Repressor induced site-specific binding of HU for transcriptional regulation

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Transcription from two overlapping *gal* promoters is repressed by Gal repressor binding to bipartite *gal* operators, O_E and O_I , which flank the promoters. Concurrent repression of the *gal* promoters also requires the bacterial histone-like protein HU which acts as a co-factor. Footprinting experiments using iron–EDTA-coupled HU show that HU binding to *gal* DNA is orientation specific and is specifically dependent upon binding of GalR to both O_E and O_I . We propose that HU, in concert with GalR, forms a specific nucleoprotein higher order complex containing a DNA loop. This way, HU deforms the promoter to make the latter inactive for transcription initiation while remaining sensitive to inducer. The example of *gal* repression provides a model for studying how a ‘condensed’ DNA becomes available for transcription.

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Introduction

The catalysis and regulation of a variety of DNA transactions, such as site-specific recombination, transcription, replication and packaging, involves formation of higher order DNA–multiprotein complexes (Echols, 1986; Grosschedl *et al.*, 1994). Proteins that bind to DNA with high specificity and play a principal role, as well as proteins that bind to DNA with broad specificity and play an architectural role, participate in forming such complexes (Nash, 1996). In *Escherichia coli*, the latter group includes HU, IHF, H-NS and Fis. Both the formation and the nature of the nucleoprotein complexes are determined by the ability of the architectural proteins to deform DNA and facilitate critical DNA–protein and protein–protein contacts, which are otherwise prohibitory (Adhya, 1989; Pérez-Martín *et al.*, 1994; Nash, 1996). The structural determination of such complexes will help researchers understand how they function. In transcriptional regulation such structures must possess an additional feature: responsiveness to external regulatory signals. As described here, the multiple regulation of transcription initiation in the galactose (*gal*) operon in *E. coli* is such an apparatus (Adhya, 1996). The *gal* transcription starts from two overlapping promoters, *P1* and *P2*, which are subject to, among others, negative control by Gal repressor (GalR). GalR represses both promoters *in vivo* by binding to two operators, O_E and O_I , which flank the two

promoters (Musso *et al.*, 1978; Adhya and Miller, 1979; Aiba *et al.*, 1981; Irani *et al.*, 1983; Kuhnke *et al.*, 1986). It was proposed that repression requires an interaction between the two operator-bound repressors, and thereby formation of a loop of the intervening 113 bp DNA encompassing the promoters (Irani *et al.*, 1983; Majumdar and Adhya, 1984). In contrast to the *in vivo* observation, GalR represses *P1* inefficiently and activates *P2* *in vitro* with purified components, and does not form the proposed DNA loop (Mandal *et al.*, 1990; Choy and Adhya, 1992; Goodrich and McClure, 1992). Because of this discrepancy between the *in vivo* and *in vitro* observations, we predicted that efficient and coordinated repression of the two promoters requires another factor that acts by assisting GalR in looping the DNA (Choy and Adhya, 1992). We recently discovered and purified an additional factor that co-operates with GalR for the concomitant repression of two *gal* promoters *in vitro* and identified the accessory factor as the bacterial histone-like protein HU (Aki *et al.*, 1996). The HU protein from *E. coli* is predominantly a heterodimer of α and β subunits, each with a molecular weight of 9 kDa. It is an abundant DNA-binding protein associated with the bacterial nucleoid (Rouvière-Yaniv, 1978; Rouvière-Yaniv and Kjeldgaard, 1979). HU is not known to show DNA sequence specificity and has a tendency to bind to distorted regions of DNA, e.g. kinks, bends, single-stranded gaps and cruciforms (Bianchi *et al.*, 1989; Pontiggia *et al.*, 1993; Bianchi, 1994; Bonnefoy *et al.*, 1994; Castaing *et al.*, 1995). HU also binds to a transcription terminator which has a dyad symmetry followed by a stretch of AT base pairs (see below). The terminator attracts HU very likely by forming a hairpin in supercoiled DNA. In most cases of DNA transactions involving HU, it acts architecturally by assisting spatially separated sequence-specific DNA-bound proteins to associate by binding to and bending the intervening DNA region (Craigie *et al.*, 1985; Johnson *et al.*, 1986; Bramhill and Kornberg, 1988; Hodges-Garcia *et al.*, 1989; Ogawa *et al.*, 1989; Wada *et al.*, 1989; Baker and Mizuuchi, 1992; Hwang and Kornberg, 1992; Lavoie and Chaconas, 1990; Lavoie *et al.*, 1991; Lavoie and Chaconas, 1994; Segall *et al.*, 1994; Bètermier *et al.*, 1995; Pérez-Martín and deLorenzo, 1995). We report here that in restoring repression of transcription to *P1* and *P2* by GalR, HU binds site-specifically between the two *gal* operators. HU binding is entirely dependent upon binding of GalR to the two operators and is sensitive to exogenous signal D-galactose, thus revealing a novel way by which a HU-containing higher-order complex responds to transcriptional regulation.

Results

gal repression requires HU

In a purified transcription system, GalR activates the *P2* promoter and represses, albeit less efficiently, the *P1*

Fig. 1. Effect of GalR and HU on transcription initiation from *gal* promoters. *In vitro* transcription assays were conducted using supercoiled *gal* DNA template (2 nM) carrying wild type operators (lanes 1–6 and 11–14, $O_E^+ O_I^+$) or mutant operators (lanes 7 and 8, $O_E^+ O_I^-$ and lanes 9 and 10, $O_E^- O_I^+$). The DNA template used in lanes 11–14 was $P1^+ P2^+$. The concentrations of GalR were as indicated; HU was 80 nM; and D-galactose was 10 mM. RNA transcripts were analyzed by electrophoresis on an 8% polyacrylamide–urea gel followed by autoradiography. The RNA1 transcripts shown as controls were made from the *rep* promoter present in the plasmid DNA used as templates.

promoter (Choy and Adhya, 1992; Goodrich and McClure, 1992; Choy *et al.*, 1995a; Figure 1, lane 2). As demonstrated previously, the HU aids GalR to repress both *gal* promoters *in vitro* (Aki *et al.*, 1996; Figure 1, lane 4). Since HU by itself does not alter the levels of *gal* transcripts, it acts as a co-factor in this system (Figure 1). The repression of *gal* transcription observed in the presence of HU is lifted by the addition of the inducer, D-galactose (lane 6). The coordinated repression of $P1$ and $P2$ requires the binding of GalR to both *gal* operators, O_I and O_E ; mutation of either results in loss of the coordination (lanes 8 and 10). These results are entirely consistent with the *in vivo* observations. We have proposed that the repression is exerted by formation of an intricate nucleoprotein complex of higher-order structure that contains a DNA loop (Irani *et al.*, 1983; Majumdar and Adhya, 1984; Choy *et al.*, 1995b; Aki *et al.*, 1996).

Site-specific binding of HU is promoted by GalR binding and supercoiled DNA

Affinity cleavage mapping by chemically converting HU into a nuclease gives information about the position of the reactive groups in HU in the DNA–protein complex (Lavoie and Chaconas, 1993; Lavoie *et al.*, 1996). In order to analyze the presence of HU in the proposed nucleoprotein complex, we used HU modified with (EDTA-2-aminoethyl)-2-pyridyl disulfide–iron complex [EPD–Fe(III)] (Ebright *et al.*, 1992; Ermácora *et al.*, 1992) to locate the binding site of HU (Lavoie and Chaconas, 1993). The iron, Fe(III), chelated with EDTA is reduced by ascorbate to EDTA–Fe(II) which in turn reduces hydrogen peroxide, creating hydroxyl radicals (Tullius *et al.*, 1987). Thus, the DNA regions near the amino acid residues derivatized by the reagent are cleaved through hydroxyl radical reactions indicating the binding of the modified HU in the vicinity. By converting HU into a ‘nuclease’ in this way, the HU binding site has been located in the transpososome recombination complex of bacteriophage Mu DNA (Lavoie and Chaconas, 1993; Lavoie *et al.*, 1996). More than 60% of the HU molecules

Fig. 2. HU-nuclease footprinting on *gal* DNA. HU-nuclease used was made by coupling of HU with iron–EDTA in the presence (modified; lanes 1, 3–5, 6 and 8–10) or absence (unmodified; lanes 2 and 7) of a cross-linking agent 2-iminothiolane. Supercoiled *gal* DNA template (2 nM) was incubated with different combinations of GalR (40 nM), HU (80 nM) and D-galactose (10 mM), as indicated, under conditions of the *in vitro* transcription. After a hydroxyl radical reaction as described in Materials and methods, nicked DNA was purified and used as a template to synthesize complementary DNA strands with 32 P-end labeled oligonucleotide primers. Dideoxy chain termination reactions were performed using wild type *gal* DNA and the same oligonucleotide primer (lanes G and C on bottom and top strands respectively). Nucleotide positions relative to the start site of the transcription from the $P1$ promoter are indicated on both sides. Closed and open vertical bars indicate the position of *gal* operators and transcription terminator respectively.

we derivatized contained one or more molecule of EPD–Fe(III) as judged by an electrophoretic analysis on an SDS–polyacrylamide gel. The ‘HU-nuclease’ preparations did not show any detectable reduction in its co-factor activity in the GalR-mediated repression of transcription *in vitro* (data not shown).

Figure 2 shows the cleavage pattern of the two strands of DNA by HU-nuclease. The results clearly demonstrate HU binding to *gal* DNA at a specific region between the two operators. Although weak cleavages by HU-nuclease were observed in the absence or presence of the reducing agent Na-ascorbate, some of which could be artifacts of primer extension assay (lanes 1, 3, 6 and 8), the addition of GalR dramatically enhanced site-specific cleavages on both *gal* DNA strands at specific sites (lanes 4 and 9). Cleavage sites ranged from the nucleotide position –8 to

+35 relative to the start point of transcription from the promoter *P*₁. The site-specific cleavages were attenuated by the addition of inducer, D-galactose (lanes 5 and 10), clearly indicating that GalR binding to the operators stimulates HU binding to specific sites in *gal*. Since no significant cleavages were made by HU-nuclease prepared without the cross-linker, 2-iminothiolane, it argues against the possibility that a nuclease contamination of EPD-Fe(III) resulted in non-specific cleavages (lanes 2 and 7). This was also supported by hydroxyl radical DNA cleavage experiments which showed that HU did not create additional hypersensitive cleavage sites for DNA nicking reactions similar to that with HU-nuclease both in the presence or absence of GalR (data not shown). Incidentally, we have also observed that HU-nuclease enhances cleavages at the Rho-independent transcription terminator sequence (*r*^{poC}) present in the *gal* DNA (lanes 3–5). Such a sequence, which contains a dyad symmetry followed by a stretch of AT base pairs, very likely generates a cruciform structure that attracts HU. This is consistent with the previous reports that HU prefers to bind to distorted DNA.

HU has been described as an accessory factor that assists the formation of specific nucleoprotein complexes in many other DNA transactions (Johnson *et al.*, 1986; Bramhill and Kornberg, 1988; Hodges-Garcia *et al.*, 1989; Ogawa *et al.*, 1989; Baker and Mizuuchi, 1992; Lavoie and Chaconas, 1994; Segall *et al.*, 1994; Betermier *et al.*, 1995; Pérez-Martín and deLorenzo, 1995). In some of these cases, HU could be either displaced by high concentrations of salt or replaced by other proteins such as IHF or HMG1. We tested the stability of the GalR-induced DNA binding of HU to *gal* DNA by competition assays (Figure 3). Interestingly, the DNA cleavages caused by the EPD-Fe(III)-derivatized HU pre-bound to the DNA in the presence of GalR were not affected by the subsequent addition of a 5-fold excess of 'cold' HU before the Na-ascorbate treatment (lane 4), whereas pre-bound 'cold' HU inhibited significantly the cleavages by a 5-fold excess of derivatized HU added later (lane 3). The inhibition of intensities of cleavages in the latter case was quantified to be ~75%. IHF and HMG1 did not compete effectively with the derivatized HU (lanes 5–8); the reduction of cleavages were up to 30% at specific spots. We have previously shown that IHF does not replace HU in repressing *gal* transcription (Aki *et al.*, 1996). Thus, the GalR-dependent HU binding to *gal* is specific and relatively stable. GalR dimer is also specific for inducing site-specific binding of HU. The binding of homologous GalS (an isorepressor of GalR with 86% amino acid similarities) to *O*_E and *O*_I did not show the enhanced DNA cleavages by HU-nuclease. More interestingly, when wild type tetrameric LacI which generates DNA looping in *gal*, or dimeric LacI^{adi} which does not, was used in HU binding experiments where *gal* operators were replaced by *lac* operators, neither protein supported HU binding. Apparently HU does not prefer or recognize a LacI-mediated DNA loop (see Discussion).

We also observed that HU binding to *gal* DNA did not occur when using a relaxed (topoisomerase I-treated) or a linearized (restriction enzyme-digested) *gal* DNA template (data not shown). This suggests either an energetic or a specific topological requirement for DNA in one of

Fig. 3. Competition assays on HU-nuclease footprinting. The footprinting reaction was performed in the presence of GalR with cold HU (100 nM; lane 1) or iron-EDTA-derivatized HU (~20 nM; indicated in box in lanes 2–8). For competition assays, cold HU (100 nM; lanes 3 and 4), IHF (100 nM; lanes 5 and 6), or HMG1 (300 nM; lanes 7 and 8) was added before (lanes 3, 5 and 7) or after (lanes 4, 6 and 8) the addition of derivatized HU and incubated for 10 min. The DNA cleavages by the addition of ascorbate were detected by the primer extension reactions as described in the legend of Figure 2. The result shown here is from the top strand of wild type *gal* DNA. Lane G shows the sequencing ladder obtained by dideoxy termination reaction with the same DNA template and primer.

the steps leading to the assembly of HU and GalR on *gal* DNA.

The GalR-dependent 'HU-nuclease' DNA cleavage sites, which were clearly observed, and the intensities with which they were cleaved on two *gal* DNA strands are diagrammed in Figure 4. Although the cleavage sites were distributed from position -8 to position +35, the major impact was in the region -5 to +23. The cleavage sites can be grouped approximately into four clusters. The four sets of cleavage sites overlapped three incomplete dyad symmetry sequences. Since the approximate centers of the four clusters of the cleavage sites were at 8–10 bp intervals, all of them would be more or less on one face of the DNA double helical cylinder (10.5 bp/turn) but somewhat distributed around the cylinder. The results were similar to that observed in Mu transpososome complex containing HU (Lavoie and Chaconas, 1993). We also note that the center of the entire region containing the cleavage sites in *gal* was at the nucleotide position +6.5 and not at the center (-5) of the two operators, i.e. the center of the proposed DNA loop. A nuclease cleavage experiment was performed without the use of 2-iminothiolane in which the α -subunit of the HU heterodimer became the only source of the nuclease activity because of a A43C substitution. In this experiment only the -12 to +6 segment of *gal* DNA showed HU cleavage patterns (Figure 4).

HU-nuclease Attack Sites Observed in the Presence of GalR

Fig. 4. Map of HU-nuclease cut sites observed in the presence of GalR. (Upper figure) Control region of the *E. coli gal* operon showing the location of the two promoters, P_1 and P_2 , and two operators, O_E and O_I . (Lower figure) Helical representation of HU-nuclease cleavage sites aligned with nucleotide sequence of *gal* DNA (-15 to +39). Horizontal comb-like bars indicate incomplete dyad symmetric sequences. Intensities of the bands detected in lanes 4 and 9 in Figure 3 were quantified. Only strong cleavage sites with at least 40% intensities were considered. Large and small circles indicate the sites with 70–100% and 40–70% intensities of nuclease cleavages respectively. The marked segment below the horizontal double helix indicates the region -12 to +6 in contact with the HU α -subunit as discussed in the text. The filled circle shown in the double helix represents the center of the segment that is in contact with HU heterodimer.

These results strongly suggest that only one molecule of HU is responsible for all of the *gal* specific cleavages and the binding of the heterodimer is orientation specific. Since an HU heterodimer contacts two minor grooves in DNA through polypeptide loops, the center of *gal* DNA occupied by HU would be located at position +6.5 (Figure 4).

HU binding requires occupancy of both operators by GalR

The repression of the two overlapping *gal* promoters both *in vivo* and *in vitro* requires GalR binding to both *gal* operators (Adhya and Miller, 1979; Irani *et al.*, 1983; Haber and Adhya, 1988; Aki *et al.*, 1996; Figure 1, lanes 3, 8 and 10). To test the contribution of the operators in site-specific HU binding, HU-nuclease cleavage experiments were conducted using operator mutant DNAs (Figure 5). It is clear that if one of the operators, O_I , is deleted from the *gal* DNA, 'HU-nuclease' fails to show any site-specific *gal* DNA cleavage. This was true for DNA templates with two different O_I deletions (line 1 versus lines 3 and 4). This and the following results suggest that the binding of GalR to both O_E and O_I is required to facilitate HU binding. HU binding, in turn, results in co-operative binding of GalR to O_E and O_I , presumably by an interaction between two DNA-bound GalRs as shown below.

Although the binding of GalR to O_E and O_I by plasmid-borne operator titration experiments has been indicated to be co-operative *in vivo* (Irani *et al.*, 1983; Haber and Adhya, 1988), *in vitro* binding of GalR to the two operators is non-co-operative (Brenowitz *et al.*, 1990). Since site-specific HU binding to *gal* DNA is strictly dependent upon GalR interaction with both operators, we reinvestigated the co-operative binding of GalR using the 'HU-nuclease'-dependent DNA cleavage as a diagnosis of GalR co-

Fig. 5. Effect of mutations or deletion of *gal* operator on HU-nuclease footprinting. DNA cleavages were detected using HU-nuclease as in Figure 3. Supercoiled DNA templates used were (from top to bottom) pSA509, pSA532, pSA-GD1, pSA-GD2, pSA512 and pSA510 (see Materials and methods). Closed and shaded boxes indicate *gal* and *lac* operators respectively. The results shown are for the top strand of DNA. Experiments that showed strong or weak cleavages are represented with ++ or + respectively.

operativity. We nevertheless observed significant site-specific HU-nuclease cleavages of *gal* DNA carrying one critical change in each half of the dyad symmetry at O_E or O_I (lines 2 and 5). We have previously shown that such mutations abolish its intrinsic binding to GalR as judged by DNase I protection assays (Majumdar and Adhya, 1986; Brenowitz *et al.*, 1990). When both operator loci carried the mutations, the cleavages were no longer significant (line 6). These results clearly show that in the presence of HU, GalR binding to a wild type operator can rescue repressor binding to a mutant operator. Site-specific HU binding to *gal* is essential for such GalR co-operativity; the repressor did not bind to the mutant operators if HU was absent (data not shown). HU binding with GalR occupying two wild type operators is more stable than that with GalR occupying one wild type and the other mutant operator; HU binding in the former case is resistant to heparin challenge whereas in the latter it is not (data not shown). The simplest interpretation of such co-operative binding is an interaction between two GalR dimers in the presence of HU. Thus, an interaction of two DNA bound GalRs stabilizes site-specific binding of HU and vice versa.

The nucleoprotein complex of GalR, HU and gal DNA prevents open complex formation

We tested whether RNA polymerase binds to *gal* promoter in the presence of GalR and HU by DNase I protection analysis. DNA-protein complexes were formed by using a template DNA carrying a promoter mutation at P_1 ($P_1^-P_2^+$) in the presence of heparin. The P_1^- mutation (a G→A transition at the nucleotide position -14) while abolishing any detectable P_1 transcription, did not affect the action of GalR and HU in repression of *gal* transcription from the P_2 promoter (Figure 1, lanes 11–14). DNase I experiments show the protection of the P_2 promoter (-40 to +20) by RNA polymerase (Figure 6, lanes 1 and 5). The same protection pattern was obtained when GalR or HU was also present (lanes 6 and 7). RNA polymerase binding to P_2 by DNase I protection assay was not observed in the presence of both GalR and HU (lane 8). Similar inhibition of RNA polymerase binding was also obtained by using wild type DNA ($P_1^+P_2^+$) (data not shown). Under such conditions, RNA polymerase, nevertheless, is able to bind and initiate transcription from RNAI promoter present elsewhere in the plasmid DNA

Fig. 6. DNase I protection of *gal* DNA. Supercoiled $P1$ - $P2^+$ *gal* template was incubated in the presence or absence of GalR, HU or RNA polymerase as indicated under conditions of the *in vitro* transcription except that dNTPs were omitted. After probing of the DNA-protein complex with DNase I, the nicked DNA was purified and used as a template for synthesizing complementary strands with 32 P-end labeled oligonucleotide primers. Results shown are from the top strand of DNA. Dideoxy chain termination reactions were performed using wild type *gal* DNA and the same oligonucleotide primer (lanes G, A, T and C). Nucleotide positions relative to the start site of the transcription from the $P1$ promoter are indicated on the right. Closed vertical bars indicate the positions of *gal* operators.

(Figure 1, lane 4). Thus, we conclude that the *gal* DNA-HU-GalR complex hinders heparin-resistant open complex formation by RNA polymerase at the *gal* promoters. If RNA polymerase binds to the promoter, such a complex is unstable under the conditions used. Interestingly, HU binding in the presence of GalR neither showed protection from nor generated hyper-reactive sites by DNase I treatment (lanes 4 and 8). Footprinting experiments probed by free iron-EDTA complex were also unable to detect HU binding (data not shown).

Discussion

HU is an operon-specific transcriptional regulator

It has been proposed previously that an interaction of two GalR dimers bound to spatially separated operators forming a DNA loop is a determinant of repression of the intervening promoters (Irani *et al.*, 1983; Haber and Adhya, 1988). Indeed, DNA looping occurs frequently

in multipartite operator systems for regulation of gene expression (Adhya, 1989). In an experimental *gal* system in which the two *gal* operators were replaced by *lac* operators, it has been demonstrated both *in vivo* and *in vitro* that Lac repressor (LacI) binding to the operators represses the initiation of transcriptions from the two *gal* promoters via DNA looping (Haber and Adhya, 1988; Mandal *et al.*, 1990; Choy and Adhya, 1992). As expected, such an effect is abolished if a mutant LacI (LacI^{adi}), which fails to tetramerize but interacts with *lac* operator normally, is used (Mandal *et al.*, 1990; Alberti *et al.*, 1991; Brenowitz *et al.*, 1991; Choy and Adhya, 1992).

GalR, like LacI^{adi}, is a dimer in solution; it does not tetramerize under physiological conditions *in vitro* (Brenowitz *et al.*, 1990). Furthermore, GalR, like LacI^{adi}, represses transcription from $P1$ somewhat inefficiently and activates that from $P2$ (Choy and Adhya, 1992; Figure 1). *In vivo*, however, GalR, like LacI and unlike LacI^{adi}, represses *gal* transcription from both $P1$ and $P2$ (Haber and Adhya, 1988; Mandal *et al.*, 1990). We have demonstrated that the histone-like protein HU restores the ability of GalR to coordinately repress the *gal* promoters *in vitro* (Aki *et al.*, 1996). The GalR and HU mediated repression of the two *gal* promoters requires both *gal* operators and is sensitive to the addition of inducer, D-galactose (Figure 1). HU failed to assist LacI^{adi} in bringing about the concomitant repression of the *gal* promoters. The results of DNA cleavage by HU-nuclease showed that HU binds, or at least closely approaches, *gal* DNA in a site-specific way. The properties of site-specific stable binding of HU parallel the properties of HU in transcriptional repression—the requirement for binding of GalR to both operators, the requirement for supercoiled DNA and the sensitivity to the presence of inducer, D-galactose. The total dependency of site-specific HU binding for *gal* repression and its responsiveness to inducer shows for the first time that HU acts as an operon specific transcriptional regulator.

Co-operative binding of HU, GalR and DNA looping

Whereas HU has been shown to bind to 9 bp segments of DNA nonspecifically and transiently at micromolar concentrations (Broyles and Pettijohn, 1986; Bonnefoy *et al.*, 1994), HU bound to -10 to +30 segment of *gal* DNA with an affinity in the nanomolar range (Figure 2). HU binding to *gal* is not only site-specific but also orientation-specific. The center of HU heterodimer binding is position +6.5 with the α subunit preferring the O_E side on *gal* DNA. In spite of such orientation-specific binding, it is not clear whether HU binding to *gal* is sequence-specific. This region is AT-rich and contains three overlapping weak dyad symmetries. Such sequences have the potential for making a distorted structure when the DNA is supercoiled and thus becomes a target of HU.

The site-specific stable binding of HU to *gal* DNA is totally dependent upon GalR binding to both operators, O_E and O_I . In turn, HU helps co-operative binding of GalR to O_E and O_I . A typical explanation of co-operative binding of proteins to multipartite DNA sites is protein-protein interactions between the co-operative partners (Hochschild and Ptashne, 1986). In this model, co-operative GalR binding is brought about by an interaction

between O_E - and O_I -bound GalR molecules, resulting in the formation of a DNA loop. Loop formation in *gal* DNA has been independently confirmed by imaging GalR–HU–DNA complex by atomic force microscope (Lyubchenko *et al.*, 1997).

Architecture of GalR–HU–*gal* DNA complex

We have considered different roles of HU in forming the GalR–HU–*gal* DNA complex responsible for repression of transcription (Aki *et al.*, 1996). (i) HU molecules wrap solenoidally around the promoter DNA. White *et al.* (1989) have proposed such a structure in which several HU dimers bind to DNA side by side. (ii) HU helps DNA looping by acting as a DNA bender, as a protein adaptor, or both. As a bender, it binds to a DNA site between O_E and O_I and stabilizes a transient interaction between two operator-bound GalR dimers. As an adaptor, HU binds simultaneously to O_E - and O_I -bound GalR, which are otherwise incapable of interaction. The architecture of GalR and HU binding to *gal* DNA complex is somewhat similar to the architecture of site-specific recombination complexes in bacteriophage Mu and λ (Goodman *et al.*, 1992; Lavoie and Chaconas, 1994). In the case of MuA–HU–DNA transpososome structure, Lavoie and Chaconas (1993, 1994) and Lavoie *et al.* (1996) have shown that one HU heterodimer binds around the center of the two MuA binding sites, creating a footprint of ~30 bp because of HU-induced DNA bending. HU binding and bending of the intervening DNA is proposed to stabilize a weak interaction between the two MuA monomers. A similar architecture has also been described in the formation of the intasome complex in bacteriophage λ , in which a bivalent Int protein contacts two distal DNA sites with an IHF molecule protein binding in the intervening region and bending the DNA (Segall *et al.*, 1994). Such a co-operative effect of HU (or IHF) upon building of higher order structure to permit long distance DNA–protein or protein–protein interaction is termed the compass model of binding (Grosschedl *et al.*, 1994; Segall *et al.*, 1994). Our results with *gal* DNA are inconsistent with a solenoidal or simple adaptor role for HU and strongly favor a GalR–HU–DNA architecture that contains two GalR molecules bound to O_E and O_I and an HU molecule bound (or located very close) to the DNA segment centered at position +6.5.

The multiprotein complex containing HU in Mu DNA and *gal* DNA differs in one respect. HU can be displaced from the Mu transposome complex after the assembly steps by high salt (Lavoie and Chaconas, 1990). Once HU binds to *gal* DNA, however, the HU binding is stable and cannot be displaced by the addition of heparin (25 μ g/ml) or by chasing with excess HU (Figure 5).

The HU–nuclease cleavage patterns show several cluster cleavages at about 10 bp intervals, suggesting that HU occupies more or less the same face of DNA centered around position +6.5. The face of HU occupancy is the same as occupancy of RNA polymerase at the $P2$ promoter and opposite to that of RNA polymerase at the $P1$ promoter. Note that GalR binding to both O_E and O_I occurs on the same face of DNA as RNA polymerase at $P1$ (Majumdar and Adhya, 1989). We propose that in the GalR–HU–*gal* DNA complex, the two bound GalR dimers achieve a physical contact by (i) overtwisting or unwinding

Fig. 7. Models of the nucleoprotein complexes with (A) GalR and HU or (B) LacI negatively regulating the initiation of *gal* transcription. The cartoons represent the face of the DNA double helix and the location of DNA binding of proteins, as identified in the box. The order of assembly is not considered.

DNA, or (ii) causing the intervening DNA to cross (Figure 7A).

Strikingly, HU binding was not found to be enhanced by LacI⁺ tetramer binding to *gal* DNA carrying two *lac* operator sequences (data not shown). Thus, GalR is specific in assisting HU binding. HU cannot be replaced by IHF, Fis or HNS proteins. LacI tetramer forms a DNA loop and concurrently represses $P1$ and $P2$ transcription without HU (Mandal *et al.*, 1989; Choy and Adhya, 1992). Unlike that in the proposed DNA loop formed by GalR and HU, the face of DNA at position +6.5 and the $P2$ promoter is on the outside of the loop generated by LacI alone. In the LacI-generated DNA loop, DNase I hypersensitive sites appeared at an interval of ~10 bp on the $P2$ side of the DNA (Brenowitz *et al.*, 1991; Choy *et al.*, 1995b). Considering the fact that LacI forms stable tetramers, the LacI mediated DNA loop provides an unfavorable structure for HU binding; HU binding to the outside face of the loop would prohibit HU from binding and bending the DNA (Figure 7B).

Whereas LacI forms tetramers through an antiparallel four-helix bundle model, located at the C-end of the protein (Alberti *et al.*, 1991; Lewis *et al.*, 1996), GalR is devoid of such a motif and must tetramerize in a different way. In one model, GalR tetramerization involves a specific stacking of the core of the O_E and O_I bound dimers, O_I on top of O_E (Figure 7A). This model would make the DNA segment between O_E and the HU binding center longer than that between O_I and the HU site in the loop, which is experimentally true. In an alternative model, as discussed, HU helps GalR tetramerization in a more direct way by playing the role of an adaptor.

Role of DNA supercoiling

The role of DNA supercoiling in forming the GalR–HU–*gal* DNA architecture may be topological or energetic. (i) The stability of the *gal* DNA loop created together by GalR and HU is optimized by the plectonemic geometry

of the supercoiled DNA (Lyubchenko *et al.*, 1997).
 (ii) Supercoiling DNA may compensate for the energy shortage for DNA binding by HU and interactions between GalR. DNA supercoiling and HU action are closely related (Rouviere-Yaniv *et al.*, 1992; Pruss and Drlica, 1989).
 (iii) The proposed DNA twisting or unwinding by GalR, discussed above, needs to overcome a kinetic barrier, which may also be compensated by DNA supercoiling.
 (iv) Supercoiling may rephase the two *gal* operators.

Materials and methods

Plasmids and bacterial strains

Plasmid pSA509 contained a 288 bp segment of the *gal* control region (−197 to +91) followed by a transcription terminator (Choy and Adhya, 1993). Plasmids pSA532 ($O_E^+ O_I^-$), pSA512 ($O_E^- O_I^+$) or pSA510 ($O_E^- O_I^-$) were identical to pSA509 except for the nucleotide sequences of O_I , O_E or of both (Choy and Adhya, 1992). Plasmid pSA562, that contained a 188 bp segment of *gal* DNA (−97 to +91) carrying mutant *gal* promoter ($P1^+P2^+$), was constructed by a G→A conversion at the nucleotide position −14 by site-directed mutagenesis (Bingham *et al.*, 1986; Choy *et al.*, 1995a). The internal operator (O_I ; +46 to +61)-truncated DNA templates were made by digesting pSA509 DNA with *Bsr*EII and *Bam*HI (pSA-GD1) or *Bsr*EII and *Hind*III (pSA-GD2), followed by filling in the cohesive ends with Klenow fragment and by self-ligation. On both DNA templates, a segment containing a *gal* DNA from +44 to +91 is deleted and replaced by unrelated sequences.

E. coli strains carrying plasmids that overproduce wild type HU were from Roger McMacken and α -A43C/ β -wild type HU were from Brigitte Lavoie and George Chaconas.

Reagents and proteins

EPD-Fe(III) was kindly provided by Dr Mario Ermcora and Dr Robert Fox (Ebriht *et al.*, 1992; Ermcora *et al.*, 1992). Thiourea and 2-iminothiolane were obtained from Sigma and Pierce respectively. Purification of GalR has been described before (Majumdar *et al.*, 1987). FPLC-purified rabbit anti-GalR antibody was prepared by Thomas Soares. Rabbit anti-HU antibody was donated by Roger McMacken. *E. coli* RNA polymerase and Klenow fragment were purchased from Pharmacia Biotech. IHF and HMG proteins were gifts from Howard Nash and Michael Bustin respectively.

Purification of HU proteins

Wild type protein HU or α -A43C/ β -wild type mutant HU-hyperexpressing cells were grown at 30C in 200 ml of LB medium containing ampicillin (100 g/ml) to A_{600} of 1.5. The culture was quickly placed in a water bath at 42C and was shaken for 30 min. The cells were washed once and suspended in 50 ml of HK [20 mM HEPES-NaOH, pH 7.5, 60 mM KCl, 1 mM dithiothreitol (DTT)] and disrupted by passing through a French Press three times. After removing the cell debris by centrifugation for 10 min at 7000 g, the supernatant was fractionated by ammonium sulfate precipitation at 70–90% saturation. The ammonium sulfate precipitation was repeated twice. IHF and nuclease activities were effectively removed at this step (data not shown). The fraction rich in HU was loaded on a 10 ml Q-Sepharose column (Pharmacia Biotech) pre-equilibrated with HK. A flow-through fraction was taken and was directly applied on a 5 ml heparin-Sepharose column (Pharmacia Biotech) pre-equilibrated with HK. After thoroughly washing the column with HK containing 400 mM KCl, HU was eluted by a linear gradient with HK containing 1.2 M KCl. Eluate at 0.6–0.8 M of KCl was taken and stored at −80C.

In vitro transcription assays

In vitro transcription assays were performed as described by Choy and Adhya (1992) with slight modifications. Supercoiled DNA template (2 nM) was preincubated at 37C successively for 10 min at each protein concentration in 20 mM Tris acetate, pH 7.8, 10 mM magnesium acetate, 220 mM potassium glutamate, 0.3 mM DTT, 2% glycerol, 1 mM ATP, 0.1 mM GTP, 0.1 mM CTP, 0.01 mM UTP and 10 Ci of [α -³²P]UTP (800 Ci/mmol; 1 Ci = 37 GBq). Transcription (30 l) was initiated by the addition of RNA polymerase to a final concentration of 20 nM. After an incubation period of 5 min, an equal volume of RNA loading buffer [80% (v/v) deionized formamide, 1 TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA), 0.025% bromophenol blue, 0.025% xylene cyanol]

was added to quench the reaction. RNA product was analyzed on an 8% polyacrylamide–urea gel followed by autoradiography.

DNase I footprinting

Supercoiled DNA–protein complex was formed in the same manner as described in the *in vitro* transcription assay without the addition of ribonucleotide triphosphates. DNase I (0.5 g/ml) was added to the complex, incubated with the complex at 37C for 1 min and inactivated by the addition of an equal volume of 20 mM EDTA, followed by a heat treatment at 85C for 3 min. The resultant DNA was purified by QIA quick DNA purification kit (QIAGEN) and mixed with the ³²P-end-labeled primer, TGCAGGCATGAAACCGCGTC or CGTGAAA-GGTAGCGGATCC, which was complementary to the bottom strand of the upstream region or to the top strand of the downstream region of the *gal* promoters respectively. The DNAs were annealed in distilled water by heating at 95C for 4 min and at 45C for 10 min. After the addition of concentrated buffer to become 50 mM Tris–HCl, pH 7.2, 10 mM MgCl₂, 1 mM DTT and 5 mM each of four kinds of deoxyribonucleoside triphosphates, 4–8 units of Klenow fragment were added and incubated at 45C for 10 min. The extension reaction was terminated by the addition of an equal volume of stop solution containing 1 M ammonium acetate, 1 mM EDTA and 0.5 mg/ml yeast tRNA. The DNA was precipitated with ethanol and analyzed on a 6% polyacrylamide–urea gel followed by autoradiography.

HU-nuclease footprinting

Derivatization of HU with EPD-Fe(III) was performed according to Lavoie and Chaconas (1993). Briefly, HU preparation (~50 g) in Chelex 100 treated buffer containing 50 mM HEPES-NaOH, pH 7.8 and 50 mM KCl was applied on a 50 l DNA–cellulose column (Pharmacia Biotech) in an eppendorf yellow tip with glass wool. After washing the column exhaustively with the same buffer, an aqueous solution containing EPD-Fe(III) (500 M) and 2-iminothiolane (1 mM) was loaded. The latter was omitted when the α -43C/ β -wild type HU was used. The column was flushed with nitrogen gas, sealed and placed at room temperature for 4 h. After washing the column with 5 volumes of the buffer, HU was eluted with the buffer containing 0.5 M KCl. More than 60% of the contents of wild type HU and 30% of HU mutants were modified as judged by (i) analysis on SDS–polyacrylamide (10%) gel electrophoresis in Tris–tricine buffer system in the absence of DTT, and (ii) following disulfide bond cleavage after an incubation in buffer containing 0.3 mM DTT.

The supercoiled DNA–protein complex was formed as in DNase I footprinting analysis except that the EPD-derivatized HU was used where indicated. Hydroxyl radical reaction was initiated by the addition of sodium ascorbate and hydrogen peroxide to a final concentration of 1 mM and 0.03% respectively. After incubation at room temperature for 2–3 min, thiourea and EDTA were added to a final concentration of 100 mM and 20 mM respectively, to quench the reactions. Under this condition, more than 90% of the supercoiled DNA was nicked and relaxed as judged by an agarose gel electrophoresis (data not shown). The DNA was purified and treated as performed in DNase I footprinting analysis. The Klenow-synthesized complementary fragments were analyzed on a 6% polyacrylamide–urea gel followed by autoradiography. Relative intensities of individual bands on the gel were measured by PhosphorImager (Molecular Dynamics).

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