0×10^{-6} M and D-galactose at 2.0×10^{-3}
M (⋯⋯). The bandwidth was 1.0 nm, and the scan speed was 200
nm/min. Ten spectra were averaged to improve signal-to-noise ratio.

presence of 2×10^{-3} M D-galactose did not cause any
significant change in the CD spectrum of the repressor–
operator complex. Because the D-galactose concentration was
10-fold higher than the apparent dissociation constant of the
D-galactose from the operator-repressor complex (Fig. 1), it
was evident that the binding of D-galactose to the operator-
repressor complex at micromolar concentrations did not lead
to the dissociation of repressor from the operator.

A control experiment focused on a DNA duplex of the same
size that included a mutant O_E^C sequence (2). Because of a
change of 2 bp, the nature of the spectra of the O_E^C DNA is
slightly different from that of O^+ DNA. Fig. 4B shows the CD
spectra of the mutant operator, the mutant operator in the
presence of repressor, and the mutant operator in the presence
of repressor and D-galactose. The presence of GalR led to only
a very small change in the CD spectrum. The addition of
D-galactose did not cause any detectable CD change, indicat-
ing that the change in the CD spectrum of O_E is a consequence
of specific binding of GalR. Furthermore, binding of D-
galactose to this complex preserved the nature of the complex.

O_E-Induced Conformational Change in GalR. Similar to the
reduction in the affinity of inducer for the operator–repressor
complex compared with that for free repressor, the affinity of
the operator for the inducer–repressor complex is lower than
for free repressor. We tested the possibility that DNA binding
induces an allosteric change in repressor conformation by
measuring the tryptophan fluorescence of GalR at 2.0×10^{-6}

FIG. 3.   Titration of fluorescein-labeled O_E DNA with GalR in the
absence (*A*) and in the presence (*B*) of 1.0×10^{-3} M D-galactose.
Fluorescein-labeled oligonucleotides (29-mer) were prepared as de-
scribed in the text. The O_E concentration was 2×10^{-9} M. Each
reading was time-averaged for 100 sec. The solution conditions are
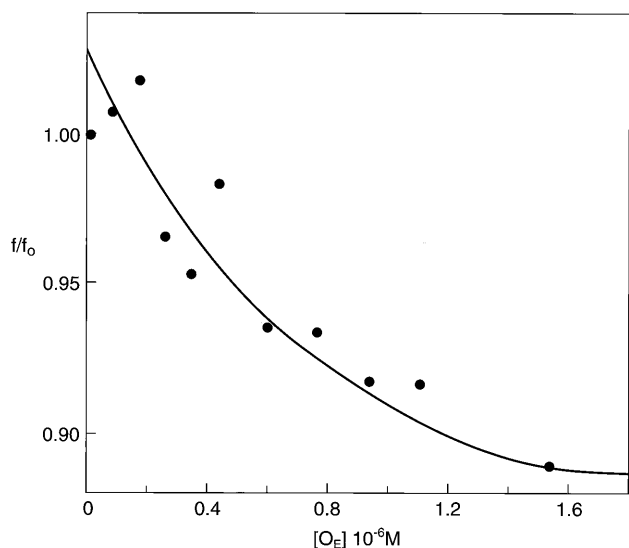described in *Materials and Methods*.

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FIG. 5. Effect of O_E DNA binding on the tryptophan fluorescence (f/f_o) of GalR. GalR at a concentration of 2.0×10^{-6} M was titrated with increasing concentrations of the operator (20-mer). The total emission spectra were integrated.

M concentration in the presence of increasing concentrations of O_E DNA. As shown in Fig. 5, binding of O_E reduced the fluorescence by more than 11%.

Measurements were taken of the distance of the tryptophan residue in GalR from the AEDANS placed at one end of the operator DNA. AEDANS has a large overlap in its excitation spectra with the tryptophan emission spectra. Fig. 6 shows the excitation spectra of the AEDANS-labeled O_E and of a stoichiometric complex of AEDANS-O_E and GalR. At 295 nm, the fluorescence of the complex was slightly higher than that of the free repressor, suggesting a small, but significant, energy transfer. The calculated energy transfer efficiency was 0.09, which corresponds to a distance of 38.6 Å between the tryptophan and the AEDANS label. Mutational and modeling studies also have placed the tryptophan residue in the C domain and the DNA-binding motif in the N domain of GalR (19, 20). Because the DNA-binding site and the tryptophan residue in GalR are far from each other and in different domains, quenching of tryptophan fluorescence in GalR by operator binding strongly suggested that a DNA-induced conformational change is transmitted from the N domain to
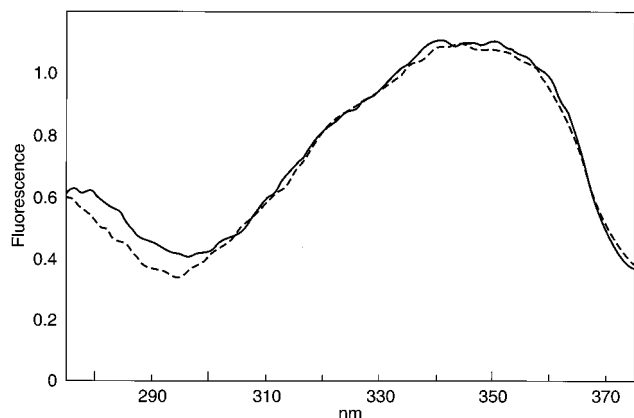
the C domain in GalR. A similar change has been documented in λ repressor (21).

***In Vitro* Transcription.** Because the fluorescence anisotropy and CD results indicated that GalR was bound to the operator in the presence of the inducer, we studied derepression of transcription of the *gal P*1 promoter *in vitro* as a function of D-galactose concentration under conditions used for ligand binding studies, except the KCl concentration was 200 mM. The slightly lower salt concentration was chosen to avoid a possible decrease in transcription. The lower salt concentration is expected to further tighten the binding of GalR to the operator. The results are shown in Fig. 7. In the absence of inducer, GalR at 1.6×10^{-7} M transcription initiation from the *P*1 promoter repressed by about 70% (Fig. 7*A*, lane 1 vs. 2). In the presence of 2×10^{-3} M D-galactose, transcription was derepressed 78%, whereas in the presence of a saturating concentration of the inducer (3×10^{-2} M) the *P*1 promoter was derepressed to 99% (lanes 2 and 3). When transcription was studied at 8×10^{-7} M GalR, the presence of 3×10^{-2} M D-galactose derepressed transcription by 38% (Fig. 7*B*). This result is significant considering the fact that at this high GalR concentration, the repressor also inhibited transcription from nonspecific promoters, such inhibition not being lifted by D-galactose. Given the determined values of the K_Ds, we calculated the fractional occupancy, Y, of operator by repressor from the equation

$$Y = \frac{1}{1 + K_D/R_f} \qquad [1]$$

at 1.6×10^{-7} M and 8.0×10^{-7} M GalR. At 2×10^{-9} M DNA and such high repressor concentrations, the free repressor concentration, R_f, is practically the same as the total repressor concentration. At 1.6×10^{-7} M and 8.0×10^{-7} M GalR and saturating inducer concentration, transcription is 99% and 38%, respectively, of the unrepressed levels and the calculated occupancy of operator by repressor is 86% and 94%, respectively.
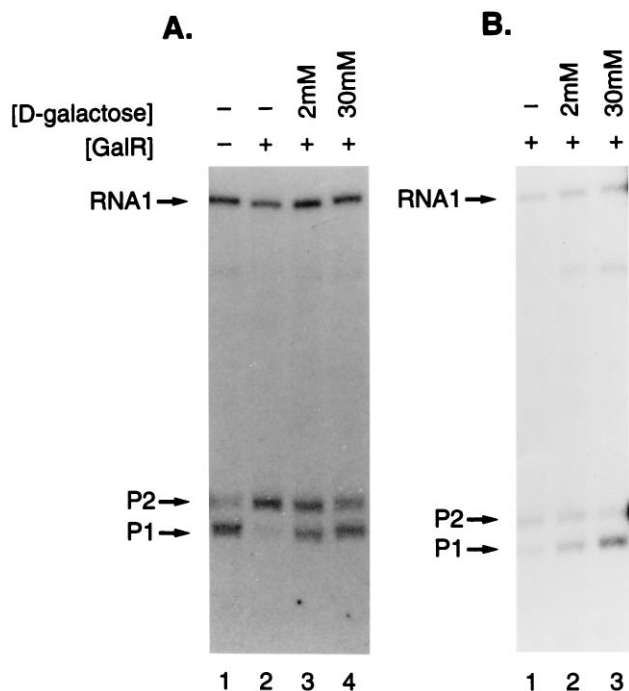

FIG. 6. Fluorescence energy transfer between AEDANS-O_E and tryptophan of the GalR. The excitation spectra of 20-mer AEDANS-labeled O_E (0.5×10^{-6} M) (– – – –), and that of the same DNA complexed with GalR (1.0×10^{-6} M) (———) are shown.

FIG. 7. The effect of D-galactose on *galP*1 transcription in the presence of GalR. *In vitro* transcription assays were carried out as described in the text. GalR concentrations were 160 (*A*) and 800 nM (*B*).

DISCUSSION

In negative control, binding of repressor to a cognate operator inhibits RNA polymerase action at the promoter, and an allosteric modification of repressor by a small-molecule inducer inhibits repressor action. Likely mechanisms by which the inducer can inhibit repressor action include:

(*i*) Inducer binds only to free repressor, allosterically inactivating its operator binding site. In this model, induction follows the rate of spontaneous dissociation of operator from repressor.

(*ii*) Inducer forms an inducer–repressor–operator ternary complex which, because of allosteric change, dissociates into inducer-repressor complex and free operator more readily than into repressor–operator complex and free inducer. In this case, induction follows the rate of dissociation of repressor–inducer complex from free operator.

(*iii*) Inducer forms an inducer–repressor–operator ternary complex which, because of allosteric change, allows RNA polymerase to act without repressor being physically dissociated from operator.

GalR binds to operator O_E and inhibits transcription from the $P1$ promoter of the *gal* operon (6, 22). We showed that D-galactose and the O_E operator antagonize the binding of each other to GalR. Consistently, both D-galactose and O_E DNA binding to GalR exhibit allosteric changes in the protein (ref. 13; results reported in this paper). Despite such an antagonistic effect, inducer binding to the operator-bound repressor was found to be only 10-fold less than the extent of binding to free repressor under conditions of repression and induction of *gal* transcription. Similarly, the affinity of O_E to the inducer–repressor complex is reduced by 7-fold compared with the binding of O_E to the free repressor. The results of GalR binding to fluorescein-labeled DNA in the presence of D-galactose also showed the existence of stoichiometric amounts of inducer–repressor–operator ternary complex. The existence of stable ternary complex under physiological conditions also has been confirmed by comparing CD spectra of the free operator, operator bound to repressor, and operator bound to repressor in the presence of inducer. Comparable reduction in the affinity of inducer for repressor when complexed with operator was found in the *lac* system (23, 24).

In vitro transcription results presented here clearly demonstrated that full derepression of *gal* $P1$ occurs under conditions of binding in which (*i*) the repressor is still bound to the DNA, i.e., the concentrations of inducer–repressor binary complex are severalfold higher than the dissociation constant of the inducer–repressor–operator ternary complex into the inducer–repressor complex and free DNA; (*ii*) the RNA polymerase concentration was significantly less than the dissociation constant of the RNA polymerase-promoter complex (K_B^{-1}) (25, 26), and (*iii*) nonspecific DNA concentration was negligible. These results strongly suggest that previous dissociation of repressor is not obligatory for full derepression of transcription from the $P1$ promoter of the *gal* operon. Whether *gal* derepression occurs by a similar mechanism *in vivo* remains to be investigated.

It is unclear how repressor inhibits transcription. Operator-bound repressor either occludes RNA polymerase binding to the promoter or inhibits RNA polymerase activity at one or more of the postbinding steps (27). We previously demonstrated that GalR and RNA polymerase coexist on the DNA, and GalR inhibits the $P1$ promoter by freezing the RNA polymerase at an intermediate step between closed and open complex formation (22). GalR does so by modulating the activity of the α subunit of the DNA-bound RNA polymerase. As discussed above, dissociation of repressor from the operator is not obligatory for transcription from the $P1$ promoter. We propose that because a direct contact between DNA-bound GalR and RNA polymerase brings about repression of the $P1$ promoter, then binding of inducer to the operator-bound repressor neutralizes the inhibitory contact without dissociating the repressor from DNA. A role of repressor at a post-RNA polymerase binding level also has been suggested in the *E. coli lac* promoter and *Bacillus subtilis* phage ϕ 29 A2c promoter (28–30). As in the *gal* $P1$ promoter, MerR repressor and RNA polymerase coexist at the repressed promoter in the *mer* operon (31, 32). Binding of inducer Hg^{2+} to MerR derepresses transcription without Hg^{2+}–MerR being dissociated from the promoter. But unlike the repressor *gal* $P1$ complex, the protein-DNA linkage is obligatory for *mer* transcription, because the Hg^{2+}–MerR complex is an activator of the *mer* promoter.

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