Interactions between RNA Polymerase and the Positive and Negative Regulators of Transcription at the *Escherichia coli gal* Operon[†]

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ABSTRACT: The simultaneous binding of Gal repressor (GalR), catabolite activator protein (CAP or CRP), and RNA polymerase (RNAP) to the promoter region of the *Escherichia coli gal* operon has been analyzed thermodynamically, by quantitative DNase I "footprint" titration analysis, and structurally, by the use of hydroxyl radical (•OH) and 5-phenylphenanthroline (5OPP) "footprinting". In the absence of regulatory proteins, the preference of RNAP for one (P1) of the two *gal* operon overlapping promoters (P1 and P2) is −0.4 ± 0.2 kcal/mol, indicating only a small energetic preference for P1. The simultaneous binding of CAP and RNAP occurs with 10-fold cooperativity, with greater than 99% of the CAP−RNAP complex present at the P1 promoter. This cooperativity is inhibited by the binding of GalR to the upstream operator, O_E, but does not result in the repartitioning of RNAP between the P1 and the P2 promoters. These results suggest that the CAP−RNAP cooperativity and promoter partitioning are not linked and are consistent with a mechanism by which GalR binding to O_E represses transcription by inhibiting the CAP−RNAP cooperativity. It is suggested that the CAP−RNAP cooperativity is dependent upon contacts made by the complex with the upstream DNA and that GalR binding to O_E prevents these contacts from occurring. Changes in nuclease reactivity at the internal operator O_I (centered at +53.5) take place upon RNAP binding. These changes are dependent on the DNA sequence present at O_I and on the presence or absence of CAP. They are independent of the helical phasing between the promoters and O_I and of the distance between them. These results suggest that RNAP can directly communicate with events occurring at both the external and the internal operator sequences without direct contact between repressor molecules bound at their cognate sites.

The *gal* operon of *Escherichia coli* is regulated by a small ensemble of proteins that utilize interdependent pathways of positive and negative transcriptional regulation. Transcription of this operon occurs at two partially overlapping, mutually exclusive promoters, P1 and P2 (Figure 1; Adhya & Miller, 1979), and is regulated by at least two proteins (Figure 1), the catabolite activator protein (CAP;[1] also known as the cAMP receptor protein, CRP) and the Galactose repressor (GalR). The binding of the cAMP−CAP complex to its cognate site at the *gal* operon activates transcription from P1 while transcription from P2 is repressed (Irani et al., 1989; Shanblatt & Revzin, 1986; Goodrich & McClure, 1992). Because of the location of the CAP binding site relative to P1, this promoter has been classified as a class II promoter (Gaston et al., 1990). GalR binds to two operators,

O_E and O_I, that flank the transcription start sites (Figure 1). Simultaneous binding of GalR to both operators is required for full repression *in vivo* (Haber & Adhya, 1988; Choy & Adhya, 1992) although no cooperativity has been detected for GalR binding *in vitro* (Brenowitz et al., 1990; Dalma-Weiszhausz & Brenowitz, 1992). The binding of CAP to its specific sequence in the *gal* operon has no effect on the binding of GalR to either or to both operator sequences (Goodrich & McClure, 1992; Dalma-Weiszhausz & Brenowitz, 1992).

Since the regulation of the *gal* operon is carried out by the specific binding of several proteins to multiple DNA sites (Figure 1), this operon is an excellent model system to study the interplay of positive and negative transcriptional regulators. It has become increasingly clear that, in addition to the binding of regulatory proteins, DNA conformational changes play a key role in transcriptional regulation [reviewed by Tjian and Maniatis (1994)]. In order to study the energetic protein−protein interactions between the *gal* operon transcriptional regulators and RNAP, a thermodynamic analysis of the simultaneous binding of GalR, CAP, and RNAP has been conducted. In parallel with these thermodynamic studies, an analysis of DNA conformation upon multiple protein binding has been obtained through the use of hydroxyl radical (•OH) and 5-phenylphenanthroline (5OPP) "footprinting" (Lavigne et al., 1992a; Strahs & Brenowitz, 1994). These studies seek to establish a correlation between the structural and the energetic changes that result from the simultaneous binding of RNAP and the regulatory proteins.

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[1] Abbreviations: RNA, ribonucleic acid; RNAP, RNA polymerase; 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; BSA, bovine serum albumin; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Bis-tris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane; DNase I, deoxyribonuclease I (EC 3.1.21.1); DNA, deoxyribonucleic acid; cAMP, adenosine 3′5′-cyclic monophosphate.
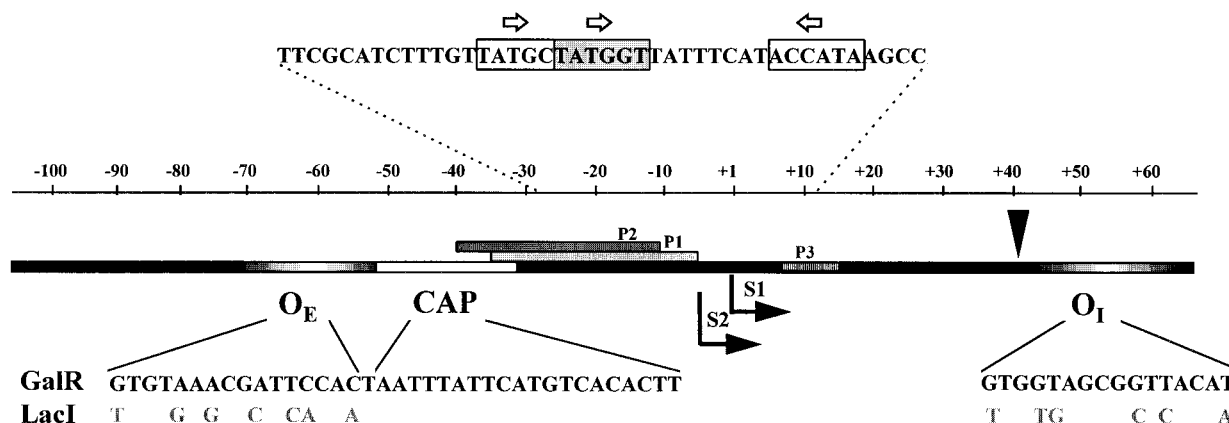
FIGURE 1: Schematic representation of the *gal* operon. Numbers are relative to the S1 start site of transcription. The CAP binding site and the operator sites, O_E and O_I, are indicated. The two alternate promoter sequences (P1 and P2) as well as their respective transcription start sites, S1 and S2, are depicted. P3 shows the localization of the putative promoter, which shares sequence homology to the Pribnow box. The DNA sequences of each of the known transcriptional regulator binding sites are shown in the line marked GalR. The base pairs that were changed to alter O_E and O_I from GalR to LacI recognition sequences are shown in the line marked LacI. There are 10.9 helical turns from the center of the external operator sequence to the center of the internal one. The number of turns was calculated assuming 10.4 bp/turn of canonical B-DNA. The site for the 15 or 30 bp insertion between the P1/P2 promoter region and O_I is shown by an inverted triangle.

MATERIALS AND METHODS

Reagents. The DNA restriction and modifying enzymes were purchased from New England Biolabs except for calf intestinal phosphatase (CIP), *Taq* polymerase, and kinase, which were purchased from Promega. DNase I (EC 3.1.21.1) was purchased from Sigma and stored as described in Dalma-Weiszhausz and Brenowitz (1992). Radiolabeled nucleotides (^{35}S and ^{32}P) were purchased from Amersham. The pGEM-7Zf (+) plasmid was obtained from Promega. Sequenase 2.0 was obtained from United States Biochemicals. Non-radioactive deoxyribonucleotides, as well as ribonucleotides, were purchased from Boehringer Manheim. *E. coli* wild-type K12 cells were purchased from Grain Processing, Muscatine, IA. 1,10-Phenanthroline, 5OPP, and 2,9-dim-ethyl-1,10-phenanthroline were purchased from GFS Chemicals. Cupric sulfate and ammonium sulfate were purchased from Mallinckrodt.

DNA Restriction Fragments. The production of 633 bp linear DNA restriction fragments that contain the sequences from −199 to +434 relative to the *gal* operon S1 transcription start site has been previously described (Brenowitz et al., 1990, 1991). The base pair numbers cited in the text are relative to the S1 start site of transcription (Adhya & Miller, 1979; Irani et al., 1989). The construction of DNA fragments in which the GalR recognition sequences (designated O_E^G and O_I^G) were altered to Lac repressor (LacI) recognition sequences (designated O_E^L and O_I^L) has also been described (Haber & Adhya, 1988). The protein binding studies presented here are conducted either with DNA in which both operators are recognized by GalR (O_E^G/O_I^G) or with DNA in which only one site is recognized by GalR (O_E^G/O_I^L or O_E^L/O_I^G). Even though the CAP binding site lies adjacent to O_E, the affinity of CAP for this sequence remains unaltered by the base pair substitutions that alter O_E^G into O_E^L (Dalma-Weiszhausz & Brenowitz, 1992). The CAP consensus recognition sequence (Ebright et al., 1984) is unchanged by these substitutions.

Plasmids containing the *gal* promoter sequences from −96 to +89, in which the *Eco*RI and the *Hind*III restriction sites were inserted 36 bp away from O_E and O_I, respectively

(measured from the center of the restriction site to the center of the operator sequence), were constructed (Dalma-Weiszhausz, 1995). Restriction fragments of 185 bp were excised from the resulting plasmids for footprinting experiments. Plasmids pDW001, pDW002, and pDW003 contain the O_E^G/O_I^L, O_E^L/O_I^G, and O_E^G/O_I^G sequences, respectively. Plasmid pDW003 was further modified to contain either a 15 or a 30 bp insert between the RNAP binding sequence and the O_I operator (Dalma-Weiszhausz, 1995). The resulting plasmids, pDW005 and pDW007, have the sequence GTTACAGATCTGACC inserted once or twice at the *Bste*II site, respectively. The *Bste*II site is centered at position +42 of the *gal* promoter sequence.

DNA Radiolabeling. Five picomoles of the appropriate plasmid(s) was linearized by *Eco*RI or by *Hind*III digestion (for labeling of the template or the nontemplate strand, respectively). The Klenow fragment of *E. coli* DNA polymerase I was used to fill the recessed strand site with the incorporation of the complementary [α-^{32}P]deoxynu-cleotides. The plasmids were restricted again with *Hind*III or *Eco*RI to produce fragments that were uniquely labeled at one terminus. Incorporation of the ^{32}P label at the 5′ end of the restriction fragments was accomplished using T4 kinase as has been described (Strahs & Brenowitz, 1994).

Proteins. The CAP protein used in these studies was a gift from James C. Lee. GalR was purified as described (Hsieh et al., 1994). Protein concentrations were corrected for the specific DNA binding activities as determined from titrations conducted under conditions where the DNA concentration exceeded the DNA binding dissociation constant (Heyduk & Lee, 1990; Senear et al., 1993).

RNAP was purified as described by Burgess and Jendrisak (1975) and Hager et al. (1990) and stored at −70 °C in 100 mM NaCl, 0.01 M Tris-HCl, pH 7.9 (at 20 °C), 0.1 mM EDTA, 0.1 mM DTT, and 50% glycerol. RNAP sigma factor was purified by a simplification of the procedure of Gribskov and Burgess (1983) (Dalma-Weiszhausz, 1995) and stored at −70 °C in the same storage buffer as the core enzyme. RNAP was reconstituted by the addition of a 1:1.4 stoichiometric ratio of RNAP to sigma factor. After the sample was incubated at 4 °C overnight, the core and the

holoenzyme were separated on a MonoQ column (Pharmacia; Hager et al., 1990). The stoichiometry and purity of the protein were determined by densitometric quantitation of an SDS−PAGE gel. Protein concentrations were determined by spectrophotometry ($\epsilon_{260(1\%)} = 6.5$; Richardson, 1966) and by the Bio-Rad protein assay using BSA as a standard.

Quantitative DNase I Footprint Titration Assay. The quantitative DNase I footprint titration experiments were conducted as described in Brenowitz et al. (1986, 1993) and Dalma-Weiszhausz & Brenowitz (1992). 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 7.0 with HCl. The protein(s) and DNA were equilibrated for at least 45 min prior to the addition of DNase I. Presiliconized microfuge tubes were used at all steps in the experimental protocols. The assumption that [protein]$_{total}$ ≈ [protein]$_{free}$ was made since the concentration of operator sites in the equilibrium mixtures (<10 pM) was low relative to the protein−DNA dissociation constants. In all experiments in which CAP was included, the buffer used was the same *assay buffer* except that it contained 100 μM cAMP. Protein concentrations of GalR and of CAP are expressed in units of dimer.

Experiments in which one protein was titrated in the presence of a constant concentration of another protein or proteins, 125 nM RNAP, 200 nM CAP (100 μM cAMP), and 23.5 nM GalR were the constant "saturating concentrations" used. In all the experiments, the protein(s) being held at constant and saturating concentration(s) was (were) added to the DNA buffer mixture prior to addition of the protein being titrated. Electrophoresis was conducted on a 6% acrylamide/urea sequencing gel. Autoradiography and quantitation of the fractional occupancy were carried out as described (Dalma-Weiszhausz & Brenowitz, 1992).

The cooperative energies describing DNA-bound proteins were obtained from the difference between the intrinsic binding free energy of one protein when it binds to the DNA and its binding free energy in the presence of a saturating amount of another DNA-bound protein. Therefore, in the titration of a DNA binding site with its cognate protein, when one (or more) other protein is present at a concentration sufficient to saturate its respective binding site or sites, the Gibbs binding free energy of the first protein will be a composite of its intrinsic binding free energy ($\Delta G_{intrinsic}$) and the cooperative interaction energy (ΔG_{coop}; Dalma-Weiszhausz & Brenowitz, 1992). Values of ΔG_{coop} are determined by the comparison between titrating one protein in the absence, and separately in the presence, of a constant and saturating concentration(s) of the other protein(s). This can be expressed as the $\Delta\Delta G$ of binding

$$\Delta\Delta G = (\Delta G_{intrinsic} + \Delta G_{coop}) - (\Delta G_{intrinsic})$$

or

$$\Delta\Delta G = \Delta G_{coop}$$

Since the Gibbs free energy is a state function, ΔG_{coop} should be identical, irrespective of the order in which the proteins are added and of which protein is being titrated (Dalma-Weiszhausz & Brenowitz, 1992). The reversibility of the interactions was verified by titrating each protein in the presence of constant and saturating concentration(s) of the other protein(s), as well as by explicit order of addition experiments.

The binding curves obtained for the independent binding of CAP and GalR were described, as expected, by the Langmuir binding expression (Dalma-Weiszhausz & Brenowitz, 1992; Brenowitz et al., 1991). All parameters were estimated using methods of nonlinear least-squares analysis (Johnson & Faunt, 1992). The 65% confidence limits estimated in this manner correspond to approximately one standard deviation.

•OH and 5OPP Footprinting. Labeled DNA fragments and proteins were prepared in the same manner as for DNase I footprinting except that only a single ^{32}P nucleotide was incorporated. The procedures were carried out as previously described (Tullius et al., 1987; Spassky & Sigman, 1985) with the modifications described in Strahs and Brenowitz (1994). The reactions were quenched by adding 20 μL of thiourea−EDTA solution (8 mg/mL thiourea, 200 mM EDTA) followed by the addition of 500 μL of a mixture that contained 92% ethanol, 570 mM ammonium acetate, and 0.004% linear polyacrylamide (Gaillard & Strauss, 1990). After DNA precipitation, the same protocol was followed as for the DNase I footprinting. The samples were resolved by electrophoresis on a 6% (for the 633 bp DNA fragments) or on a 10% (for the 185, 200, and 215 bp fragments) acrylamide/urea sequencing gel. The Maxam and Gilbert *G reaction* of the appropriate sequences was carried out (Maniatis et al., 1982) and run on the same gel for sequence determination.

RESULTS

Thermodynamic Analysis. DNase I "footprints" of CAP, GalR, and RNAP binding independently or simultaneously to the *gal* operon DNA fragments are shown in Figures 2 and 3. These studies extend the chemical protection studies of Lavigne et al. (1992a,b) by including the binding of GalR to both operator sequences and by including the downstream DNA sequences that encompass O_I. They also provide additional information to that obtained from the abortive initiation assays as well as the gel mobility-shift analysis of Goodrich and McClure (1992). The structural detail of the DNase I, as well as the •OH and 5OPP footprints presented in Figure 3, will be presented following the results of the thermodynamic analysis.

The energetic interactions between GalR, CAP, and RNAP were determined by conducting a series of DNase I footprint titration experiments of each protein in the absence and separately in the presence of a saturating concentration of the other protein(s). This experimental design enables the separate quantitation of the intrinsic binding free energy of one protein and its interaction with another DNA-bound protein(s). The $\Delta\Delta G$ values determined from the titrations of CAP binding to its specific site are summarized in Figure 4. The titration of CAP in the presence of a saturating concentration of RNAP occurs with -1.4 ± 0.3 kcal/mol of cooperativity. Cooperativity between RNAP and CAP has been formerly reported [*e.g.*, Lavigne et al. (1992b)].

The effect of GalR binding to O_E, and separately to O_I, and the synergy resulting from the binding of GalR to both operators simultaneously are distinguished by analyzing experiments conducted with DNA fragments in which only
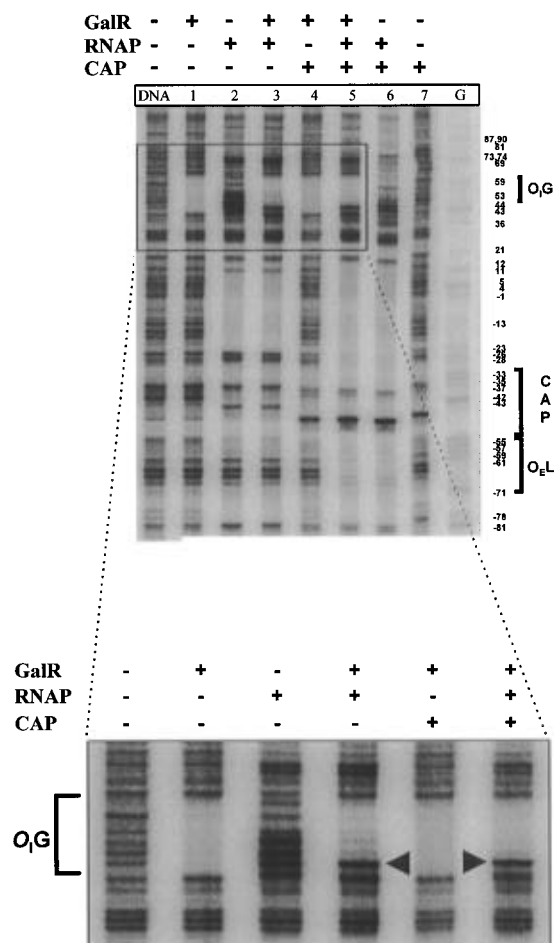
FIGURE 2: Digital representation of an autoradiogram showing the DNase I cutting pattern of combinations of RNAP, CAP, and GalR bound to the O_E^L/O_I^G sequence. The operator sequences, as well as the CAP binding site, are shown on the right. A (+) sign indicates the presence of the appropriate protein at a saturating concentration (see Materials and Methods), while a (−) sign indicates its absence. The lane designated DNA shows the DNase I-treated DNA in the absence of any regulatory proteins. G refers to the Maxam−Gilbert sequencing reaction for guanine, in order to provide reference bands for the location of the DNA cleavage products. The image shown was generated from the 16 bit PhosphorImager file following reduction of the image density to 256 gray levels using the parameters determined by the ImageQuant software. The insert magnifies the effect of GalR binding on the RNAP-induced hypersensitivity. The location of the O_I^G site is shown. Arrows indicate the RNAP-induced hypersensitive base pair which remains hypersensitive even in the presence of GalR binding to O_I.

one of the operators is competent to bind GalR and comparing those to the results obtained from the DNA fragment where both operators are competent to bind GalR (see Methods and Materials; Ackers et al., 1982). Previous studies have shown that CAP and GalR bind to the *gal* operon independently (Goodrich & McClure, 1992; Dalma-Weiszhausz & Brenowitz, 1992). The CAP−RNAP cooperativity is completely inhibited by the presence of a saturating concentration of GalR only when this repressor is bound to the O_E sequence (O_E^G). Within experimental error, GalR binding at O_I has no effect on the CAP−RNAP binding cooperativity. The simultaneous binding of GalR to both operator sequences has no synergistic effect on the CAP−RNAP cooperativity.

The GalR DNase I footprint titration experiments are summarized in Figure 5. The binding of GalR to O_E and

O_I, or to each operator separately, is independent of RNAP except for the GalR titration of O_E on the single-site template conducted in the presence of RNAP (Figure 5, lane 2). The inhibition of GalR binding to this template by RNAP slightly exceeds the confidence limits of the measurements. However, since inhibition of GalR binding is not observed on the two-site template (Figure 5, lane 8), and RNAP titrations are unaffected by GalR bound at O_E (Dalma-Weiszhausz, 1995), the preponderance of evidence points to RNAP and GalR binding occurring with $\Delta\Delta G$ values close to zero. The GalR-induced inhibition of the CAP−RNAP cooperativity could not be confirmed by GalR titrations on the external operator, since the DNA-bound CAP−RNAP complex shows an extended protection from DNase I cleavage which encompasses O_E (Figure 5, lanes 3 and 9). However, the inhibition of the CAP−RNAP cooperativity by GalR binding at O_E observed in the RNAP titration experiments corroborates this result (Dalma-Weiszhausz, 1995).

Binding of RNAP and CAP Probed by DNase I. RNAP induces an extensive pattern of DNase I protection and hypersensitivity encompassing the DNA from the proximal side of O_E through the distal side of O_I. Multiple regions of DNase I protection are observed on the template and nontemplate strands that include regions as far upstream as −73 and as far downstream as +75 (Figure 2, lane 2, and Figure 3, lane 19; summarized in Figure 6). DNase I hypersensitive bands are observed upstream at −38, −48, and −63 and downstream in the region of O_I (Figure 6). This pattern of DNase I protection and hypersensitive sites suggests that RNAP makes extensive contacts with the DNA and that the conformation of the DNA is altered.

The concomitant binding of CAP and RNAP results in an extended footprint from −75 to +12 on the template strand (Figure 2, lane 6) that is more extensive than the simple sum of the protection patterns of the individual proteins. Several regions between bp −75 and bp +30 are also protected by the CAP−RNAP complex in the nontemplate strand. O_E is completely protected by the CAP−RNAP complex as has been observed previously (Lavigne et al., 1992a,b). Because of this protection, the detection of GalR binding to O_E in the presence of the CAP−RNAP complex cannot be observed by this method (Figure 3, lanes 21 and 22). The simultaneous binding of GalR at O_I and of RNAP (or the CAP−RNAP complex) can be detected in the DNase I footprints (Figure 2, lane 3). However, the binding of GalR to O_I does not completely obscure the RNAP-induced hypersensitivity in this region (Figure 2, lanes 2−4). Footprinting studies of the CAP−RNAP−DNA ternary complex by alternate nucleolytic probes will be discussed below.

Partitioning of RNAP between the P1 and P2 Promoters. In addition to the RNAP-induced DNA protections and hypersensitivities observed with DNase I and •OH, the 5OPP protection pattern reveals hypersensitive bands centered at −2 and at +3 that correlate with open complexes formed at the P1 and the P2 promoter sequences (Lavigne et al., 1992a; Figure 3). Quantitative analysis of these unique 5OPP reactivities reveals that, in the absence of either regulatory protein, 68.5 ± 6.5% of the bound RNAP is located at P1. This partitioning corresponds to only a −0.4 ± 0.2 kcal/mol difference in the intrinsic affinities of RNAP for P1 and for P2. The presence of CAP shifts the distribution to >99.0% P1. The binding of GalR at O_E decreases the
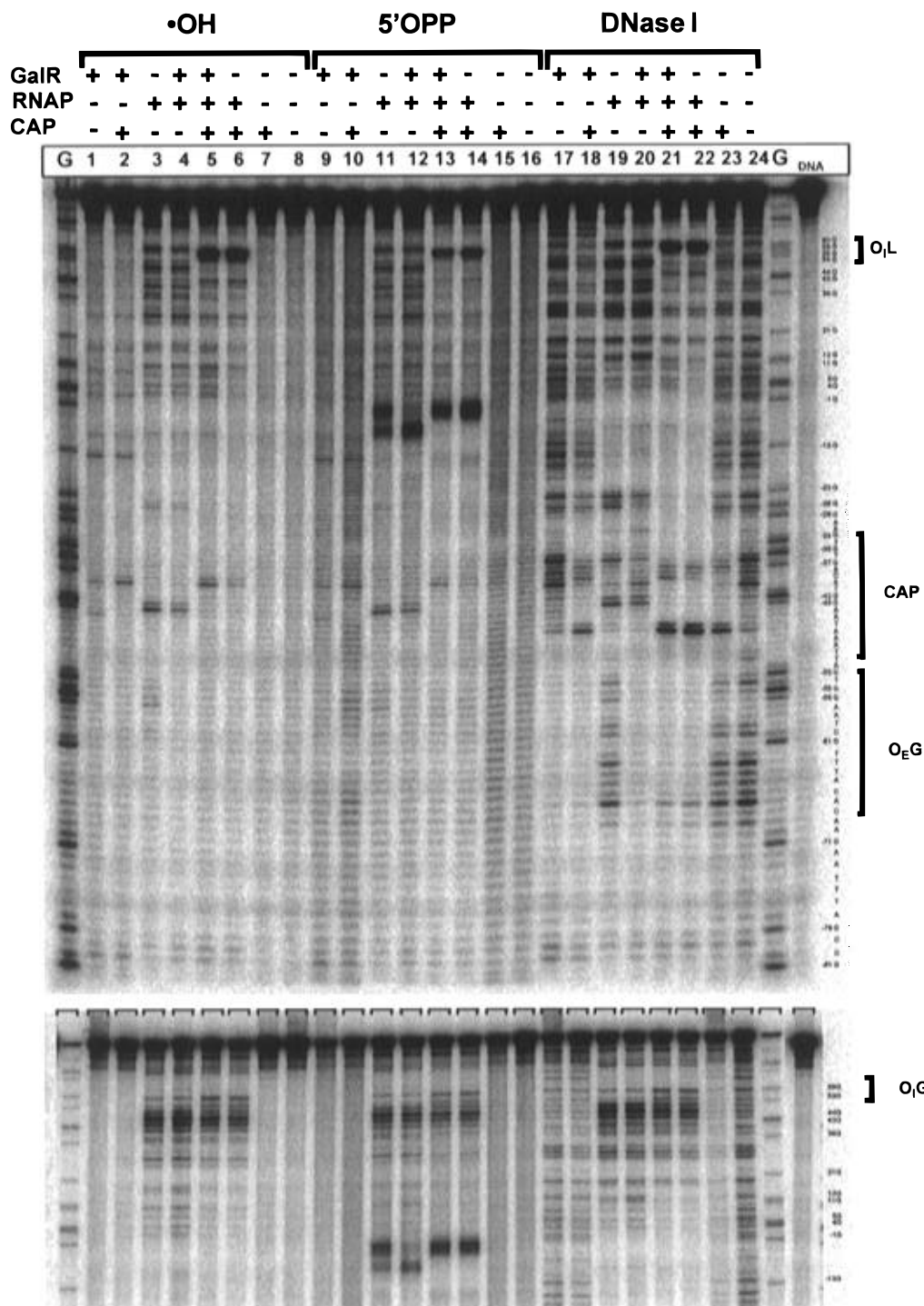
FIGURE 3: Autoradiograph showing DNA reagent susceptibilities upon protein binding. The top gel shows the cleavage patterns of the $O_E{}^G/O_I{}^L$ DNA fragment using different reagents as indicated. Only the template strand is shown. The presence (+) or absence (−) of each protein is indicated. The lane designated DNA shows the untreated DNA as a control for the integrity of the fragments. G refers to the Maxam−Gilbert sequencing reaction for guanine, in order to provide reference bands for the location of the DNA cleavage products. The CAP and the repressor binding sequences are indicated. The bottom gel shows only the downstream region of the same experiment conducted on the $O_E{}^L/O_I{}^G$ sequence, for comparison of the sequence-dependent hypersensitivity at O_I. The 5OPP hypersensitivities at the promoter sequences were used for the quantitation of the partitioning of RNAP between P1 and P2. The CAP dependence, as well as the repressor dependence of this partitioning, was also quantitated.

proportion of complexes at P1 to 29.0 ± 2.0%. However, the distribution of complexes remains >99.0% P1 when both CAP and GalR are simultaneously bound with RNAP, even though the CAP−RNAP cooperativity is inhibited when GalR is bound at O_E.

Nuclease Protection Analysis of the Simultaneous Binding of RNAP, CAP, and GalR. The •OH, 5OPP, and DNase I protection patterns of RNAP and the regulatory proteins are

shown in Figures 2 and 3 and summarized in Figure 6. The restriction fragments used in these studies contain three sequences that resemble a consensus Pribnow box (TATXXT; Pribnow, 1975). The protected region from −23 to +12 encapsulates the two functional promoters, P1 and P2. There is no evidence of RNAP-dependent protection at the Pribnow-like sequence located 14/15 base pairs downstream of P1 (P3, Figure 1), although mutations that render P1 and P2
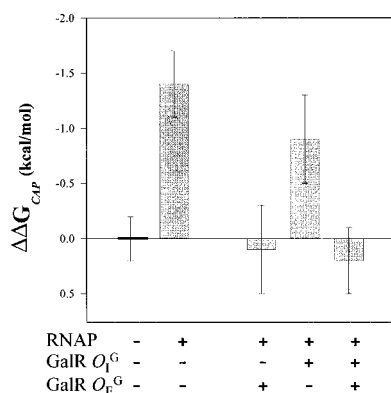
FIGURE 4: Histogram of the free energy differences of binding CAP in the presence and absence of RNAP and of GalR bound to one or two of the operator sequences at the *gal* operon. Negative values depict *cooperativity* between simultaneously bound proteins, while positive values show the negative interaction(s) between these proteins. A (+) sign refers to the presence of a constant and saturating concentration of the respective protein, while a (−) sign symbolizes its absence. The first bar (shown in black), represents CAP binding to this operon in the absence of any other protein (−11.0 kcal/mol). The second bar shows the difference between CAP binding in the absence of RNAP and CAP binding in the presence of the holoenzyme. The following three bars represent the difference in free energy of CAP binding in the presence of RNAP and GalR bound at O_E (third bar), O_I (fourth bar), and both operators simultaneously (fifth bar).
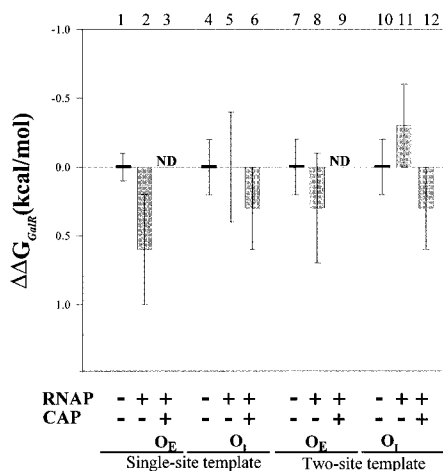

FIGURE 5: Histogram of the free energy differences of GalR binding in the presence and absence of RNAP and CAP. The left half of the histogram shows GalR binding to the one-site templates, O_E^L/O_I^G or O_E^G/O_I^L. The right half separately shows the results of GalR binding to each operator when the two-site template is used. The first bar (black) represents GalR binding to the O_E sequence in the absence of any other DNA-bound protein. The second bar represents the difference between GalR binding to O_E in the absence and in the presence of RNAP. Even though the third bar should represent the difference of GalR binding to the same operator in the presence and absence of DNA-bound CAP and RNAP, it is labeled ND, for not determinable, due to the reasons mentioned in the text. Bars 4−6 depict the corresponding differences of GalR binding to the O_I. Independent quantitation of each site on experiments conducted with the two-site template gives rise to the following six bars. Bars 7−9 refer to GalR binding to O_E in the absence of any other protein, in the presence of RNAP, and in the presence of the RNAP−CAP complex. The same relative order is used in bars 10−12 for the binding of GalR to the O_I operator sequence in the two-site template.

nonfunctional have identified the third sequence as a weak promoter (Figure 1; Ponnambalam et al., 1987). Identical nuclease protection and hypersensitivity results within base

pairs −96 to +89 were obtained from experiments conducted on the 633 bp restriction fragments, which encompass base pairs −199 to +434 (data not shown), eliminating the possibility that the RNAP-induced nuclease hypersensitivities are due either to binding of additional RNAP molecules to the DNA termini or to redistribution of the footprinting probes.

Striking changes in nuclease reactivity occur within and surrounding O_I upon RNAP binding. The base pairs that are protected or become hypersensitive are comparable, in position and magnitude, for all three nucleases (Figures 3 and 6). The protection of base pairs downstream of O_I occurs with both the native O_I (O_I^G) and the altered O_I sequence, which has been modified to a LacI recognition site, O_I^L. The downstream protected region does not resemble a typical RNAP footprint, and shows an RNAP concentration dependence identical to that of the P1/P2 promoter region (Dalma-Weiszhausz, 1995). These observations argue against the binding of additional RNAP molecules in this region of the DNA fragment. However, the CAP-induced effect on the RNAP-dependent hypersensitivity of the O_I^L sequence is different and significantly greater than on the O_I^G sequence (Figure 3; compare top and bottom panels). An intense hypersensitivity is observed at position +58, within O_I^L, on the template strand following the binding of the CAP−RNAP complex.

The binding of GalR to DNA containing the O_E^G sequence has no detectable effect on the downstream nuclease reactivities of RNAP and the CAP−RNAP complex (Figure 3). When GalR is bound to O_I, the RNAP-induced hypersensitivity at that site is obscured by the GalR-induced protection, except for a single base pair at the 5′ border of O_I (Figure 2, insert). Upon RNAP binding, sequences with altered reagent susceptibilities downstream of O_I remain visible even in the presence of GalR at O_I.

In order to investigate the helix phase and the distance dependence of the RNAP-induced nuclease reactivities at O_I, DNA molecules were constructed that have a 15 bp insertion between the P1/P2 promoter region and O_I. This insertion has no detectable effect on the nuclease reactivity of the base pairs within O_I; the RNAP-induced hypersensitivity remains constant (Figure 7). In addition, the inserted sequence does not become hypersensitive to nuclease upon RNAP binding. Comparable results are observed for a DNA construct containing a 30 bp insertion (Dalma-Weiszhausz, 1995). The CAP dependence of the intensity of the downstream hypersensitive sites is qualitatively unchanged by the sequence insertions. We conclude from these data that the hypersensitivity of these bases is dependent upon the specific DNA sequences at and downstream of O_I and not upon either the helical phasing of the promoter region and O_I or the number of bases away from the promoter.

DISCUSSION

In vivo, the binding of GalR solely to O_E modestly represses transcription. GalR binding to O_I alone has no detectable effect on transcriptional regulation while the simultaneous binding of GalR to both operators results in a 15-fold repression of transcription (Adhya & Majumdar, 1987; Haber & Adhya, 1988). In order to explain this synergistic interaction, repression by a GalR-mediated DNA "looped complex" was postulated (Adhya & Majumdar,
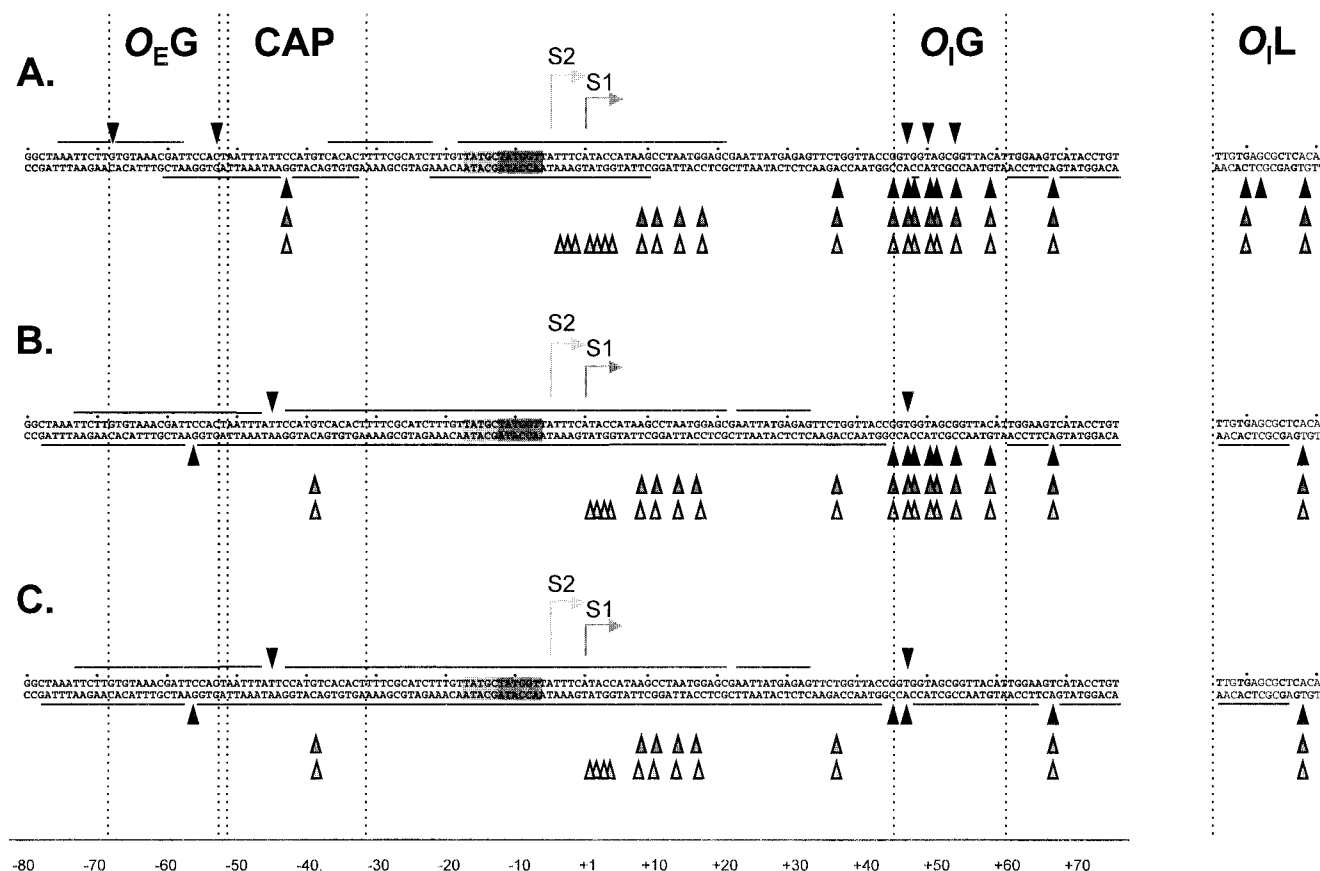
FIGURE 6: Schematic representation of reagent susceptibilities to *gal* promoter DNA upon the simultaneous binding of CAP, RNAP, and GalR. Bars indicate the limits of nuclease protection, while arrowheads represent hypersensitive sites. The DNase I data are shown in black. •OH data are shown in gray, while 5OPP data are shown in light gray. The cognate binding sites, as well as the transcription start sites S1 and S2, are shown. Panel A shows the DNA cleavage profile induced by the binding of RNAP to the O_E^{G}/O_I^{G} sequence. Panel B represents the DNA nuclease susceptibilities upon the simultaneous binding of CAP and RNAP. Panel C shows the data for GalR, RNAP, and CAP binding simultaneously to the same DNA fragment.

1987). However, *in vitro* studies have shown that the mechanism of repression of the *gal* operon is unlikely to be simply a GalR-mediated looped complex. In cell extracts, GalR binding solely to O_E can partially repress transcription from P1 in the presence and in the absence of CAP (Kuhnke et al., 1986). *In vitro* transcription studies with purified proteins have shown that GalR is not, by itself, sufficient to repress transcription either on linear or on supercoiled minicircle DNA (Goodrich & McClure, 1992; Choy & Adhya, 1993). It has also been shown that GalR binding to O_E and O_I on linear DNA is noncooperative (Brenowitz et al., 1990) and that the protein itself does not self-associate to tetramers in solution (Hsieh et al., 1994). Gel retardation assays, as well as quantitative footprinting experiments, have shown that the presence of DNA-bound CAP has no effect upon GalR binding to either or both operators simultaneously (Dalma-Weiszhausz & Brenowitz, 1992).

One of the goals of the present studies was to determine whether the binding of RNAP and GalR to the *gal* operon occurred in a cooperative manner. Several studies have shown that GalR and RNAP can bind simultaneously to the *gal* regulatory region (Busby et al., 1987; Kuhnke et al., 1989; Adhya & Majumdar, 1987; Goodrich & McClure, 1992). Abortive initiation studies further show that GalR has a small effect on the formation of "closed complexes" (Goodrich & McClure, 1992). The DNase I footprint titration studies presented in this paper demonstrate independent binding of RNAP and GalR, within experimental

error, when the latter binds to each operator separately or to both operators simultaneously. However, since GalR affects the partitioning of RNAP between the P1 and the P2 promoters (Figure 3; Goodrich & McClure, 1992), some interaction between the two proteins must occur. It is likely that the absence of detectable cooperativity is due to the small energy difference (-0.4 ± 0.2 kcal/mol) that characterizes RNAP binding to the P1 and the P2 promoters. This small value is comparable to the precision of the individual measurements of $\Delta\Delta G$ and sets an upper limit for an interaction energy between GalR and RNAP binding simultaneously to the DNA. GalR–RNAP interactions are thus unlikely to be a major contributor to the repression mechanism mediated by the simultaneous binding of GalR to O_E and to O_I.

The fact that the simultaneous binding of GalR to O_E and O_I does not inhibit RNAP binding (Figure 5) is direct evidence that a GalR-mediated looped complex does not form on these DNA restriction fragments. This conclusion is supported by the presence of a positive control demonstrating that a protein-mediated looped complex is inhibited by RNAP binding to the *gal* promoters (Dalma-Weiszhausz, 1995). A LacI-induced repressor looped complex was observed for the chimeric control region containing LacI recognition sequences at O_E and O_I (Brenowitz et al., 1991; Brenowitz & Jamison, 1993). Experiments comparable to those shown for GalR in Figure 5 were conducted with LacI on the chimeric *gal* control region.
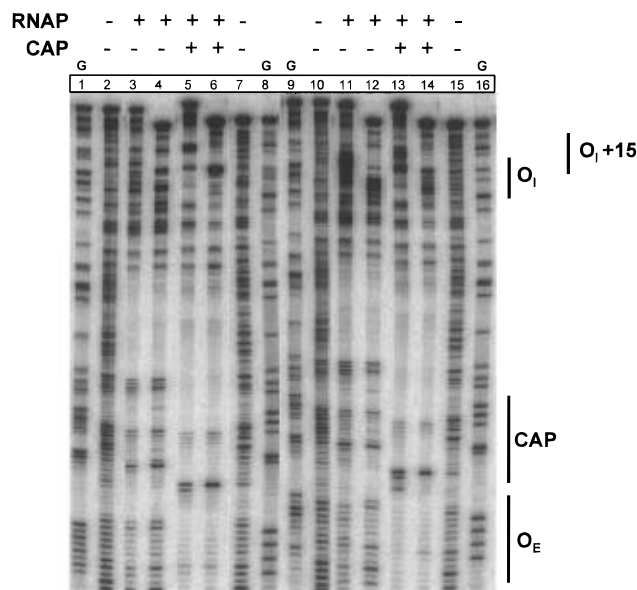
FIGURE 7: Denaturing gel electrophoresis of the DNase I footprint of RNAP binding to the 185 bp fragments that contain the *gal* operon regulatory sequences (O_E^L/O_I^L and O_E^L/O_I^G) and to the altered fragments that contain a 15 bp insertion between O_I and the P1/P2 promoters ($O_E^L/+15$-O_I^L and $O_E^G/+15$-O_I^G). Results are shown for RNAP binding in the presence (+) and absence (−) of CAP. The localization of the upstream regulatory sequences, as well as the O_I sequence for each fragment, is depicted on the right. The location of O_I on the 15 bp insertion fragments is also shown. Lanes 1, 8, 9, and 16 designate the Maxam−Gilbert G reaction for the fragments $O_E^L/+15$-O_I^L, O_E^L/O_I^L, $O_E^G/+15$-O_I^G, and O_E^L/O_I^G, respectively. Lanes 2, 7, 10, and 15 represent the corresponding DNase I cutting pattern for the DNA fragments in the absence of protein. The DNase I footprint of RNAP binding to the $O_E^L/+15$-O_I^L (lane 3) and to the O_E^L/O_I^L (lane 4) fragments is shown. The same two DNA fragments were used to exhibit the simultaneous binding of RNAP and CAP in lanes 5 and 6. Lanes 11 and 12 compare the binding of RNAP alone on the $O_E^G/+15$-O_I^G and on the O_E^L/O_I^G fragments, respectively. Lanes 13 and 14 show the simultaneous binding of CAP and RNAP to the same DNA fragments.

These studies showed that the LacI-mediated looped complex is inhibited by the binding of RNAP (Dalma-Weiszhausz, 1995). Thus, although a LacI-mediated looped complex can inhibit RNAP binding and repress transcription at the *gal* operon (Haber & Adhya, 1988; Choy & Adhya, 1993), GalR does not function by this mechanism. Further studies are required in order to determine whether additional components contribute to the O_E- and O_I-mediated repression mechanism.

The inhibition of the CAP−RNAP cooperativity by GalR does *not* require the synergistic interaction between GalR dimers bound at O_E and O_I (Figures 4 and 5). The separation of these two functions suggests that the mechanisms of repression mediated by the inhibition of CAP-induced activation and by the synergistic action of GalR dimers bound to O_E and O_I have evolved separately. The fact that the CAP−RNAP cooperativity can be inhibited by GalR binding to the upstream operator, O_E, without a repartitioning of RNAP between the P1 and P2 promoters shows that binding cooperativity and promoter partitioning are not linked. This conclusion is supported by the •OH protection patterns in which no detectable GalR-dependent change in the position of the CAP−RNAP complex is observed (Figure 6). The thermodynamic data are consistent with several mechanisms by which GalR bound at O_E might inhibit the CAP−RNAP

binding cooperativity: GalR binding to O_E might (1) directly interfere with protein−protein contacts between CAP and RNAP, (2) alter the geometry of the CAP−RNAP complex thereby inhibiting protein−protein interactions, or (3) alter the trajectory of the DNA and prevent the CAP−RNAP complex from making contact with the upstream DNA. Upstream "A-tract" sequences, located between −85 and −63 (contained on the restriction fragments used in our studies), contribute to RNAP partitioning and CAP-induced activation (Lavigne et al., 1992a). An upper limit to the upstream sequence likely to interact with RNAP can be made since binding studies conducted with restriction fragments terminating at −96 and −199 (the 185 and 633 bp restriction fragments, respectively) yield identical results. It should also be noted that the participation of a second CAP molecule in CAP−RNAP cooperativity has been suggested (Shanblatt & Revzin, 1983, 1986). Further studies would be required to distinguish this possibility from mechanism 3 described above.

In order to facilitate the evaluation of these mechanisms, a model of the simultaneous binding of GalR and CAP to the *gal* operon has been constructed (Figure 8). The model is based on the atomic resolution cocrystal structures of CAP (Schultz et al., 1991) and of PurR (Schumacher et al., 1994). Three assumptions are made in its construction: (1) The structure of the two-protein complex is the sum of the individual structures; (2) the DNA bend induced by CAP at its cognate sequence at the *gal* operon can be modeled as the bent DNA structure in the CAP−DNA cocrystal; (3) PurR is an appropriate structural model for GalR. The first assumption is clearly a first approximation, as is the second. Although it is known that the extent of the CAP-induced DNA bend is sequence-dependent, the overall direction of the bend is unaltered, which argues that the overall conformation of CAP in this model is correct (Liu-Johnson et al., 1987; Gartenberg & Crothers, 1988). Furthermore, inversion or substitution of the CAP binding sequence in the *gal* operon has no discernible effect on transcription activation (Zhou et al., 1994). Several lines of evidence suggest that the third assumption is valid. GalR and PurR are members of the "Gal and Lac repressor" family of bacterial regulatory proteins and possess amino acid sequences that are 31% identical and 53% similar (Weickert & Adhya, 1992). The inducer binding domains of this family are also homologous with the *E. coli* periplasmic binding proteins. On the basis of this homology, three-dimensional models of LacI and of GalR have been constructed (Nichols et al., 1993; Hsieh et al., 1994) that are structurally homologous with the inducer binding domain present in the PurR crystal structure (Schumacher et al., 1994). Moreover, both GalR and PurR are known to bend their cognate sequences (Schumacher et al*.*, 1994; Kuhnke et al., 1989; Zwieb et al., 1989). Leucine 54 plays a key role in sequence-specific binding of PurR; this same amino acid is conserved in GalR. These findings suggest that PurR and GalR are structurally very similar.

Figure 8 was constructed by merging the cocrystal structures of the CAP−DNA and PurR−DNA complexes using Insight II (BIOSYM Technologies, 1993). The sequence GCGAA of the CAP binding site in the cocrystal structure was deleted in order to reproduce the spacing between the two binding sites present in the *gal* operon. There is no obvious overlap of the two proteins in the model,
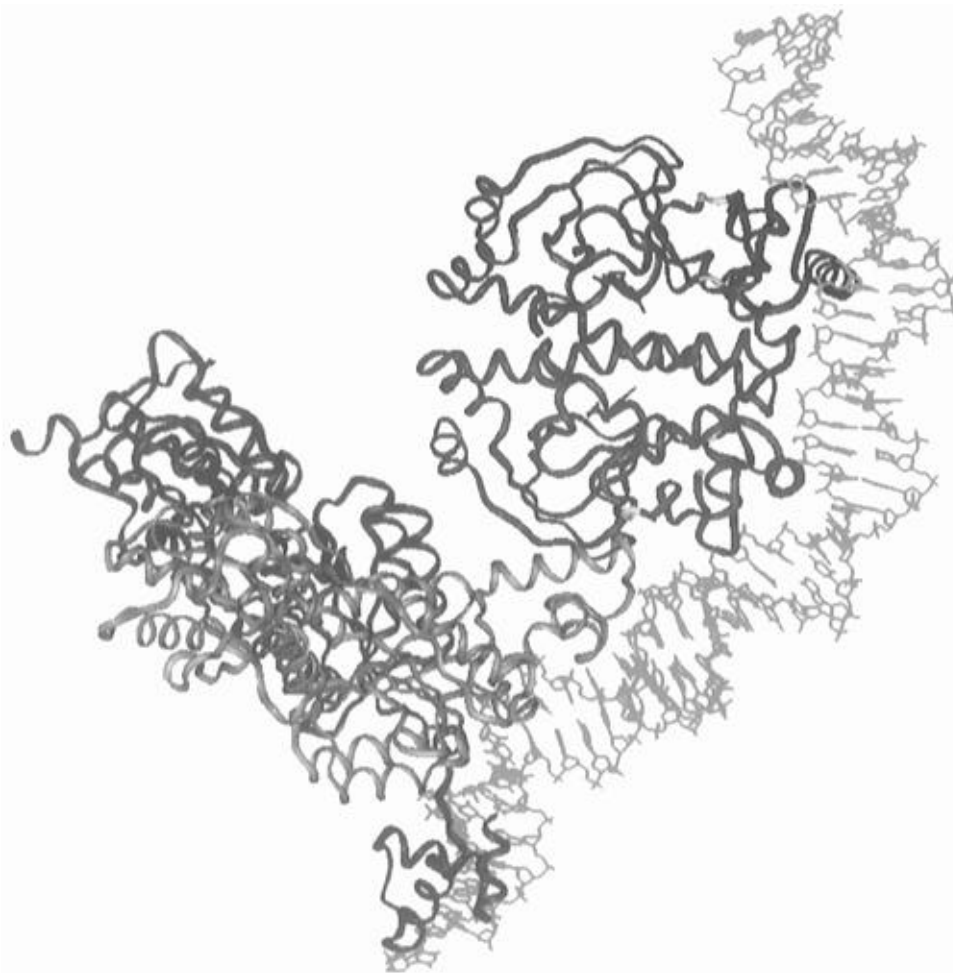
FIGURE 8: A model of the simultaneous binding of the cAMP−CAP complex and GalR. This is a composite model of the DNA-bound ribbon representations of the CAP and the PurR cocrystal structures, showing the spatial positioning of the two proteins on a linear DNA fragment. This view highlights the DNA distortions brought about by the binding of CAP and of PurR. The gold and green show the individual monomers of the dimeric PurR molecule. The magenta and violet structures show the monomers of the CAP dimer. The DNA molecule is shown in blue. The yellow residues on CAP are the 158−159 as well as the 52 loops, implicated in protein−protein interactions with RNAP.

consistent with the absence of any detectable interaction between GalR and CAP upon binding to the *gal* operon (Dalma-Weiszhausz & Brenowitz, 1992). CAP bends the DNA toward itself by about 90° while PurR introduces a 45° kink in the center of its operator sequence, and the DNA bends *away* from the protein (Figure 8). The CAP and PurR bends are oriented in opposite phases, which results in the DNA following a tortuous trajectory. A result of the protein binding sites being out of phase is that the CAP residues that are implicated in activation at class II promoters are not occluded by the O_E-bound GalR from potentially interacting with RNAP (Figure 8, greenish yellow residues). Overall, this picture is consistent with the available thermodynamic data (Dalma-Weiszhausz & Brenowitz, 1992).

Several lines of evidence including (i) the presence of upstream nuclease protections upon RNAP binding (*e.g.*, Figures 2 and 3), (2) the ability of RNAP to bend DNA (as demonstrated by circular permutation assays; Kuhnke et al., 1989), (3) the effect of upstream intrinsically curved sequences on transcription activation at the *gal* promoter (Lavigne et al., 1992b), and (4) the finding of a third RNAP recognition element in bacterial promoters (Ross et al., 1993) suggest that the DNA wraps around the RNAP and the RNAP−CAP complex (Crothers & Steitz, 1992; Kolb et al.,

1993). An additional consequence of DNA conformational changes is that the upstream subunit of CAP, which mediates transcription activation of class II promoters such as the *gal* operon (Zhou et al., 1993), gets positioned closer to RNAP. In the context of the model shown in Figure 8, the simplest interpretation of the thermodynamic data is the hypothesis in which the DNA trajectory is altered by GalR binding, thus preventing the RNAP−CAP protein−protein contacts to form. At least a 90° change in the phase of the bound CAP and GalR dimers is required to juxtapose GalR near the "activation residues" of CAP, arguing against GalR sterically blocking the RNAP−CAP contacts. It can be seen in Figure 8 that the binding of GalR changes the trajectory of the upstream DNA sequence. This could clearly disrupt the interactions between CAP and RNAP and between RNAP and the DNA within or upstream of O_E, as proposed in hypothesis 3. The fact that GalR does not detectably inhibit RNAP binding (Figure 5) is evidence that the proposed interactions of RNAP with the upstream DNA are CAP-dependent. Furthermore, the DNase I footprint of RNAP extends through O_E only in the presence of CAP. Finally, it should be noted that hypotheses 2 and 3 are not completely independent since putative upstream interactions may contribute to the stability of the CAP−RNAP protein−protein interactions. In summary, the available structural data

support a significant role for upstream DNA contacts by RNAP in the mechanism of CAP−RNAP binding cooperativity and its inhibition by GalR. On the basis of previous studies that linked the CAP−RNAP cooperativity to transcriptional activation (Lavigne et al., 1992a), we propose that the GalR-induced inhibition of the CAP−RNAP cooperativity is at least partially responsible for the overall repression of this operon.

The nuclease protection studies reveal complex patterns of protection and hypersensitivity. However, the position of each protein on its cognate site is not detectably dependent on the simultaneous binding of the other proteins. In addition to upstream interactions discussed above, the binding of RNAP or of the CAP−RNAP complex changes the reactivity of the downstream sequences in the region of O_I (Figure 3) to DNase I, 5OPP, and $^\bullet$OH. These nuclease reactivities are CAP and DNA sequence-dependent but are independent of the helical phase and distance (within three helical turns) relative to the P1/P2 promoters. These results provide direct evidence that RNAP is in "communication" with the region of DNA encompassing O_I. The presence of nuclease protection (Figures 3 and 6) is suggestive of protein−DNA contacts. However, the phase and distance independence of the downstream nuclease reactivities suggests that the intervening DNA would have to be distorted if downstream RNAP−DNA contacts were to be maintained (Figure 7). An additional, but puzzling, aspect of this downstream interaction is that the binding of GalR to O_I and of RNAP is energetically independent (Figure 5), despite the fact that some of the RNAP-induced hypersensitive base pairs are within O_I (Figure 2, insert). The observation that a base pair within O_I, which is usually protected upon GalR binding, remains hypersensitive when RNAP is present suggests that GalR at O_I and RNAP can bind simultaneously to this DNA, perhaps on opposite sides of the DNA helix. The data establish that the promoter binding of RNAP has a *direct* effect on O_I although the mechanism underlying this effect is uncertain. This conclusion differs from the prevailing view of O_I, that its synergistic effect with O_E is strictly through protein−protein interactions between two DNA-bound GalR dimers. Even though GalR binding to O_I is energetically independent of RNAP, the conformation of the DNA between these two proteins is altered. GalR-induced repression through the participation of the two operator sequences need not require a GalR-mediated looped complex.

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