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Operator-bound GalR dimers close DNA loops by direct interaction: tetramerization and inducer binding

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The assembly of the Gal repressosome, a higher order nucleoprotein complex that represses transcription of the *gal* operon in *Escherichia coli*, involves the formation of a DNA loop encompassing the promoter segment. GalR dimers bound to two spatially separated operators, O_E and O_I, specifically interact with the histone-like protein HU and close the loop in supercoiled DNA. We isolated and characterized a GalR mutant containing an amino acid substitution (R282L) that can repress transcription in the absence of HU and supercoiled DNA both *in vivo* and *in vitro*. Repression involves the same DNA looping; deletion of either O_E or O_I makes the mutant GalR ineffective in repression. This and other results suggest that the R282L substitution increases the normal affinity between two DNA-bound GalR dimers, allowing looping. We conclude that GalR dimers interact directly and do not use HU as an adaptor in loop closure; HU and DNA supercoiling act in concert to stabilize the GalR tetramer. The stronger GalR–GalR interaction also made the *gal* transcription non-inducible, suggesting that the inducer binding acts by modulating tetramerization.

Keywords: gal regulation/protein–protein interface/transcription repression

Introduction

DNA–multiprotein complexes of different types participate in carrying out various DNA transaction reactions, transcription, replication and repair recombination (Echols, 1990). Many of these complexes have a common theme: they contain topologically independent DNA domains composed of loops formed by bringing two non-contiguous points of a DNA molecule together by sequence-specific DNA-binding proteins. As shown in Figure 1, the sequence-specific DNA-binding proteins, which close the DNA loop, may do so by using one of the following strategies: (I) two similar or dissimilar proteins bind to cognate DNA sites and interact with each other directly (Irani *et al*., 1983; Dunn *et al*., 1984; Su *et al*., 1990; Roy *et al*., 1991; de Beer *et al*., 2002); (II) a bidentate protein molecule simultaneously binds to two DNA sites (Kramer *et al*., 1987; Nash, 1996); (III) in example (I), the two DNA-bound proteins, instead of interacting directly, close the loop by both interacting with an adaptor (Kallipolitis *et al*., 1997); (IV) in the above examples, DNA looping may be aided energetically by another DNA-binding protein bound to an architecturally critical position (Santero *et al*., 1992; Aki *et al*., 1996; Nash, 1996; Wassem *et al*., 2000; Thomas and Travers, 2001; de Beer *et al*., 2002); or (V) the adaptor helps loop closure by acting as an architectural protein as well (Cosma *et al*., 2001; Kar and Adhya, 2001). In the Gal repressosome, which represses transcription in the *gal* operon in *Escherichia coli*, two GalR dimer proteins bind to two operator elements, O_E and O_I, and form a DNA loop encompassing a 113 bp DNA segment comprising the *gal* promoters (Irani *et al*., 1983; Majumdar and Adhya, 1984; Adhya *et al*., 1998; Figure 3A). Formation of the loop by the two operator-bound GalR dimers requires the architectural protein, HU, and supercoiled DNA (Aki *et al*., 1996; Lewis *et al*., 1999). The 113 bp DNA loop in the Gal repressome is refractory to transcription initiation from the two *gal* promoters, *P*1 and *P*2 (Choy *et al*., 1995). Repression is abolished when D-galactose binds to GalR (Nakanishi *et al*., 1973; Majumdar and Adhya, 1984). During repressosome formation, HU specifically interacts with a segment of the looped DNA, centered at position +6.5, and to GalR, thus suggesting the possibility of both an architectural and an adaptor role for HU (Aki and Adhya, 1997; Kar and Adhya, 2001). An adaptor role for HU raised the question of whether DNA-bound GalR directly interacts at all in loop closure. The results reported here show that operator-bound GalR forms the DNA loop by direct interaction, and that the inducer may act by targeting the tetramerization step.

Results

GalR super-repressor mutants

In an *in vivo* system, the DNA looping and DNA binding activities of GalR were monitored by following the repression of the *P*2 and *P*1 promoters, respectively. This was based on the fact that the repression of *P*2 is entirely dependent upon the formation of a DNA loop, whereas binding of GalR to the O_E operator is sufficient for repression of *P*1 (Aki *et al*., 1996; Choy *et al*., 1997). Repression of *P*2 requires the formation of the repressosome through cooperative binding of GalR to both O_E and O_I, and of HU to the interoperator segment. The tester strain, DM0026, with two corresponding reporter genes for the two promoters, contains a chromosomal $O_E^+O_I^+P1^-P2^+$–*gusA* transcriptional fusion, an $O_E^+O_I^-P1^+P2^-$–*lacZ* translational fusion and a deletion of the *galR* gene (Lewis *et al*., 1999). Wild-type or mutant GalR protein was expressed from a plasmid (pSEM1029 or pSEM1050) in the cell. Assay of β-glucuronidase and β-galactosidase either on indicator plates or in cell extracts

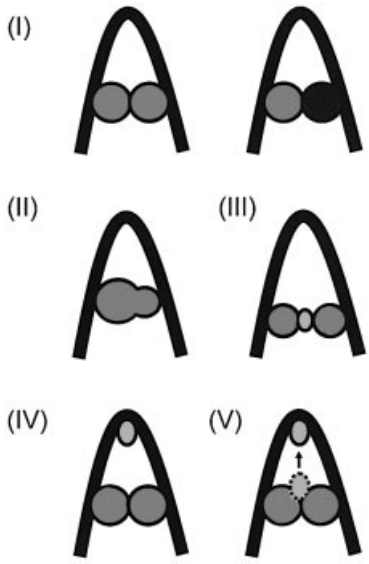


Fig. 1. Modes of DNA looping discussed in the text.

reflected the effect of the GalR protein on DNA looping and operator binding, respectively. By screening of site-directed substitutions in a defined surface area of GalR previously surmised to participate in a GalR–GalR interaction, we isolated a GalR mutant, R282L, which showed stronger repression of the *P*2 promoter relative to that of wild-type GalR without changing *P*1 repression on indicator plates. As shown in Figure 3A, the expression of either wild-type GalR or R282L mutant protein genes from a plasmid resulted in strong reduction of the β-glucuronidase activity compared with that in the total absence of GalR (Δ*galR*). However, the R282L mutant reproducibly repressed the *P*2 expression more than that of the wild-type GalR. Efficient repression of the *P*2 promoter is totally dependent upon the presence of the HU protein (Lewis *et al*., 1999). Deletion of *hupA* (encoding HUα) and *hupB* (encoding HUβ) in the cells carrying wild-type *galR* in the chromosome fully derepresses the *P*2 promoter. We observed similar derepression of *P*2, by measuring β-glucuronidase synthesis when the *galR*$^+$ gene was present in the plasmid (pSEM1029) in the strain deleted for the two *hup* genes (Figure 3B). However, the same strain showed a significantly lower β-glucuronidase level when the plasmid-borne *galR* gene was the mutant R282L. Thus, R282L mutant specifically repressed *P*2 in an HU-independent manner. Another arginine to leucine substitution, R279L, located in the vicinity of R282L in the GalR structure behaved like wild-type repressor, i.e. showed derepression of *P*2 in the absence of HU (data not shown).

In vitro properties of GalR R282L

To study the behavior of the R282L mutant *in vitro*, we overexpressed the wild-type and the R282L mutant GalR proteins, and purified them to >95% homogeneity as described in Materials and methods. *In vitro* transcription was performed using supercoiled DNA template as described previously (Geanacopoulos and Adhya, 1997). The results are shown in Figure 3B. The wild-type GalR protein, as expected, enhanced *P*2 and repressed *P*1 transcription. The presence of GalR (wild-type or R282L) and HU resulted in complete repression of both promoters. However, as was found *in vivo*, the purified R282L mutant GalR repressed transcription in the absence of HU. Addition of 80 nM HU did not interfere with the repression of *P*2 by R282L mutant.

DNA templates with an O_E or O_I substitution/deletion were used to study the nature of *P*2 repression by the mutant GalR protein. As mentioned, DNA looping-mediated *P*2 repression requires the binding of GalR to both *gal* operators (Irani *et al*., 1983; Choy and Adhya, 1992; Aki and Adhya, 1997). In the template pSA886, in which the O_I operator was replaced by a random sequence, the R282L mutant did not show repression but activated transcription from *P*2 and repressed that from *P*1 as did the wild-type protein (Figure 3C). Neither the wild-type nor the mutant GalR had a significant effect on *gal* transcription in the DNA template in which O_E was absent. These results demonstrate that the repression of *P*2 by R282L in the absence of HU involves DNA looping by the bipartite operators bound by the mutant GalR.

Effect of supercoiling

DNA looping-mediated repression of the *P*2 promoter requires a supercoiled DNA template (Aki *et al*., 1996). If GalR mutant R282L resulted in increased affinity of the bound dimers to form a tetrameric structure, one may expect to form the DNA loop and thus bring about *P*2 repression with reduced DNA supercoiling or improper helical phasing of the operator sites. Negative supercoiling can be decreased by the addition of coumermycin, an antibiotic that inactivates DNA gyrase (Gellert *et al*., 1976). Coumermycin inhibits *P*2 repression by GalR without affecting the intrinsic strength of the promoter (Lewis *et al*., 1999). Repression of *P*2 in cells expressing the wild-type and R282L mutant GalR was compared in the presence of coumermycin (Figure 2C and D). In contrast to wild-type, the mutant GalR showed repression of *P*2 in the presence of coumermycin. This suggests that DNA supercoiling may not be mandatory for DNA looping in the latter case. We tested this hypothesis by studying *P*2 repression *in vitro* on linear *gal* DNA templates with the R282L protein (Figure 3D). As expected, the wild-type GalR enhanced *P*2 transcription with a concurrent partial repression of *P*1. The presence of wild-type GalR and HU had the same effect, since the repressosome structure cannot be formed on linear template (Aki *et al*., 1996). In the presence of mutant GalR protein, however, transcription from both promoters decreased dramatically, in both the absence and presence of HU.

Effect of changing the DNA helical phasing

DNA looping-mediated repression of *P*2 depends on the correct helical phasing of the two operator sites (Lewis and Adhya, 2002). Derepression occurred if the relative angular orientation of O_E and O_I was changed by the addition or deletion of non-integral DNA helical turns between the two operators. We inserted 5 bp between O_E and O_I (at position +9) and tested the template for *P*2 repression by R282L GalR (Figure 4A). The improper helical phasing of the operators inhibited the looping-mediated repression of *P*2 with wild-type GalR in the presence of HU (Figure 4B). However, the mutant GalR

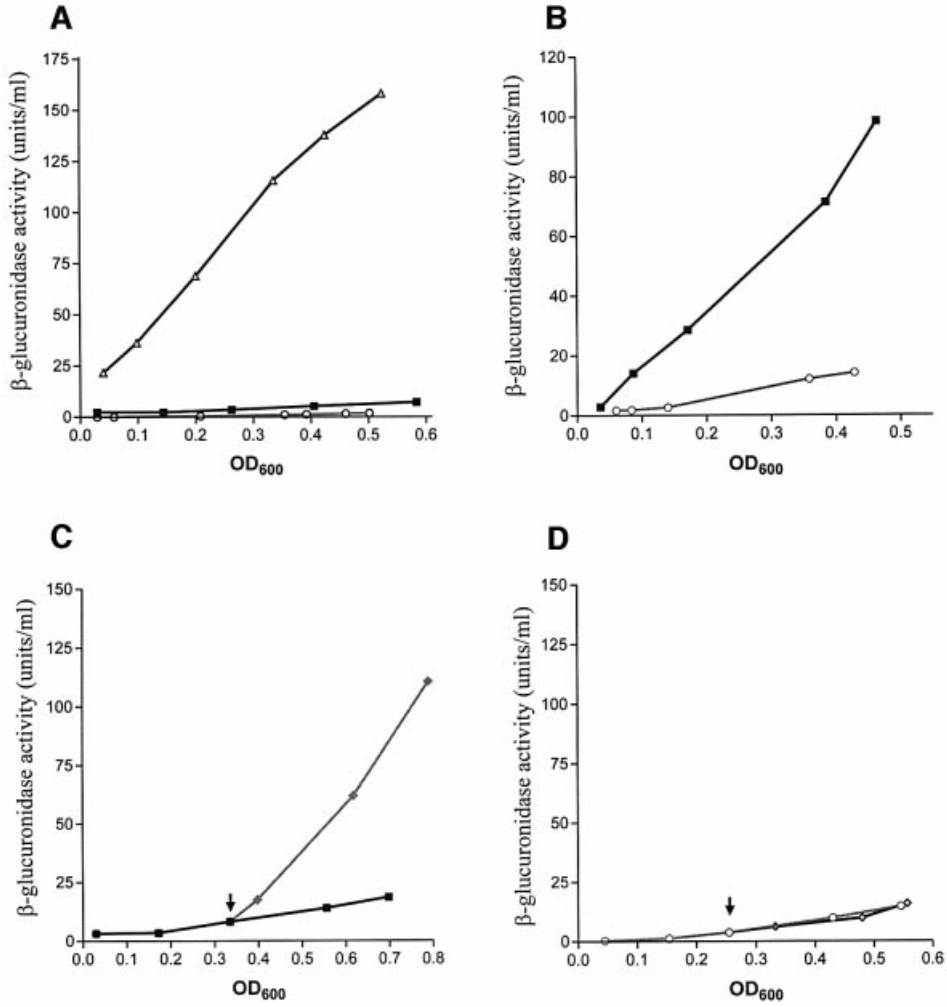


Fig. 2. (**A**) Effect of the GalR R282L mutation on the looping repression of the *galP*2 promoter. Differential rates of β-glucuronidase synthesis from *P*2–*gusA* in the presence of the plasmids pEM7/Zeo (Δ*galR*, triangles), pSEM1029 (*galR*+, squares) and pSEM1050 (*galR* R282L, circles). Cells were grown in minimal medium supplemented with 0.4% (w/v) D-fructose, 0.1% (w/v) casamino acids and 0.004 (w/v) vitamin B1. (**B**) Effect of *hup* deletions on the looping repression of the *P*2 promoter in the presence of the wild-type GalR (squares) and R282L GalR (circles). DM0100 cells bearing the *P*2–*gusA* chromosomal fusion and lacking both the *hupA* and *hupB* genes were transformed with the pSEM1029 and pSEM1050 plasmids, carrying the wild-type and the R282L *galR* gene, respectively. Strains were assayed for the rates of β-glucuronidase synthesis in the *P*2–*gusA* fusions. Conditions for cell growth were as in (A). (**C**) Differential rates of β-glucuronidase synthesis from *P*2–*gusA* in DM0026 (pSEM1029) cells, expressing the wild-type *galR* gene, in the absence (squares) and presence (diamonds) of coumermycin A_1. (**D**) Differential rates of β-glucuronidase synthesis from *P*2–*gusA* in DM0026 (pSEM1050) cells, expressing the R282L mutant *galR* gene, in the absence (circles) and presence (diamonds) of coumermycin A_1. Cells were grown in minimal medium supplemented with 0.4% (w/v) D-fructose, 0.1% (w/v) casamino acids and 0.004% (w/v) vitamin B1. The arrowhead indicates the point of addition of 50 μg/ml coumermycin A_1.

protein, R282L, efficiently repressed transcription from the altered DNA template regardless of the presence or absence of HU. These results demonstrate the capability of the mutant protein to close the DNA loop in spite of improper helical phasing of the operators presumably because of its increased strength in tetramerization.

Interaction of the GalR dimers

GalR was shown to form a stable dimer in solution (Majumdar *et al*., 1987). To investigate the potential tetramerization of the GalR dimers, chemical cross-linking experiments were performed. The purified protein preparations were treated with dimethyl suberimidate (DMS), a homobifunctional imidoester that can react with primary amine groups to form stable covalent bonds. The products of the cross-linking reactions were separated by SDS–PAGE (Figure 5). In the presence of DMS, GalR resulted in protein bands with molecular weights of monomers, dimers, trimers and tetramers. The pattern and intensity of the bands representing the GalR oligomers were similar in the case of the wild-type and the R282L mutant protein preparations, clearly demonstrating the ability of both proteins to tetramerize under the conditions of cross-linking.

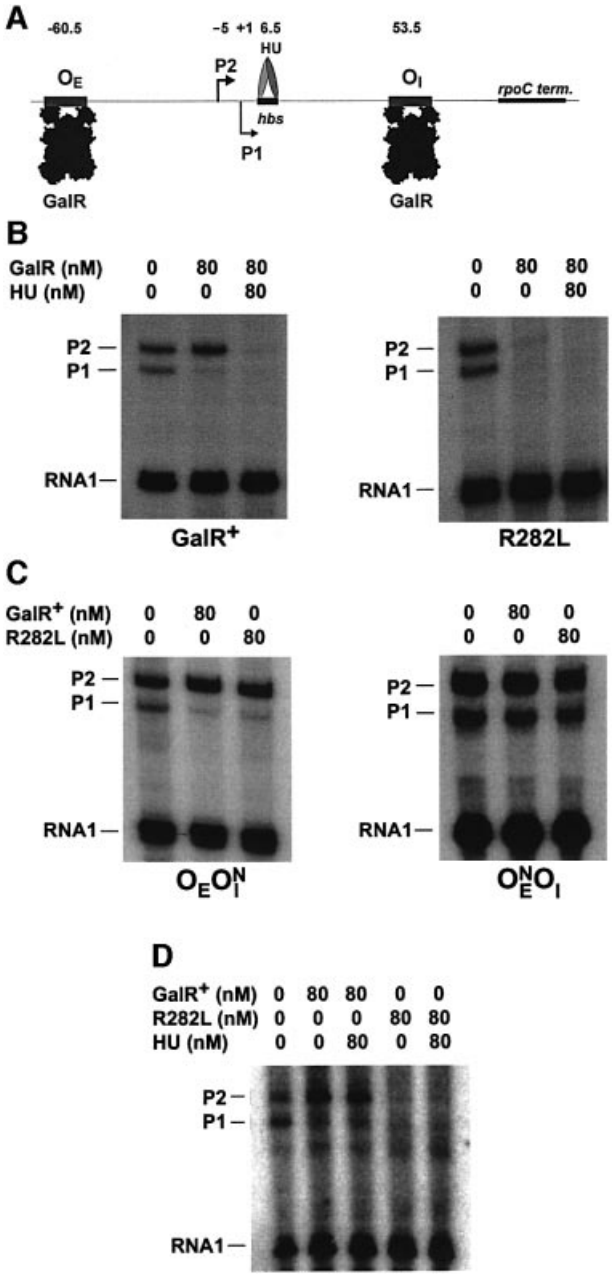
Non-inducibility of GalR R282L

The addition of inducer D-galactose to cells expressing the wild-type GalR derepressed *P*2 transcription, as expected (Lewis *et al*., 1999; Figure 6A). However, the R282L mutant GalR showed a non-inducible phenotype; the mutant protein maintained repression of *P*2 in the presence of D-galactose (Figure 6B). Because of this non-inducible

nature, we tested the effect of the inducer on the O_E–GalR complex *in vitro* by electrophoretic mobility shift assay (EMSA; Majumdar and Adhya, 1984). GalR was bound to the operator DNA, followed by the addition of different amounts of D-galactose. Compared with the wild type, the R282L mutant GalR protein bound to O_E with ~10% higher efficiency in the absence of the inducer (data not shown). The percentage of DNA remaining bound to GalR after the addition of different concentrations of D-galactose is shown in Figure 7A and B. The amount of D-galactose needed to reduce the amount of O_E–GalR complexes by 50% was ~0.12 mM for wild-type GalR and 8 mM for R282L.

Discussion

In our attempt to define the structure of the Gal repressosome, we previously described the isolation and characterization of GalR mutants that bind to DNA operators normally but are defective in potential GalR–GalR or GalR–HU contacts during DNA loop



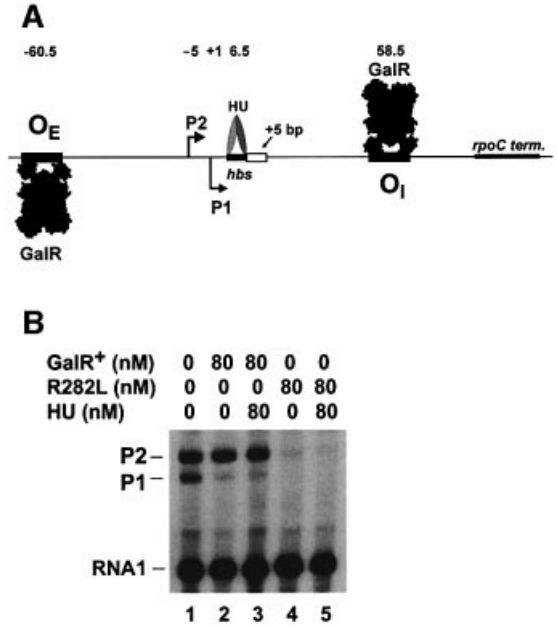


Fig. 4. The results of the *in vitro* transcription assay performed on DNA template with a 5 bp insertion. (**A**) Alteration of the topology of the *gal* regulatory region upon a 5 bp insertion. The sequence of 5 bp was inserted at position +9. The operator-bound GalR proteins occupy the opposite face of the DNA molecule. (**B**) Transcription of *galP*1 and *galP*2 on supercoiled pSA907 DNA. Proteins were used at the concentrations indicated on the top. Transcripts, marked *P*1 and *P*2, are RNAs made from the *gal* promoters. The transcript, marked RNA1, served as an internal control.

Fig. 3. Effect of the GalR mutant R282L on the transcription of the *P*1 and *P*2 promoters *in vitro*. (**A**) Schematic drawing of the *gal* regulatory region in plasmid pSA850 used as a template DNA in the *in vitro* transcription assay. The wild-type regulatory region, containing the external (O_E) and the internal (O_I) operator sites and the *P*1 and *P*2 promoters, is followed by the *rpoC* terminator. Transcription termination at the *rpoC* terminator results in a 125 nucleotide RNA from *P*1 and a 130 nucleotide RNA from *P*2. The numbering system used the +1 start site of *P*1 as reference. The HU-binding site (*hbs*) is centered at position 6.5. (**B**) *In vitro* transcription assay performed on supercoiled pSA850 DNA in the presence of wild-type (left panel) and R282L mutant (right panel) GalR protein. The RNA product was resolved on 8% polyacrylamide gels. The concentration of GalR and HU was 80 nM as shown on the top. The 80 nucleotide RNA1 transcript, which is independent of HU and GalR concentrations, served as an internal control between lanes. (**C**) Effect of operator deletions on the transcription of the *gal* promoters. Transcription on supercoiled pSA886 ($O_EO_I^N$) DNA (left panel) and pSA887 ($O_E^NO_I$) DNA (right panel) in the presence of the wild-type and R282L mutant GalR protein. The concentration of the repressor proteins was 80 nM, as indicated on the top. Transcripts, marked *P*1 and *P*2, are RNAs made from the *gal* promoters. The transcript, marked RNA1, serves as an internal control. (**D**) Transcription of the *gal* promoters on linear pSA850 DNA. pSA850 was treated with the restriction enzyme *Hin*dIII. The *Hin*dIII restriction site is located 491 bp downstream of the O_I operator site. The total length of the plasmid is 3.5 kb. Transcription was performed as described in Materials and methods. Proteins were used at the concentrations indicated on the top. Transcripts, marked *P*1 and *P*2, are RNAs made from the *gal* promoters. The transcript, marked RNA1, served as an internal control.

formation (Geanacopoulos *et al*., 1999, 2001). These looping-defective amino acid substitutions in the GalR dimer were located in the DNA-distal domain, which preferably defined a GalR–GalR rather than a GalR–HU contact area. Further genetic investigations of the potential GalR–GalR contacts by isolation and characterization of intragenic suppressors and by alanine scanning of looping-defective GalR mutations suggested the existence of a GalR tetramerization interface (Geanacopoulos and Adhya, 2002). In this study, we report the physiological and biochemical characterization of a GalR mutant protein, which can repress *gal* transcription and thus form the Gal repressosome in the absence of HU and supercoiled DNA. Since the mutant GalR behaved in this way not only *in vivo*, but also in assays with purified proteins, the repressosome formation in cells by HU and supercoiled DNA independent of GalR was not because of substitutions of HU by an unknown protein capable of working in concert with the mutant protein. We also showed the ability of both the wild-type and the mutant GalR to tetramerize by chemical cross-linking experiments. These results demonstrate that tetramerization is an intrinsic property of GalR; it closes the DNA loop by direct GalR–GalR contact. Since the substituted amino acid residue (R282L) is located within the genetically defined area of the tetramerization interface (Geanacopoulos *et al*., 2001), and the GalR containing both R282L and another looping-defective substitution failed to form the repressosome (data not shown), we conclude that the R282L change did not create a new GalR–GalR tetramer interface, but enhanced the strength of the pre-existing interface. This conclusion is consistent with the results that GalR with the single R282L substitution: (i) showed stronger repression than the wild-type protein; (ii) could withstand a distortion of the relative angular alignment of the two operator-bound GalR dimers on DNA; and (iii) was not dependent upon the architectural protein HU and supercoiled DNA to stabilize energetically a presumably weaker GalR–GalR interaction. The R282A substitution also showed the same phenotype as the R282L change. It seems likely that a non-hydrophobic residue at position 282 in wild-type GalR contributes to the reversibility of the system by making the dimer–dimer interaction weaker. Strong hydrophobic interactions are not favored in reversible protein–protein interactions (Jones and Thornton, 1996; Gerk *et al*., 2000). The R282L/A changes in the mutant GalR did just that. The predicted quantitative difference in the dimer–dimer interaction between the wild type and mutant in the transcription system was not investigated.

The R282L/A substitutions also make the GalR protein, as shown above, insensitive to the effect of D-galactose, which acts by interacting at a distant domain of the protein. In the closely related LacI protein, the mechanism of inducer binding was explained by the allosteric transition model (Lewis *et al*., 1996; Bell and Lewis, 2000). In this model, inducer and DNA binding exert their effects by shifting the protein between two basic states. These states differ in the relative rotation of the two highly similar globular subunits of the monomer core. Sugar binding in the cleft between these two globular domains causes a rotation of the two subunits with respect to one another that alters the position of the DNA-binding domain so that in the dimeric protein the orientation of the two headpieces or DNA-binding domains is less favorable for operator binding. In the GalR mutant R282L, non-inducibility resulting from strong tetramerization was coupled with a

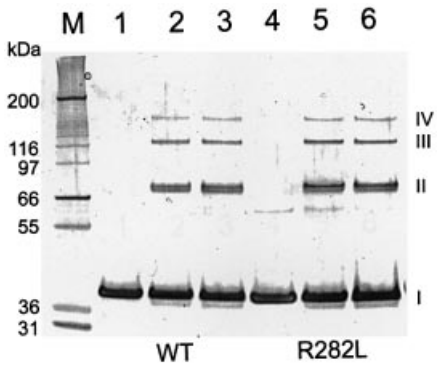


Fig. 5. Cross-linking of the GalR protein. Wild-type (lanes 1–3) and R282L mutant (lanes 4–6) proteins were cross-linked using DMS in the presence of 250 mM (lanes 2 and 5) or 500 mM (lanes 3 and 6) NaCl. In lanes 1 and 4, no DMS was added. Reaction products were separated by SDS–PAGE on a 4–12% gel. The positions of monomers (I), dimers (II), trimers (III) and tetramers (IV) of GalR are marked. Mark 12™ Standard (M; Invitrogen) was used to estimate the molecular weight of the products.

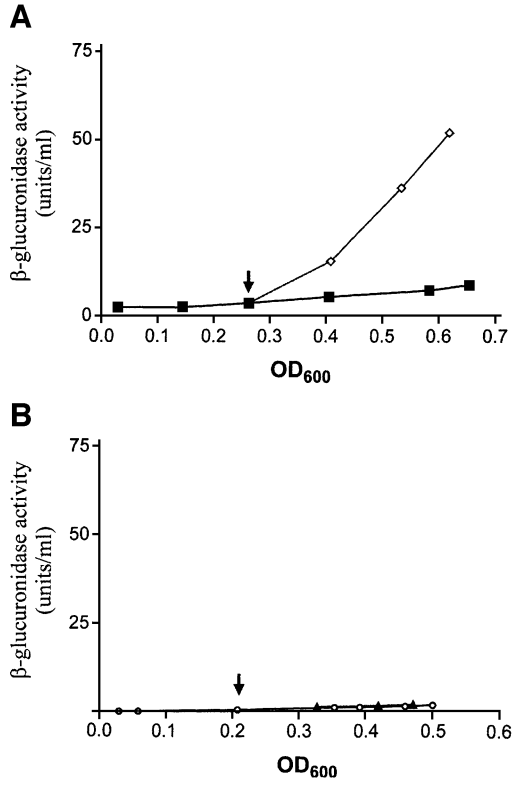


Fig. 6. (**A**) Differential rates of β-glucuronidase synthesis in the DM0026 (pSEM1029) cells expressing the wild-type *galR* gene in the absence (squares) and presence (diamonds) of the inducer D-galactose. (**B**) Differential rates of β-glucuronidase synthesis in the DM0026 (pSEM1050) cells expressing the R282L mutant *galR* gene in the absence (circles) and presence (triangles) of the inducer D-galactose. The arrow indicates the time of addition of the inducer at 5 mM final concentration.

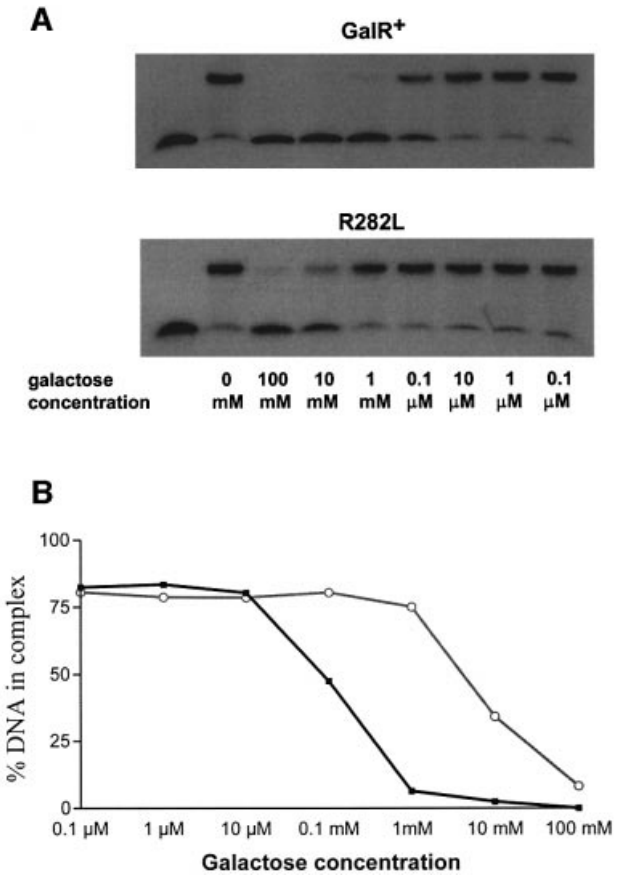


Fig. 7. Effect of D-galactose on the stability of the GalR–O_E complex. (**A**) GalR binding to the ^{32}P-labeled O_E operator sequence in the presence of varying amounts of D-galactose in a gel retardation assay. (**B**) Percentage of the O_E DNA bound by the wild-type (squares) and the R282L mutant (circles) GalR proteins in the presence of the indicated amounts of D-galactose.

slight increase in the stability of the GalR–O_E complex, strongly suggesting that the R282L mutation acts by stabilizing the DNA-binding conformation and increasing the energy required for the structural rearrangement in the sugar-binding state. The multiple effects of the R282L substitution indicate that tetramerization as well as DNA and inducer binding are coupled properties of the GalR protein. In LacI, inducer binding does not affect protein tetramerization because the two protein dimers are linked by the relatively flexible four helix bundle (Alberti *et al*., 1993). The V-shaped stacked structure in the modeled GalR tetramer does not allow such flexibility of the dimers (Geanacopoulos *et al*., 2001). Based on our results, we propose that the allosteric transition resulted from inducer binding affecting not only the DNA binding but also tetramerization of the GalR protein. Because of the very weak dimer–dimer interaction, induction by D-galactose is likely to involve breaking of the loop by altering the tetramerization interface before GalR is removed from the operators. Because, in the absence of DNA looping, GalR activates transcription from the *P2* promoter without the inducer being present (Choy and Adhya, 1992; Aki and Adhya, 1997), induction of the *gal* operon by this scenario would be considerably more rapid than in the case where the stability of the GalR–GalR interface is not modulated by inducer binding.

In summary, we report that the DNA loop in the Gal repressosome closes by tetramerization of two DNA-bound GalR dimers [model (I); see Introduction] without using HU as an adaptor in between [model (III)] although the latter is recruited by GalR [model (V)] (Kar and Adhya, 2001). Wild-type GalR, because of weak dimer–dimer interaction, needs the aid of supercoiled DNA and binding of HU as an architectural protein during DNA loop formation. A weak dimer–dimer interaction makes the DNA looping-mediated transcription repression, as argued above, quickly responsive to cellular need for *gal* operon expression.

Materials and methods

DNA manipulation methods

Plasmid manipulations followed protocols described in Sambrook and Russel (2001). Transformations were performed with Max Efficiency DH5α competent cells (Invitrogen). Restriction endonucleases were purchased from Invitrogen, DNA oligonucleotide primers from BioServe Biotechnologies, PCR (GeneAmp XL) and sequencing (ABI Prism) kits from Applied Biosystems, and DNA purification kits from Qiagen. DNA sequencing reactions were analyzed in a Perkin-Elmer/Applied Biosystems (model 373A) automated sequencer.

Plasmid constructions

To create plasmid pSEM1026, the *galR* gene was PCR amplified to allow for the addition of a sequence encoding the His_6 tag (GSSHHHHHHSS) at the C-terminal end of the *galR* open reading frame. The PCR product contained an *Nco*I site coincident with the ATG start codon and an *Xba*I site downstream of the stop codon of the *galR* gene. The amplified DNA fragment was inserted into the pBAD24 vector using the *Nco*I and *Xba*I sites (Guzman *et al*., 1995). The vector contained a Shine–Dalgarno sequence upstream of the translation start codon of the *galR* gene. The R282L mutation in the *galR* gene in plasmid pSEM1051 was created by PCR. The DNA sequence of the gene in the plasmid was verified. To create pSEM1029, the *Nhe*I–*Pst*I fragment of pSEM1026 containing the ribosome-binding site and the *galR* gene was inserted between the *Xba*I and *Pst*I sites of plasmid pEM7/Zeo (Invitrogen). Similarly, the *Nhe*I–*Pst*I fragment containing the R282L substitution from pSEM1051 was cloned into pEM7/Zeo to generate pSEM1050. Plasmid pSA886 ($O_EO_I^N$) was generated by replacing the *Bst*EII–*Hin*dIII fragment of pSA850 (Lewis and Adhya, 2002) by a PCR fragment, which contained a random 16 bp sequence (5′-CACTATGGCGAACGTC-3′) at the O_I operator site. This site will be referred to as O^N. It was shown by gel retardation assay that GalR does not bind to this sequence (data not shown). Similarly, the O_E operator of pSA850 was replaced by the same random 16 bp sequence to generate pSA887 ($O_E^NO_I$). Plasmid pSA907 contained a 5 bp (GATCT) insertion at +9 to generate a *Bgl*II site in pSA850. It was constructed by two overlapping PCR amplifications: (i) pSA509-1 (5′-GAGCTCGTCGACCCGGGTACCG-3′) and DLinsert 5D (5′-AATTCGCTCCATTAGGCAGATCTTATGGTATGAAATAACC-ATAGCA-3′); and (ii) DLinsert 5C (5′-TTTCATACCATAAGAT-CTGCCTAATGGAGCGAATTATGAG-3′) and BAMH-1 (5′-AAG-ACTCTATGGGATCCAGATAAGCGATAAGTTTGCTCAACATCT-TCTCGG-3′). Both PCR fragments were mixed and amplified with pSA509-1 and BAMH-1. The resulting product, which was digested with *Eco*RI and *Pst*I, was cloned into pSA850, which was also digested beforehand with *Eco*RI and *Pst*I.

Expression and purification of the His_6-tagged GalR protein

Escherichia coli strain DH5α bearing pSEM1026 or pSEM1051 was grown in superbroth containing 50 µg/ml ampicillin. After the optical density at 600 nm reached 0.4, the culture was induced with 0.2% arabinose for 5 h. The cells were harvested by centrifugation and stored at –80°C. The frozen cells were resuspended in 1/40 volume of lysis I buffer (50 mM sodium phosphate pH 8.0, 0.5 mg/ml lysozyme) and stored on ice for 30 min. An equal volume of lysis II buffer (50 mM sodium phosphate pH 8.0, 2 M NaCl, 8 mM imidazole, 20% glycerol, 1% Triton X-100) was added and incubated for 30 min on ice. The cell debris was removed by centrifugation at 10 000 *g* for 1 h. Addition of 3% Ni-NTA slurry (Qiagen) to the solution was followed by 1 h incubation at 4°C. A Poly-Prep Chromatography Column (Bio-Rad) was used to collect the protein

bound to Ni-NTA–agarose from the mixture. Twenty column volumes of washing buffer (50 mM sodium phosphate pH 8.0, 600 mM NaCl, 60 mM imidazole, 10% glycerol) were allowed to flow through the column. GalR was eluted by four column volumes of elution buffer (50 mM sodium phosphate pH 8, 600 mM NaCl, 10% glycerol) containing 250 and 500 mM imidazole for wild-type and mutant GalR, respectively, and stored at –80°C in 100 µl aliquots. Approximately 2.5 mg of protein was eluted from 1 ml of Ni-NTA–agarose in each case.

Chemical cross-linking

Repressor proteins were cross-linked using DMS (Pierce). Reactions were performed in HEPES pH 8.0 buffer containing 250 or 500 mM NaCl. The final concentration of the protein in the cross-linking reaction was 0.1 mg/ml. DMS was added at 2.5 mM concentration and the reactions were incubated at room temperature for 60 min. Reactions were terminated by addition of NuPAGE LDS sample buffer and Sample reducing agent (Invitrogen). Reaction products were separated on a 4–12% SDS–polyacrylamide gel and stained using the SilverQuest Silver Staining Kit (Invitrogen).

In vitro transcription

Transcription reactions were performed as described previously (Geanacopoulos and Adhya, 1997). The reaction mixture (50 µl) contained 20 mM Tris–acetate pH 7.8, 10 mM magnesium acetate, 100 mM potassium glutamate, 2 nM DNA template and 20 nM RNA polymerase. After incubation of the reactions at 37°C for 5 min, transcription was started by the addition of 1.0 mM ATP, 0.1 mM GTP, 0.1 mM CTP, 0.01 mM UTP and 5 µCi (for supercoiled template) or 20 µCi (for linear template) of [α-^{32}P]UTP (3000 Ci/mmol). The reaction was terminated after 10 min by addition of an equal volume of transcription loading buffer (0.025% bromophenol blue, 0.025% xylene cyanol, 0.01 M EDTA and 90% deionized formamide). After heating at 90°C for 3 min, the samples were loaded onto 8% polyacrylamide–urea DNA sequencing gels. To generate linear templates for transcription, pSA850 DNA was digested with *Hin*dIII, which cuts the plasmid 491 bp downstream of O_I. Successful linearization was verified by agarose gel electrophoresis.

DNA binding of GalR in the absence and presence of inducer

The 88 bp *Eco*RI–*Bgl*II DNA fragment containing the O_E operator from pSA907 was labeled with [α-^{32}P]dATP by end filling, separated by PAGE and purified using a Qiaex II gel extraction kit (Qiagen). Proteins (2.5 µg/ml) and labeled DNA fragments (2 pM) were mixed in the same buffer used for the *in vitro* transcription assay together with 10% glycerol and 2.5 ng/µl sonicated salmon sperm DNA. Aliquots of 19 µl of the binding reaction mixture were added to Eppendorf tubes containing 1 µl of D-galactose solutions to give the final concentrations of the inducer as indicated in Figure 6, and incubated at room temperature for 6 min. The reaction mixtures were loaded on a 4% polyacrylamide gel and electrophoresed with 1.5 mA/cm of gel in 1× TBE buffer for 2 h at room temperature. DNA bands were quantified using the ImageQuant™ PhosphorImager (Molecular Dynamics).

Assay of β-glucuronidase and β-galactosidase activities

Cells were grown overnight in LB medium and diluted 50-fold for further growth in M63 supplemented with 0.4% (w/v) D-fructose, 0.1% (w/v) casamino acids and 0.004% (w/v) vitamin B1. At various times, aliquots of cells were removed, pelleted and resuspended in M63 medium containing 100 µg/ml chloramphenicol, and stored on ice (Wilson *et al*., 1992). The optical densities of the cells were measured at 600 nm. The activity of β-glucuronidase from the *P2–gusA* fusion was determined by the Softmax microplate spectrophotometer system. Aliquots of 50 µl of permeabilization buffer [100 mM Tris pH 7.8, 32 mM sodium phosphate, 8 mM dithiothreitol (DTT), 8 mM CDTA and 4% Triton X-100] containing 200 µg/ml polymyxin B (Schupp *et al*., 1995) were placed in the wells of a microtiter plate, followed by the addition of 100 µl of cells. Cells were allowed to permeabilize at room temperature for 15 min before 50 µl aliquots of GUS assay buffer (0.5 mM DTT, 1 mM EDTA, 50 mM sodium phosphate pH 7) containing 1.25 mM α-*p*-nitrophenyl β-D-glucuronide were added (Wilson *et al*., 1992). The rate of β-glucuronide hydrolysis was determined at 405 nm and 37°C. The activity of β-galactosidase from the *P1–lacZ* fusion was measured as described above for β-glucuronidase activity except that 50 µl of *o*-nitrophenyl β-D-galactoside solution (4 mg/ml in 2 mM sodium citrate) was used as a substrate. The rate of β-galactoside hydrolysis was determined at 420 nm and 28°C.

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