Antagonistic Role of H-NS and GadX in the Regulation of the Glutamate Decarboxylase-dependent Acid Resistance System in Escherichia coli*

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One of the most efficient systems of acid resistance in Escherichia coli, the gad system, is based on the coordinated action of two isosforms of glutamate decarboxylase (GadA and GadB) and of a specific glutamate/γ-aminobutyrate antipporter (GadC). The gadA/BC genes, activated in response to acid stress and in stationary phase cells, are subjected to complex circuits of regulation involving σ70, σ8, cAMP receptor protein, H-NS, EvgAS, TorRS, GadE, GadX, GadW, and YdeO. Herein, we provide evidence that the nucleoid-associated protein H-NS directly functions as repressor of gadA, one of the structural genes, and gadX, a regulatory gene encoding one of the primary activators of the gad system. Band shift and DNase I footprints reveal that H-NS indeed binds to specific sites in the promoter regions of gadA and gadX and represses the transcription of these genes both in an in vitro system and in vivo. Moreover, we show that a maltose-binding protein MalE-GadX fusion is able to stimulate the promoter activity of gadA/BC, thus indicating that GadX is by itself able to up-regulate the gad genes and that a functional competition between H-NS and GadX takes place at the gadA promoter. Altogether, our results indicate that H-NS directly inhibits gadA and gadX transcription and, by controlling the intracellular level of the activator GadX, indirectly affects the expression of the whole gad system.

Colonization of the mammalian gastrointestinal tract by enteric bacteria requires the induction of genes, the protein products of which participate, either as regulators or as structural components, in survival mechanisms aimed at countering the life-threatening consequences of internal pH acidification (1–5). Escherichia coli have evolved three systems of acid resistance (4, 5), but the most effective of these is dependent on glutamate and comprises at least the genes gadA and gadB, encoding two isosforms of glutamate decarboxylase, and gadC, encoding a glutamate/γ-aminobutyrate antipporter (6, 7). The enzyme glutamate decarboxylase incorporates protons during production of γ-aminobutyrate, which is then exported by the antipporter GadC in exchange for new substrate. The net effect is that protons leaking into the cell during acid stress are consumed and released by this system, thus contributing to pH homeostasis also in terms of restoring the negative-internal electrical potential (2).

The structural gadA/BC genes are subjected to a complex regulatory network involving the following proteins: the vegetative and stationary phase σ factors, σ70 and σ8, the cAMP receptor protein (CRP),1 the histone-like protein H-NS, the EvgAS and TorRS two-component systems, the TrmE GTPase, the AraC-like regulators GadX, GadW, and YdeO, and the LuxR-family regulator GadE (Ref. 2 and references therein). The degree of involvement of each regulator varies depending upon growth condition and phase of growth, thus adding a further level of complexity to the regulation of the gad system. Transcriptional analyses have demonstrated that the gadB and gadC genes are cotranscribed as a gadBC dicistronic mRNA from the gadB promoter (7), gadA is cotranscribed with gadX as a dicistronic gadAX mRNA (8), and gadX can also be cotranscribed with gadW (9). In addition, gadA and gadX can be synthesized as monocistronic transcripts starting from their own promoters (8). Two main activation circuits were identified, a σ8-dependent circuit and a σ8-independent circuit. Within the σ8-dependent circuit during the stationary phase of growth, either in rich or in minimal medium, the increase in gadA and gadBC transcription requires GadX, which directly binds the gadA and gadB regulatory regions and, under some circumstances, acts in combination with GadW (8–12). The σ8 dependence of gadX expression (8) partially explains the involvement of CRP and H-NS in the regulation of the gad genes. In fact, this alternative σ factor is known to be repressed by CRP at the transcriptional level and by H-NS at the post-transcriptional level (12, 13). Different is the scenario when the expression of the gad system is analyzed at acidic pH in exponentially growing cells in minimal glucose medium (σ8-independent circuit). Under these growth conditions, the GadE activator is primarily implicated in gadA and gadBC transcription (its overexpression causes GAD production even in cells lacking both GadX and GadW regulators), and gadE itself turns out to be acid-induced (14, 15). A cascade reaction model, in which EvgA stimulates gadE expression directly and indirectly via ydeO, was proposed to explain the induction of GadE during acid challenge (16, 17).

The observation that the levels of gadA and gadBC transcripts are greatly enhanced in an hns-defective strain when compared with that found in wild-type E. coli cells led several

* This work was supported by grants from MIUR (to M. F. and D. D. B.) and from the Istituto Pasteur-Fondazione Cenci Bolognetti (to D. D. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

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1 The abbreviations used are: CRP, cAMP receptor protein; MalE, maltose-binding protein.
groups to assume that H-NS exerts a negative control over the expression of gad genes during the exponential phase (7, 8, 10, 18). Since mutations in hns are highly pleiotropic and there have been no attempts to systematically identify the target genes of the H-NS-mediated repression, so far, a direct role of this histone-like protein on the regulation of the gad system can only be hypothesized. The DNA-binding protein H-NS, one of the major components of bacterial chromatin, is recognized as a global regulator of bacterial cell physiology, being involved in the modulation, primarily at transcriptional level, of a fairly large number of genes. Most of the genes belonging to the H-NS regulon are linked to the stress response or to changes in the environmental conditions such as temperature, osmolarity, growth phase, pH, and availability of oxygen and nutrients (10, 19, 20). The structural basis for H-NS function as a gene silencer seems to reside in its capacity to induce bending of non-curved DNA (24), possibly with ampicillin 50

Band Shift Assay—Each reaction mixture contained ~5 ng of PCR-generated DNA fragments (end-labeled with [32P]dATP by fill-in reac-
tion using the Klenow fragment of E. coli DNA polymerase I, 50 ng of poly(dI-dC) as DNA competitor, and the indicated concentrations of purified H-NS in a total volume of 15 μl of 10 mM Tris-HCl, pH 8.0, 10 mM MgCl₂, 100 mM NaCl, 1 mM KCl, 1 mM spermidine, 0.5 mM dithiothreitol, and 5% glycerol. After 10 min of incubation at 25 °C, the DNA-protein complexes were analyzed by 7% PAGE under native conditions in TAE buffer, pH 7.4 (40 mM Tris, 5 mM sodium acetate, 1 mM EDTA). The two gadA DNA fragments of 154 and 256 bp were generated by PCR amplification using as template the pKgadA-179 DNA, used as forward primers AB69 (from –69 to –53, 5’-GGGAATTCGAGTATTTTTTTTTCTTTTTTTTT-3’) and AB179 (from –179 to –158, 5’-GGGAATTCTGCGGACAGAACGATG-3’), respectively, and using AB77 (from +77 to +61, 5’-GGGGATCCGGATATATGTTATATGAATGTTTATTC-3’) as reverse primer. The two gadX DNA fragments of 198 and 325 bp were generated by PCR amplification using as template the pKgadX-222 DNA, used as forward primers X67B (from –70 to –45, 5’-CCGG-GATCCCGCTTCTGTGAAATAATTTTATTTTATCT-3’) and X222B (from –222 to –197, 5’-GGGGATCCGATATATGTTATATGAATGTTTATTC-3’), respectively, and using X121H (from +121 to +99, 5’-CCCCAGCTTGGCATTTAATAGGATATTTTATTTTATCT-3’) as reverse primer.

In Vitro Transcription—In vitro transcription from supercoiled pKK232.8 derivatives as templates (200 ng) was carried out at 37 °C in the presence of increasing amounts of the indicated purified protein. Each reaction contained mixture (40 μl) contained 40 mM Tris-HCl, pH 7.5, 150 mM KCl, 10 mM MgCl₂, 10 mM dithiothreitol, 0.01% Triton X-100, 0.5 mM each of NTPs, and 2 units of ribonuclease inhibitor. After preincubating the samples for 20 min, 0.3 units of α-saturated E. coli RNA polymerase (United States Biochemical) were added, and the incubation was prolonged for an additional 30 min. The reaction was stopped on ice, and RNA was precipitated with ethanol in the presence of 1 μg of RNase inhibitor. The gad:cat transcription were detected by Northern analysis using as probe a 32P-labeled cat gene essentially as described previously (37). The radioactivity present on the filter was quantified by Molecular Imager (Bio-Rad model GS-250).

DNase I Footprinting—Supercoiled plasmid DNA (200 ng/sample) was preincubated 20 min at 25 °C with the indicated concentrations of purified H-NS in 30 μl of binding buffer (40 mM Hepes-HCl, pH 8, 100 mM NaCl, 0.1 mM EDTA) at magnesium, strate, and 0.5 mM spermidine. After the addition of 1 unit of DNase I, incubation was continued for 40 s, and the reactions were stopped by placing the samples on ice and by the addition of 8 μl of EDTA 250 mM and 5 μl of sodium acetate (3 M). Each DNA sample (~50 μl), precipitated with 2.5 volumes of ethanol in the presence of 1 μg of RNA carrier, was resuspended in 10 μl of Polymer PCR buffer supplemented with 3 mM MgCl₂, 100 mM each dNTP, 4 pmol of the 5’-32P end-labeled primer, and 0.5 units of Taq polymerase (Polynzym) and subjected to 30 cycles of linear PCR (denaturation, 30 s at 95 °C; annealing, 30 s at temperatures between 40 and 50 °C depending on the primer; extension, 30 s at 72 °C). The extension products were separated on 7% sequencing gel. The oligonucleotides A179 (see above) and AB77 (see above) were used to detect H-NS protections at both non-template and template strand, respectively. The two gadX non-coding and the gadBC coding strand were separated on 7% sequencing gel. The oligonucleotides X771 (from above) and AB77 (see above) were used to detect H-NS protections at both non-template and template strand, respectively.

Construction of Single-copy gadA and gadB:lacZ Transcriptional Fusions and β-Galactosidase Assay—The gadB fragment spanning from –244 to +77, relative to the transcription start site, was excised from the plasmid pKKgadB-244 with BamHI and HindIII restriction enzymes, filled in, and cloned into the Smal site of pRS415 (34). The transcriptional fusions of gadA-179/+77 (7) and gadB-244/+77, cloned into the vector pRS415, were transferred to phase vectors ARS45 by in vivo homologous recombinatioin in E. coli MC4100, according to standard protocol (35). Lysogens were obtained by infecting the E. coli strains MC4100 carrying the empty plasmid pBS or pBSX with (8) the recombinant fusions phages as described (34). β-Galactosidase activity was measured according to Miller (36) and expressed as follows: 1000 × (ΔA420 nm - 1.75 × A550 nm)/A600 nm culture × reaction time × volume.

RESULTS

H-NS Specifically Interacts with the Promoter Region of gadA and gadX Genes—Although many authors have shown that gadA and gadBC genes are derepressed in an hns genetic background during the exponential growth in neutral medium, a condition in which these genes are normally silenced (7, 10, 18), so far, no evidence has been provided that H-NS is directly involved in repressing transcription of gadA, gadBC, and other...
genes belonging to the *gad* system. To determine whether 
H-NS interacts with one (or more) *gad* gene(s), we have 
screened by band shift assays the promoter regions of the two 
structural genes *gadA* and *gadBC* and of the regulatory gene 
*gadX*. As shown in Fig. 1, H-NS is able to specifically interact 
with different DNA fragments encompassing the promoter re-
gions of *gadA* (Fig. 1A) and *gadX* (Fig. 1B). Under the same 
experimental conditions, very weak or no interaction of H-NS 
was detected with the DNA fragment of the *gadBC* promoter 
spanning the nucleotide region -307/+77 relative to the gadB 
transcriptional start point (data not shown). Thus, H-NS shifts 
the gadA (−69/+77) and gadX (−67/+121) DNA fragments 
encapsulating the corresponding promoter regions as well as 
the gadA (−179/+77) and gadX (−222/+121) fragments carrying 
an additional region upstream the promoter elements, al-
beit with different affinities. In fact, although shifts of the 
smaller fragments are observed only with the highest amounts 
of H-NS tested (1.6–2 μM), less protein (0.8–1.2 μM) is required 
to retard the longer DNA fragments. These results indicate 
that H-NS binds the longer fragments, carrying the entire 
regulatory region, of *gadA* and *gadX* with an affinity that is 
−2-fold greater than that displayed for the shorter ones, which 
only contain the minimum promoter. Furthermore, the nature 
of the interaction of H-NS with PgadA and PgadX is different 
depending on the length of the promoter region tested (Fig. 1). 
In fact, H-NS can give rise to two types of retardation patterns 
producing a high and low mobility complex, named C1 and C2, 
respectively. A unique nucleoprotein complex (C1) is formed 
with the shorter DNA fragments, carrying the proximal-pro-
moter region of *gadA* (−69/+77) and *gadX* (−67/+121), indi-
cating the presence of a single H-NS binding site. A further 
complex (C2), presumably resulting from the occupancy by 
H-NS of an additional binding site, appeared only with the 
longer gadA (−179/+77) and gadX (−222/+121) fragments. 

To deeply characterize the nature of C1 and C2 complexes, 
the interaction of H-NS with the regulatory regions of *gadA* 
and *gadX* was analyzed by DNase I footprinting experiments. 
According to results of electrophoretic gel shift experiments, 
two H-NS binding sites, numbered I and II, have been identi-
fied both on *gadA* and on *gadX* regulatory regions (Fig. 2, A–D). 
H-NS protections show an average length of about 60 bp and, 
within the limit of accuracy of this analysis, are reasonably 
well matching on both DNA strands. H-NS protections on *gadA* 
span from +22 to −50 on template strand and from +43 to −55 
on non-template strand (site I), and from −89 to −133 on 
template strand and from −86 to −120 on non-template strand 
(site II). H-NS protections on *gadX* span from −10 to −67 on 
template strand and from +1 to −59 on non-template strand 
(site I), and from −88 to −153 on template strand and from 
−86 to −134 on non-template strand (site II). The location of 
H-NS binding sites on *gadA* and *gadX* promoters regions is 
summarized in Fig. 3. 

The average size of sites (−60 bp) and the finding that H-NS 
has a similar affinity for all four sites (as shown in Fig. 2, A–D, 
a remarkable protection is obtained within the range of 0.3–0.4 
μM of protein) suggest that both sites, either on *gadA* or on 
gadX, might be occupied simultaneously and somewhat coop-
eratively by several H-NS molecules. Thus, the two sites may 
be not entirely independent of each other, and the existence of 
long range protein–protein interactions among H-NS molecules 
bound to sites I and II can be rationally supposed. This hypo-
thesis is supported by the observation that H-NS, upon interac-
tion with one site, efficiently binds to the other site so that in 
the presence of the entire regulatory region of *gadA* and *gadX*, 
the predominant complex formed is C1 (Fig. 1).

Computer-generated prediction of DNA curvature reveals 
that regulatory regions of *gadA*, *gadBC*, and *gadX* are not 
edowed with an intrinsic bending, often found in H-NS-re-
sponsive promoters (data not shown). The H-NS binding model 
observed for *gadA* and *gadX* for many aspects (i.e. binding sites 
location, reciprocal influence between different sites, long 
range protein–protein interactions) resembles those proposed 
for the regulation of other genetic systems characterized at the 
molecular level, such as *hns*, *proU*, *rrnB*, and *virF* (32, 37–40), 
and is consistent with the property of H-NS to actively induce 
DNA bending as well as with the essential role attributed to 
oligomerization in the correct functioning of this DNA-binding 
protein (24).

Finally, for the interpretation of functional data (see below), 
it seems relevant to notice that H-NS site I, centered approxi-
amately at positions −10 and −32 on *gadA* and *gadX* genes, 
respectively, includes the conserved promoter elements. Also, it 
is reasonable to assume that this site is responsible for tran-
scriptional repression by H-NS, the interaction of which with 
DNA might occlude the access of RNA polymerase to the −10 
and −35 consensus sequences.

**H-NS Functions Directly as Transcriptional Repressor of** 
*gadA* and *gadX*—To determine whether H-NS is directly re-
sponsible for *gad* gene repression, the effect of this histone-like 
protein on transcription was investigated in a purified in vitro 
system programmed with pKK232-8 derivatives in which the 
gada, gadBC, and gadX DNA fragments analyzed by gel retar-
dation and DNase I footprinting were fused to the promoterless 
cat gene. As shown in Fig. 4, transcription from pKgada-179 
and pKgadX-222, carrying the entire promoter regions of the 
genes of interest, was inhibited by H-NS in a dose-dependent 
manner, and in the presence of −1 μM H-NS, residual promoter 
activity of *gadA* and *gadX* was 30 and 25%, respectively. Under
the same experimental conditions, the shorter gadA and gadX constructs, lacking the distal promoter region, showed instead a reduced sensitivity to H-NS repression. In fact, the promoter activity directed by pK\textsubscript{gadA}\textsubscript{-69} and pK\textsubscript{gadX}\textsubscript{-67}, was decreased by H-NS to 85 and 52% of the starting values, respectively. Moreover, consistently with the failure of several attempts to detect any interaction of H-NS with the gadBC promoter, no H-NS repression was observed when the pK\textsubscript{gadB}\textsubscript{-244} construct was used as DNA template in \textit{in vitro} transcription assays (Fig. 4). Taken together, these results indicate that H-NS acts as a transcriptional repressor of gadA and gadX but not of gadBC genes. Moreover, the most efficient repression is only attained with the DNA fragments of the target genes containing both of the H-NS binding sites identified by DNase I footprints. Interaction only with site I, which overlaps the conserved promoter elements, generates in the absence of the upstream binding site II an unstable nucleoprotein complex (presumably C\textsubscript{1} in Fig. 1) that cannot efficiently compete with the RNA polymerase.

**FIG. 2.** Identification of H-NS binding sites on gadA and gadX promoters by DNase I footprints. Plasmid pK\textsubscript{gadA}\textsubscript{-179} (A and B) and pK\textsubscript{gadX}\textsubscript{-222} (C and D) were incubated at \(-25^\circ\text{C}\) with or without the indicated amounts of H-NS, and the samples were processed as described under “Experimental Procedures” using the oligonucleotides A179 (A), AB77 (B), X771 (C), and X772 (D) as primers. Lanes G and A represent the Taq polymerase sequencing reactions using the same primers. The H-NS-protected sites are indicated with vertical lines and labeled I and II.

**FIG. 3.** H-NS binding sites within gadA and gadX regulatory regions. The H-NS binding sites within the nucleotide sequence of gadA (from \(-183\) to \(+97\)) and gadX (from \(-228\) to \(+121\)), as determined by DNase I footprinting, are indicated by dotted lines. The bold letters indicate a translational initiation triplet, a hypothetical ribosome binding site sequence, transcriptional start sites, and \(-10\) and \(-35\) promoter consensus sequences. The horizontal arrows, marked by a number, identify the boundaries of the DNA region carried by pK\textsubscript{gadA}\textsubscript{-69} (1 and 3), pK\textsubscript{gadA}\textsubscript{-179} (2 and 3), pK\textsubscript{gadX}\textsubscript{-67} (4 and 6), and pK\textsubscript{gadX}\textsubscript{-222} (5 and 6). Underlined sequences are the GadX binding sites on the gadA regulatory region (8).
The above observations were also confirmed by in vivo assays. The levels of gadA:cat mRNAs synthesized from the same fusions used for the in vitro tests were monitored in E. coli strains, either wild-type (HMG1) or carrying an hns null allele (HMG9). In line with previous studies (7, 8, 10, 18), the expression from all gadA and gadX constructs (either short or long ones) was greatly enhanced in the hns background during the exponential phase of growth in rich LB medium, a condition in which the gad system is normally maintained silent (Fig. 5, A and B). The analysis of the ratio of cat mRNA levels expressed by the hns mutant over that expressed by wild-type cells, containing the same plasmids, are given (C).

The minimum promoter region (Fig. 5C). The in vivo measurements also confirm that in the wild-type background, the gadA and gadX long constructs are less efficiently transcribed than the corresponding short constructs and are consistent with the way of action of H-NS as deduced by in vitro data. Our model indeed involves the simultaneous interaction of this DNA-binding protein with its sites I and II, similarly on gadA or gadX promoters, resulting in the formation of high order aggregates, which ultimately represents a fundamental event to achieve a full repression of the target genes by H-NS.

GadX Stimulates the Transcription of gadA and gadBC Promoters—Several authors, comparing the expression pattern of wild-type cells and gadX mutants, have suggested that the AraC-like transcriptional regulator GadX is implicated in the activation of the structural genes gadA and gadBC (8–12). However, despite the notable amount of data accumulated so far, not only has evidence for a direct involvement of GadX in the activation of gadA and gadBC promoters not yet been provided, but its role was even proposed to be indirect (2). In a previous study from this same laboratory, the MalE-GadX fusion protein was used in DNase I footprinting analysis to identify the binding sites on the promoter regions of both gadA and gadBC (8).

Thus, the functional relevance of the interaction of GadX with the promoters of gad genes was evaluated in an in vitro transcription assay, in the attempt to reproduce the GadX-dependent up-regulation of gadA and gadBC, as observed in vivo. As shown in Fig. 6, A and B, the addition of the MalE-GadX fusion protein causes a stimulation of both gadA and gadBC transcription when plasmids pKgadA-179 and pKgadB-244

- FIG. 4. Effect of H-NS on the promoter activity of gadA, gadBC, and gadX genes. In vitro transcription from supercoiled pKgadA-69 (open square), pKgadA-179 (filled square), pKgadB-244 (filled triangle), pKgadX-67 (open circle), and pKgadX-222 (filled circle) was carried out in the presence of increasing concentrations of H-NS as described under “Experimental Procedures.” Values represent the average of at least three independent experiments with duplicated points, and the standard deviation is less than ± 10%.

- FIG. 5. Effect of hns mutation in the in vivo level of gadA and gadX mRNAs. E. coli wild type (wt) (HMG11) and its isogenic hns strain (HMG9), harboring pKgadA-69, pKgadA-179 (A), and pKgadX-67, pKgadX-222 (B), were grown at 37 °C in LB broth until A600 = 0.5. Aliquots of each culture were processed for total RNA extraction, and mRNA samples (10 μg) were analyzed by Northern blotting. Filters were hybridized with a 32P-labeled cat gene, and signals were quantified by Molecular Imager (Bio-Rad model GS250). C, β-galactosidase levels directed by gadA(−179/−67)/lacZ and gadB(−244/−77)/lacZ chromosomal fusions in MC1410 carrying pBs or pBsX grown in LB medium to the exponential phase (A600 = 0.7). Values represent the average of 10 independent experiments, with a mean standard deviation of 25%.

- FIG. 6. Stimulation of gadA and gadBC transcription by GadX. In vitro transcription from pKgadA-179 and pKgadB-244 (200 ng) was carried out at 37 °C in the presence of the indicated concentrations of purified MalE-GadX fusion protein, as described under “Experimental Procedures.” A, Northern hybridization of mRNA transcribed from the gadA:cat fusions using a 32P-labeled cat probe. Samples are in duplicate. B, radioactivity associated with gadA (filled circle) and gadBC (open circle) transcripts was quantified using a Molecular Imager (Bio-Rad model GS250). C, β-galactosidase levels directed by gadA(−179/−67)/lacZ and gadB(−244/−77)/lacZ chromosomal fusions in MC1410 carrying pBs or pBsX grown in LB medium to the exponential phase (A600 = 0.7). Values represent the average of 10 independent experiments, with a mean standard deviation of 25%. The above observations were also confirmed by in vivo assays. The levels of gad::cat mRNAs synthesized from the same fusions used for the in vitro tests were monitored in E. coli strains, either wild-type (HMG1) or carrying an hns null allele (HMG9). In line with previous studies (7, 8, 10, 18), the expression from all gadA and gadX constructs (either short or long ones) was greatly enhanced in the hns background during the exponential phase of growth in rich LB medium, a condition in which the gad system is normally maintained silent (Fig. 5, A and B). The analysis of the ratio of cat mRNA levels expressed by the hns mutant over that expressed by wild-type strain, both transformed with the same plasmids, revealed that the long constructs pKgadA-179 and pKgadX-222 display an ~2-fold higher sensitivity to H-NS inhibition than the deletion constructs pKgadA-69 and pKgadX-67, which contain solely the minimum promoter region (Fig. 5C). The in vivo measurements also confirm that in the wild-type background, the gadA and gadX long constructs are less efficiently transcribed than the corresponding short constructs and are consistent with the way of action of H-NS as deduced by in vitro data. Our model indeed involves the simultaneous interaction of this DNA-binding protein with its sites I and II, similarly on gadA or gadX promoters, resulting in the formation of high order aggregates, which ultimately represents a fundamental event to achieve a full repression of the target genes by H-NS.

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transcriptional fusions (Fig. 6) up-regulates Transcription—study) and GadX (8) on the gadA activity in the presence of both purified proteins. Although gene, have been investigated GadX and H-NS, resulting in activation/repression of H-NS (Fig. 3). Therefore, possible antagonistic effects between partially covered by the two extended regions protected by fact, all four GadX binding sites on these two proteins in the regulation of tractions of GadX (below 25 nM), thus significantly counteract-

were used as templates in the purified in vitro system. The extent of GadX-dependent stimulation (3- and 5-fold for gadA and gadBC, respectively) closely corresponds to that observed in vivo when the gadA gene was supplied in trans in E. coli MC4100 carrying chromosomal gadA179::lacZ and gadB244::lacZ transcriptional fusions (Fig. 6C), indicating that GadX directly up-regulates gadA and gadBC genes.

H-NS and GadX Functional Competition on in Vitro gadA Transcription—The overlap of the binding sites for H-NS (this study) and GadX (8) on the gadA regulatory region suggests a physical basis for a possible functional competition between these two proteins in the regulation of gadA transcription. In fact, all four GadX binding sites on gadA are completely or partially covered by the two extended regions protected by H-NS (Fig. 3). Therefore, possible antagonistic effects between GadX and H-NS, resulting in activation/repression of gadA gene, have been investigated in vitro by monitoring promoter activity in the presence of both purified proteins. Although gadA transcription is stimulated more than 2-fold by GadX alone and strongly repressed (up to ~70%) by H-NS alone, as also shown in Figs. 4 and 6, the effect of simultaneously adding both proteins to the reaction mixture gives rise to the gadA transcription profiles shown in Fig. 7A. H-NS is still capable of repressing gadA transcription in the presence of low concentrations of GadX (below 25 nM), thus significantly counteracting the GadX-dependent activation. However, when GadX is brought to 45 nM, it is able to efficiently relieve H-NS inhibition even at the highest H-NS concentration tested (936 nM). In fact, gadA transcription attains 226% in the presence of GadX alone (45 nM) and 189 and 135% in samples containing the same amount of GadX and 468 and 936 nM H-NS, respectively (Fig. 7A). The GadX-dependent stimulation, without and with different amounts of H-NS, is more apparent when data from Fig. 7A are plotted as the relative increase (fold) of gadA promoter activity versus gadA vs H-NS. Under these conditions, a higher concentration of gadA (144 nM) is required to detect a significant increase in gadA promoter activity (transcription attains 120 and 75% at 468 and 936 nM H-NS, respectively) (data not shown), indicating that the GadX-H-NS competition is influenced, albeit moderately, by the order of the addition of the two regulators. However, data similar to those shown in Fig. 7A are obtained reversing the order of addition (samples preincubated with GadX) (data not shown). These results confirm that H-NS and GadX actively compete and that GadX is able to remove H-NS from gadA promoter even when this is already bound, a condition that likely mimics the one in vivo.

DISCUSSION

In the present study, we focused on the molecular mechanism adopted by the nucleoid-associated protein H-NS and the AraC-like activator GadX to cause antagonistic effects, negative and positive, respectively, on the expression of gad genes that play a major role in E. coli acid resistance. Despite possible pleiotropic effects, first of all, we provide strong experimental evidence that H-NS plays a direct role in the regulation of this acid resistance system, identifying gadA and gadX as target genes responsive to H-NS control. In fact, it was found that H-NS repression, exerted mainly at the transcriptional level, similarly for gadA and gadX, requires the interaction of H-NS with two binding sites, mapped by DNase I footprints, on the promoter regions of these genes. Results of in vitro and in vivo transcription, gel retardation, and DNase I footprinting experiments are consistent with one another and support an H-NS binding model in which a fundamental prerequisite is the simultaneous interaction of several H-NS molecules with two sites, which results in the formation of a stable higher order nucleoprotein structure (Fig. 1, complex CII), finally accounting for successful inhibition of gadA and gadX transcription. Compatible with this mode of action are the following findings. (i) H-NS shows a lower binding affinity and is impaired in producing complex CII with DNA fragments, derived from gadA and gadX promoters, which contain only site I (Fig. 1). (ii) Although site I overlaps the −10 and −35 consensus sequences of gadA and gadX promoters and H-NS bound at this site may compete with RNA polymerase, the deletion of distal site II (i.e. pKgadA-69 and pKgadX-67) significantly reduces the ability of H-NS to inhibit gadA and gadX promoter activity both in vitro and in vivo. This “clamping” model, based on the formation of bridging interactions between H-NS molecules sitting at sites I and II, is reminiscent of those proposed for the regulation of other H-NS-sensitive genes such as hns, proU, rnb, and virF (32, 37–40) and is consistent both with the ability of H-NS to bend DNA that is not intrinsically curved and with the relevance of long range protein-protein interactions in the correct functioning of H-NS as transcriptional repressor (24). The fact that H-NS directly controls at the transcriptional level the production of the activator GadX emphasizes the regulatory role of this protein over the expression of the whole gad system and suggests a scenario in which H-NS is indeed at the crux of the complex regulatory network governing, at least, growth phase-dependent activation/repression of gadA and gadBC.

Literature data already reported that in an hns background, derepression of gad genes occurs regardless of growth conditions, i.e. log phase cells in rich neutral medium (7, 8, 10, 18), thus suggesting that α70-RNA polymerase can initiate transcription from gad promoters in the absence of H-NS. This hypothesis is supported by the observations made in this study that, although to different extents, all gad genes tested (gadA, gadBC, and gadX) are transcribed in vitro by a commercial α70-RNA polymerase and that gadA and gadBC transcripts can be detected in an hns-ropS double mutant (41). Thus, in cells growing in the exponential phase, H-NS plays a key role in silencing the gad system, presumably preventing promoter recognition by α70. Our results indicate that H-NS can do this at different levels as follows. (i) It directly acts as transcriptional repressor of gadA and gadX promoter; (ii) it exhibits an additional negative control over gadX expression possibly by reducing its transcription from the gadA promoter; (iii) unlike gadA, gadB does not display any specific H-NS binding, and its
promoter activity is not affected in vitro by H-NS; however, H-NS might indirectly inhibit the GadX-mediated expression of gadBC by repressing gadX. Moreover, it was suggested that H-NS might doubly affect gad expression (7, 8), first via the well known negative post-transcriptional regulation of $\sigma^0$ (13) and second by preventing a successful transcription-inducing complex with $\sigma^70$ during the exponential growth. Therefore, the data herein reported are in line with the latter hypothesis and suggest that depending on growth conditions, a structural/functional competition between H-NS and both $\sigma^0$ and $\sigma^70$ factors could occur at gad promoters. In particular, during the logarithmic phase of growth, H-NS dominates, whereas increased levels of $\sigma^70$ during stationary phase might overcome H-NS repression primarily at the responsive gadX promoter, leading to induction of the whole gad system.

In a previous study, we identified by DNase I footprints GadX binding sites on the promoter regions of gadA and gadBC (8). Here, we provide the first experimental evidence that a MalE-GadX fusion protein is capable of directly stimulating gadA expression (8). Here, we provide the first experimental evidence that a MalE-GadX fusion protein is capable of directly stimulating gadA expression (8). Moreover, it was suggested that H-NS might doubly affect gad expression (7, 8), first via the well known negative post-transcriptional regulation of $\sigma^0$ (13) and second by preventing a successful transcription-inducing complex with $\sigma^70$ during the exponential growth. Therefore, the data herein reported are in line with the latter hypothesis and suggest that depending on growth conditions, a structural/functional competition between H-NS and both $\sigma^0$ and $\sigma^70$ factors could occur at gad promoters. In particular, during the logarithmic phase of growth, H-NS dominates, whereas increased levels of $\sigma^70$ during stationary phase might overcome H-NS repression primarily at the responsive gadX promoter, leading to induction of the whole gad system.

In a previous study, we identified by DNase I footprints GadX binding sites on the promoter regions of gadA and gadBC (8). Here, we provide the first experimental evidence that a MalE-GadX fusion protein is capable of directly stimulating transcription of both gadA and gadBC in a purified in vitro system. Likewise, up-regulation of gadAB/C was also observed when the gadX gene was supplied in trans. Interestingly, the gadBC operon coding for both glutamate descarboxylase and cyanate antiporter, necessary for gad system functioning, is more stimulated than gadA by GadX, thereby providing a prompt response to the acid stress. These results indicate that the presence of GadX alone is a sufficient condition to activate gadA and gadBC and fits with the observation that overexpression of GadX confers the acid resistance phenotype (10). Moreover, the finding that a functional competition between H-NS and GadX occurs at gadA promoter can help to explain how one of the multiple pathways leading to gad induction takes place. Indeed, as GadX is synthesized and its concentration rises over a threshold level, it can successfully relieve H-NS repression at the gadA promoter (as shown in Fig. 7A, a ratio GadX/H-NS ranging between 1:10 and 1:20 is required to alleviate the H-NS-dependent inhibition), further causing its own production from the dicistronic gadAX mRNA. Therefore, the increased level of GadX would ensure an appropriate expression of both gadA and gadBC. Interestingly, the indirect effect, by counteracting H-NS inhibition, is more productive than the direct stimulation of gadA promoter activity by GadX. In fact, the relative increase of gadA transcription, which is comparable among samples containing GadX alone and GadX with a low amount of H-NS, becomes 2-fold greater in the presence of a higher concentration of H-NS (Fig. 7B), suggesting a role of GadX as an antirepressor, a finding that is in line with several others in which sequence-specific transcriptional activators have already been reported to play an active role in disruption of H-NS-DNA complexes (42–44). Taking into account that during the exponential phase of growth gadA is repressed by H-NS and that GadX is only synthesized upon entry into the stationary phase, we suggest that the observed in vivo activation of gadA more closely resembles the one obtained in vitro at 936 nm H-NS (a value close to the estimated intracellular concentration of H-NS) and that repression/stimulation of this gene is likely to arise from the physiological fluctuations in the relative levels of the two regulators and from their different binding affinities to target sequences. Furthermore, it is important to notice that the activation of gadA transcription by GadX in the absence of H-NS is not cooperative, whereas it shows a substantial degree of cooperativity in terms of effects in the presence of H-NS. This finding might be correlated with the progressive occupancy of the four binding sites by GadX and consequent displacement of H-NS from gadA promoter. In fact, it is reasonable to imagine that at low concentrations of GadX (0–25 nm), heterologous H-NS-GadX-DNA complexes, which are transcriptionally inactive, are formed. Over a threshold level (25 nm), GadX causes a shift from a repressed to an activated state, possibly destabilizing the H-NS-DNA complex (see the clamping model described above), thus facilitating the access of RNA polymerase to gadA promoter. This hypothesis is supported by the appearance, in preliminary band shift assays performed by combining GadX and H-NS, of characteristic retarded bands not seen in the single GadX and H-NS gel retardation patterns (data not shown). This observation is in line with the finding that even at the highest GadX concentration tested, in vitro gadA promoter activity in the presence of H-NS never attains the values obtained with GadX alone. It can be reasonably hypothesized that the molecular mechanism of GadX/H-NS antagonism, as deduced from in vitro experiments, is further complicated in vivo by the possible participation of GadW, which was found to interact with the gadA promoter (12), so that an even more complex interplay among H-NS, GadX, and GadW can be supposed.

In the present work, we have provided evidence that H-NS directly affects the $\sigma^0$-dependent circuit of induction because it controls the expression of GadX, the primary activator of this regulatory network. It is thus clear that the $\sigma^0$-dependent circuit relies on the increased levels of this alternative $\sigma$ factor, which occur upon entry into the stationary phase and in acidic environments. Using a transcriptomic approach, Hommam et al. (10) found that H-NS also represses genes such as evgA, ydeO, and gadE, which belong to the GadE-mediated pathway of the activation ($\sigma^0$-independent circuit) of the gad system (16, 17). This implies that H-NS might indirectly participate in the regulation of GadE-mediated expression of gadA and gadBC by repressing gadE and/or components of the EvgA/S regulatory system. Additionally, because GadE was shown to bind the promoter regions of gadA and gadX (14, 15), a structural/functional interplay between H-NS and GadE can be also hypothesized.

It is evident that the gad genes are among the most finely regulated genes in E. coli, and the large number of regulators involved probably reflects the absolute requirement for a prompt adaptation to acid stress, in order to prevent low pH damages. Further investigations are clearly necessary to define possible interactions among all the gad system regulators and to understand how the intracellular level of each component is linked to cell physiology and environmental conditions. In fact, a hypothetical sensor of protons and how the stimulus drives the activation of the target genes, in a cascade reaction manner, remain to be identified. However, our study, in demonstrating both the direct repressive activity of H-NS on gadAX and the direct GadX-mediated up-regulation of the gadA/B/C genes, assigns to H-NS and GadX precise roles, which within the global view of the gad regulatory network have not yet been fully recognized.

Acknowledgments—The help of F. Pellegrini in some of the experiments and of M. De Canio in the purification of MalE-GadX, computer prediction of intrinsic DNA curvature performed by G. Micheli, and critical reading of the manuscript and helpful discussion of R. Spurio, B. Colonna, and C.O. Gualerzi are gratefully acknowledged.

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