

Recruitment of HU by piggyback: a special role of GalR in repressosome assembly

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In Gal repressosome assembly, a DNA loop is formed by the interaction of two GalR, bound to two distal operators, and the binding of the histone-like protein, HU, to an architecturally critical position on DNA to facilitate the GalR–GalR interaction. We show that GalR piggybacks HU to the critical position on the DNA through a specific GalR–HU interaction. This is the first example of HU making a specific contact with another protein. The GalR–HU contact that results in cooperative binding of the two proteins to DNA may be transient and absent in the final repressosome structure. A sequence-independent DNA-binding protein being recruited to an architectural site on DNA through a specific association with a regulatory protein may be a common mode for assembly of complex nucleoprotein structures.

[Key Words: Transcription repression; DNA looping; protein–protein interaction; HU mutants]

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Nonspecific DNA-binding proteins are ubiquitous among all organisms. These proteins have wide-ranging effects on DNA conformation, including bending, supercoiling, and compaction (Drlica and Rouviere-Yaniv 1987; Ner et al. 1994; Werner and Burley 1997; Thomas and Travers 2001). The archetype of prokaryotic histone-like protein, HU, binds to DNA with little or no sequence specificity, although it has a preference for DNA containing cruciforms or single-stranded breaks (Bianchi 1994; Nash 1996). HU also bends the DNA to which it binds. The bending induced by HU facilitates optimal protein–protein and/or protein–DNA contacts in an otherwise physically recalcitrant structure, thereby orchestrating the regulated assembly of functional nucleoprotein complexes, such as those involved in DNA replication, recombination, transposition, transcription, and DNA repair (Skarstad et al. 1990; Dri et al. 1992; Haykinson and Johnson 1993; Manna and Gowrishankar 1994; Li and Waters 1998). By playing an architectural role, HU configures and stabilizes DNA conformations required for sustaining a higher-order complex for active DNA metabolic transactions. Consistent with the idea of HU being a passive architectural partner in the nucleoprotein complexes, there is little evidence of HU being involved in any direct contacts with other proteins.

Regulation of transcription initiation in the *gal* operon of *Escherichia coli* involves a DNA–multiprotein complex, called repressosome, in which HU plays an essential role. The *gal* operon is driven by two partially over-

lapping promoters, *P1* and *P2* (Fig. 1). The binding of two repressor (GalR) dimers to the two spatially separated operators, *O_E* and *O_I*, and of HU to a site (*hbs*) in between the two operators in negatively supercoiled DNA forms the Gal repressosome, containing a DNA loop. DNA looping, a consequence of interaction between the operator-bound GalR dimers, engenders inhibition of transcription from both promoters (Aki and Adhya 1997; Geanakopoulos et al. 1999; Lewis et al. 1999). The role of HU in the formation of the repressosome is distinguished by the following features (Aki and Adhya 1997): (1) Binding of HU to the *hbs* occurs at 20-fold lower concentration than its affinity of 10⁻⁷M for DNA (Cann et al. 1995). Once bound, HU cannot be competed out of the repressosome by excess unbound HU or heparin, indicating that HU is a stable component of the final nucleoprotein complex, unlike in the HU-containing Mu transposome (Lavoie and Chaconas 1990) or Hin invertosome (Paull et al. 1993) structures. (2) HU is specific in Gal repressosome and cannot be replaced by other bacterial histone-like proteins such as IHF, HNS, or Fis, although it can be replaced by one of the eukaryotic high-mobility group proteins, HMG-17, at a 10-fold higher molar concentration; successful substitution of HU with other bacterial architectural proteins has been shown in *hin* inversion and Mu DNA transposition (Lavoie and Chaconas, 1994; Paull et al. 1994). Conversely, homologous repressors, like GalS and LacI, when provided with their cognate operators, cannot replace GalR in bringing about HU binding. (3) Finally, there is a tripartite cooperativity between GalR and HU in binding to *gal* DNA; binding of HU to *hbs* is absolutely dependent on binding of GalR dimers to both operators and HU binding, in turn, results in increasing the strength of GalR binding.

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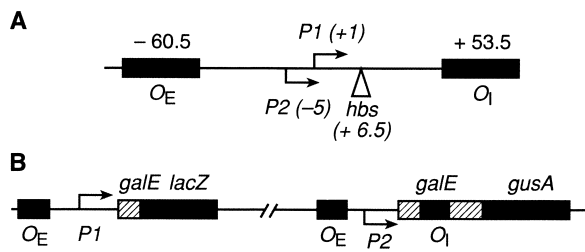


Figure 1. (A) Map of the *gal* promoter region showing the location of the *P1* and *P2* promoters and the HU binding site, *hbs*. (B) Schematic diagram of the $O_E^+ P2-P1^+ O_I^- lacZ$ and $O_E^+ P2^+ P1^- O_I^+ gusA$ fusions.

The binding of GalR dimers to O_E and O_I in the absence of HU is noncooperative (Brenowitz et al. 1990). The synergistic binding of GalR and HU, the strength of HU binding, and the specificity of GalR and HU in the repressosome may originate from a functional interaction between GalR and HU. In this paper, we provide structure-based genetic and biochemical evidence to show a specific and functional GalR–HU interaction, both in vivo and in vitro.

Results

Structure-based genetic analysis of HU α

In *E. coli*, HU is a heterodimer of two highly homologous subunits, HU α and HU β . Previous studies showed that the deletion of either of the corresponding genes, *hupA* or *hupB*, did not affect the repression of the *gal* promoters, suggesting that either HU homodimer can substitute functionally for the heterodimer (Aki et al. 1996; Lewis et al. 1999). To study any critical GalR–HU interaction, we performed site-directed mutagenesis of the *hupA* gene to identify any HU α mutants that would be specifically defective in the formation of the repressosome while retaining their ability to bind to DNA with normal affinity. Based on a modeled structure of *E. coli* HU α homodimer derived from the *Bacillus stearothermophilus* HU homodimer X-ray and nuclear magnetic resonance structure (Tanaka et al. 1984; Vis et al. 1995), we identified 10 amino acid residues for targeted mutagenesis, following two criteria: (1) the amino acid residues were surface exposed, and (2) they were not on the DNA-binding β -sheet arms or the dimerization interface of the protein (Nash 1996). The amino acid residues at the chosen positions were substituted by residues found at corresponding positions in HU of other bacterial species. The high degree of homology between their primary structures helped us to select amino acids from analogous positions in different species of HU for substitution in HU α , so as to minimize the possibility of destabilization of the mutant proteins (Bordo and Argos 1991). The list of native and substituted amino acid residues in HU α is in Table 1.

HU α mutants defective in repression of *gal* promoters

The ability of the different HU α mutants to repress tran-

scription of the *gal* operon by the formation of the DNA loop was determined in vivo in the reporter strain SK22, which carries a chromosomal fusion of the *P2* promoter of *gal* to the reporter gene *gusA*. The *P1* promoter was inactivated by mutation. In the presence of GalR and HU α , β -glucuronidase synthesis is repressed from the *P2*–*gus* fusion (Lewis et al. 1999). The level of expression of β -glucuronidase from *P2* provides a measure of the efficiency of DNA loop formation in this strain. The mutant *hupA* genes, described in Table 1, were transferred to the chromosome of strain SK22, which is deleted for *hupB*, to study their effects on *P2* repression. The results of β -glucuronidase assays in these strains are shown in Figure 2. Based on these results, we divided the HU α mutants into three groups: (1) Q5D, D8S, and K22G substitutions were as efficient as the wild type in repression of the *P2* promoter, whereas E38K repressed *P2* better than the wild type (Fig. 2A). (2) S17P, K18A, and T19D substitutions caused derepression of the *P2* promoter, presumably because of defects in the formation of the repression loop. Compared with the wild type, these three mutants showed a 2.5- to 4-fold higher rate of β -glucuronidase synthesis (Fig. 2B). (3) T4A, E12A, and A30E substitutions showed partial derepression of the *P2* promoter (Fig. 2C). Because T4A, E12A, and A30E showed significantly longer cell doubling times than the wild-type strain at 37°C and the *hupA*–*hupB* double null mutant was considerably defective in its growth rate (data not shown), the HU mutations in this class most probably resulted in nonfunctional proteins, leading to impaired growth. The *hupA* mutants in the first two groups showed no appreciable changes in growth characteristics. The three mutants in the second group, S17P, K18A, and T19D, which were defective in *galP2* repression in vivo, were studied further.

Characterization of the HU α mutants: S17P, K18A, and T19D

We measured the cellular levels of the HU α variants to verify that the results obtained in the *P2*–*gus* reporter gene assays were not due to a difference in their levels in comparison to that of the wild type. Analysis of cell ex-

Table 1. Amino acid substitutions in HU α

Position	Native amino acid	Substituted amino acid
4	threonine	alanine
5	glutamine	aspartate
8	aspartate	serine
12	glutamate	phenylalanine
17	serine	proline
18	lysine	alanine
19	threonine	aspartate
22	lysine	glycine
30	alanine	glutamate
38	glutamate	lysine

The substituted amino acids were chosen from amino acids at the corresponding positions in HU from other species (see text).

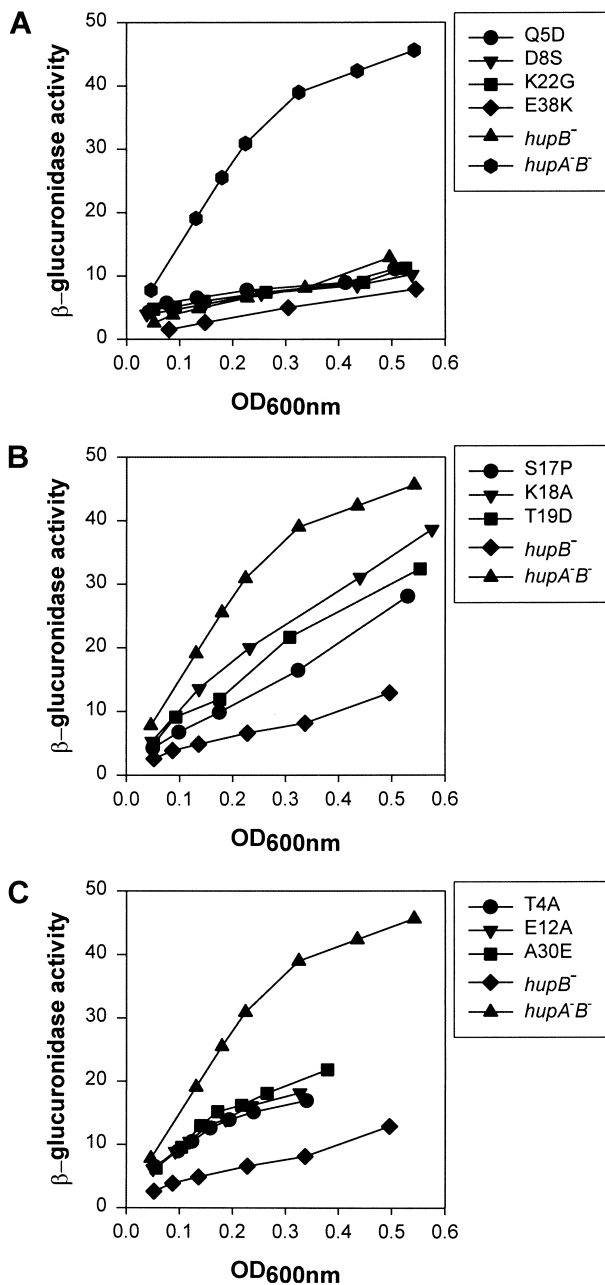


Figure 2. Effect of *hupA* mutations on DNA looping-dependent repression of the *galP2* promoter. Strains containing wild-type or mutant *hupA* genes were grown in minimal media at 37°C and assayed for expression of the *P2-gusA* fusion gene. β -Glucuronidase activities are expressed as change in optical density at 405 nm versus cell density at 600 nm during enzyme assay.

tracts of the three mutants using immunoblotting with antiserum specific to HU showed no apparent deviation in the expressed HU α levels in strains carrying S17P, K18A, or T19D mutation when compared with that in the wild type (data not shown).

Furthermore, we tested whether these HU α mutants that were impaired in *P2* repression of *gal* were compro-

mised in their other cellular functions. Mini-P1 plasmids are not stably maintained in *hupA-hupB* double null mutants because of the deficiency of *ori2*-dependent mini-P1 DNA replication in the absence of HU protein (Ogura et al. 1990). The transformation efficiency of mini-P1 plasmids on the three HU α mutants showed no difference when compared with that of the wild-type strain (Table 2, column 1). Bacteriophage Mu is also unable to replicate in *hupA-hupB* double null mutants (Kano et al. 1989). Growth of phage Mu was tested by measuring the efficiency of plating on lawns of wild type and the *P2* repression-defective HU mutants. The plaque-forming efficiency of phage Mu was virtually the same on the wild type and the three HU mutants (Table 2, column 2).

Effect of HU α mutant proteins on gal transcription in vitro

Wild-type and mutant HU α homodimers (S17P, K18A, and T19D) with a hexa-histidine tag at the N-termini were expressed in *E. coli* and purified as described in Materials and Methods. The purified proteins were used to study their effects as modulators of repression of *gal* transcription in vitro (Fig. 3A). In the absence of GalR and HU, transcription from wild-type *gal* template generated two transcripts, a 125-nucleotides long *P1* RNA and a 130-nucleotides long *P2* RNA (Choy and Adhya 1993). The presence of GalR alone caused repression of *P1* with a concurrent activation of *P2*, as expected. Presence of both GalR and wild-type HU α caused simultaneous repression of both *P1* and *P2*. Wild-type HU α repressed *P2* with a concentration-dependent profile, attaining >80% repression at 80 nM HU α (Fig. 3B). However, in the case of the three HU α mutants, S17P, K18A, and T19D, when used at similar concentrations, repression of *P2* was significantly reduced for all of them. The level of *P2* transcription in the presence of T19D, for example, was similar to that with GalR alone.

DNA binding of the HU α mutants

We performed an electrophoretic mobility-shift assay to compare the mutant HU α proteins with the wild-type HU α for their ability to bind to linear DNA. Binding of successive molecules of HU dimers generated nested

Table 2. Mini-P1 plasmid replication and Mu bacteriophage development in looping-defective HU mutants

Strain	No. of transformants/ μ g of plasmid DNA	No. of plaques/ mL of phage
<i>hupA+B+</i>	2.85×10^4	3.14×10^9
<i>hupA-B-</i>	0	$<1 \times 10^3$
SKS17P	3.18×10^4	2.78×10^9
SKK18A	2.96×10^4	3.03×10^9
SKT19D	3.15×10^4	2.82×10^9

The numbers are averages of three independent sets of experiments.

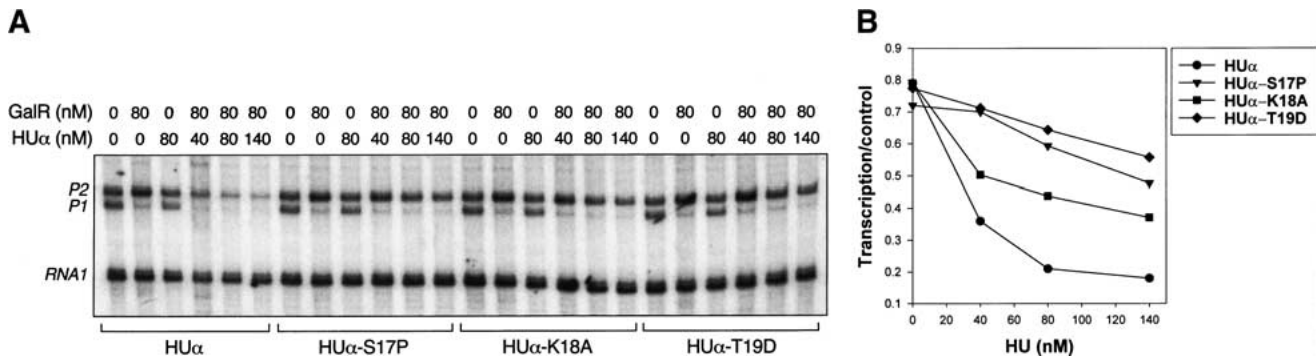


Figure 3. (A) Transcription of *galP1* and *galP2* promoters in the presence of GalR or wild-type HU α and HU α mutants, S17P, K18A, or T19D. Concentration of GalR was 80 nM; concentrations of HU were 40 nM, 80 nM, and 140 nM, as shown at the top. The 80-bp *RNA1* served as an internal control. Details are in the text. (B) Graphical representation of the repression data for *galP1* promoter. Transcription is expressed as the percentage of that which was obtained in the absence of GalR.

complexes that were progressively more retarded in electrophoretic mobilities (Fig. 4). Wild-type HU α and the three HU α mutants, at equal concentrations, gave rise to identical retarded species, indicating that within the range of sensitivity and resolution of this assay, the mutants were as proficient as the wild type in DNA-binding.

Coimmunoprecipitation of GalR and wild-type HU

We investigated the potentiality of a physical interaction between GalR and HU biochemically by coimmunoprecipitation reactions. Coimmunoprecipitation using purified GalR and HU with antiserum specific to GalR consistently revealed the presence of HU in these immunocomplexes, as ascertained by Western blotting with HU-specific antiserum. Complex formation was detected even at high salt concentration, indicating a specificity of GalR–HU interaction (Fig. 5A; lanes 3–5). As a control, we used a homologous protein, LacI^{adi} (Brenowitz et al. 1991), instead of GalR, with LacI^{adi} antiserum to pull down the complexes. As shown in Figure 5A, lane 2, LacI^{adi} and HU complex formation was barely detectable under the lowest salt concentration that was used in reactions involving GalR. To test whether GalR forms a specific complex with HU in vivo, we transformed wild-

type *E. coli* cells with an expression vector plasmid encoding GalR with a hexa-histidine tag at the C-terminal end. This was done to make the in vivo concentrations of GalR and HU comparable for easy detection of GalR–HU interaction by immunoprecipitation: The normal in vivo concentration of GalR is 40 dimers per cell (Tokeson 1989) whereas that of HU is 30,000 dimers per cell (Rouviere-Yaniv and Kjeldgaard 1979). After induction of GalR, the cellular extracts were analyzed for GalR–HU complexes by immunoprecipitating with anti-His antibody and immunoblotting with antiserum specific for HU. The histidine-tagged GalR coimmunoprecipitated with HU (Fig. 5B, lanes 2,3). The same extracts were also immunoblotted with anti-IHF antibody to check whether GalR coimmunoprecipitated with IHF, a protein that is highly homologous with HU (Oberto et al. 1994). GalR did not form any complexes with IHF either in crude extracts or with purified proteins (Fig. 5C, lanes 2–4).

Coimmunoprecipitation of HU α mutants with GalR

We determined whether the repression-defective HU α mutants were impaired in their ability to interact with GalR in immunoprecipitation reactions. Equal amounts of GalR were mixed with different concentrations of wild-type and mutant HU α and precipitated with anti-GalR antibody. Figure 6A shows that, compared with wild type, the HU α mutant T19D was nearly completely defective in interacting with GalR. Figure 6B, which shows the amount of HU protein added to each reaction, confirmed that the differences in the formation of immunocomplexes between the wild-type and mutant HU α were not due to variations in protein concentrations or inadequate antibody recognition by the mutant HU α . HU α mutants S17P and K18A also showed weak interaction with GalR by immunoprecipitation (data not shown).

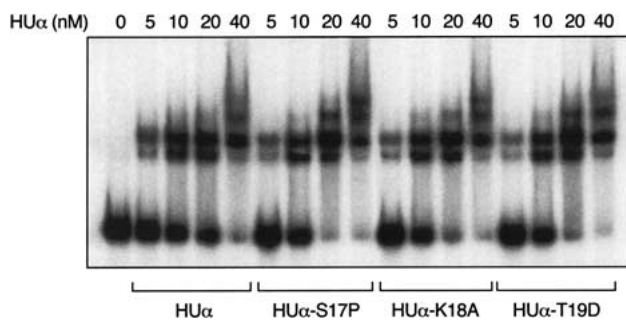


Figure 4. Electrophoretic mobility-shift assay of wild-type HU α and HU α mutants with a 266-bp *gal* DNA fragment, as described in Materials and Methods. Complexes were formed with 0.1 nM DNA and 5 nM, 10 nM, 20 nM, and 40 nM of HU α (lanes 2–4), S17P (lanes 6–8), K18A (lanes 10–12), and T19D (lanes 14–16).

Location of S17, K18, and T19 in the modeled HU α structure

The structure of HU heterodimer in *E. coli* has not yet been determined experimentally. We used the crystal

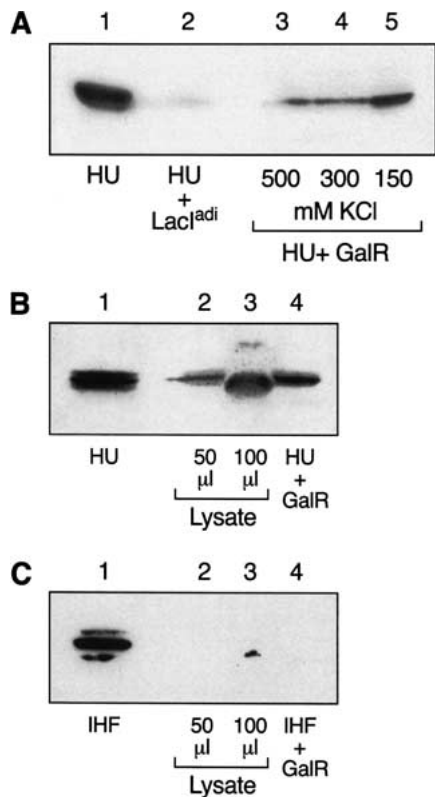


Figure 5. Immunoprecipitation of GalR and HU. (A) Purified GalR and HU. Equimolar amounts of GalR and HU were mixed together in buffer containing varying concentrations of salt. (Lane 3) 150 mM KCl; (lane 4) 300 mM KCl; (lane 5) 500 mM KCl. The proteins were immunoprecipitated with anti-GalR antibody and protein A-agarose. (Lane 2) Equimolar amounts of LacI^{adi} and HU were mixed together in 150 mM KCl and precipitated with anti-LacI^{adi} antibody. (Lane 1) HU. Samples were separated by SDS-PAGE and immunoblotted with anti-HU antibody. (B) GalR and HU in crude cell extracts. BL21(DE3) cells were transformed with plasmid pAP2 containing histidine-tagged GalR coding region under the T7 promoter. Following IPTG induction, cell extracts were made and immunoprecipitated with anti-His monoclonal antibody and protein A-agarose. Samples were divided in two, separated by SDS-PAGE, and immunoblotted either with anti-HU antibody (B) or anti-IHF antibody (C). (Lane 1) HU or IHF; (lane 2) 20 μ L cell extract; (lane 3) 40 μ L cell extract; (lane 4) Purified GalR with either HU or IHF.

structure of the HU homodimer (HBs) from *B. stearotherophilus* to model the *E. coli* HU α . Because the HU α of *E. coli* and HBs from *B. stearotherophilus* share a 59% sequence homology (Drlica and Rouviere-Yaniv 1987), the actual three-dimensional structure of HBs was used as a prototype for HU α . The amino acid residues S17, K18, and T19 in HU α , which when substituted showed defect in repression of *gal* transcription and in interaction of GalR with HU, are located contiguously in a small turn between the first and second alpha helices (Fig. 7). This region lies on the opposite face of the DNA-binding surface, in a prominently accessible portion of HU. All three amino acids have solvent-exposed side chains and are likely candidates to contact GalR.

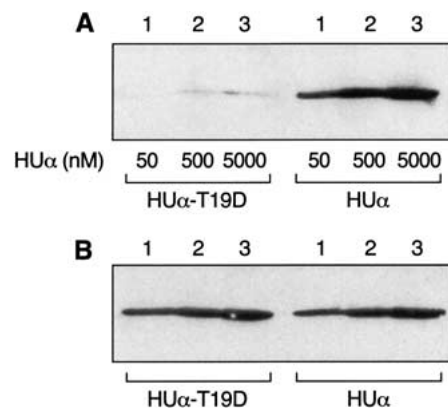


Figure 6. Immunoprecipitation of HU α and T19D with GalR. (A) A fixed concentration of GalR (5 nM) was mixed with increasing concentrations of wild-type and mutant HU α , as indicated in the figure. The proteins were immunoprecipitated with anti-GalR antibody followed by immunoblotting with anti-HU antibody. (B) Equivalent amount of HU added to each immunoprecipitation reaction was run on SDS gel and immunoblotted with anti-HU antibody.

Discussion

A piggyback model for HU recruitment

In the absence of a stable interaction between the two DNA-bound GalR dimers, the formation of a DNA loop must overcome the energetic barrier imposed by the axial rigidity of the 113-bp DNA segment between the

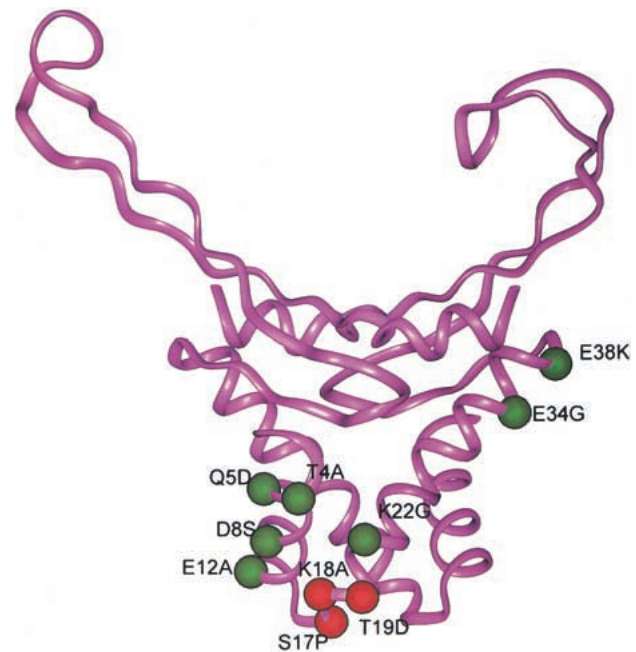


Figure 7. Three-dimensional model of the HU α dimer showing the location and spatial arrangement of the substituted residues. The amino acid substitutions are shown in green or red in one subunit. The three substitutions, which specifically disrupted looping-mediated repression, are shown in red. Mutations that had no effect on repression are shown in green.

two *gal* operators. The small nonspecific DNA-binding histone-like protein HU engenders DNA looping and hence, repressosome formation, by binding to and bending the intervening DNA segment by a tripartite cooperativity between itself and the two DNA-bound GalR dimers. The role of HU in the cooperative binding of HU and GalR to DNA can be explained by two models. In model A, the two operator-bound GalR dimers transiently interact and bend the intervening DNA region, facilitating HU binding to the bent region (Fig. 8A). HU binding energetically stabilizes the interaction between the two DNA-bound GalR dimers, resulting in the observed cooperativity. This is the preferred model in the formation of several repressosome-like structures, for example, intasome, transpososome, and enhanceosome. In model B, a direct protein-protein interaction between HU and GalR brings about cooperative binding (Fig. 8B). In this model, HU-bound GalR binds to the *gal* operators, thereby bringing HU to the vicinity of the *gal* region and facilitating its DNA binding, which in turn stabilizes DNA-bound GalR tetramerization. We investigated the potentiality of a GalR-HU interaction in the formation of the cooperative complex both genetically and biochemically. By site-directed mutagenesis, we scanned the surface of the HU α homodimer to identify amino acid residues that, when altered, would be defective in DNA looping without the loss of their intrinsic DNA-binding activity. Our experiments discovered three substitutions of HU α —S17P, K18A, and T19D—that were unable to repress the *P2* promoter of *gal* in vivo but retained their ability to support several HU-dependent cellular functions; cells carrying the substitutions showed normal bacterial growth, mini-P1 plasmid replication,

and bacteriophage Mu growth. In vitro, the altered HU α proteins showed substantial defect in repression of *P2* transcription and exhibited no discernible difference in their DNA-binding ability. We think that the altered function of the mutant proteins originate from a failed interaction with GalR, a specific interaction essential for the assembly of the repressosome structure. Consistently, we demonstrated a complex formation between GalR and wild-type HU by coimmunoprecipitation reactions using both crude extracts and purified proteins. A GalR-HU interaction was also demonstrated by sedimentation ultracentrifugation analysis and fluorescence studies (S. Roy and M. Geanacopoulos, in prep.) and by SELDI protein chip assays (S. Kar and B. Martin, unpubl.). Next, we showed that the repression-defective HU α proteins are defective in forming the immunocomplexes with GalR. In the modeled structure of the HU α dimer, the three residues, S17, K18, and T19, whose replacements resulted in defective transcriptional repression and GalR interaction, are prominently exposed in a peptide turn between two alpha helices on the face of HU near the N-terminal region and farthest from the DNA-binding C-domain and are accessible for interaction with other proteins. From the results described earlier, we conclude that HU specifically interacts with GalR in repressosome formation. The demonstration of an essential GalR-HU contact in the repressosome assembly supports model B described earlier. This is the first instance where a DNA sequence-independent, nucleoid-associated protein has been shown to have a functional interaction with another protein to perform its architectural role in the formation of a higher-order nucleoprotein structure. The formation of complex nucleoprotein structures in which nonspecific DNA-binding proteins are targeted to specific DNA locations by interaction with sequence-specific DNA-binding proteins has been implicated in other cases; for example, the interactions of the HMG1 and HMG2 proteins with p53 (Jayaraman et al. 1998), with steroid hormone receptors (Boonyaratanakornkit et al. 1998), with Hox proteins (Zappavigna et al. 1996), and with Oct-1 and -2 (Zwilling et al. 1995) in the eukaryotic systems.

In model B, the delivery of HU by piggyback to its destination on the DNA may be followed by one of the two scenarios (Fig. 8B): (1) After HU binds to the specified position on the DNA, it dissociates from GalR, leaving behind a GalR dimer-dimer contact required for DNA looping; (2) HU binds to DNA while remaining GalR bound. In the proposed models for the assembly of Gal repressosome, the stoichiometry of GalR and HU was not taken into account. Although further studies are needed to distinguish between these two models, we note that energetic considerations favor a model in which HU is not in contact with GalR in the final repressosome structure (Geanacopoulos et al. 2001). Nevertheless, the ability of architectural proteins without any sequence preference to function in specific DNA contexts by virtue of piggyback delivery by a sequence-specific regulatory partner may be a common theme for the assembly of higher-order nucleoprotein structures.

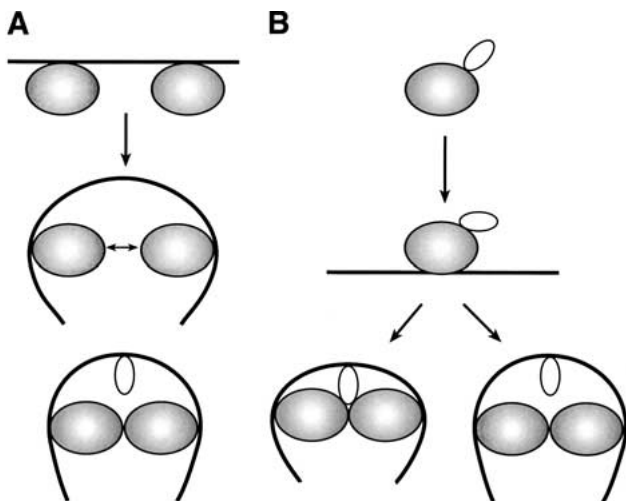


Figure 8. Role of HU and GalR in the formation of Gal repressosome. (A) Two GalR dimers interact transiently to bend the DNA for the HU dimer to bind. (B) HU forms a complex with GalR and is delivered to a specific site on the DNA. The DNA-bound HU could then either maintain or lose contact with GalR in the final repressosome structure. The larger, shaded molecule represents GalR dimer and the smaller molecule represents HU dimer.

Materials and methods

Bacterial strains and plasmids

DM025 ($O_E^+ P2^+ P1^- O_I^- \sim lacZ$ and $O_E^+ P2^+ P1^- O_I^+ \sim gusA$, ap^R , tet^R , cm^R , $\Delta cya854$, $\Delta hupB$) has been described previously (Lewis et al. 1999). KM22, $\Delta[recC\ ptr\ recB\ recD]::P_{lac}\text{-bet}\ exo\ kan$, was obtained from K.C. Murphy. Strain SK22 was constructed by transduction of DM025 by bacteriophage P1 grown on strain KM22 and selection for kanamycin-resistant colonies. Plasmid pSA509, containing a 288-bp segment of the *gal* control region (–197 to +91), has been described previously (Choy and Adhya 1993). Maintenance of mini-P1 plasmid and growth of bacteriophage Mu were performed using standard microbiological methods.

Site-directed mutagenesis of HU α

PCR was used to amplify the upstream region (from –1038 to –95) of the *hupA* gene from wild-type *E. coli* MG1655 with an *EcoRI*-containing primer at the 5' end and a *BamHI*-containing primer at the 3' end. The fragment was cloned between the *EcoRI*–*BamHI* sites of plasmid pEM7(Zeo) (pSKHU/US). The downstream region of the *hupA* gene, from +299 (17 bp downstream of the stop codon) to +1005, was amplified using a 5' end PCR primer containing an *HindIII* site and a 3' end PCR primer containing a *KasI* site. The purified PCR product was subcloned into pSKHU/US at the *HindIII*–*KasI* segment, creating the plasmid pSK/USDS. The wild-type *hupA* and the *hupA* containing point mutations, generated by PCR, were cloned into pSK/USDS between the upstream and downstream sequences described earlier. The wild-type *hupA* was amplified from MG1655 using a forward primer, HU/pR3, which contained a *BamHI* restriction site, and a reverse primer, HU/pR4, which contained a *HindIII* site. The *hupA* mutants were generated by a two-step PCR recombination process. The first round of PCR was performed in two reactions, one using the HU/pR3 primer along with one of the mutant downstream primers and the other using the HU/pR4 along with one of the mutant upstream primers. The two PCR products were used in a second round of PCR with the original HU/pR3 and HU/pR4 primers to generate the final mutant *hupA* fragments. The PCR products containing the wild-type and mutant *hupA* genes were cleaved with *BamHI* and *HindIII* and cloned in pSK/USDS. To clone an antibiotic resistance gene upstream of *hupA*, we cleaved the plasmids with *StuI* at a site +248 from the start codon. Plasmid pSE418 (a kind gift from Dr. D. Chattoraj) was used to generate a spectinomycin-resistance cassette. A 2.4-kb *BamHI* fragment containing the entire spectinomycin resistance gene was cleaved from pSE418, end-filled, and ligated to the *StuI* site in each of the individual *hupA* plasmids. The entire cloned DNA region was verified by DNA sequencing in each plasmid.

Chromosomal construction

The mutant *hupA* genes were transferred from the plasmid into the bacterial chromosome as described by Murphy (1998). A 4.2-kb fragment containing the wild-type or mutant *hupA* was amplified from the respective plasmid and electroporated into *E. coli* strain SK22. Spectinomycin-resistant colonies were selected and purified. The chromosomal *hupA* gene from each spectinomycin-resistant recombinant was sequenced to confirm that the mutations have been transferred to the chromosome.

Assay of β -glucuronidase activity

β -Glucuronidase activities were measured as described previously (Lewis et al. 1999) in log-phase cells growing in M63

supplemented with 0.4% (w/v) D-fructose, 0.1% casamino acids, and 0.0004% (w/v) vitamin B₁. The activity of β -glucuronidase was measured by Softmax microplate spectrophotometer system. The rate of β -glucuronide hydrolysis was determined at 405 nm at 37°C.

Purification of HU α

The wild-type *hupA* and *hupA* genes containing the mutations S17P, K18A, and T19D, after PCR amplification with primers containing *NdeI* and *BamHI* sites at the N-terminal and C-terminal ends, respectively, were cloned in expression vector plasmid pET15b (Novagen), which contains an N-terminal hexa-histidine tag. The cloned plasmids were transformed into BL21(DE3) cells (Stratagene). The proteins were induced with 1 mM IPTG for 3 h at an optical density of 0.7. After induction, the cells were harvested by centrifugation and resuspended in ice-cold Buffer A containing 5 mM imidazole, 500 mM NaCl, 10 mM HEPES (pH 7.9), 2 mM PMSF, and 0.1% TritonX-100. The bacteria were lysed by sonication and centrifuged again to remove the cellular debris. The His-tagged proteins were purified by Buffer A equilibrated Ni-NTA agarose (Qiagen). The gel matrix was washed extensively with Buffer B containing 20 mM imidazole, 500 mM NaCl, 10 mM HEPES (pH 7.9), 0.2 mM PMSF, and 0.1% TritonX-100. Proteins were eluted with a step gradient of imidazole ranging from 50 to 250 mM and the fractions were run on 4%–20% Tris-glycine gels. Fractions containing pure HU α proteins were pooled and dialyzed against 2 mM HEPES (pH 7.9).

In vitro transcription

Transcription reactions were performed as described by Geanacopoulos et al. (1999). Supercoiled DNA template (2 nM) was preincubated with or without proteins at 37°C in a 45- μ L reaction mixture containing 20 mM Tris acetate (pH 7.8), 10 mM magnesium acetate, 100 mM potassium glutamate, 1 mM ATP, 1 mM DTT, and 20 nM RNA polymerase. After incubation for 5 min, transcription was initiated by the addition of 5 μ L of NTP mix containing 0.1 mM GTP, 0.1 mM CTP, 0.01 mM UTP, and 20 μ Ci of [α -³²P] UTP (3000 Ci/mmol) (ICN). Reactions were terminated after 10 min by addition of equal volume of RNA loading buffer (80% [v/v] deionized formamide, 1 \times Tris-borate-EDTA [TBE], 0.025% bromophenol blue, 0.025% xylene cyanol). The reactions were analyzed on an 8% polyacrylamide-urea gel followed by autoradiography. The transcription products were quantified with Phosphorimager, using the *RNAI* transcript as an internal control for each lane.

Electrophoretic mobility-shift assay

Gel retardation was performed in 10 μ L final volume of 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 5 mM MgCl₂, 10% glycerol, 1 mM EDTA, and 0.5 mM DTT. A 266-bp *gal* DNA fragment was isolated from plasmid pSA509 by cleaving with restriction enzymes *EcoRI* and *PstI*. The purified DNA fragment was labeled with (α -³²P) dATP by end-labeling with Klenow fragment. Labeled DNA fragments (5 ng and 1000 cpm) and proteins (5 nM, 10 nM, 20 nM, and 40 nM) were incubated at room temperature for 15 min. The reactions were loaded onto a 5% nondenaturing polyacrylamide gel. After completion of electrophoresis, the gels were dried and autoradiographed.

Coimmunoprecipitation

For immunoprecipitation reactions with purified proteins, 5 μ M HU and 5 μ M GalR or LacI^{adi} were incubated in 20 mM Tris-

HCl (pH 7.5), 10 mM MgCl₂, 0.2% Tween 20, 0.02% NP-40, 1 mM DTT, and varying concentrations of KCl, as indicated, in a total volume of 20 μ L at room temperature for 30 min. The mixtures were diluted 10-fold with buffer containing appropriate concentrations of KCl, and 10 μ g of anti-GalR or anti-LacI^{adi} antibody was added to each mixture. Precipitation was performed at 4°C for 4 h. Fifty microliters of buffer-equilibrated Protein-A agarose (Sigma) was added next and incubations continued at room temperature for two more hours on a rotary shaker. The slurries were then poured in small disposable columns and washed extensively with immunoprecipitation buffer, as described earlier. After transferring the slurry to microfuge tubes, the beads were pelleted and suspended in 50 μ L SDS sample buffer. Following boiling for 5 min, the reactions were separated on 4%–20% Tris-glycine gels (Invitrogen). The proteins were then transferred to PVDF membranes and probed with anti-HU antibody. Immunoprecipitation of purified HU α and T19D proteins with GalR was done basically as described earlier except a fixed concentration of GalR (5 μ M) was mixed with varying concentrations (50 nM, 500 nM, and 5 μ M) of either HU α or T19D proteins.

For immunoprecipitations using cell extracts, plasmid pAP2, which carried the *galR* gene with a hexa-histidine tag at the N-terminal end and situated downstream of a *lac* promoter, was used. After induction of the *galR* gene using 0.4 mM IPTG for 3 h, cell extracts were made by sonication and centrifuged to remove the cellular debris. Twenty microliters of monoclonal anti-His antibody coupled to protein A-agarose (Sigma) was added to lysate volumes of 50 and 100 μ L. Immunoprecipitation was performed essentially as described earlier for purified proteins. After transfer, the blots were developed with anti-HU or anti-IHF antibodies. Immunoprecipitation of purified HU α and T19D proteins with GalR was done basically as described earlier except a fixed concentration of GalR (5 μ M) was mixed with varying concentrations (50 nM, 500 nM, and 5 μ M) of either HU α or T19D proteins.

Western blotting

Proteins were transferred to PVDF membranes, which were blocked with 5% nonfat dry milk in TBST (10 mM Tris-HCl at pH 7.5, 150 mM NaCl, and 0.1% Tween 20). The blots were then incubated with primary antibody at 4°C overnight. They were then incubated with horseradish peroxidase (HRP)-conjugated antibody (Sigma) and developed with Supersignal (Pierce). The dilutions of the antibodies were anti-HU antibody (1:60,000), anti-IHF antibody (1:100,000), HRP conjugated anti-rabbit antibody (1:120,000), and HRP conjugated anti-mouse antibody (1:10,000).

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