Interactions between DNA-Bound Transcriptional Regulators of the *Escherichia coli gal* Operon†

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ABSTRACT: Regulation of the initiation of gene transcription from the *gal* operon of *Escherichia coli* is activated by the binding of CAP (catabolite activator protein) to a site centered at base pair −41.5 relative to the S1 start site of transcription. This operon is repressed by the specific binding of Gal repressor (GalR) to two operators, O_E and O_I, centered at −60.5 and +53.5, respectively. It has been proposed that this negative regulation results from the interaction of GalR dimers bound to O_E and O_I to form a protein-mediated "looped complex" [cf. Adhya, S. (1989) *Annu. Rev. Genet. 23*, 207–230]. In order to test whether DNA-bound CAP would facilitate or inhibit the binding of GalR, the simultaneous binding of these proteins was studied by quantitative DNase I footprint titration analysis. These studies demonstrate that GalR binding is noncooperative in the presence and in the absence of CAP and that GalR and CAP bind to the *gal* operon independently. No evidence was found that CAP stabilizes a putative Gal repressor-mediated protein–DNA looped complex. It has been shown that the *gal* operon can be negatively regulated by the binding of Lac repressor (LacI) to a *gal* operon in which O_E and O_I were both modified to be recognized by LacI [Haber, R., & Adhya, S. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 9683–9687]. In contrast to GalR, LacI binds to the chimeric *gal* operon with moderate cooperativity via the formation of a stable protein–DNA looped complex. In order to assess the effect of CAP on a LacI-mediated looped complex at the *gal* operon, the simultaneous binding of these two proteins to DNA was studied. The simultaneous binding of CAP and Lac repressor has no significant effect on the cyclization probability of the LacI-mediated "looped complex". However, while no interaction was observed between bound CAP and LacI bound to the downstream operator, O_I, an antagonistic interaction was observed when LacI was bound to the O_E operator, which is adjacent to the CAP binding site. The observation that LacI binding at O_E modulates the DNase I hypersensitivity induced by CAP within its binding site suggests that the CAP–DNA interaction is disrupted, potentially decreasing the magnitude of the CAP-induced DNA bend. This disruption may account for the absence of an effect on the cyclization probability of the LacI-mediated looped complex by CAP.

The initiation of gene transcription is regulated by nucleoprotein complexes in which both protein–DNA and protein–protein interactions play key roles. When two proteins bind to widely separated sites on the DNA and interact with each other, the intervening DNA is often extruded, resulting in a protein–DNA "looped complex" [see Adhya (1989) and Bellomy and Record (1990) for reviews]. The stability of these looped complexes is critically dependent on the affinity of the proteins for the DNA, on the association of the bound proteins for each other, and on the flexibility of the intervening DNA sequence [Mossing & Record, 1986; Bellomy et al., 1988; Brenowitz et al., 1991b; reviewed in Bellomy and Record (1990)]. Studies have shown that the binding of proteins that bend DNA or the presence of intrinsically bent DNA sequences can stabilize protein-mediated looped complexes [cf. Goodman and Nash (1989) and Moitoso de Vargas et al. (1989)]. In vivo quantitative analysis of the regulation of transcription initiation by Lac repressor (LacI)[1] binding to two separated operators revealed a value of the apparent persistence length of the intervening DNA that is one-fifth of that determined for free DNA in vitro (Bellomy et al., 1988). It was suggested that the DNA bending induced by the binding of CAP to a site located between the two operator sites could be the molecular mechanism underlying this observation (Bellomy et al., 1988).

Transcription from the *gal* operon is initiated from two partially overlapping promoters which are shifted 5 base pairs from each other with respect to the S1 transcription start point. This operon is activated by CAP and negatively regulated by GalR, which has been proposed to act by mediating the formation of a DNA loop [reviewed in Adhya (1989)]. There are two operator sites for GalR, O_E and O_I, neither of which overlaps the two promoters (Figure 1). CAP binds to a single site adjacent to the 3′ end of O_E and overlaps the −35 region of the RNA polymerase binding site of both promoters (Figure 1). In vivo transcription studies have shown that both O_E and O_I are required for negative regulation and that the simple occupancy of these operators by a repressor protein is not sufficient to achieve full repression [Haber & Adhya, 1988; Mandal et al., 1990; reviewed in Adhya (1989)]. In vitro DNase I footprinting experiments have shown that CAP, GalR, and RNAP can bind simultaneously to the *gal* operon [unpublished results, summarized by Adhya and Ma-

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[1] Abbreviations: LacI, Lac repressor; GalR, Gal repressor; CAP, catabolite activator protein (also known as CRP, cAMP receptor protein); O_E, "external" operator; O_I, "internal" operator; bp, base pair(s); BSA, bovine serum albumin; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane; DNase I, deoxyribonuclease I (EC 3.1.21.1); DNA, deoxyribonucleic acid; cAMP, adenosine 3′5′-cyclic monophosphate.

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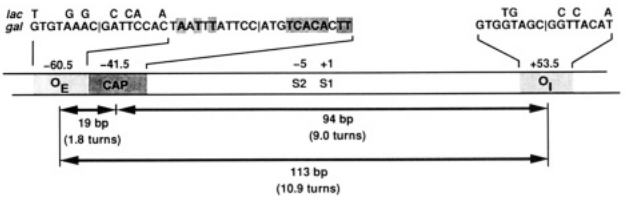

FIGURE 1: Schematic representation of the *gal* operon. Distances are relative to the S1 start site of transcription. They are calculated from the dyad symmetry axis for each of the protein binding sites and are shown above each site. (The ".5" values indicate that the center of dyad symmetry lies between two base pairs.) Operator sites O_E and O_I as well as the CAP binding site are shown. The DNA sequences of each of the protein binding sites are shown in the line marked *gal*. The base pairs that were converted to alter O_E and O_I from *gal* to *lac* recognition sequences are depicted in the line marked *lac*. The boxed bases within the CAP site denote the bases that are identical to the *consensus* sequence. The diagram is not to scale. The number of turns was calculated assuming 10.4 bp/turn of canonical B-DNA.

jumdar (1987)]. However, the in vitro regulation of transcription from the *gal* promoters by GalR is both qualitatively and quantitatively different from the regulation observed in vivo (Kuhnke et al., 1986; Goodrich & McClure, 1992; H. S. Choy and S. Adhya, unpublished results).

Repression of a chimeric *gal* promoter by LacI was demonstrated by the conversion of O_E and O_I from GalR to LacI recognition sequences (Haber & Adhya, 1988). This result, together with the observation that a LacI mutant (Lac^{adi}) deficient in the dimer to tetramer association was incapable of negatively regulating this chimeric gene, suggested that a protein-mediated looped complex, rather than a unique property of GalR, was sufficient to repress transcription initiation from the *gal* promoters (Mandal et al., 1990). However, the analysis of the binding of GalR to O_E and O_I by electron microscopy (Mandal et al., 1990) as well as by DNA binding titration assays (Brenowitz et al., 1990) suggests that GalR-mediated looped complexes do not form on linear DNA fragments in the absence of other regulatory components.

As the first step in evaluating the role of other regulatory factors on the energetic stability of repressor-mediated looped complexes, quantitative DNase I titrations were conducted to probe for heterologous interactions between CAP and either GalR or LacI at the *gal* promoter. The *individual-site binding curves* (Ackers et al., 1982, 1983) determined from this analysis allow the differentiation of the effect of CAP on the *intrinsic* binding of repressor bound at each or both operators from its effect on the flexibility of the intervening DNA sequence. By conducting these studies with both GalR and LacI, we hope to clarify the roles of protein association and DNA structure in the formation of protein–DNA looped complexes that are involved in the regulation of transcriptional initiation. A preliminary account of some of this work has been published (Dalma-Weiszhausz et al., 1991).

MATERIALS AND METHODS

DNA Operators. The preparation of linear DNA restriction fragments of 635 bp containing the sequences from −199 to +434 relative to the S1 transcription start site of the *gal* operon has been described (Brenowitz et al., 1990, 1991b). These linear restriction fragments were used in all DNA binding experiments. The construction of operators in which the GalR recognition sequences (designated $O_E{}^G$ and $O_I{}^G$) were altered to LacI recognition sequences (designated $O_E{}^L$ and $O_I{}^L$) has also been described (Haber & Adhya, 1988) and is shown in Figure 1. Protein binding studies were conducted with DNA in which both operators were recognized by GalR or LacI ($O_E{}^G/O_I{}^G$ or $O_E{}^L/O_I{}^L$) or with DNA in which each site was recognized by a different repressor ($O_E{}^G/O_I{}^L$ or $O_E{}^L/O_I{}^G$). The *consensus* CAP binding site (Ebright et al., 1984) is unchanged by the base-pair substitutions in the operators (Figure 1).

Protein Preparation. LacI and Lac^{adi} were purified as described in Brenowitz et al. (1991a). The specific DNA binding activity of the LacI and Lac^{adi} preparations is assumed to be 100%. The CAP protein used in these studies was prepared as described by Heyduk and Lee (1989) and has a specific DNA binding activity of 100% (Heyduk & Lee, 1990). Gal repressor was purified as has been described (Majumdar et al., 1987). The specific DNA binding activity of the GalR preparations used in these studies is 100% on the basis of gel-mobility shift titrations conducted under conditions where the DNA concentration exceeds the DNA binding dissociation constant (unpublished data). The activity determinations were conducted utilizing the commercially prepared presiliconized tubes noted below.

Quantitative DNase I Footprint Titration Assay. The quantitative DNase I footprint titration experiments were conducted as described in Brenowitz et al. (1991b) and the references cited therein. The degree of nicking by DNase I was carefully controlled so that, on the average, each DNA molecule that was nicked, was nicked only once (Brenowitz et al., 1986). This control minimizes the possibility that the DNase I exposure perturbed the protein–DNA equilibrium by, for example, compromising the integrity of the double-stranded DNA located between O_E and O_I. It is clear from controls in which DNA that was not exposed to DNase I was subjected to electrophoresis on denaturing sequencing gels that the phosphate backbones of the DNA fragments used in these studies were intact (Figure 2; lane N). All experiments were conducted at 20 °C in an *assay buffer* containing 25 mM Bis-Tris, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM DTT, 50 μg/mL BSA, 2 μg/mL calf thymus DNA, and 100 mM KCl titrated to pH 7.00 with HCl. The protein and DNA were equilibrated for 30–45 min prior to the addition of DNase I. Presiliconized microfuge tubes [low binding "Lube Tubes" (catalog no. T6050G) from Marsh Biomedical Products] were used at all steps in the experimental protocols. The assumption that $[\text{protein}]_{\text{total}} \approx [\text{protein}]_{\text{free}}$ was made in all experiments since the concentration of operator sites in the equilibrium mixtures was low relative to the protein–DNA dissociation constants.

Analysis of LacI Binding to DNA. The formulation of a statistical mechanical model describing the interaction of the bidentate LacI tetramer to two binding sites widely separated on the DNA and the individual-site binding equations appropriate for these titration experiments has been described (Brenowitz et al., 1991b). In this model, the binding of LacI is described by four free energies. Two *intrinsic* binding free energies, ΔG_E and ΔG_I, describe the binding of LacI to $O_E{}^L$ and $O_I{}^L$, respectively, in the absence of other interactions. ΔG_j represents the "free energy of cyclization" and is related to the "cyclization probability" [or "j-factor"; cf. Shore and Baldwin (1983) and Shimada and Yamakawa (1984)] of the DNA within the protein–DNA complex by $\Delta G = -RT \ln j$ where R is the gas constant and T is the temperature in degrees Kelvin. The fourth energy that describes the system is the dimer to tetramer association free energy. The concentrations of LacI dimers and tetramers were calculated by assuming that $2[\text{dimer}] \rightleftarrows [\text{tetramer}]$ is the sole equilibrium necessary to describe the self-association of the protein in the concen-

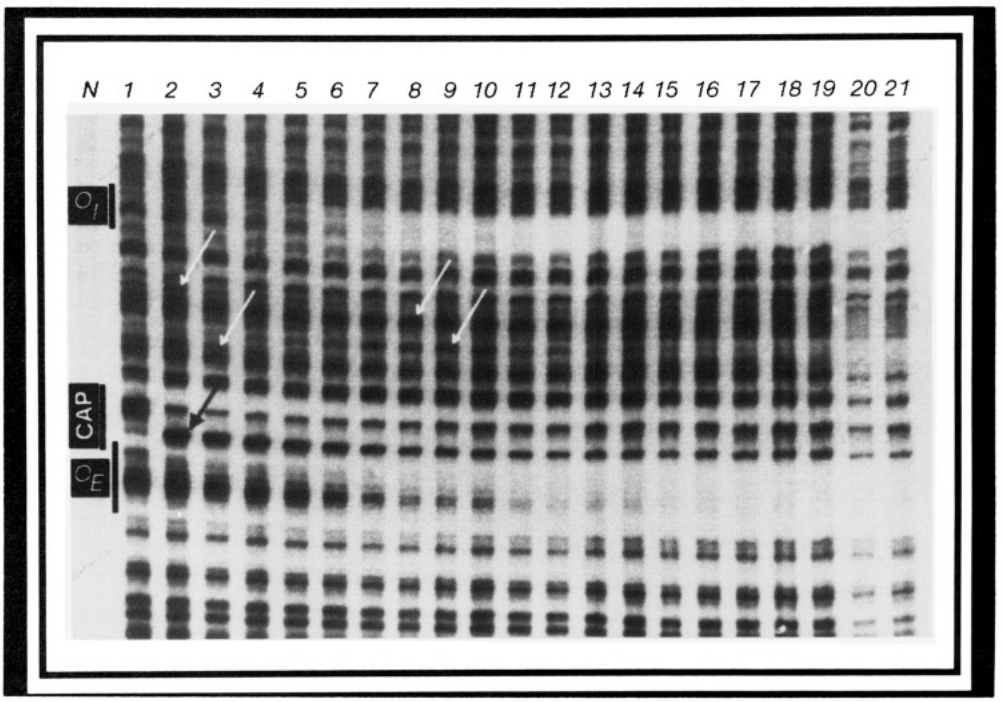

FIGURE 2: Representation of an autoradiograph of a DNase I footprint titration of LacI conducted in the presence of a saturating concentration of CAP (192.5 nM, dimer). The band within the CAP site that becomes hypersensitive to DNase I is marked with a black arrow. Bands located between O_E^L and O_I^L (and outside the CAP consensus sequence) that become hypersensitive to, or protected from, DNase I due to the formation of a LacI-mediated looped complex are marked by white arrows. Lane N was not treated with DNase I. All lanes except lane 1 contain CAP. Lanes 1 and 2, no LacI; lane 3, 1.5 pM LacI, lane 4, 3.0 pM; lane 5, 4.4 pM; lane 6, 5.9 pM; lane 7, 14.8 pM; lane 8, 29.6 pM; lane 9, 59.2 pM; lane 10, 148.0 pM; lane 11, 296.0 pM; lane 12, 444.0 pM; lane 13, 1.5 nM; lane 14, 3.0 nM; lane 15, 4.4 nM; lane 16, 5.9 nM; lane 17, 14.8 nM; lane 18, 29.6 nM; lane 19, 44.4 nM; lane 20, 59.2 nM; lane 21, 148.0 nM. Techniques of image analysis were used to alter the contrast and aspect ratio of the autoradiogram image in this figure. However, densitometric analysis of this (and all other) autoradiograms was conducted on unprocessed digital images.

tration range used (Royer et al., 1990). A value for the dimer to tetramer association free energy, ΔG_{tet}, of −13.2 kcal/mol was used in all calculations. This value was determined from an analysis of DNA binding titrations and "looped complex"-induced DNase I hypersensitivity (Brenowitz et al., 1991b). It should be noted that the determination of this value of ΔG_{tet} is subject to the assumption that LacI dimers and tetramers bind DNA equivalently and that the LacI tetramer binds DNA noncooperatively.

The resolution of these microscopic free energies requires the simultaneous analysis of the titrations of the DNA containing two binding sites for LacI (O_E^L/O_I^L) as well as of DNA containing a single site for LacI (O_E^G/O_I^L and O_E^L/O_I^G). Common values of the free energies are obtained which describe the least-squares minima for the analysis of the appropriate binding equations against all four titration curves [cf. Ackers et al. (1982) and Senear and Ackers (1990)].

Analysis of GalR Binding to DNA. The formulation of a statistical mechanical model describing the interaction of GalR dimers to two binding sites on the DNA and *the individual-site binding equations* appropriate for these titration experiments has also been described (Brenowitz et al., 1990). In this model, the two *intrinsic* binding constants (defined as described above), also denoted ΔG_E and ΔG_I, describe the binding of GalR to O_E^G and O_I^G, respectively. However, in the absence of a demonstration of bidentate tetramer formation, cooperative interactions are described phenomenologically as the difference in binding GalR to both sites simultaneously and to each site individually. An interaction free energy, ΔG_{IE}, is defined as $\Delta G_{IE} = \Delta G_{total} - (\Delta G_E + \Delta G_I)$ where ΔG_{total} is the energy of binding GalR to the two sites at the same time. Simultaneous minimization of multiple titration curves is also used for the GalR binding experiments to resolve the free energies as well as for the determination of the $\Delta\Delta G$'s representing the interaction energies between either repressor and CAP.

A value of the monomer to dimer association free energy, ΔG_{dim}, of −11.6 kcal/mol was determined previously from analysis of the shape of the GalR titration curves to single operators (Brenowitz et al., 1990). Because of the adsorption of GalR to microfuge tube walls that occurred in these early studies (see Results), the apparent specific DNA binding activity of GalR was greatly underestimated. Although the titrations for GalR binding to single-site DNA conducted in this study appear to be steeper than those predicted assuming a nondissociable dimer, they do not define the shape of the curves with sufficient precision to estimate a value of ΔG_{dim}. Therefore, GalR is assumed to be a nondissociable dimer in the analysis presented herein (Majumdar et al., 1987).

Analysis of CAP Binding to DNA. CAP binds to a single site at the *gal* promoter centered at −41.5 from the S1 transcription start site (Figure 1). CAP is assumed to be a nondissociable dimer within the concentration range used (Brown & Crothers, 1989). The Langmuir binding expression $\bar{Y} = k_{CAP}[CAP]/(1 + k_{CAP}[CAP])$, where k_{CAP} is the association constant for the CAP–DNA interaction and [CAP] is the free CAP dimer concentration, was used in order to estimate the *individual-site loading energies* of CAP in the presence and absence of repressor [cf. Ackers et al. (1983)]. All CAP binding experiments were conducted in the *assay buffer* to which 100 μM cAMP was added.

Simultaneous Binding of Two Different Proteins to DNA. Titrations of either GalR or LacI were conducted in the presence and absence of a saturating concentration of CAP. Corresponding titrations of CAP were also conducted in the presence of a saturating concentration of either GalR or LacI.

In each experiment, only one protein is titrated. The other protein is mantained at a constant and saturating concentration. This experimental design enables the cooperative energies, whether positive or negative, to be visualized by a shift of the binding curve of the protein being titrated (Ackers et al., 1983). Since the Gibbs free energy is a state function and hence independent of the path taken to reach it, the free energy that describes the formation of a repressor–CAP–DNA complex will be identical regardless of the order in which the proteins are added.

Thus, the "heterologous cooperative energies" representing interactions between CAP and either GalR or LacI can be expressed as energy differences ($\Delta\Delta G$'s) between titrations of one protein conducted in the presence and absence of the other protein. The *intrinsic* binding free energy differences are

$$\Delta\Delta G_E \equiv \Delta G_E^{CAP} - \Delta G_E = \Delta G_{CAP}^{O_E} - \Delta G_{CAP} \quad (1)$$

and

$$\Delta\Delta G_I \equiv \Delta G_I^{CAP} - \Delta G_I = \Delta G_{CAP}^{O_I} - \Delta G_{CAP} \quad (2)$$

where the superscript "CAP" indicates the free energy of binding GalR or LacI to O_E or O_I in the presence of a saturating concentration of CAP and the superscripts "O_E" and "O_I" indicate the free energy of binding CAP in the presence of either GalR or LacI saturating the indicated operator. Correspondingly, the effect of the heterologous interaction on either ΔG_j (for LacI) or ΔG_{IE} (for GalR) can be expressed as

$$\Delta\Delta G_j \equiv \Delta G_j^{CAP} - \Delta G_j \quad (3)$$

and

$$\Delta\Delta G_{IE} \equiv \Delta G_{IE}^{CAP} - \Delta G_{IE} \quad (4)$$

Values for $\Delta\Delta G_j$ and $\Delta\Delta G_{IE}$ can only be determined from repressor titrations conducted in the presence and absence of CAP. However, while it is not possible to partition $\Delta\Delta G_{CAP}$ into values of $\Delta\Delta G_E$, $\Delta\Delta G_I$, and $\Delta\Delta G_j$, the relationships

$$\Delta\Delta G_{CAP} \equiv \Delta G_{CAP}^{O_EL/O_IL} - \Delta G_{CAP} = \Delta\Delta G_E + \Delta\Delta G_I + \Delta\Delta G_j \quad (5)$$

and

$$\Delta\Delta G_{CAP} \equiv \Delta G_{CAP}^{O_EG/O_IG} - \Delta G_{CAP} = \Delta\Delta G_E + \Delta\Delta G_I + \Delta\Delta G_{IE} \quad (6)$$

hold true for the binding models being employed for these studies.

All data were fit to the appropriate equations by methods of nonlinear least-squares parameter estimation to determine the best-fit values of the parameters, their 65% confidence limits, and the variance of the fit (Johnson & Frasier, 1985). Free energy values were directly determined as fitting parameters.

RESULTS

CAP Binding to DNA. CAP binds to a single site adjacent to the 3′ end of O_E (Figure 1). DNase I footprint titrations of CAP binding to the *gal* promoter in the absence of other proteins reveal both DNase I protection and hypersensitivity within its binding site (Ren et al., 1988; Figure 2). This DNase I hypersensitivity has been observed upon CAP binding to other operons as well (Taniguchi et al., 1979; Valentin-Hansen, 1982; Plumbridge & Kolb, 1991; Vidal-Ingigliardi & Raibaud, 1991; Schmitz, 1981). Quantitation of density changes of bands that were either protected from or made hypersensitive to DNase I upon CAP binding resulted in binding curves that are identical within experimental error (Figure 3). In addition, the conversion of the O_E^G sequence to the O_E^L sequence had no detectable effect on CAP binding (Figure 3). Note that the *consensus* binding site for CAP is unaltered by these base-pair substitutions (Figure 1). Simultaneous analysis of both the DNase I protection and the hypersensitivity titration data shown in Figure 3 was conducted in order to determine a value of ΔG_{CAP} of -11.1 ± 0.1 kcal/mol.

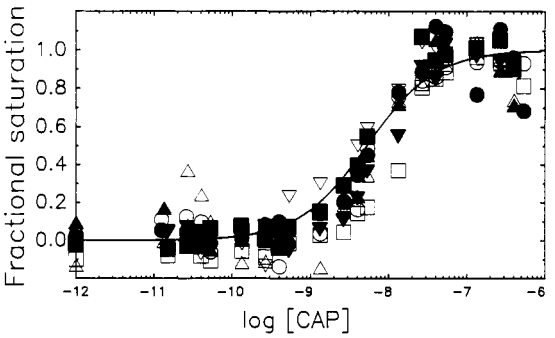

FIGURE 3: Titration of CAP in the absence of repressor proteins. All titration experiments whose resolved free energies, in kilocalories per mole, are as follows: on the O_E^G/O_I^G DNA sequence, (○) protection = 10.8 ± 0.30 ($\sigma = 0.160$) and (●) hypersensitivity = -11.0 ± 0.30 ($\sigma = 0.115$); on the O_E^G/O_I^L DNA sequence, (△) protection = -10.9 ± 0.20 ($\sigma = 0.093$) and (▲) hypersensitivity = -11.1 ± 0.30 ($\sigma = 0.125$); on the O_E^L/O_I^G DNA sequence, (□) protection = -10.5 ± 0.20 ($\sigma = 0.073$) and (■) hypersensitivity = -11.0 ± 0.20 ($\sigma = 0.055$); on the O_E^L/O_I^L DNA sequence, (▽) protection = -11.1 ± 0.30 ($\sigma = 0.080$) and (▼) hypersensitivity = -10.5 ± 0.20 ($\sigma = 0.059$). Open and solid symbols represent fractional saturation separately determined from protein-induced DNase I protection and hypersensitivity, respectively. The solid line depicts the curve calculated for the Langmuir isotherm using the value of ΔG_{CAP} shown in the first row of Table II. The concentration of CAP is in protein dimers.

LacI Binding to DNA. A detailed analysis of the binding of LacI to the chimeric *gal* promoter in which O_E and O_I were altered to LacI recognition sequences (Figure 1) has been published (Brenowitz et al., 1991b). The intrinsic binding free energies and the free energy of cyclization resolved from the simultaneous analysis of a series of titrations of LacI to the O_E^L/O_I^L, O_E^L/O_I^G, and O_E^G/O_I^L promoters are shown in the top row of Table I. These values differ slightly from those published previously (see footnote *d* of Table I).

Interaction of CAP and LacI. Titrations of the chimeric *gal* promoters O_E^L/O_I^L, O_E^L/O_I^G, and O_E^G/O_I^L with LacI were done in the presence of a saturating concentration of the cAMP–CAP complex. Corresponding titrations of CAP on these sequences in the presence of a saturating concentration of LacI were also conducted. The fact that the CAP binding site remains fully protected throughout the LacI titrations, and that the LacI site(s) remain(s) fully protected throughout the CAP titrations, was confirmed by densitometric analysis for all the titration experiments (data not shown). This can be seen in the autoradiograph of a representative titration of LacI conducted in the presence of CAP (Figure 2). However, as will be discussed below, the magnitude of the DNase I hypersensitivity induced by CAP is dependent upon LacI binding to O_E^L.

The titration of only O_E^L with LacI in the presence of CAP results in a decrease in the DNA binding affinity of LacI (Figure 4, upper left panel; Table I, compare rows 1 and 2). In contrast, the titration of only O_I^L with LacI in the presence of CAP resulted in a slight increase in the DNA binding affinity of LacI (Figure 4, upper right panel; Table I, compare rows

Table I: Free Energies of Binding LacI to One or Two Operator Sites in the Presence and Absence of Saturating Concentrations of CAP[a]

binding site competency[b]	CAP	ΔG_E	ΔG_I	ΔG_j	σ[c]
simultaneous[d]	–	−13.9 ± 0.1	−13.7 ± 0.1	11.7 ± 0.3	0.078
O_E^L/O_I^G	+	−13.1 ± 0.1			0.044
O_E^G/O_I^L	+		−14.0 ± 0.1		0.050
O_E^L/O_I^L	+	−12.3 ± 0.3	−14.4 ± 0.4	(11)	0.087
		−13.3 ± 0.3	−14.5 ± 0.4	(12)	0.087
		−14.1 ± 0.2	−14.6 ± 0.2	(13)	0.088
simultaneous	+	−13.1 ± 0.2	−14.0 ± 0.1	11.5 ± 0.4	0.079

[a] These values were calculated by assuming that the dimer to tetramer association free energy, ΔG_{tet}, is −13.2 kcal/mol and that dimers and tetramers bind DNA with equivalent affinity (Brenowitz et al., 1991b; see text). Free energies are in kilocalories per mole. Values in parentheses were fixed during numerical analysis. [b] The "binding site competency" refers to which DNA binding site is capable of binding to either the GalR or the LacI. O_E^L and O_I^L denote O_E and O_I operators that have been altered to be recognized by Lac repressor. Correspondingly, the native sequences of O_E and O_L that are recognized by Gal repressor are denoted O_E^G and O_L^G. [c] Square root of the variance of the fitted curve. [d] "Simultaneous" refers to the simultaneous analysis of repressor titrations to the two-site "wild-type" DNA and to the two single-site DNAs (see Materials and Methods for a complete description). The values reported here for the binding of Lac repressor in the absence of bound CAP differ slightly from the values reported in Brenowitz et al. (1991b) for two reasons. (1) Additional data sets were included in this analysis; four O_E^L/O_I^L, three O_E^L/O_I^G, and three O_E^G/O_I^L titrations were simultaneously analyzed. (2) An error in the calculation of the probability of the "heterogeneous tandem complexes" [configurations 9 and 10 in Table I of Brenowitz et al. (1991b)] in the computer function used in the original analysis has been corrected. However, the equations presented in Brenowitz et al. (1991b) are correct as written. The difference in the cyclization free energy of 0.5 kcal/mol corresponds to a change in the cyclization probability, j, of approximately a factor of 2.

1 and 3). The simultaneous titration of O_E^L and O_I^L with LacI in the O_E^L/O_I^L promoter yields two individual-site binding curves, one for O_E^L and one for O_I^L. The affinity of LacI for the O_E^L site decreases while the affinity of LacI for O_I^L remains unchanged (Figure 4, lower panels). The free energies that describe the binding of LacI to this promoter, the intrinsic energies, ΔG_E and ΔG_I, and the free energy of cyclization, ΔG_j, cannot be resolved from these experiments independently (Table I, rows 4–6). However, unique values of these three free energies can be determined by the simultaneous analysis of the titrations of Lac repressor on O_E^L/O_I^L, O_E^L/O_I^G, and O_E^G/O_I^L (see Materials and Methods). The results of this analysis are shown in the bottom row of Table I. The values of ΔG_j calculated in the presence and absence of CAP are identical within experimental error.

In the converse titrations of CAP conducted in the presence of LacI, an identical decrease in the DNA binding affinity is observed when LacI is bound to O_E^L alone and when it is bound to O_E^L and O_I^L simultaneously (Figure 5, upper and lower panels; Table II, rows 1, 3, and 4). In contrast, LacI bound at O_I^L has no detectable effect on the DNA binding affinity of CAP (Figure 5, middle panel; Table II, rows 1 and 2).

As described under Materials and Methods, the heterologous interaction between LacI and CAP can be expressed as free energy differences, $\Delta\Delta G$'s. An important check of the internal consistency of these determinations is the thermodynamic constraint that the formation of the heterologous complex be independent of the path taken for its formation. It is clear that for the two control regions containing only a single LacI binding site (O_E^L/O_I^G and O_E^G/O_I^L) the converse titrations of LacI and CAP yield values of $\Delta\Delta G_E$ and $\Delta\Delta G_I$ that are identical within experimental error. The values of $\Delta\Delta G_E$ and $\Delta\Delta G_I$ of 0.8 ± 0.3 and −0.3 ± 0.2 kcal/mol calculated from the LacI titrations are identical within experimental error with the values of $\Delta\Delta G_E$ = 0.6 ± 0.4 and $\Delta\Delta G_I$ = 0.1 ± 0.3 kcal/mol calculated from the CAP titrations (eq 1 and 2). However, the value of $\Delta\Delta G_{CAP}$ of 0.7 ± 0.4 kcal/mol, determined for the titration of O_E^L/O_I^L by CAP in the presence of saturating LacI, is not equal to the value of $\Delta\Delta G_{CAP}$ of −0.2 ± 0.4 kcal/mol calculated from the sum of the $\Delta\Delta G$ values determined for the LacI titration in the presence of saturating CAP (eq 4; $\Delta\Delta G_E$ = 0.8 ± 0.3 kcal/mol, $\Delta\Delta G_I$ = −0.3 ± 0.3 kcal/mol, and $\Delta\Delta G_j$ = 0.2 ± 0.5 kcal/mol). This discrepancy can be explained by the fact that at the high concentration of LacI present in the binding mixture for the CAP titrations, only 10% of the complexes are looped [calculation not shown; see Brenowitz et al. (1991b)]. At this LacI concentration, the LacI–DNA complexes are predominantly in the form of "tandem complexes" in which each operator is occupied by a LacI tetramer. This predominance also explains the result that the CAP titrations with LacI bound to O_E^L/O_I^G and O_E^L/O_I^L are identical within experimental error (Table II, rows 3 and 4).

Periodic DNase I protection and hypersensitivity are observed in the intervening DNA between O_E^L and O_I^L upon the formation of a LacI-mediated looped complex and have been used as a quantitative measure of the fraction of looped complexes present in solution [see Brenowitz et al. (1991b) and references cited therein]. Quantitative analysis of these bands in the LacI–CAP–DNA complex (Figure 2, bands indicated by white arrows) yielded results identical to those obtained for the LacI–DNA looped complex in the absence of CAP; both the distribution and the magnitude of the densities of the DNase I hypersensitive bands are unaffected by bound CAP (data not shown). These results confirm that CAP does not significantly affect the probability of LacI-mediated DNA cyclization at the *gal* operon.

GalR Binding to DNA. GalR binds noncooperatively to the O_E^G and O_I^G operators in the absence of CAP (Table III, top row), a result that was obtained previously (Brenowitz et al., 1990). However, the intrinsic binding free energies, ΔG_E and ΔG_I, determined in the present studies are more negative than the previously published values. Standard precautions to minimize protein adsorption, such as the addition of BSA to assay buffers and the siliconization of microfuge tubes with dimethyldichlorosilane (Maniatis et al., 1982), were included in the original experimental protocol (Brenowitz et al., 1990). However, a series of controls have demonstrated that substitution of the commercially prepared siliconized microfuge tubes ("Lube tubes") in place of microfuge tubes that were siliconized in the laboratory is the cause of the increased binding affinity that is observed in these studies (D. Dalma-Weiszhausz, M. Hsieh, and M. Brenowitz, unpublished data). The high degree of adsorption of GalR to the walls of microfuge tubes resulted in the previous underestimation of its DNA binding affinity.

Interaction of CAP and GalR. It has been previously shown that CAP and GalR can bind simultaneously to the *gal* operon [summarized in Adhya and Majumdar (1987)]. In order to determine the heterologous interactions between GalR and CAP, a series of binding experiments were conducted using the same protocols as described for LacI. Separate titrations of each protein were conducted in the presence of a saturating concentration of the other protein using the O_E^G/O_I^G DNA as well as each of the two single-site (O_E^G/O_I^L and O_E^L/O_I^G) control regions. No evidence for GalR–CAP interactions was observed in any of the titrations (Figures 6 and 7). The free energies calculated from these data are the same within

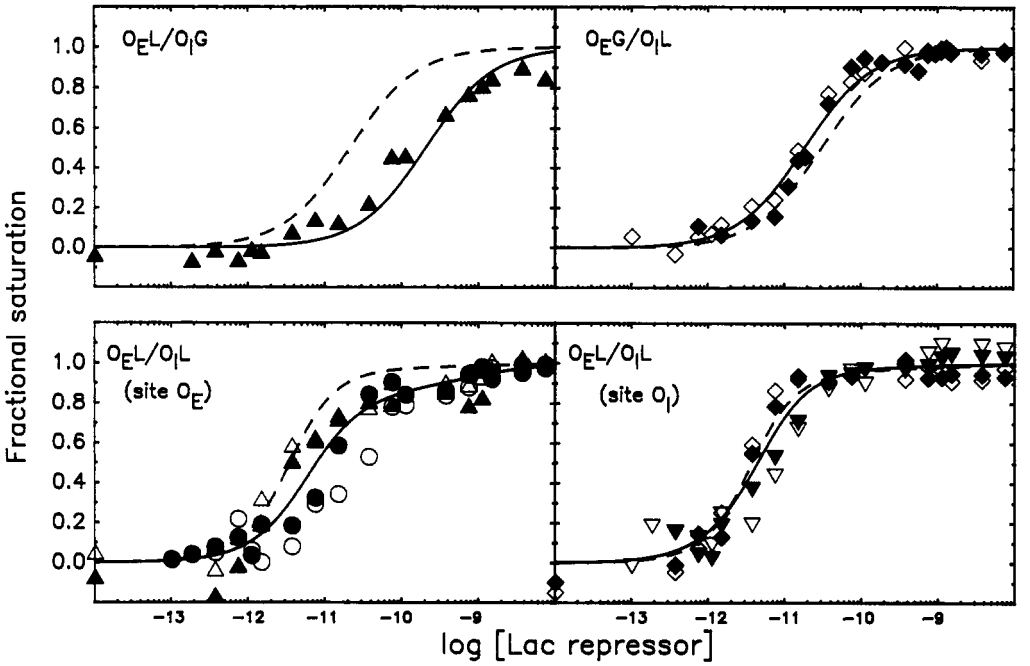

FIGURE 4: Fractional saturation of LacI binding to O_E^L and O_I^L on the O_E^L/O_I^L and the two single-site (O_E^L/O_I^G and O_E^G/O_I^L) promoters in the presence of a saturating amount of CAP protein (192.5 nM, dimer). The top panels represent the titration of each site on the separate single-site promoters. The bottom panels separately represent the titration of each site of the two-site O_E^L/O_I^L promoter. Different symbol types denote replicate experiments. The solid lines depict the best-fit curves for the simultaneous analysis of LacI titrations of the three promoters (Table I, bottom row). The dashed lines depict the curves calculated for the titration of LacI in the absence of saturating CAP (Table I, top row). Concentrations are in terms of LacI tetramer.

experimental error in the presence and in the absence of CAP (Table II, rows 1 and 5–7, and Table III). The presence of CAP does not result in cooperative binding of GalR. In addition, the base pairs located between O_E and O_I, whose rate of DNase I cleavage is altered (hypersensitivity or protection) upon formation of the LacI–CAP–DNA looped complex (Figure 2), are unaffected by the formation of the GalR–CAP–DNA complex (data not shown). The $\Delta\Delta G$ values calculated for the GalR/CAP experiments are all zero within experimental error (eq 1, 2, 5, and 7).

LacI-Dependent Modulation of CAP-Induced DNase I Hypersensitivity. CAP bends the DNA to which it site-specifically binds (Schultz et al., 1991; Zinkel & Crothers, 1990). The bending loci of the DNA correlate with the base pairs that become hypersensitive to DNase I upon the binding of CAP (Schultz et al., 1991). Such DNase I hypersensitivity is also observed for CAP binding to the *gal* promoter (Figure 2, black arrow). The magnitude of this DNase I hypersensitivity is quantitatively diminished by the binding of LacI to O_E^L, but not O_I^L (Figure 8, top and bottom panels). No additional effect was observed upon the simultaneous binding of LacI to both sites (Figure 8, middle panel). In contrast, GalR binding to either O_E^G or O_I^G independently or together has no effect on CAP-induced DNase I hypersensitivity (data not shown).

In order to distinguish whether the modulation of the CAP-induced DNase I hypersensitivity by LacI was a function of its binding to the O_E^L site, or due to its dimer to tetramer association, a titration of the O_E^L/O_I^G DNA sequence with the dimeric LacI mutant Lac^{adi} [cf. Brenowitz et al. (1991a) and references cited therein] was conducted in the presence of a saturating concentration of CAP. A value of ΔG_E of -13.3 ± 0.2 kcal/mol ($\sigma = 0.074$) for Lac^{adi} was determined in comparison to the value of -14.1 ± 0.1 kcal/mol reported for its binding to O_E^L/O_I^G in the absence of CAP (Brenowitz et al., 1991a). This result indicates that Lac^{adi} shows a similar decrease in the free energy of binding at the O_E site as LacI does in the presence of CAP. In addition, the magnitude of the CAP-induced hypersensitivity is quantitatively diminished by the binding of Lac^{adi} to O_E^L as described above for LacI (data not shown). These results suggest that the modulation of the CAP-induced DNA hypersensitivity by LacI is a result of the LacI dimer–DNA interaction and is not a function of the LacI dimer to tetramer oligomerization.

DISCUSSION

An understanding of the interactions that occur among transcriptional regulators and RNA polymerase when these proteins are bound to promoters is central to a determination of the molecular mechanism(s) by which they regulate transcription initiation. The approach to this problem that we have taken is to characterize the binding of proteins to the *gal* promoter, first individually and then in concert. GalR binds to O_E^G and O_I^G noncooperatively (Adhya, 1989; Brenowitz et al., 1990). The cooperative binding of LacI to O_E^L/O_I^L via the formation of a protein-mediated looped complex demonstrated that the noncooperative binding of GalR was not due to unusual stiffness of the intervening DNA sequence (Brenowitz et al., 1991b) but reflected the inability of GalR to form bidentate tetramers. Additional evidence that supports this conclusion includes electron microscopic analysis of GalR–DNA complexes (Mandal et al., 1990), sedimentation equilibrium analysis of GalR in solution (P. Hensley and M. Brenowitz, unpublished data), in vitro "abortive initiation" (Goodrich & McClure, 1991) and "run off" transcription assays (H. S. Choy and S. Adhya, unpublished results), and the absence, on GalR, of the heptad repeat of leucines at the C-terminus of LacI that has been shown to mediate dimer to tetramer association (Chakerian et al., 1991; Alberti et al., 1991). The fact that identical results were obtained utilizing both circular relaxed and supercoiled templates in the "abortive initiation" and the "run off" transcription assays shows that, unlike many systems, DNA supercoiling does not appear to play an important role in the negative regulation of the *gal*

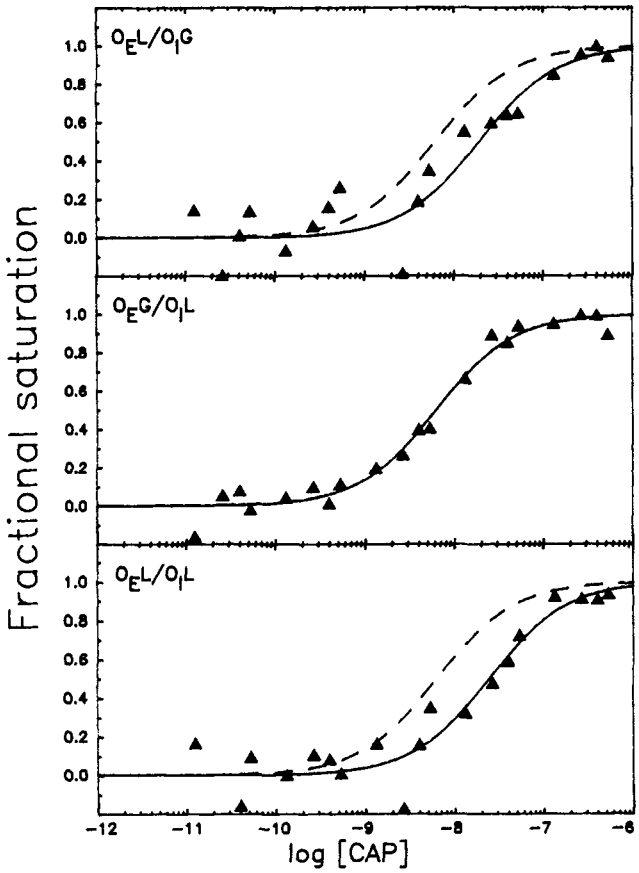

FIGURE 5: Fractional saturation of the CAP binding site by CAP in the presence of saturating LacI (5.92 nM, monomer). Titrations were conducted utilizing promoters with either one (O_E^L/O_I^G, upper panel, or O_E^G/O_I^L, middle panel) or two binding sites (O_E^L/O_I^L, lower panel) competent to bind LacI. The solid lines depict the curves calculated for the Langmuir isotherm using the best-fit values shown in Table II. The dashed lines depict the curve calculated for the titration of CAP in the absence of LacI (Figure 3). The concentration of CAP is in protein dimers.

Table II: Free Energies of Binding CAP to the *gal* Promoter in the *Presence* of Saturating Concentrations of LacI or GalR

binding site competency[a]	LacI	GalR	ΔG_{CAP}[b]	σ[c]
all[d]	–	–	−11.1 ± 0.1	0.111
O_E^G/O_I^L	+	–	−11.0 ± 0.2	0.063
O_E^L/O_I^G	+	–	−10.3 ± 0.4	0.152
O_E^L/O_I^L	+	–	−10.2 ± 0.3	0.119
O_E^G/O_I^L	–	+	−11.3 ± 0.4	0.160
O_E^L/O_I^G	–	+	−11.0 ± 0.1	0.043
O_E^L/O_I^L	–	+	−11.0 ± 0.3	0.119

[a] See footnote *b* of Table I. [b] Free energies are expressed in kilocalories per mole. [c] Square root of the variance. [d] "All" refers to the simultaneous analysis of the eight titrations shown in Figure 4. This results from separate analysis of the protected and hypersensitive bands of the following sequences: O_E^G/O_I^L, O_E^G/O_I^G, O_E^L/O_I^G, and O_E^L/O_I^L.

operon, a result that is consistent with those obtained in vivo (Menzel & Gellert, 1987).

The fact that tetrameric LacI, but not the dimeric mutant Lac^{adi}, can negatively regulate a chimeric *gal* operon both in vivo (Haber & Adhya, 1988) and in vitro (H. S. Choy and S. Adhya, unpublished results) suggests that the formation of a repressor-mediated looped complex is sufficient for repression of this operon. However, is a looped complex necessary for regulation? If GalR participates in the formation of a stable looped complex, it must do so by a mechanism other than the "mini leucine zipper" by which LacI has been

Table III: Free Energies of Binding GalR to One or Two Operator Sites in the Presence and Absence of a Saturating Concentration of CAP[a]

binding site competency[b]	CAP	ΔG_E	ΔG_I	ΔG_{IE}	σ[c]
simultaneous[d]	–	−13.5 ± 0.2	−13.4 ± 0.2	−0.2 ± 0.3	0.110
O_E^L/O_I^G	+		−13.6 ± 0.1		0.079
O_E^G/O_I^L	+	−13.8 ± 0.2			0.097
O_E^L/O_I^L	+	−13.9 ± 0.3	−13.6 ± 0.2	(0.0)	0.081
simultaneous	+	−13.8 ± 0.2	−13.5 ± 0.2	−0.2 ± 0.3	0.091

[a] Free energies are in kilocalories per mole. Values in parentheses were fixed during numerical analysis. [b] See footnote *b* of Table I. [c] Square root of the variance of the fitted curve. [d] "Simultaneous" refers to the simultaneous analysis of repressor titrations to the two-site "wild-type" DNA and to the two single-site DNAs (see Materials and Methods for a complete description).

shown to associate into a tetrameric form (Chakerian et al., 1991; Alberti et al., 1991). A plausible macromolecular participant in the formation of a GalR-mediated looped complex is CAP. This protein is composed of two subunits with identical amino acid sequence which bind to two successive major grooves of the DNA (Schultz et al., 1991; Zinkel & Crothers, 1990). Site-specific binding of CAP results in a 90–100° bend in the DNA. The magnitude of the bend is dependent upon the DNA sequence to which it binds (Liu-Johnson et al., 1986; Gartenberg & Crothers, 1988).

DNA-bound CAP alters the rate of transcription of a number of catabolite-sensitive genes, including the *gal* operon. The mechanism by which CAP exerts its action is not conclusively known and may vary depending on the promoter to which it is bound. Evidence has been presented for mechanisms involving direct CAP–RNA polymerase interactions [cf. Ren et al. (1988), Li and Krakow (1988), Ushida and Aiba (1990), Blazy et al. (1980), and Wu et al. (1978)] as well as CAP-induced DNA conformational change [Dickson et al., 1977; reviewed in Gilbert (1976)]. In the *gal* operon, CAP binds to a region encompassing at least 22 base pairs (Figure 1) and shifts transcription from the S2 to the S1 start site of transcription. CAP induces a 30-fold increase in the apparent binding constant of RNA polymerase for DNA, resulting in an increase in the number of open complexes formed (Goodrich & McClure, 1992).

When a CAP binding site is properly phased with regard to a protein-mediated looped complex, significant stabilization of this complex is observed, presumably due to the CAP-induced DNA bend [cf. Goodman and Nash (1989) and Moitoso de Vargas et al. (1989)]. Correspondingly, the binding of CAP to "out of phase" sites destabilizes protein-mediated looped complexes. A recent demonstration of this phasing relationship is found in the studies of the effect of bound CAP on AraC-mediated looped complexes (Lobell & Schleif, 1991). The AraC studies also show that the distance between the CAP and AraC binding sites is a critical factor in determining whether CAP will stabilize or destabilize an AraC protein-mediated looped complex.

The results presented in this paper clearly show that the binding of CAP does not facilitate the formation of either a LacI- or a GalR-mediated looped complex at the *gal* operon, a result consistent with gel-mobility shift experiments in which GalR and CAP were found to bind independently (Goodrich & McClure, 1992). It is clear that CAP is not the single "missing component" of the negative regulatory apparatus of the *gal* operon. The fact that bound CAP has *no net effect* on the cyclization probability of the LacI-mediated looped complex (Table I) is somewhat surprising given the 90° DNA bend induced by this protein. One explanation for this result

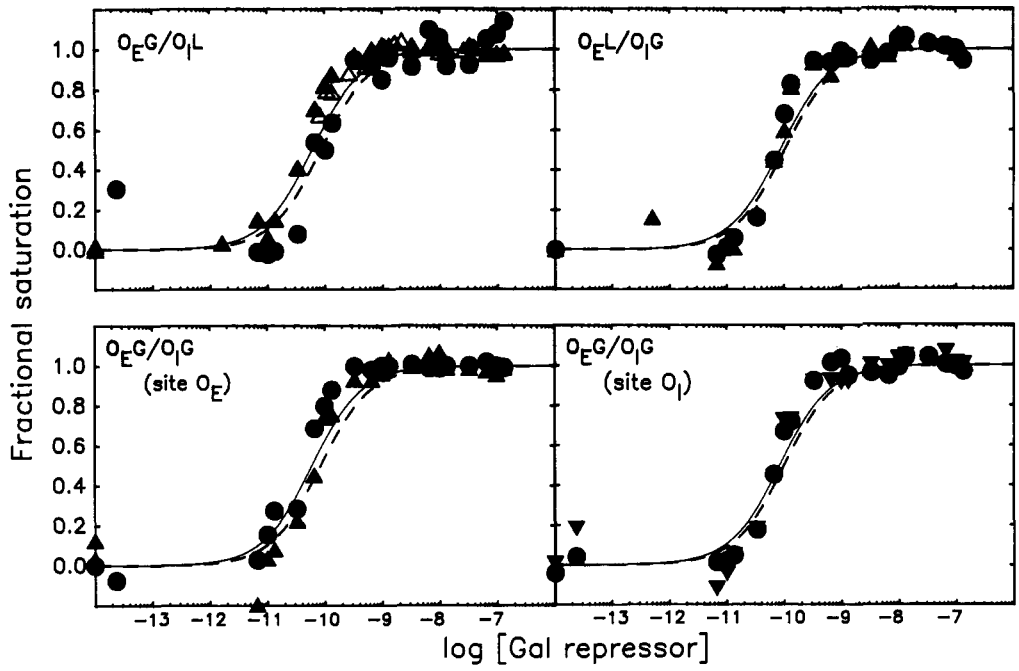

FIGURE 6: Fractional saturation of O_E^G and O_I^G by GalR on the O_E^G/O_I^G promoter and the two single-site (O_E^G/O_I^L and O_E^L/O_I^G) control regions in the presence of saturating CAP protein (192.5 nM, dimer). The top panels separately represent the titration of each of the single-site promoters. The bottom panels separately represent the titration of each site of the two-site O_E^G/O_I^G DNA. Different symbol types denote replicate experiments. The solid lines depict the best-fit curves for the simultaneous analysis of GalR titrations of the three promoters (Table III, last row). The dashed lines depict the curves calculated for the titration of GalR in the absence of saturating CAP (Table III, first row). Concentrations are in terms of GalR dimer.

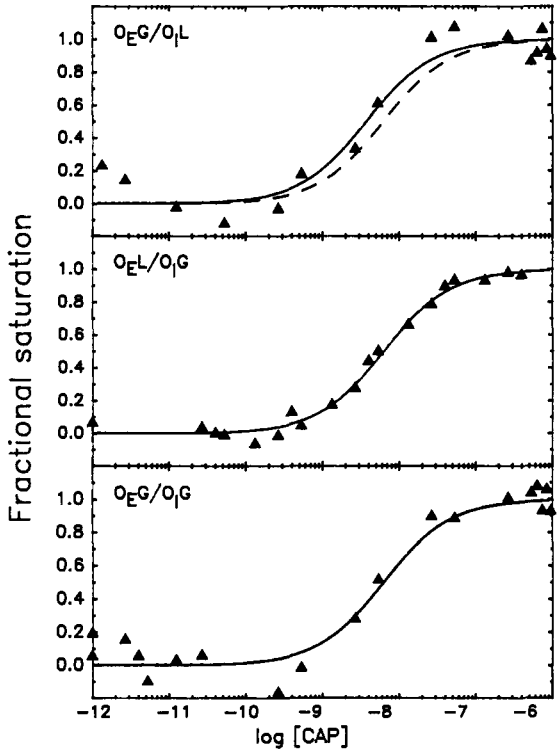

FIGURE 7: Titration of CAP in the presence of a saturating concentration of GalR (88.2 nM, monomer). Titrations were conducted utilizing promoters with either one (O_E^G/O_I^L, top panel, or O_E^L/O_I^G, middle panel) or two (O_E^G/O_I^G, bottom panel) binding sites competent to bind GalR. The solid lines depict the curves calculated for the Langmuir isotherm using the best-fit values shown in Table III. The dashed lines depict the curve calculated for the titration CAP in the absence of GalR (Figure 3; Table II, top row). The concentration of CAP is in protein dimers.

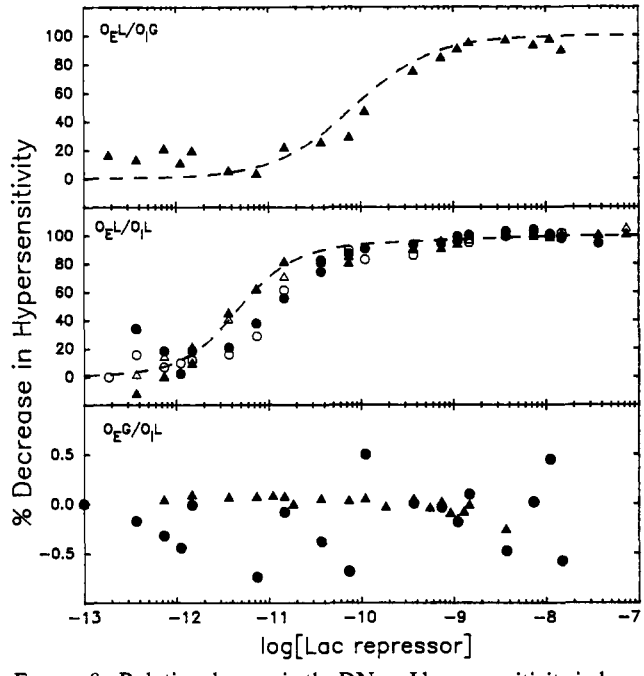

FIGURE 8: Relative changes in the DNase I hypersensitivity induced by CAP as a function of the binding of LacI. The dashed lines in the top two panels indicate the LacI titration curves for O_E^L for the indicated promoter (Table I). The DNase I hypersensitivity data were scaled to these curves by floating the upper and lower plateaus as adjustable parameters in the nonlinear analysis. The percent decrease is calculated relative to the level of DNase I hypersensitivity present when CAP is bound in the absence of LacI. No discernible transition was observed for the CAP-induced DNase I hypersensitivity when only O_I^L was titrated with LacI (lower panel). Different symbols in middle and lower panels represent replicate experiments.

is that an increase in the cyclization probability of the DNA due to the CAP-induced bend would be compensated by its less than optimal phasing with the LacI-mediated "looped complex". However, calculation of the trajectory of the DNA in a model of a LacI-mediated looped complex (Figure 9A) upon which a 90° DNA bend is superimposed suggests that CAP would *destabilize* the looped complex (Figure 9B). The addition of the CAP-induced bend causes O_E to be rotated out

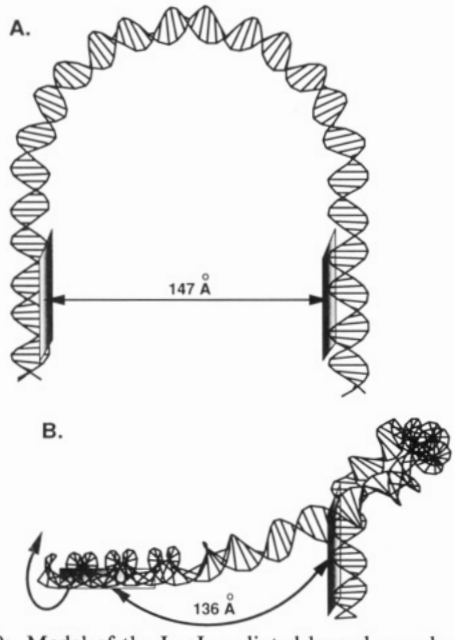

FIGURE 9: Model of the LacI-mediated looped complex that was generated subject to the simplifying assumptions of a planar loop and a symmetric 180° bend within a 130 bp DNA fragment (A). The size of the loop was chosen to give approximately the correct operator–operator separation (147 Å, helix axis to helix axis). The DNA bends by about 28° per helical turn within the loop. This model was used as the starting point for the addition of a CAP-induced bend. The superimposition of the CAP-induced bend upon the looped complex is intended to illustrate the effect that a full CAP-induced DNA bend would have on the trajectory of the DNA if one end of the loop was not constrained. The CAP-induced bend was modeled by the addition of two 45° kinks (Schultz et al., 1991) within the CAP binding site (B). This figure does not represent a proposed model of the LacI–CAP–DNA looped complex. Superimposition of a full 90° bend on DNA whose ends are constrained would clearly result in significant local changes in the structure of the DNA.

of phase relative to O_I and the distance between O_E and O_I to be reduced. The predictions of this structural model are clearly inconsistent with the absence of an effect on the cyclization probability of the LacI-mediated looped complex by CAP.

An alternative explanation is that the antagonistic interaction of CAP with LacI bound at $O_E{}^L$ (Table I) reduces the magnitude of the CAP-induced bend by partially displacing CAP from its binding site. Evidence for this explanation is found in the diminution of the CAP-induced DNase I hypersensitivity observed upon LacI binding to $O_E{}^L$ (Figure 8). This result suggests that even though CAP remains bound to the DNA, its binding mode changes. This modulation on CAP binding by LacI is dependent only on LacI binding to $O_E{}^L$ and is independent of looped complex formation.

The titration autoradiograms show that the CAP footprint overlaps that of LacI bound to $O_E{}^L$ (Figure 2, lanes 2 and 3) and GalR bound to $O_E{}^G$ (data not shown). However, the fact that GalR and CAP bind independently (Tables II and III) and that GalR binding to $O_E{}^G$ has no effect on the CAP-induced DNase I hypersensitivity suggests that there are significant differences either in the GalR–DNA and LacI–DNA complexes or in the interaction of CAP with $O_E{}^G$ and $O_E{}^L$. Examination of the wild-type and modified *gal* DNA sequences shows that even though the consensus binding site for CAP (Ebright et al., 1984) was not modified in order to convert $O_E{}^G$ to $O_E{}^L$, base pairs upstream of it were altered (Figure 1). A 35 bp "thermodynamic binding domain" surrounds the consensus sequence, which affects both the binding affinity of CAP and the magnitude of the CAP-induced DNA bend (Liu-Johnson et al., 1986; Gartenberg & Crothers, 1988; Dalma-Weiszhausz et al., 1990). Specifically, sequences located 11 and 16 bases upstream of the pseudodyad axis of symmetry of the CAP binding site in the *lac* operon, corresponding to the minor groove and the major groove of the DNA facing the protein, respectively, are important modulators of CAP binding. In general, AT-rich sequences at position 11 and GC-rich regions at position 16 favor CAP binding and bending (Gartenberg & Crothers, 1988; Dalma-Weiszhausz et al., 1990). The dinucleotides that were altered in changing $O_E{}^G$ to $O_E{}^L$ are CT to AT (positions −12, −11), TC to CA (positions −16, −15), and TT to TC (positions −17, −16) with respect to the CAP binding site pseudodyad axis of symmetry. These data predict that CAP would have an increased affinity for the site carrying the $O_E{}^L$ modifications. It is known that CAP has a higher affinity for its consensus sequence than for its binding site at the *gal* or *lac* operons (Ebright et al., 1989). The consensus sequence is composed of a symmetrized AATGTGATCTA nucleotide series. The CAP binding sites at both the *gal* and the *lac* operons contain a half-site that closely ressembles the consensus sequence, while the other half-site does not. The absence of a measurable effect by the base-pair substitutions at the *gal* promoter (see Results) may be due to the fact that the half-site that most closely matches the consensus sequence is downstream and away from O_E. Nucleotides that are adjacent to the other half-site may not be as critical determinants of CAP binding. Thus, the differences observed in the GalR–CAP and LacI–CAP experiments appear to be due to a complex set of protein–protein as well as protein–DNA interactions.

Hudson and Fried (1990) have suggested that the simultaneous binding of CAP and LacI to sites separated by 6.9 helical turns (72 bp center to center distance at 10.4 bp/turn of B-form DNA) at the *lac* promoter is 5-fold cooperative. In the *gal* operon, the CAP binding site and $O_I{}^L$ are separated by 94 bp (9.0 helical turns of 10.4 bp/turn, Figure 1). Significant cooperativity was not observed between DNA-bound CAP and LacI bound to $O_I{}^L$ (Tables I and II). In addition, DNase I hypersensitivity in the region between the CAP binding site and $O_I{}^L$ was not observed upon LacI titration of $O_I{}^L$ in the presence of saturating concentrations of CAP (data not shown). Since the CAP and LacI binding sites are "in phase" in both sets of experiments, it would be predicted that the increased spacing of the two sites in the *gal* operon would be more favorable to the formation of a putative CAP–LacI looped complex [cf. Brenowitz et al. (1991b) and references cited therein]. The absence of an interaction between CAP and LacI bound to the *gal* operon suggests that the cooperative binding observed by Hudson and Fried (1990) on the *lac* operon is not due to looped complex formation between these two proteins.

CONCLUSIONS

Quantitative determination of the heterologous interactions between CAP and GalR, and between CAP and LacI, was obtained from footprint titration analyses of CAP, GalR, and LacI. These results highlight the value of individual-site binding curves in evaluating potential molecular mechanisms of binding cooperativity and transcriptional regulation. By conducting footprint titrations of the proteins individually and then in concert, it was possible to unequivocally distinguish the effect of CAP on the binding of LacI to $O_E{}^L$ and $O_I{}^L$ from any effect on the cyclization probability of a LacI-mediated looped complex. These studies show that CAP and GalR bind independently at the *gal* operon. In contrast, it is shown

that DNA-bound CAP and LacI bound to O_E^L have an antagonistic interaction. These results also suggest that activation by CAP and repression by GalR at the *gal* operon occur independently and that caution must be taken in utilizing LacI as a model for looped complex-mediated repression of the *gal* operon.

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REFERENCES

Ackers, G. K., Johnson, A. D., & Shea, M. A. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 1129–1133.
Ackers, G. K., Shea, M. A., & Smith, F. R. (1983) *J. Mol. Biol. 170*, 223–242.
Adhya, S. (1989) *Annu. Rev. Genet 23*, 207–230.
Adhya, S., & Majumdar, A. (1987) in *RNA Polymerase and the Regulation of Transcription* (Reznikoff, W. S., Burgess, R. R., Dahlberg, J. E., Gross, C. A., Record, M. T., & Wickens, M. P., Eds.) pp 129–135, Elsevier Science Publishing Co., Inc., New York.
Alberti, S., Oehler, S., von Wilcken-Bergmann, B., Kramer, H., & Muller-Hill, B. (1991) *New Biol. 3*, 57–62.
Bellomy, G. R., & Record, M. T. (1990) *Prog. Nucleic Acid Res. Mol. Biol. 39*, 81–128.
Bellomy, G. R., Mossing, M. C., & Record, M. T. (1988) *Biochemistry 27*, 3900–3906.
Blazy, B., Takahashi, M., & Baudras, A. (1980) *Mol. Biol. Rep. 6*, 39–43.
Brenowitz, M., Senear, D. F., Shea, M. A., & Ackers, G. K. (1986) *Methods Enzymol. 130*, 132–181.
Brenowitz, M., Jamison, E., Majumdar, A., & Adhya, S. (1990) *Biochemistry 29*, 3374–3383.
Brenowitz, M., Mandal, N., Pickar, A., Jamison, E., & Adhya, S. (1991a) *J. Biol. Chem. 266*, 1281–1288.
Brenowitz, M., Pickar, A., & Jamison, E. (1991b) *Biochemistry 30*, 5986–5998.
Brown, A. M., & Crothers, D. M. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 7387–7391.
Chakerian, A. E., Mattice, V., Manly, S. P., Brackett, J. K., Lynch, M. J., Hoh, J. T., & Matthews, K. S. (1991) *J. Biol. Chem. 266*, 1371–1374.
Dalma-Weiszhausz, D. D., Gartenberg, M. R., & Crothers, D. M. (1990) *Nucleic Acids Res. 19*, 611–616.
Dalma-Weiszhausz, D. D., Jamison, E., Pickar, A., & Brenowitz, M. (1991) *Biophys. J. 59*, 602a.
Dickson, R., Abelson, J., Johnson, P., Reznikoff, W., & Barnes, W. (1977) *J. Mol. Biol. 111*, 65–79.
Ebright, R. H., Cossart, P., Gicquel-Sanzey, B., & Beckwith, J. (1984) *Nature 311*, 232–235.
Ebright, R. H., Ebright, Y. W., & Gunaskera, A. (1989) *Nucleic Acids Res. 17*, 10295–10305.
Gartenberg, M. R., & Crothers, D. M. (1988) *Nature 333*, 824–829.
Gilbert, W. (1976) in *RNA Polymerase* (Losick, R., & Chamberlin, M., Eds.) pp 192–205, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Goodman, S. D., & Nash, H. A. (1989) *Nature 341*, 251–254.
Goodrich, J. A., & McClure, W. R. (1992) *J. Mol. Biol. 224*, 15–29.
Haber, R., & Adhya, S. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 9683–9687.
Heyduk, T., & Lee, J. C. (1989) *Biochemistry 28*, 6914–6924.
Heyduk, T., & Lee, J. C. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 1744–1748.
Hudson, J. M., & Fried, M. G. (1990) *J. Mol. Biol. 214*, 381–396.
Johnson, M. L., & Frasier, S. G. (1985) *Methods Enzymol. 117*, 301–342.
Kuhnke, G., Krause, A., Heibach, C., Gieske, U., Fritz, H.-J., & Ehring, R. (1986) *EMBO J. 5*, 167–173.
Li, X.-Y., & Krakow, J. S. (1988) *J. Biol. Chem. 263*, 3448–3453.
Liu-Johnson, H.-N., Gartenberg, M. R., & Crothers, D. M. (1986) *Cell 47*, 995–1005.
Lobell, R. B., & Schleif, R. F. (1991) *J. Mol. Biol. 218*, 45–54.
Majumdar, A., Rudikoff, S., & Adhya, S. (1987) *J. Biol. Chem. 262*, 2326–2331.
Mandal, N., Su., W., Haber, R., Adhya, S., & Echols, H. (1990) *Genes Dev. 4*, 410–418.
Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Menzel, R., & Gellert, M. (1987) *J. Bacteriol. 169*, 1272–1278.
Moitoso de Vargas, L., Kim, S., & Landy, A. (1989) *Science 244*, 1457–1461.
Mossing, M. C., & Record, M. T. (1986) *Science 233*, 889–892.
Plumbridge, J., & Kolb, A. (1991) *J. Mol. Biol. 217*, 661–679.
Ren, Y. L., Garges, S., Adhya, S., & Krakow, J. S. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 4138–4142.
Royer, C. A., Chakerian, A. E., & Matthews, K. S. (1990) *Biochemistry 29*, 4959–4966.
Schmitz, A. (1981) *Nucleic Acids Res. 9*, 277–292.
Schultz, S. C., Shields, G. C., & Steitz, T. A. (1991) *Science 253*, 1001–1007.
Senear, D. F., & Ackers, G. K. (1990) *Biochemistry 29*, 6568–6577.
Shimada, J., & Yamakawa, H. (1984) *Macromolecules 17*, 689–698.
Shore, D., & Baldwin, R. L. (1983) *J. Mol. Biol. 170*, 957–981.
Taniguchi, T., O'Neill, M., & deCrombrugghe, B. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 5090–5094.
Ushida, C., & Aiba, H. (1990) *Nucleic Acids Res. 18*, 6325–6330.
Valentin-Hansen, P. (1982) *EMBO J. 1*, 1049–1054.
Vidal-Ingigliardi, D., & Raibaud, O. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 229–233.
von Wilcken-Bergmann, B., & Muller-Hill, B. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2427–2431.
Wu, F. Y.-H., Nath, K., & Wu, C.-W. (1974) *Biochemistry 13*, 2567–2572.
Zinkel, S. S., & Crothers, D. M. (1990) *Biopolymers 29*, 29–38.