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GalR mutants defective in repressosome formation

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Transcription repression of the galactose operon of *Escherichia coli* requires (1) the binding of the GalR repressor to tandem operators flanking the promoters, (2) the binding of histone-like protein, HU, to a site between the GalR-binding sites, and (3) negatively supercoiled DNA. Under these conditions, protein–protein interactions mediate the formation of a nucleoprotein complex in the form of a DNA loop, which we have termed a repressosome. To analyze the structure of the repressosome, we have screened and isolated *galR* mutants in which single amino acid substitutions in GalR lead to defects in loop formation while the protein's operator-binding activity is retained. The mutant proteins were purified and their properties confirmed in vitro. We verified that in the case of the two stronger mutations, the proteins had secondary structures that were identical to that of wild-type GalR as reflected by circular dichroism spectroscopy. Homology-based modeling of GalR by use of the crystal structures of PurR and LacI has enabled us to place the three sites of mutation in a structural context. They occur in the carboxy-terminal subdomain of the GalR core, are surface exposed, and, therefore, may be involved in protein–protein interactions. On the basis of our model of GalR and its structural alignment with LacI and PurR, we have identified additional residues, the substitution of which leads to a specific defect in repression by looping. The effects of the mutations are the same in the presence of HMG-17, a eukaryotic protein unrelated to HU, which can also mediate GalR-dependent repression of the *gal* promoter. This observation suggests that the mutations define sites of GalR–GalR interaction rather than HU–GalR interaction in the repressosome.

[*Key Words*: Repressosome; transcription; *galR* mutants; protein–protein interactions; DNA looping; GalR; repression]

DNA looping is a general phenomenon in the control of transcription in which two or more DNA-binding proteins recognize DNA sequences flanking a promoter and, by contacting each other, cause a reversible transition to a nucleoprotein complex in which the intervening DNA is looped out and the promoter activity is altered (Adhya 1989; Martin et al. 1990). The relative simplicity of these complexes in prokaryotes compared with their counterparts in eukaryotes has facilitated the investigation by genetic and physicochemical methods of questions critical to the understanding of regulation by DNA looping. Some of these questions are structural in nature: What are the protein–protein and protein–DNA contacts that stabilize these loops? What are the symmetries, if any, of the protein complexes? Other questions are framed in terms of thermodynamics and seek to understand the origin of the negative-free energy of the looped structure.

Critical mechanistic questions include how loop formation modulates transcription activity and by what means the stability of the loop is sensitive to environmental conditions.

We have used the nucleoprotein complex formed at the galactose (gal) operon of *Escherichia coli* by the *gal* operon repressor, GalR, and a bacterial histone-like protein, HU, as a model system for the investigation of the structure and function of a repression loop. GalR, a member of the LacI family of gene regulatory proteins (Weickert and Adhya 1992), binds as a dimer with high and approximately equal affinity to two operator sequences separated by 113 bp, encompassing the two *gal* promoters (Fig. 1A; Majumdar and Adhya 1984; Brenowitz et al. 1990). It lacks the tetramerization domain found in LacI and forms a tetramer in solution, if at all, with much weaker affinity (Majumdar et al. 1987). The formation of a loop absolutely requires HU, an abundant component of the bacterial nucleoid, which, in the presence of DNA supercoiling and occupancy by GalR of both operators, binds to a specific site, hbs, located between the operators (Aki et al. 1996; Aki and Adhya
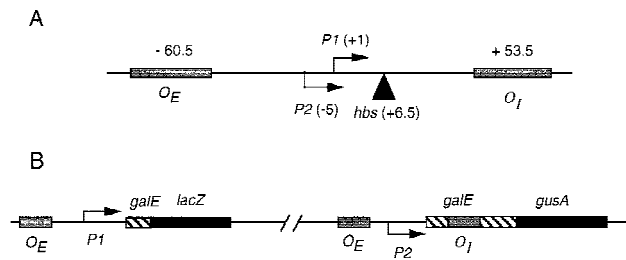
A

B

Figure 1. (*A*) Map of the *gal* promoter region showing the location of the *P1* and *P2* promoters, the tandem *gal* operators, and the center of the HU-binding site *hbs*. (*B*) Schematic drawing of the $O_E^+P2^-P1^+galE–lacZ$ and $O_E^+P2^+P1^-galEO_I^+–gusA$ fusions. The latter is present as a lysogen.

1997). We have termed the nucleoprotein complex that represses the *gal* promoters a repressosome, because its formation is by site-specific binding of a histone-like component of the bacterial nucleoid as well as a repressor (Adhya et al. 1998). In this report we describe a genetic screen to identify residues in GalR that are critical to repressosome formation and that we propose define sites of protein–protein contact between GalR dimers. We characterized the mutant proteins both in vivo and in vitro. Homology modeling of GalR with two highly conserved members of the LacI family for which the X-ray crystal structures have been reported, PurR and LacI, permitted us to place the mutated residues in a structural context and generate additional mutations that specifically disrupt loop formation. The usefulness of our findings in formulating a model for the structure of the repressosome is discussed.

Results

Genetic screen for galR *mutants defective in DNA looping*

To isolate mutations in *galR*, the gene encoding the repressor protein, that prevent the formation of a DNA loop while not affecting the interaction of GalR with the operator, we devised a genetic screen that made use of the fact that the two *gal* promoters, *P1* and *P2*, are regulated differently. GalR binding to the upstream *gal* operator, O_E, is sufficient for repression of *P1*. *P2*, located 5-bp upstream from *P1*, is activated under these conditions (Choy et al. 1997). The repression of *P2* requires the formation of a loop through simultaneous binding of GalR to both O_E and the downstream operator, O_I, as well as binding of HU to hbs. As shown schematically in Figure 1B, two chromosomal fusions were constructed in the reporter strain (DM0022). In one of these, the *gal* promoter was mutated to inactivate *P2* transcription while retaining *P1* transcription (Bingham et al. 1986) and fused to the *lacZ* gene such that O_I was deleted. This fusion, in which *lacZ* transcription from *P1* is repressed by GalR binding to O_E, was used to measure GalR-binding activity. To measure loop formation, the *gal* pro-

moter in a second fusion was mutated so that transcription occurred only from *P2* (Bingham et al. 1986) and joined to the *gusA* reporter gene such that O_I was retained. The efficacy of the promoter mutations in completely inactivating *P1* or *P2* while retaining transcription from the other promoter was verified by measuring RNA synthesis in vivo by primer extension. When cells containing a wild-type *gal* promoter were induced in the absence of CRP binding, the level of the expected 64- and 69-base products of reverse transcription from a primer complementary to *galE* was equivalent, indicating that the levels of *P1* and *P2* transcripts are equal (Fig. 2, lanes 3 and 4). The G-to-A transition at position –14 completely eliminated transcription from *P1* (Fig. 2, lane 8), whereas a G-to-T transversion at position –19 abolished transcription from *P2* (Fig. 2, lane 6). Galactose-sensitive repression is unaffected by both mutations (Fig. 2, lanes 5 and 7).

Localized mutagenesis of *galR* was performed by hydroxylamine treatment of a bacteriophage *P1* lysate of a strain in which *galR* was tightly linked to a kanamycin resistance cassette. The mutagenized phage lysate was used to transduce the *kan* allele to the reporter strain, DM0022. Screening was carried out for transductants that were derepressed for *gusA* expression, and, therefore, looping deficient but were still repressed for *lacZ* expression, indicating that GalR retained operator-binding activity. Of 6000 transductants screened, 350 colonies showed increased levels of *P2* transcription as measured by their blue color on indicator plates for β-glucuronidase, and these were assayed for expression of the *galP1–lacZ* fusion. Six of these mutants were found to be repressed for β-galactosidase expression to levels observed in a *galR*⁺ strain. Two mutants were identical and
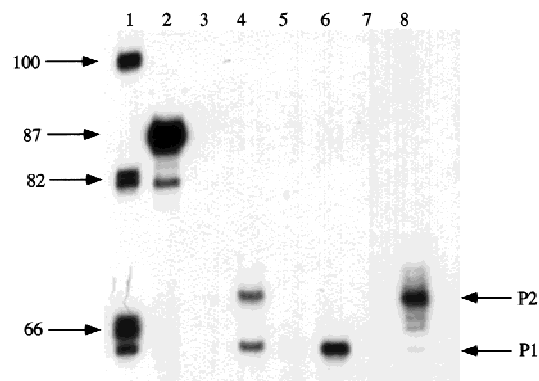
Figure 2. Effect of *gal* promoter mutations on in vivo transcription at *P1* and *P2* promoters. Primer extension of *gal*-specific DNA was performed in strain DM0013 containing the wild-type *gal* promoter (lanes *3,4*), DM0012 in which the *gal* promoter contains a G-to-T transversion at position –19 (lanes *5,6*), or DM0011 containing a G-to-A transition at –14 (lanes *7,8*). Cells were grown in the absence (lanes *3,5,7*) or presence (lanes *4,6,8*) of the inducer D-galactose. (Lane *1*) Phosphorylated DNA standards; (lane *2*) expected 87-base primer-extension product from a control reaction with a specific primer with homogeneous RNA.

coded for an aspartate-to-asparagine change at residue 258 (D258N). Two mutations resulted in redundant codons causing a lysine substitution at glutamate 230 (E230K). The remaining two mutants had a histidine substitution at arginine 325 (R325H), with one having an additional substitution of valine for isoleucine at position 323. The double mutant was not studied further.

In sequencing the mutants, we observed that the strain JT30 used to mutagenize *galR* contained an insertion of a *kan* cassette at position 1067 of the gene corresponding to residue 328. This insertion caused a very slight reduction in the efficiency of *P2* repression when compared with wild type (data not shown). We subsequently introduced an amber mutation in *galR* by site-directed mutagenesis so that the protein terminated at residue 328 and found that deletion of the 15 carboxy-terminal amino acids of GalR had little, if any, effect on nonlooping-mediated repression of *P1* or looping-mediated repression of *P2* (Table 1). The three mutations discussed above were cloned into a wild-type (full-length *galR*) background by site-directed mutagenesis and transferred to the chromosome. Their phenotypes are reported below.

The mutants were assayed under conditions of exponential growth for expression from the *P1* and *P2* *gal* fusions. As shown in Figure 3a, the D258N mutant of GalR was nearly completely defective for repression of *P2* expression exhibiting a level of β-glucuronidase activity that was comparable with that of the Δ*galR* strain. The *galR* mutants R325H and E230K showed derepression of *P2* expression to levels that were 78% and 50%, respectively of that of the Δ*galR* strain. In wild-type cells in the absence of the downstream *gal* operator, O_I, the *P1*

Table 1. *Effect of GalR mutations on operator interaction and looping-mediated repression*

GalR[a]	β-glucuronidase[b] $(O_E^+P2^+P1^-O_I^+–gusA)$	β-galactosidase[b] $(O_E^+P2^-P1^+–lacZ)$
Wild type	1.9	15
W165R	2	16
L166K	2.1	14
Q173A	2	18
Q174L	2.5	17
F216Q	3	10
G233N	3.5	10
T322R	50	16
N259M	30	13
H327R	50	15
S222E,Q226R	2.8	23
Δ329-343	2.8	21
E230K	15	19
D258N	72	18
R325H	64	17
galR[c]	2.5	45
Δ*galR*[c]	100	100

[a]GalR expressed from multicopy plasmids.
[b]Enzyme activities are expressed as a percent of that obtained in the Δ*galR* strain. The wild-type results are the mean of four separate experiments.
[c]*galR* or Δ*galR* in single chromosomal copy.

promoter as assayed by β-galactosidase activity was repressed such that expression was 40% of the level in the Δ*galR* strain, suggesting that the intracellular concentration of GalR is one that makes the fusion a sensitive reporter of affinity (Fig. 3b). Overexpression of GalR forms a multicopy plasmid in which *galR* is under the control of an IPTG-inducible *Trc* promoter-abolished expression of the *P1–lacZ* fusion (data not shown). The E230K mutant caused a slight increase in *P1* expression to 49% of that obtained in the Δ*galR* strain. D258N and R325H caused barely measurable increases to levels that were 41% and 44%, respectively, of the expression in the Δ*galR* strain (Fig. 3b). When the mutant and the wild-type GalR proteins were expressed from plasmids, all showed identical levels of repression of the *P1–lacZ* fusion, whereas the three mutants remained defective for repression of *P2* (Fig. 3c,d).

In vitro properties of GalR-looping mutants

Wild-type GalR and mutant GalR proteins E230K, D258N, and R325H were purified following overexpression to >95% homogeneity as described in Materials and Methods and assayed as regulators of in vitro transcription with a containing the wild-type *gal* promoter with both operators present as the template. The products of the transcription reactions after resolution by gel electrophoresis are shown in Figure 4. The results are expressed graphically in Figure 5. In vitro, the binding of GalR to O_E is sufficient for repression of transcription of *P1* and simultaneous activation of the *P2* promoter (Choy and Adhya 1993). At higher concentrations of GalR, transcription termination begins to occur at O_I, and, because the resulting short transcripts are not observed in 8% gels, the GalR titration appears biphasic. Looping-mediated repression of the promoters requires HU, which on supercoiled templates in the presence of GalR, binds to the DNA at *hbs* between the two operators. Figure 5a shows that in the absence of HU, wild-type GalR, E230K, and R325H repressed the *P1* promoter with approximately equal efficiency, whereas D258N required about twofold higher concentration to achieve the same level of repression. Wild-type GalR and all of the mutants activated transcription from *P2* approximately twofold (Fig. 5b). In the presence of 80 nM HU, the wild-type protein repressed both *P1* and *P2* efficiently with half-maximal repression of *P2* achieved at a slightly higher concentration than that required for *P1* (Fig. 5c,d). Under similar conditions, D258N and R325H behaved as they did in the absence of HU as activators of *P2* expression, although the level of activation was somewhat less. E230K, which had the smallest effect on *P2* repression in vivo, was only partially defective in HU-dependent repression of *P2* compared with the wild type.

We had determined previously that an abundant eukaryotic protein, HMG-17, although not affecting *gal* transcription by itself, can substitute for HU in mediating GalR-dependent repression of *P2* transcription (T. Aki, unpubl.). HMG-17 is an 89-amino acid DNA-binding protein of unknown structure that has been localized
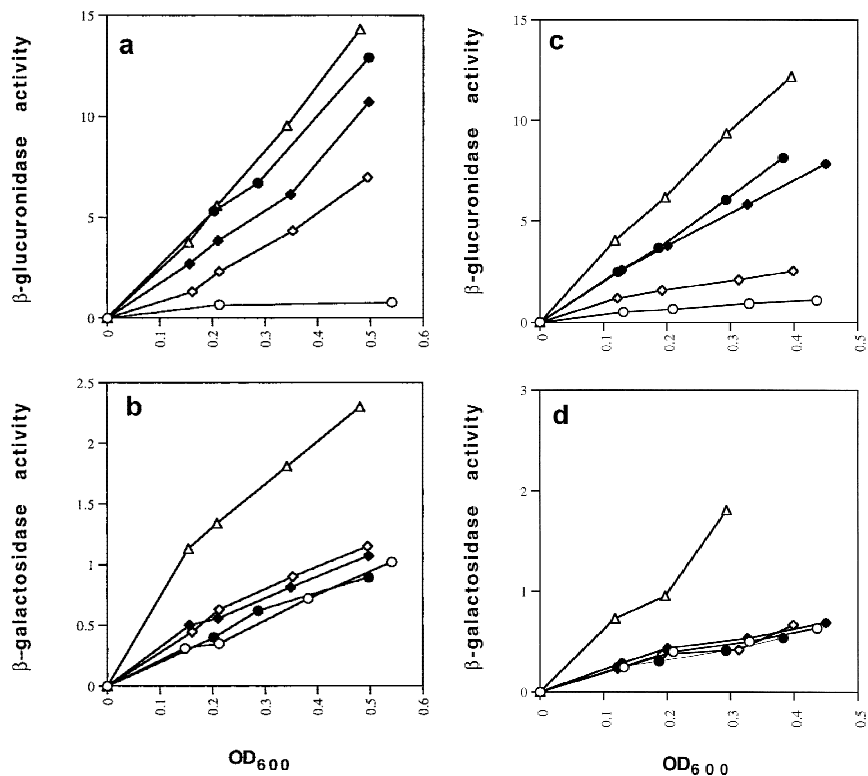
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Figure 3. Effect of GalR mutations E230K, D258N, and R325H on looping-dependent repression of *galP2* (*a*) and looping-independent repression of *galP1* (*b*). *galR*+ (○), *galR* D258N (●), *galR* E230K (◇), *galR* R325H (◆), and Δ*galR* (○) strains were grown in minimal medium and assayed for expression of the *P2–gusA* fusion containing both operators (*a*) and the *galP1–lacZ* fusion containing only O_E (*b*). β-Galactosidase and β-glucuronidase activities are expressed as the change in optical density/min during enzyme assay. (*c,d*) Expression of the *gusA* and *lacZ* reporter genes when the same set of *galR* alleles were expressed from a multicopy plasmid vector in a host deleted for *galR*.

to sites at which DNA exits from the nucleosome core (Bustin and Reeves 1996). In spite of their shared function, HMG-17 and HU have limited sequence homology (see below). As shown in Figure 5e, HMG-17 mediates complete repression of *P2* transcription in the presence of wild-type GalR. The defects in the mutant GalR proteins in repressing transcription from *P2* in the presence of HMG-17 were indistinguishable from those observed in the presence of HU; E230K was only moderately defective for *P2* repression, whereas D258N and R325H remained activators of *P2* transcription (Fig 5f). The significance of this finding with respect to the structure of the repressosome is discussed below.

Secondary structure of D258N and R325H

The mutations described above could indicate sites of protein–protein contact as long as they did not lead to large structural alterations. We used circular dichroism spectroscopy, which is sensitive to the secondary structure of proteins, to determine whether such alterations had occurred in the two mutants with the strongest derepression phenotype, D258N and R325H. As shown in Figure 6, the far-UV CD spectra of wild-type, R325H, and D258N proteins are identical, suggesting that these mutations did not detectably alter the GalR secondary structure.

Molecular modeling of GalR

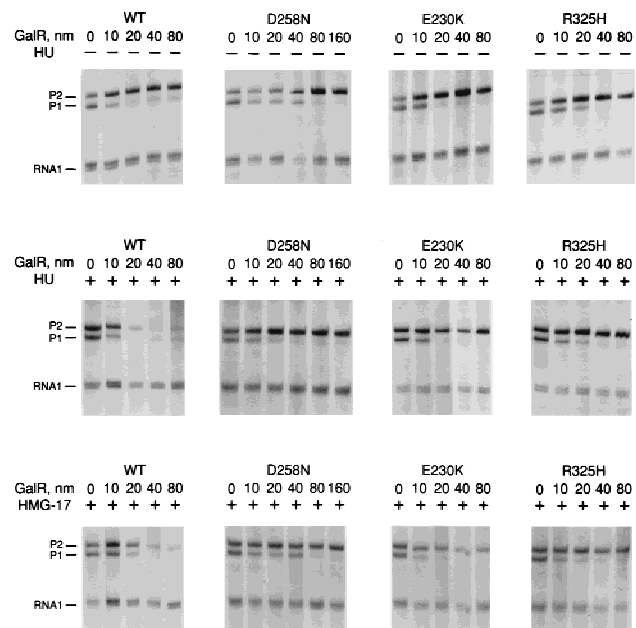The structure of GalR has not been determined experimentally. To situate the amino acid changes described

Figure 4. Transcription of *galP1* and *galP2* promoters on the supercoiled template pSA509 in the presence of wild-type GalR and GalR mutants E230K, D258N, and R325H. (*Top*) Titration with GalR proteins in the absence of HU or HMG-17; (*middle, bottom* the same reactions in the presence of 80 nM HU dimer or 500 nM HMG-17 (monomer), respectively. The 80-bp RNA 1 transcripts, which do not vary as a function of GalR concentration, served as an internal control between lanes.
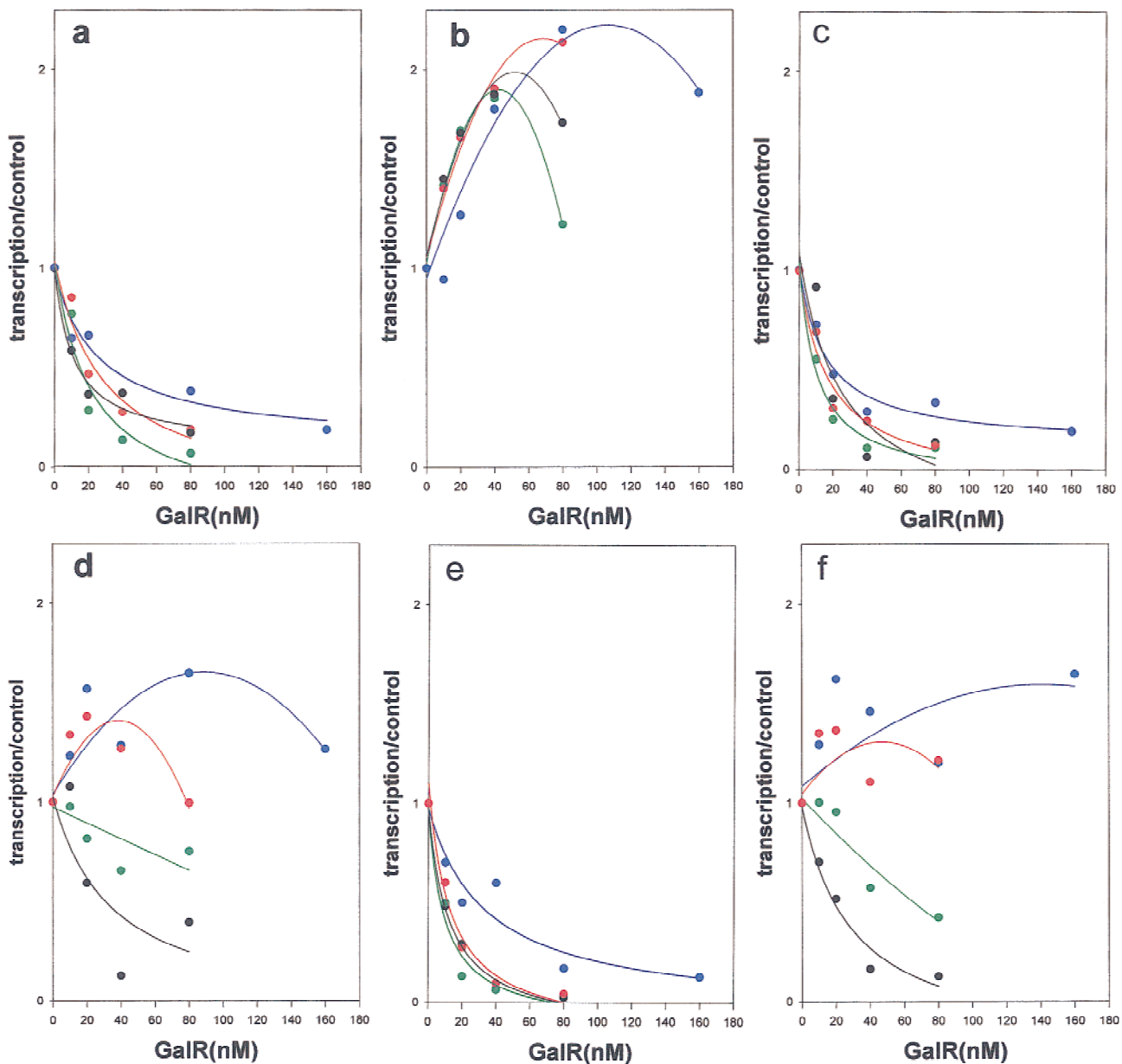
Figure 5. Regulation of the *galP1*(*a,c,e*) and *galP2* (*b,d,f*) promoters in the absence of cofactors (*a,b*) or in the presence of HU (*c,d*) or HMG-17 (*e,f*) by wild-type GalR (black), or GalR mutants E230K (green), D258N (blue), or R325H (red). RNA from gels in Fig. 4 was quantified with a PhosphorImager, and transcription is expressed as the fraction of that which was obtained in the absence of the repressor protein. Repression data for *P1* and for *P2* with wild-type GalR in the presence of HU or HMG-17 were fitted to the function $1/(1 + Kx)$ in which x is the concentration of the repressor and K is an apparent association constant. All other data were fitted to a second-order polynomial.

above in a structure, we modeled GalR by homologous extension using the X-ray crystal structures of two repressors, PurR and LacI (Schumaker et al. 1994; Lewis et al. 1996), which have a significant homology with GalR [31% and 27% sequence identity, respectively (Weickert and Adhya 1992)]. We confined our model to the core of the protein in which all of the mutations occurred. In members of the LacI family, the core is comprised of two globular subdomains (residues 52–343 in GalR) in which inducer binding and dimerization occur. It is separated

from the amino-terminal DNA-binding domain by trypsin cleavage. The cores of LacI and PurR have a high degree of structural similarity allowing a close structural alignment [root mean square deviation (RMSD) 1.1 Å]. This structural alignment can improve the sequence alignment of LacI and PurR, and, therefore, a more reliable sequence alignment of these two with GalR can be obtained.

The method used for modeling of GalR is that used for the modeling of the T-cell receptor from the structures of
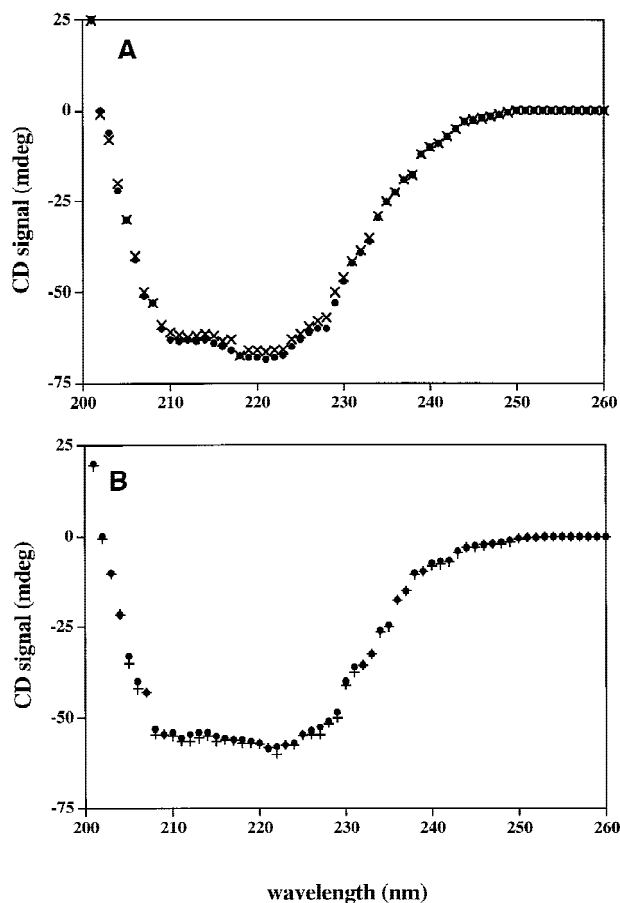
Figure 6. Far-UV CD spectra of GalR wild type (●) and GalR mutant D258N (×) (*A*), and GalR wild type (●) and GalR mutant R325H (+) (*B*).

ing to regions in which the backbone deviated <1.5 Å after superposition between PurR and LacI (the template structure) were used to form a portion of the GalR backbone. To fill in the gaps, we picked the corresponding segments from either PurR or LacI that have the same length as the GalR segments and pasted them on the modeled GalR backbone with priority given to the segments of PurR. If there was no appropriate segment from LacI or PurR, we filled the gap with the GalR segment using a loop-closure algorithm (Vasmatzis et al. 1996). The side chains of the final structure were then mutated to the corresponding side chains of GalR.

The GalR dimer was also modeled on the basis of the PurR and LacI structures. Both PurR and LacI dimerize in a similar manner with the two monomers in a symmetrical topology with an axis of symmetry that runs between the molecules along the larger dimension of the core. The PurR and LacI dimers superimpose with ~4 Å RMSD with each other. We assumed that the GalR dimer forms in a manner similar to the PurR dimer. Figure 6 shows the structure of the modeled GalR core with the mutated residues described above highlighted in red. The three residues are surface exposed and reside on the carboxy-terminal subdomain of the core.

Site-directed mutagenesis of GalR

The mutations we identified suggested that domains in the carboxy-terminal subdomain of the protein core made GalR–GalR and/or HU–GalR contacts in the repressosome. To obtain a clearer picture of these domains, we performed site-directed mutagenesis of amino acids, which roughly spanned a region defined by the initial three mutations. We attempted to introduce amino acids that would alter the surface of the GalR dimer but not disrupt the structure of the protein. Utilizing the structural alignment of the PurR and LacI crystal structures and the GalR model described above, we mutated the surface residues in GalR to only those amino acids that were found at corresponding positions (i.e., they were superimposed) in PurR or LacI after structural alignment. For example, the nonconservative substitution of arginine for threonine at position 322 in GalR might disrupt any protein–protein contact in which this residue was involved; however, because the structural alignment we performed indicates that arginine occurs at the corresponding structural position in LacI, its substitution for threonine in GalR would be unlikely to disrupt the overall structure. Ten such substitutions carried on a plasmid vector containing the *galR* gene and its promoter were assayed in the double-reporter strain deleted for *galR* (Table 1). In one case, a double mutation was made so that a close interaction between glutamate 223 and arginine 227 of PurR was preserved. All of the mutants were indistinguishable from wild-type GalR for repression of the *P1–lacZ* fusion, suggesting that the mutations did not destabilize the protein or alter the operator-binding activity to an appreciable extent. Three of the site-directed mutants, N259M, T322R , and H327R derepressed expression

immunoglobulins (Vasmatzis et al. 1996) and consists of the following steps. First, the LacI and PurR amino acid sequences were aligned by the BESTFIT program of the GCG Wisconsin Sequence Analysis Package. This tactic permitted an initial structural alignment in which the backbone atoms of all of the aligned positions of LacI were superimposed on the backbone atoms of PurR. This operation was performed by minimizing the RMSD of the backbone atoms at the aligned positions with a rotation/translation algorithm (Cornette et al. 1993). This initial alignment was not optimal because of the presence of mismatches in the sequence alignment, and therefore, using the aligned structures, we constructed a new sequence alignment that made use of the structural alignment. A second structural alignment was then performed on the basis of the aligned positions in the new sequence alignment. The structures of the cores of LacI and PurR were found to be very similar (1.1 Å RMSD) except for four short segments in domain 2 where the ligands bind and for the carboxyl terminus of the proteins where the unique tetramerization domain of LacI is found. In all other domains, the proteins are highly similar, and we expect that the predicted model of GalR will be more accurate. The PurR backbone atoms correspond-

from the *P2* promoter and are shown in orange in Figure 7. Mutations that had no discernible affect on repression are shown in green. As indicated in Figure 7, residues 259 and 327 are immediately adjacent to 258 and 325, respectively, according to the model of GalR. T322 is located on the same face of the carboxy-terminal subdomain as the other mutations, but somewhat farther from the DNA distal edge of this surface. Because the expression of the *P1–lacZ* fusion appears to be a more sensitive assay of GalR-binding activity when *galR* is present in a single chromosomal copy (Fig. 3a,c), we tested the looping-defective mutants, N259M, T322R, and H327R after transfer to the chromosome. All three mutants were in-

Figure 7. Model of the GalR dimer showing the location of mutations affecting repressosome formation in GalR. The dimeric of the GalR core (above the gray line) was modeled by homologous extension with the PurR and LacI structures. The DNA-binding domain and DNA from the PurR X-ray structure (Schumacher et al. 1994) are shown (below gray line) for reference. The β-carbon positions of the amino acids identified in our screening are shown in red. The location of the site-directed mutations, which specifically disrupted looping-mediated repression, are shown in orange. Shown in green are the α carbons of the nonconservative site-directed substitutions that had no effect on looping-mediated repression. Note that because the structure has C2 symmetry, the same arrangement of residues (not highlighted) occurs on the opposite face of the molecule. Carboxy-terminal residues 329–343, which could not be modeled by homologous extension and whose deletion did not have a marked effect on repression, are not shown.
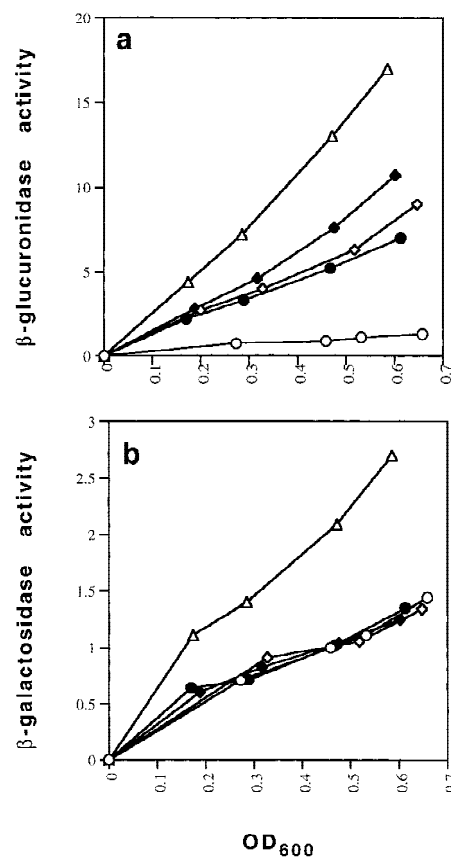

Figure 8. Effect of the site-directed chromosomal *galR* mutations N259M (●), T322R (◇), and H327R (◆) on looping-dependent repression of *galP2* (*a*) and independent repression of *galP1* (*b*) compared with that obtained in wild-type (○) and *galR* deletion (◇) strains.

distinguishable from the wild type for *P1* repression while showing pronounced defects in *P2* repression (Fig. 8).

Discussion

Evidence for the existence of a DNA loop encompassing the *gal* promoter has been obtained by several different means of investigation. In vitro, the repression of *galP2* depends on GalR binding to both operators, and HU binding to a specific site with *hbs* between them (Aki et al. 1996). Deletion of either operator abolishes HU binding and repression, a finding that indicates that tripartite cooperativity among the protein components of the loop leads to the formation of a new structure (Aki and Adhya 1997). The requirement of HU and negative supercoiling for *P2* repression in vitro is also observed in vivo. In a Δ*hupA*Δ*hupB* strain, the *P2* promoter is completely derepressed, and addition of the DNA gyrase inhibitor coumermycin, although not affecting the intrinsic activity of the promoter, also leads to complete derepression, suggesting that the structure that forms in the reconstituted system is similar to the intracellular one (Lewis et

al. 1999). Atomic force microscopy with a supercoiled minicircle as the DNA template has allowed the direct visualization of a DNA loop comprising HU and GalR (Lyubchenko et al. 1997). The proteins in the loop are visualized as bright blobs at the intersection of the DNA strands, and the size of this loop indicates that the two operators are brought into close proximity where two DNA strands cross over one another.

The genetic screen described above was undertaken to identify amino acid residues in GalR that might make protein–protein contacts in the repressosome. The purified mutant GalR proteins behaved qualitatively differently from the wild type in a way that would be expected of mutants with a specific defect in loop formation. E230K and R325H repressed *P1* in the absence of looping with approximately the same concentration dependence as the wild type protein; approximately twofold more of D258N was required to achieve the same level of repression as the wild type. However, even at the highest concentrations, D258N and R325H failed to repress *P2*. The mutant E230K, which had the weakest effect on *P2* repression in vivo, was only slightly defective in *P2* repression in vitro. In the case of the two mutants D258N and R325H, which had the strongest *P2* derepression phenotypes, we addressed the question as to whether the secondary structure of the proteins was maintained by circular dichroism spectroscopy. This technique has proven useful in determining which among cooperative mutants of bacteriophage λ cI repressor have disrupted structures (Deb et al. 1998). We found that the GalR mutant proteins were unchanged relative to the wild type within the limits of detection of this technique, suggesting that the altered amino acids may correspond to surface-exposed residues making protein–protein contacts in the repressosome.

A molecular model of the GalR core without the DNA-binding domain has been reported on the basis of the structure of the periplasmic glucose/galactose-binding protein (Hsieh et al. 1994). The subsequent reports of the X-ray crystal structures of the PurR and LacI repressors that have significantly higher homology to GalR (31% and 27%, respectively, vs. 21% for the sugar transport protein) led us to construct a new model by homology extension to situate the mutations within a structure with the greatest possible accuracy. The alternative models derived from LacI and PurR or from the sugar-binding protein are very similar; all of the building units (helices, strands, and coils) match structurally. However, there are differences in the alignments proposed by the models, which lead to changes in the orientation of some amino acids within structural domains. According to our model (Fig. 7), the mutant amino acids identified by our screening are exclusively on the surface of the protein. They reside in regions of high structural similarity in LacI and PurR, in which the predicted structure of GalR would be most accurate. All of the amino acids are in the carboxy-terminal subdomain of the protein core—away from the DNA-binding domain, the inducer binding domain, and the dimerization interface. In LacI, tetramerization and therefore, DNA looping, is mediated by a flexible domain that extends from the carboxy-terminal β strand. GalR contains 15 amino acids extending from the this β strand to which we did not assign structure because of the absence of structural conservation in this region in PurR and LacI. The mutations we have described are not in this domain, and deletion of the 15 carboxy-terminal amino acids of GalR by insertion of a stop codon at position 328 did not increase expression of the *P2–gusA* fusion to a level comparable with that obtained with the looping-defective mutations (Table 1). The results obtained with the mutations created by site-directed mutagenesis support the idea that D258N and R325H are involved in protein–protein contacts. Substitution of N259 immediately adjacent to D258 (Fig. 7) with methionine had a dramatic effect on in vivo *P2* repression as did replacement of H327, a residue that is very close to R325, with arginine. Neither of these GalR mutants, when expressed in single copy, affected the operator binding of GalR as reflected by repression of the *P1–lacZ* fusion (Fig. 8b). As evident in Figure 7, if the core of the dimer is approximated as a hexahedron in the shape of a flat box, all of the mutations that appear to specifically affect repressosome formation reside on the large faces distal to the DNA-binding region. On each face of the dimer, three of the residues that appear to be critical for loop formation are contributed by one of the monomers and three by the other monomer.

We have addressed the question as to whether the mutations cause a defect in a GalR–GalR contact or a GalR–HU contact by creating conditions in which GalR-dependent, looping-mediated repression of *P2* occurred in the absence of HU. We observed previously that HMG-17, a eukaryotic DNA-binding protein of unknown structure that associates with the nucleosome core could substitute for HU in permitting GalR-dependent repression of transcription from *P2*. HMG-17 has very low homology with either subunit of the HU heterodimer. HU-1 (*hupB*) and HU-2 (*hupA*), the two homologous subunits of the HU heterodimer, were compared with HMG-17 using GAP of GCG Lite. The two subunits have an identity of 11% and 13%, respectively, with HMG-17. Under conditions of in vitro transcription, GalR mutants did not discriminate between HU and HMG-17; each mutation caused an effect that was of similar magnitude whether HU or HMG-17 was the added cofactor. If one assumes that the mutations define sites of GalR–HU contact, then these results would suggest that HU and HMG-17 contact the same surface of GalR to create interfaces that respond identically to GalR mutations. This scenario seems unlikely given the lack of sequence homology between HU and HMG-17. The most likely explanation for the results is that, in both cases, the mutations are disrupting GalR–GalR contacts. It may be significant that a surface defined by all six residues that were found to specifically affect *P2* repression can be brought into close proximity with its counterpart on an opposing GalR dimer. Furthermore, this can be done in a manner that allows the bound DNA strands to crossover at a point the distance of which from the operators is consistent with the size of the repression loop measured by atomic

force microscopy. Whether the extremely high affinity of HU for *hbs* (Aki and Adhya 1997) is explained by specific contacts with GalR remains to be determined.

The *gal* repressosome appears to be stabilized by relatively weak protein–protein interactions; GalR appears to be a dimer at micromolar concentrations and lacks the tetramerization domain of LacI. The requirement of negative supercoiling and HU for loop formation may allow regulation of the operon by two additional modes beside concentration of the inducer, D-galactose, namely DNA supercoiling and HU concentration. This could be important because the *galE* gene product, uridinediphosphogalactose-4-epimerase, is required for anabolic functions as well as galactose catabolism. Interestingly, the expression of HU is subject to catabolite repression, whereas its α and β subunits are differentially regulated by the gene-regulatory protein FIS (Claret et al. 1996). Supercoiling in *E. coli* is also highly dependent on growth conditions (Balke and Gralla 1987). The question as to the structure of a repression complex, the formation of which so completely depends on an abundant component of the bacterial nucleoid as well as DNA topology, warrants continued investigation.

Materials and methods

Bacterial strains, plasmids, and bacteriophage

A list of the bacterial strains, phages and plasmids used in this study is given in Table 2. The $O_EP2^+P1^-O_\Gamma$–*gusA* and $O_EP2^-P1^+$–*lacZ* fusions were constructed from plasmid pI24, which contains the *gal* operon (*galETK*) as follows: 130 bp *Eco*R1–*Bst*EII fragments from pAA121(p14A) and pAA121(p19T), the kind gift of Steve Busby, were used to replace the wild-type *gal* promoters with $P2^+P1^-$ (pSA805) or $P2^-P1^+$ (pSA806) promoters. The *gusA* gene was amplified from pRAJ275 with PCR primers designed to create a *Hin*dIII site followed by a stop codon and then a Shine-Delgarno sequence at the 5′ end and a *Bsp*EI site at the 3′ end. It was cloned into pSA805 to create a transcriptional fusion with the first 414 bases of *galE* under control of the *galP2* promoter (pSA809). The *lacZ* gene minus the first 21 bases was amplified with PCR primers so that the 5′ and 3′ ends of the amplified fragment contained a *Bst*EII site and a *Bsp*EI site, respectively. Cloning of the restricted fragment into pSA806 created a translational fusion of *lacZ* with the first 12 bases of *galE* in which transcription occurred exclusively from *galP1*(pSA808). The *P2galE*–*gusA* fusion was subcloned to pTS8 (pSA814), which permitted its transfer to a λ phage. Transfer of the fusions to λ phages and to the chromosome was as outlined in Figure 9. Cells into which the *galE::lacZ* fusion had been transferred by two homologous recombination events between λY3001 and the chromosome were resistant to high temperature and sensitive to chloramphenicol. The *P2*–*gusA* fusion was transferred to the cell by site-specific recombination of λB3000 at the λ attachment site. Single lysogens were selected by assaying for low levels of induction by mitomycin. They were also differentiated from multiple lysogens by assaying β-glucuronidase activity, which increased as a linear function of the number of phage that had integrated into the chromosome.

The three *galR* mutations that were recloned into a wild-type (full-length *galR*) background were transferred to the reporter strain, DM0022, by the same method as described for the *galP1*–*lacZ* fusion except that the *galR* mutations were initially trans-

ferred into a non-lysogenic strain followed by transduction by bacteriophage *P1* of the *galR* allele into DM0022.

Assay of β-galactosidase and β-glucuronidase activity

Cells were grown overnight in Luria–Bertani medium and diluted 50-fold for further growth in M63 salt solution supplemented with 0.4% (wt/vol) D-fructose, 0.1% (wt/vol) casamino acids and 0.004% (wt/vol) vitamin B1. At various times, aliquots of the cells were washed in the same medium containing 100 μg/ml chloramphenicol and stored on ice (Wilson et al. 1992). β-Glucuronidase or β-galactosidase activities were measured for all cultures simultaneously by the Softmax microplate spectrophotometer system. Aliquots of 100 μl from cell cultures were added to the wells of a microtiter plate followed by solubilization in 50 μl of permeabilization buffer [100 mM Tris-HCl (pH 7.8), 32 mM NaPO₄, 5 mM DTT, 5 mM CDTA, 4% (vol/vol) Triton X-100, and 200 μg/ml polymixin B (Schupp et al. 1995)]. After incubation at room temperature for at least 10 min, 50 μl of substrate (4 mM α-p-nitrophenyl β–D–glucuronide for assay of β-glucuronidase or 16 mM o-nitrophenyl β-D-galactoside and 2 mM sodium citrate for assay of β-galactosidase) was added to each well. β-glucuronidase activity was measured at 405 nm and 37°C; β-galactosidase at 420 nm and 28°C.

Primer extension

RNA purification was performed as recommended in the RNeasy Midi Protocol (Qiagen). A ³²P-labeled probe complementary to *galE* mRNA from +48 to +64 was used in reverse transcription as outlined in the Promega Primer System. Products were resolved on 8% DNA polyacrylamide–urea sequencing gels.

GalR purification

Wild-type and mutant *galR* genes were cloned into the pPro-EXHta expression plasmid so that the amino terminus of the protein minus the amino-terminal methionine was fused to a cleavable sequence containing six histidines. After overexpression, cells were lysed by French press and the histidine-tagged GalR was bound to Ni-NTA resin and eluted with a buffer containing imidazole followed by desalting as described previously (Polayes 1996). The histidine sequence was removed by incubation of 1 mg of the purified protein in 1.5 ml of buffer [50 mM Tris-HCl (pH 8.0), 0.5 mM EDTA, 1 mM DTT, and 5% glycerol] which contained 300 units of recombinant TEV protease, for 1 hr at 4°C followed by 10 min at 30°C.

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, 1.0 mM ATP, 0.1 mM GTP, 0.1 mM CTP, 0.01 mM UTP, and 10–20 μCi of [α-³²P]UTP (3000 Ci/mmole). After incubation of the reactions at 37°C for 5 min, RNA polymerase was added to a concentration of 20 nanomolar. Reactions were terminated after 10 min by addition of an equal volume of RNA loading buffer [80% vol/vol deionized formamide/ TBE (89 mM Tris borate, 2 mM EDTA), 0.025% bromophenol blue, 0.025% xylene cyanol]. After heating at 90°C for 5 min, the samples were loaded onto 8% DNA polyacrylamide–urea sequencing gels. Radioactive transcription products were quantified with a PhosphorImager. RNA bands were normalized for varying background by subtrac-

Table 2. *Bacterial strains, phage, and plasmids*

	Relevant genotype	Source
Strains		
MG1655	F⁻	S. Adhya
CH1105	MG1655 Δ*lacZM15*	S. Adhya
CH1200	CH1105 Δ*cya854*	S. Adhya
MG0002	CH1105 Δ(*manA–uidA*) *1298 zdg-232*::Tn*10*	this study
MG0003	CH1105 *zdg-232*::Tn*10*	this study
DM0007	MG0002 Δ*galE::cat*	this study
DM0008	CH1200 Δ(*manA–uidA)1298 zdg-232*::Tn*10*	this study
DM0009	DM0008 Δ*galE::cat*	this study
DM0011	DM0009 [λ B3001]	this study
DM0012	DM0009 [λ B3002]	this study
DM0013	DM0009 [λ B3003]	this study
DM0018	DM0009 [λ*P1galE–lacZΔ(galT)galK*]	this study
DM0021	DM0009 *P1galE–lacZΔ(galT)galK*	this study
DM0022	DM0021 [λ B3001]	this study
DM0026	DM0022 Δ*galR::cat*	this study
JT7	*galR*⁺[λ Δ*galR::cat*]	S. Adhya
JT30	Δ*galR::kan*	S. Adhya
JT32	Δ*galR(A–B)::kan*	S. Adhya
DM0081a	DM0022 Δ*galR(A–B)::kan*	this study
DM0081b	DM0081a*galR*–D258N	this study
DM0082	DM0081 a*galR*–R325H	this study
DM0083	DM0081a*galR*–E230K	this study
DM0108	DM0081a*galR*–N259M	this study
DM0109	DM0081a*galR*–T322R	this study
DM0110	DM0081a*galR*–H327R	this study
Phage		
Y392	λ*c1857 gal*⁺*int*⁻	this study
Y3001	λ*P1*⁺*P2-galE–lacZ(galT)galK*	this study
B3000	λ *imm21 galK*⁺ Δ*att P′ P*⁺	this study
B3001	B3000 *P2*⁺*P1*⁻*galE–gusA bla*	this study
B3002	B3000 *P2*⁻*P1*⁺*galE–gusA bla*	this study
B3003	B3000*P2*⁺*P1*⁺*galE–gusA bla*	this study
W30	λ*cI*⁻	NIH collection
W248	λ*cI*⁻*h*⁸⁰	NIH collection
B10	λ*imm21 cI*⁻	NIH collection
B 482	λ*imm21 cI*⁻*h*⁸⁰	NIH collection
Plasmids		
pAP1	pPROEX *galR*⁺	this study
pI24	pBR322Δ(*Eco*RI–*Pvu*II) *gal*⁺	S. Adhya
pRAJ275	*gusA*⁺	this study
pAA121(p14A)	*P2*⁺*P1*⁻*galEK*	S. Busby
pAA121(p19T)	*P2*⁻*P1*⁺*galEK*	S. Busby
pSA805	pI24Δ(*Eco*RI–*Bst*EII) *galP2*⁺*P1*⁻	this study
pSA806	pI24Δ(*Eco*RI–*Bst*EII) *galP2*⁻*P1*⁺	this study
pSA809	pSA805(*Hind*III–*Bsp*EI)*gusA*⁺	this study
pSA808	pSA806(*Bst*EII–*Bsp*EI)*lacZ*⁺	this study
pTS8	*P1*⁻*P2*⁻*gal ETK P*⁻*lacZ* λ*attPOP′int*⁺*bla*	this study
pSA814	pTS8 Δ (*Pst*I–*Bsp*EI) *P2*⁺*P1*⁻*galE–gusA*	this study
pMT4	pBR322 Δ (*Pst*I–*Eco*RI) *galR*(A–B)::*kan*	J. Tokeson

tion of the total radioactivity of an equal area immediately above or below the band of interest. The *RNA1* transcript originating from the plasmid, which was not affected by GalR, HU, or HMG-17 addition, served as an internal control between lanes.

Mutagenesis of galR

GalR was mutagenized by treatment of bacteriophage *P1* lysate of a strain (JT30) in which *galR* was closely linked to a *kan* allele

with hydroxylamine as described previously (Hong and Ames 1971). Mutagenesis was allowed to proceed at 37°C until the transducing titer was reduced 100-fold (~20 hr). Transduction of the *kan* allele to the reporter strain, DM0022, was carried out by standard methods. Cells were plated on minimal glucose kanamycin plates supplemented with 0.1% casamino acids to which 5-bromo-4-chloro-3-indolyl-*N*-actyl-β-D-glucuronide had been added to a concentration of 50 µg/ml. Site-directed mutagenesis of *galR* was performed with the Quick-Change site-directed mu-
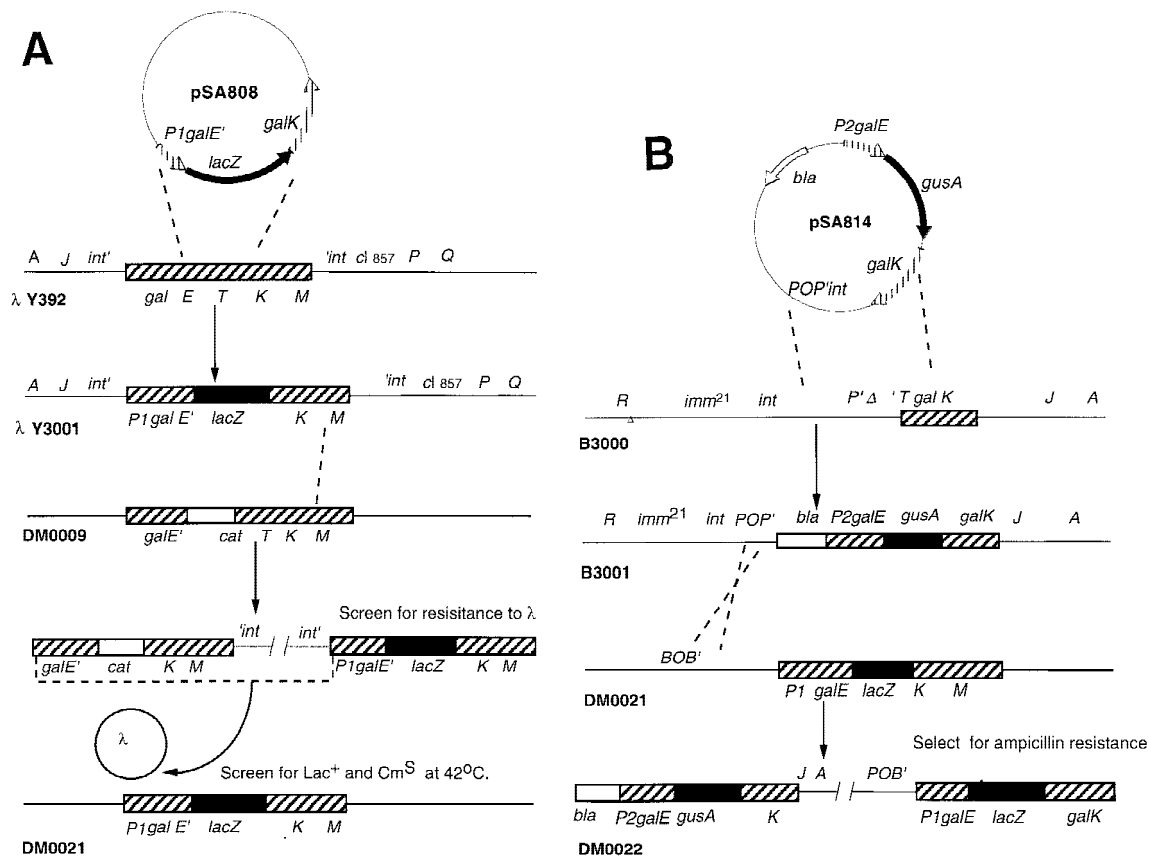
Figure 9. Construction of the dual reporter strain. (*A*) Transfer of the *galP1-lacZ* fusion to the chromosome by homologous recombination. (*B*) Construction of a second reporter fusion by homologous recombination and integration into the bacterial chromosome at the λ att site. Details are given in Materials and Methods.

tagenesis kit from Stratagene. All mutants were sequenced by standard methods.

Circular dichroism

Circular dichroism spectra were obtained in a JASCO J-600 spectropolarimeter with a 0.1-cm pathlength cuvette. The concentrations of mutant and wild-type GalR were adjusted to ~5 micromolar as measured by absorbance at 280 nm. Measurements were performed in a buffer containing 0.02 M Tris-HCl (pH 8.5) and 15% glycerol.

Molecular modeling of GalR

For the sequence alignment and similarity calculations, we used the package Bestfit program (http://molbio.info.nih.gov/molbio/gcglite/compare.html; Needleman and Wunsch 1970). Parameters used in the alignment program were as follows: gap weight 3.000, average match, 0.540, length weight 0.1, and average mismatch –0.396. The structural alignment algorithm (Cornette et al. 1993), and both the Monte Carlo algorithm for loop closure and the homologous extension procedure (Vazmatzis et al. 1994) have been described previously.

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