JOURNAL OF BACTERIOLOGY, June 1996, p. 3418–3425 Vol. 178, No. 12
0021-9193/96/$04.00+0

The Histone-Like Protein H-NS Acts as a Transcriptional Repressor for Expression of the Anaerobic and Growth Phase Activator AppY of *Escherichia coli*

TOVE ATLUNG,[1]* SUSANNE SUND,[2] KIRSTEN OLESEN,[1] AND LONE BRØNDSTED[2]

Department of Chemistry and Life Sciences, Roskilde University, 4000 Roskilde,[1] and Department of Microbiology, Technical University of Denmark, DK-2800 Lyngby,[2] Denmark

Received 12 October 1995/Accepted 9 April 1996

The transcriptional activator AppY is required for anaerobic and stationary-phase induction of the *cyx-appA* and *hya* operons of *Escherichia coli*, and expression of the *appY* gene itself is induced by these environmental conditions. The sequence of the *appY* gene and its promoter region is unusually AT rich. The nucleoid-associated protein H-NS has a DNA-binding specificity for intrinsically curved AT-rich DNA. Using a single-copy transcriptional *appY-lacZ* fusion, we have shown that *appY* gene expression is derepressed in *hns* mutants during aerobic exponential growth. In the *hns* mutant, growth phase and growth rate regulation under aerobic conditions was maintained, while ArcA-dependent anaerobic induction was greatly diminished. Judged by two-dimensional gel electrophoresis, the *appY* promoter fragment exhibits the features characteristic of curved DNA. Gel retardation assays showed that purified H-NS protein bound with high affinity to two different segments of the *appY* promoter region. The role of H-NS in the AppY regulatory cascade is discussed and compared with its function in the regulatory cascades of the AppY homologs CfaD and VirF.

The H-NS protein of *Escherichia coli* belongs to the group of small histone-like proteins which function to compact the nucleoid and which influences a variety of cellular processes such as transcription, recombination, and replication. H-NS is an abundant 15.5-kDa protein that exists predominantly as a homodimer (15). H-NS has a high affinity for double-stranded DNA (29) and shows a preference for DNA with intrinsic curvature (13, 24, 31, 38). H-NS is encoded by the *hns* gene located at 27.5 min on the *E. coli* map (18, 25). *hns* mutants show highly pleiotropic phenotypes, and *hns* mutant alleles have been isolated by using many different selections for changes in regulation of gene expression; previous gene designations include *osmZ*, *drdX*, *bglY*, *cur*, and *pilG* (see reference 37 for an overview). Two-dimensional gel analysis of protein synthesis has shown that the expression of a sizeable number of proteins is changed in *hns* mutants (5, 38).

Expression of the *hns* gene is influenced by environmental factors; it is increased three- to fourfold both by cold shock (23) and by entry into stationary phase, and it is subject to negative autoregulation (13, 35). In accordance with its role as a cold shock protein, it was recently shown that H-NS is required for efficient adaptation to growth at low temperatures (12).

It has been proposed that H-NS might exert its effect on gene expression directly by binding to the DNA either as a silencer for extended chromosomal regions (18) or as a repressor by binding to specific promoters (36). Alternatively, H-NS might affect expression of some genes indirectly through its effect on σ^S (4, 39). H-NS has been shown to repress transcription in vitro at the *hns*, *proV*, and *rrnB* P1 promoters as well as at a modified *gal* promoter, where it binds to the curved DNA (32, 35, 36, 40). H-NS is required for normal thermoregulation of different genes involved in pathogenesis, e.g., the *E. coli* fimbrial *cfa* and *pap* genes, and the *Shigella flexneri vir* genes. In these cases, the H-NS protein represses transcription specifically at low temperature and seems to act by antagonizing the transcriptional activators CfaD, cyclic AMP (cAMP)/CRP, and possibly VirF (16, 22, 34).

The *appY* gene of *E. coli* was originally identified as a gene which, when cloned on high-copy-number plasmids, led to an increase in the level of expression of AppA, the acid phosphatase (pH 2.5) (2). Subsequently, we showed that the *appY* gene product, AppY, is required for the anaerobic induction of two operons involved in energy metabolism, the *hya* operon encoding hydrogenase 1 and the *cyx-appA* operon encoding a putative third cytochrome *x* oxidase and AppA (1, 8). The AppY protein is also required for the full growth phase and phosphate starvation induction of the *cyx* promoter (1). We have recently shown that the induction of AppY-responsive operons by different environmental stimuli is partly mediated by an increase in the level of expression of the *appY* gene and that the ArcA respiration response regulator is involved in induction by anaerobiosis and growth phase (9).

The AppY protein is a member of the AraC superfamily of transcriptional activators (17) and is most homologous to the members of the family involved in pathogenesis, e.g., CfaD of *E. coli* and VirF of *S. flexneri.*

The *appY* sequence shows a very unusual base composition for *E. coli*: the 548-bp intergenic sequence upstream of *appY* contains 75% AT, and the structural gene contains 68% AT. The promoter region contains a number of A tracts, strongly suggesting that it is intrinsically curved DNA (11).

The sequence characteristics of the *appY* promoter region together with the role of H-NS in *cfa* and *vir* gene regulation inspired us to the studies reported in this paper. We have tested the effect of *hns* mutations on the expression of the *appY* gene under different environmental conditions, and we have analyzed the *appY* promoter region for the presence of bent DNA and for binding of purified H-NS protein. Finally, we also investigated the effect of H-NS on the two AppY target promoters *hya* and *cyx.*

* Corresponding author. Mailing address: Department of Chemistry and Life Sciences, Roskilde University, P.O. Box 260, 4000 Roskilde, Denmark. Phone: (45) 46 75 77 11 ext. 2402. Fax: (45) 46 75 77 21. Electronic mail address: atlung@virgil.ruc.dk.

TABLE 1. *E. coli* K-12 strains used

Strain	Genotype[a]	Reference or construction
LJ24	*thi-1 leu-6 lacY1 lacI-ZΔ* (*Mlu*) *supE44 tonA21 rpsL rfbD1*	Rasmussen et al. (28)
TC3594	Δ(*appY-ent*)[b]	Atlung and Brøndsted (1)
GM230	*araD139* Δ(*argF-lac*)*U169 deoC1 flb5301 relA1 rpsL150 ptsF25 rbsR hns-205*::Tn*10* Φ(*proU-lacZ*)hyb2 (λ *plac*Mu15)	Higgins et al. (19)
PD32	*araD139* Δ(*argF-lac*)*U169 deoC1 flb-5301 relA1 rpsL150 ptsF25 rbsR hns-206*::Ap^r	Dersch et al. (13)
BL21	*gal met* r$^-$m$^-$ *hsdS* p*lacI*^{q}/λ p*lacUV5*-T7-gene 1	Studier and Moffat (30)
TC3981	*attB*::p$_{cyx'}$-*lacZ*^{b}	Atlung and Brøndsted (1)
TC3983	*attB*::p$_{appY'}$-*lacZ*^{b}	Brøndsted and Atlung (9)
TC3985	*attB*::p$_{hya'\text{-}lacZ}$^{b}	Brøndsted and Atlung (8)
TC4032	*hns-205*::Tn*10* *attB*::p$_{cyx'}$-*lacZ*^{b}	GM230(P1) × TC3981
TC4033	*hns-205*::Tn*10* *attB*::p$_{appY'}$-*lacZ*^{b}	GM230(P1) × TC3983
TC4034	*hns-205*::Tn*10* *attB*::p$_{hya'}$-*lacZ*^{b}	GM230(P1) × TC3985
TC4117	Δ(*appY-ent*) *attB*::p$_{cyx'}$-*lacZ*^{b}	TC3981(P1) × TC3594
TC4119	Δ(*appY-ent*) *attB*::p$_{hya'}$-*lacZ*^{b}	TC3985(P1) × TC3594
TC4120	*hns-205*::Tn*10* Δ(*appY-ent*) *attB*::p$_{cyx'}$-*lacZ*^{b}	GM230(P1) × TC4117
TC4122	*hns-205*::Tn*10* Δ(*appY-ent*) *attB*::p$_{hya'}$-*lacZ*^{b}	GM230(P1) × TC4119
TC4135	*hns-206*::Ap^r *attB*::p$_{appY'}$-*lacZ*^{b}	PD32(P1) × TC3983
LB137	*arcA1* zjj::Tn*10* *attB*::p$_{appY'}$-*lacZ*^{b}	Brøndsted and Atlung (9)
TC4250	*arcA1 zjj*::Tn*10* *hns-206*::Ap^r attB::p$_{appY'}$-*lacZ*^{b}	PD32(P1) × LB137
TC4159	*attB*::p$_{appY'}$-*lacZ*^{b}/pACYC184	
TC4163	*hns-206*::Ap^r *attB*::p$_{appY'}$-*lacZ*^{b}/pACYC184	

[a] Genetic symbols are according to Bachmann (3).
[b] Genotype otherwise like that of strain LJ24.

MATERIALS AND METHODS

Bacterial strains, plasmids, growth media, and conditions and enzyme assays. The strains used for physiological experiments in this study are all derivatives of the C600 Δ*lac* strain LJ24 (28) and are listed together with the other strains in Table 1. The plasmids pTAC3953, pTAC3956, pTAC3958, and pTAC3960, which were used for analysis of curved DNA and gel retardation assays, have been described previously (1, 8, 9). Plasmid pLOB6 is a derivative of pTAC3953 (8) (see Fig. 6), which carries a modified multicloning site with *Sac*II, *Not*I, *Sfi*I, and *Spe*I sites inserted into the *Bgl*II site, and pLOB7 carries the same *Taq*I *appY* promoter fragment as pTAC3958 cloned into the *Bst*BI site.

For growth phase experiments, cultures were grown with the indicated degree of shaking in 500-ml flasks with 100 ml of AB minimal medium (10) supplemented with 1 μg of thiamine per ml, 0.2% glucose, and either 1% Casamino Acids (Bacto Difco) or a synthetic amino acid mixture (FN20) at the concentrations suggested by Neidhardt et al. (27). For anaerobic growth experiments, we used AB minimal medium supplemented with 1 μg of thiamine per ml, 0.1% glucose, 100 μg of leucine per ml, and, when required, 100 μg of isoleucine and 100 μg of methionine per ml. Anaerobic conditions were achieved as described previously (8). Antibiotics were added at 100 μg of ampicillin, 50 μg of kanamycin, and 10 μg of chloramphenicol per ml. Cell density was monitored and β-galactosidase activity was determined as described previously (8).

DNA technology and DNA gel electrophoresis. Plasmid DNA was prepared according to the method described by Birnboim and Doly (6). Restriction enzymes were used as recommended by the suppliers (New England Biolabs and Amersham). PCR amplifications were performed in a DNA Thermal Cycler (Perkin-Elmer Cetus) with AmpliTaq polymerase (Roche Molecular system) or Thermoprime Plus (Advanced Biotechnologies) in the buffers provided by the suppliers. *Eco*RI-digested plasmid pTAC3958 was used as a template. The primers for the *appY* promoter region (positions indicated in Fig. 6) were the following synthetic oligonucleotides and the -21 M13 sequencing primer:

Y1: 5′AGTCTAGAGCATACTGGTAACTCCC
Y2: 5′CGGTCGACCATCCTGATTATGATTGTG
Y3: 5′CGGTCGACAAGCATAGTAAGCGTTG
Y4: 5′AGTCTAGACAAGTTGATAGTAGTCAAA
Y5: 5′AAGTCGACGCAAGCTGGATGTTGGT
Y6: 5′CGGTCGACCAGTATGTATATCATAGG
pCB: 5′CGTGCAGCCCTTATTGC

Reactions were carried out for 30 cycles with a 1-min denaturation step at 94°C and a 1-min annealing step at 51 to 56°C, depending on the primers, and elongation for 2 min at 72°C. PCR fragments were purified with the QIAquick-spin PCR purification kit (QIAGEN).

DNA fragments exhibiting a curvature were identified by a two-dimensional electrophoresis assay (26). The DNA fragments were separated at 60°C (first dimension) on polyacrylamide gels with 0.5× TBE (Tris-borate-EDTA) as running buffer. For the second dimension at 4°C, the gel was placed in a horizontal agarose gel apparatus. The DNA was visualized by staining with ethidium bromide.

Purification of H-NS protein and gel retardation assaying. H-NS protein was overproduced and purified, and protein concentrations were determined exactly as described by Dersch et al. (13). Gel retardation assays were carried out as described there (13) with 10-μl reaction mixtures containing increasing concentrations of purified H-NS protein (0.025 to 0.5 μg). The DNA used for the reaction was either 0.25 μg of restriction enzyme-digested plasmid DNA or purified *appY* promoter PCR fragments (5 ng) mixed with the control *tet* PCR fragment (25 ng) or mixed with restriction enzyme-digested pTAC3953 vector DNA. Plasmid pTAC3958 was digested with *Bst*NI-*Pst*I-*Xho*I, and plasmids pTAC3956 and pTAC3960 were digested with *Pvu*II-*Pst*I-*Xho*I-*Bst*EII.

RESULTS

Effect of an *hns* mutation on *appY* expression during exponential growth at different temperatures. A single-copy *appY′-lacZ* operon fusion integrated into the λ attachment site was used for all studies of *appY* gene expression. The fusion was constructed by inserting the 756-bp *Taq*I fragment (shown in Fig. 7) in front of a promoterless *lacZ* gene, and the fusion thus contained the entire *appY* promoter region as well as the first 180 bp of the coding sequence.

For the initial experiments to test the possible effect of H-NS on *appY* gene expression, we used the *hns-205* mutation, which carries a Tn*10* insertion in codon 93 of the *hns* gene. We found that inactivation of *hns* led to a significant increase in *appY* gene expression (4- to 15-fold) during exponential aerobic growth conditions (Table 2). Although there was very little variation in *appY* expression in the wild type at temperatures ranging from 30 to 42°C, the effect of the *hns* mutation was more pronounced at 30°C than at higher temperatures, in agreement with the role of H-NS in thermoregulation of fimbrial and virulence genes (18, 22, 34).

Effect of *hns* inactivation on growth phase and growth rate regulation of *appY* expression. Since both *appY* and *hns* gene expression is growth phase regulated (9, 13), we investigated the effect of *hns* inactivation on *appY* gene expression during entry into stationary phase. In the experiment shown in Fig. 1, *appY′-lacZ* expression was monitored in cultures of the wild type and in an *hns-206* mutant growing in glucose amino acids medium at 37°C with good aeration. In both strains, *appY* expression was induced when the cultures reached the decel-

TABLE 2. Effect of *hns-205* mutation on expression from the $p_{appY'}$-*lacZ* fusion at different temperatures and in different media[a]

Growth temp (°C)	β-Galactosidase activity (U ml^{-1} A_{450}^{-1})[b]			
	Glucose minimal medium		Glucose Casamino Acids	
	TC3983 (*hns*$^+$)	TC4033 (*hns-205*)	TC3983 (*hns*$^+$)	TC4033 (*hns-205*)
30	4.2	51	1.8	26
37	4.5	22	1.0	4.7
42	4.3	19	0.9	5.0

[a] Cells were grown exponentially at the indicated temperatures in AB minimal medium supplemented with glucose, leucine, isoleucine, and methionine or with glucose plus Casamino Acids.

[b] β-Galactosidase activities were determined as described in Materials and Methods.

eration phase of growth. Expression was induced to nearly the same extent in both strains, fivefold in the wild type and fourfold in the mutant, suggesting that H-NS does not play a major role in stationary-phase induction of *appY*.

In the experiment presented in Fig. 1, the growth phase response of *appY* expression is probably a reflection of the decrease in growth rate taking place in the deceleration phase (9). Therefore, we tested the effect of the *hns* mutation on growth rate regulation of *appY* by performing α-methylglucoside downshift experiments. From Fig. 2, it can be seen that a downshift caused an immediate induction in both strains and that the differential rate of β-galactosidase synthesis increased 13-fold in the wild type and 8-fold in the mutant and then decreased to 3- and 2-fold the preshift rates, respectively, when the growth rate increased moderately after an adaptation period. It should be noted that the preshift generation time of the *hns* mutant was 90 min, versus 60 min for the wild type; however, the effect of the downshift in both cases was a 4-fold increase in generation time.

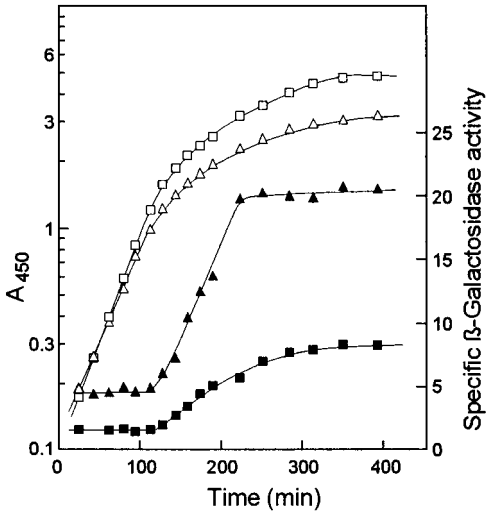

FIG. 1. Growth phase induction of *appY* gene expression in an *hns* mutant. Strains TC4159 ($p_{appY'}$-*lacZ hns*$^+$ [squares]) and TC4163 ($p_{appY'}$-*lacZ hns-206* [triangles]) were grown at 37°C with good aeration (120 rpm) in AB minimal medium supplemented with 0.2% glucose and synthetic amino acids mixture. Growth of the cultures was monitored spectrophotometrically (open symbols), and specific β-galactosidase activities (closed symbols) were determined as described in Materials and Methods.

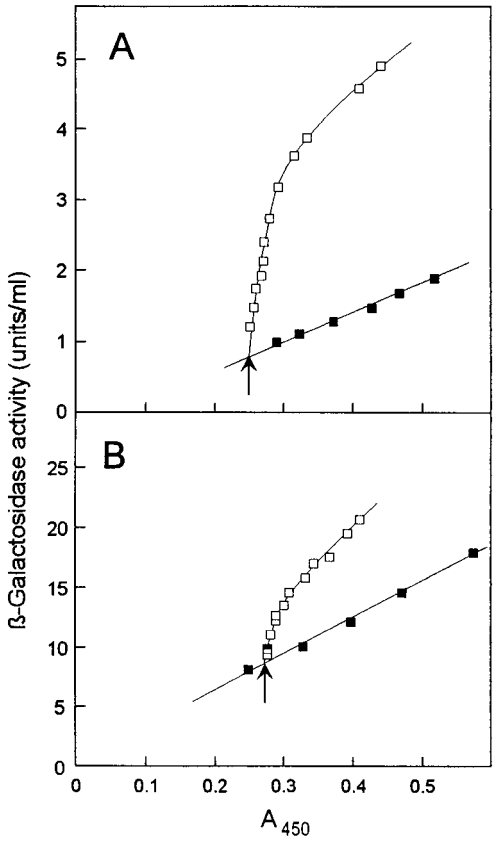

FIG. 2. Growth rate regulation of *appY* gene expression in an *hns* mutant. Strains TC4159 ($p_{appY'}$-*lacZ hns*$^+$) (A) and TC4163 ($p_{appY'}$-*lacZ hns-206*) (B) were grown at 37°C in AB minimal medium supplemented with 0.1% glucose. Growth of the cultures was monitored spectrophotometrically, and at the optical densities indicated by the arrows, the cultures were divided and one-half received α-methyl glucoside to a final concentration of 2%. β-Galactosidase activities were determined as described in Materials and Methods and were plotted versus cell mass. Closed symbols, untreated cultures; open symbols, α-methyl glucoside downshifted cultures.

Role of H-NS in anaerobic regulation of *appY* gene expression. The expression of the *appY* gene is induced by anaerobiosis (9). Surprisingly, we found that inactivation of *hns* strongly affected the regulation of *appY* gene expression upon a shift to anaerobic growth conditions (Fig. 3). Induction was delayed for half a generation in the *hns* mutant, and the induction was severely reduced; the steady-state anaerobic expression in the mutant showed only a 1.5-fold increase compared with the 6-fold increase in the wild type. The anaerobic generation times were 90 and 115 min for wild type and mutant, respectively; thus, the growth defect of the *hns* mutant is less severe under anaerobic conditions.

The ArcA regulator acts as a transcriptional activator for *appY* expression under oxygen-limiting conditions (9). The results described above suggested that H-NS might act by blocking ArcA action at the *appY* promoter region. Therefore, we compared the *appY'-lacZ* expression levels upon entry into stationary phase under oxygen-limiting conditions in the wild type, *hns* and *arcA* single mutants, and an *hns arcA* double mutant (Fig. 4). This experiment showed that introduction of the *arcA* mutation into the *hns* mutant had virtually no effect on *appY* expression, whereas the *arcA* mutation reduced the induction in the *hns*$^+$ background fourfold, as shown previously (9). Neither the *arcA* mutation (9) nor the *hns* mutation

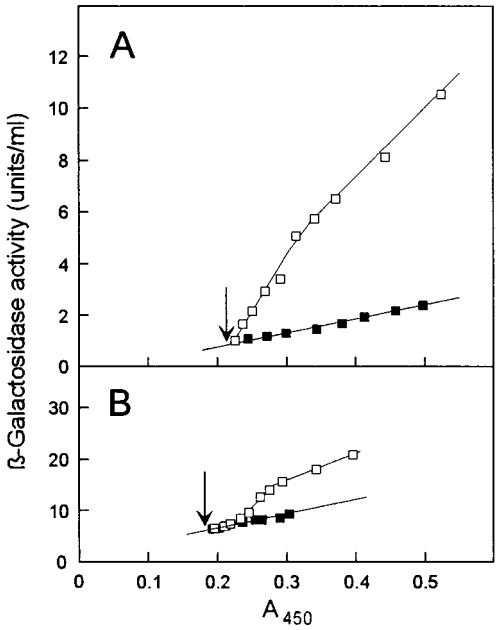

FIG. 3. Anaerobic induction of *appY* gene expression in an *hns* mutant. Strains TC4159 ($p_{appY'}$-*lacZ hns*$^+$) (A) and TC4163 ($p_{appY'}$-*lacZ hns-206*) (B) were grown at 37°C in AB minimal medium supplemented with 0.1% glucose. Growth of the cultures was monitored spectrophotometrically, and at the optical densities indicated by the arrows, the cultures were divided and one-half was shifted to anaerobic conditions. β-Galactosidase activities were determined as described in Materials and Methods and were plotted versus cell mass. Closed symbols, aerobic cultures; open symbols, anaerobic cultures.

(Fig. 1) has any effect on *appY* induction during entry into stationary phase in well-aerated cultures.

It has been reported that H-NS affects the degree of supercoiling of reporter plasmids (20, 37), and it has been shown that shift to anaerobic conditions leads to an increase in negative supercoiling (14). Increase in the osmolarity of the growth medium also increases negative supercoiling (14). However, addition of NaCl to 0.2 M had no effect on *appY'-lacZ* expression levels, neither in the wild type nor in the two *hns* mutants (data not shown), indicating that *appY* is not subject to osmoregulation and suggesting that the effect of H-NS on *appY* is independent of possible effects on supercoiling.

Analysis of intrinsically curved DNA in the *appY* promoter region. The H-NS protein has been shown to bind preferentially to intrinsically curved DNA (38), for which the sequence characteristic is phased A tracts of 4 to 6 bp (11). As expected from the extreme AT richness of the *appY* promoter region, several such A tracts are found within the 500-bp region upstream of the *appY* coding region (2). We used two-dimensional acrylamide gel electrophoresis with the first dimension at 60°C and the second dimension at 4°C to investigate the presence of bent DNA in the *appY* promoter. By this method, DNA fragments are resolved according to size and conformation. Noncurved DNA fragments migrate on a diagonal line, whereas curved fragments deviate from the diagonal because of slower migration at 4°C (26).

As an initial test for curved DNA, a restriction enzyme digest of the *appY-lacZ* fusion plasmid pLOB7 together with a digest of the vector pLOB6 was analyzed (Fig. 5A). The four pLOB7 fragments that clearly deviate from the diagonal are the 1,218-bp fragment containing the pBR322 origin, the 805-bp fragment containing the inserted *appY* promoter, the 517-bp fragment containing the *bla* promoter, and a 354-bp fragment from the *phoA* coding sequence. To localize the bend in the *appY* promoter fragment, we also analyzed two mixtures of the PCR fragments shown in Fig. 6. As seen in Fig. 5B, fragment 5, encompassing the 500 bp just upstream of the coding region, showed the most pronounced deviation from the diagonal, suggesting that the sequences responsible for the curvature are located near the middle of this fragment. The position of the bend was further localized by analysis of a *Dra*I digest of PCR fragment 0. Only the smallest of the two products of fragment 0 deviated from the diagonal (data not shown). Since PCR fragment 6 also clearly migrated as a bent fragment (Fig. 5B), this located the major bend approximately 350 bp upstream of the coding sequence.

Interaction of the H-NS protein with the *appY* promoter. To test if the H-NS protein might act as a repressor of *appY* transcription directly via binding to the curved DNA in the promoter region, we performed in vitro gel retardation assays with purified H-NS protein as described by Dersch et al. (13). pTAC3958 plasmid DNA was digested with appropriate restriction enzymes and was incubated with increasing amounts of H-NS protein (Fig. 7). Addition of very small amounts of H-NS protein was sufficient to retard the 1,328-bp fragment containing the *appY* promoter. Much higher amounts of H-NS were required to retard the 741-bp *bla* promoter and the 976-bp pBR322 origin fragments, and amounts of H-NS even higher than those used for the experiment shown in Fig. 7 were needed for retardation of the noncurved DNA fragments (data not shown).

To narrow down the regions of the *appY* promoter responsible for the high-affinity H-NS binding, we initially carried out gel retardation assays using the different PCR fragments mixed with a control fragment from the *tet* gene. These experiments (data not shown) suggested that H-NS bound specifically to all

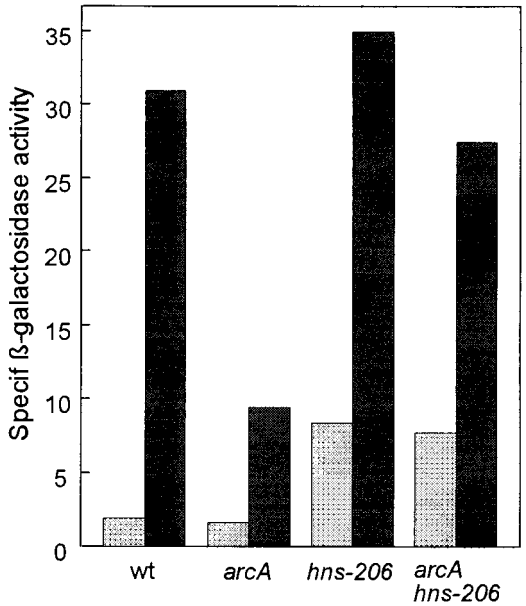

FIG. 4. Effect of ArcA and H-NS on induction of *appY* expression under oxygen-limiting conditions. The four strains TC3983 (wild type [wt]), LB137 (*arcA1*), TC4135 (*hns-206*), and TC4250 (*arcA1 hns-206*), all carrying the *appY'-lacZ* fusion, were grown at 37°C in glucose Casamino Acid-supplemented medium with moderate aeration, and the specific activities of β-galactosidase were determined in exponential phase (light-grey bars) and in early stationary phase (dark-grey bars). Early stationary phase corresponds to an A_{450} of 6 (growth curves for the wild-type and *arcA1* strains from a similar experiment are shown in Fig. 4A in reference 9).

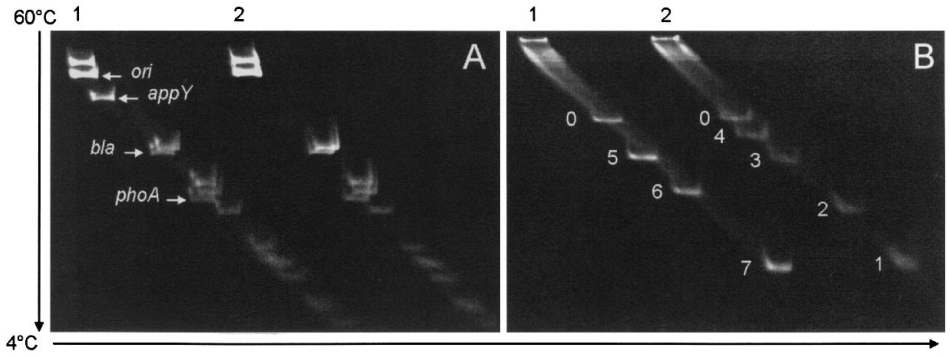

FIG. 5. Analysis of intrinsically curved DNA in the *appY* promoter region. Mixtures of DNA fragments were separated by two-dimensional polyacrylamide gel electrophoresis. The gels were run at 60°C in the first dimension and at 4°C in the second dimension. (A) Restriction enzyme digests (*Hin*dIII-*Hin*fI) of the *appY* promoter plasmid pLOB7 (lane 1) and the vector pLOB6 (lane 2) were separated on an 8% acrylamide gel. The fragments exhibiting the feature of curved DNA are indicated by arrows and are labelled by their genetic designations. *appY*, the *appY* promoter; *bla*, the *bla* promoter; *ori*, the pBR322 replication origin; *phoA*; fragment from *phoA* coding sequence. (B) Mixtures of chromosomal DNA digested with *Hin*fI and the PCR fragments, indicated by their numbers (see Fig. 6 for extents of the fragments), were separated by two-dimensional gel electrophoresis in a 6% acrylamide gel.

of the *appY* PCR fragments, although with different relative affinities. These observations were confirmed by gel retardation assays with restriction enzyme digests of pTAC3953-derived plasmids carrying the different PCR fragments (Fig. 6). An example of these analyses is shown in Fig. 8A, and the results for all PCR fragments are summarized in Fig. 6, in which affinities relative to that of the *bla* promoter fragment are assigned. H-NS binding to the *appY* promoter-carrying fragments is due to the inserted DNA, since the 440-bp fragment spanning the cloning site in pTAC3953 did not exhibit any specific H-NS binding (Fig. 8A, lanes 2 to 5, and 8B). Analysis of *Hin*cII-digested PCR fragment 0 mixed with a restriction enzyme digest of plasmid pTAC3953 (Fig. 8B) showed that there are at least two high-affinity H-NS binding sites, since both resulting fragments were specifically bound.

Effect of H-NS on expression of the *hya* and *cyx* operons. The promoters of the *hya* and *cyx* operons are the only two identified AppY targets. We tested the effect of H-NS on expression of these two promoters using single-copy *lacZ* operon fusions (Table 3). The presence of the *hns-205* mutation led to an increase in expression of both fusions; this effect was most pronounced for the *cyx* promoter. Since both promoters are

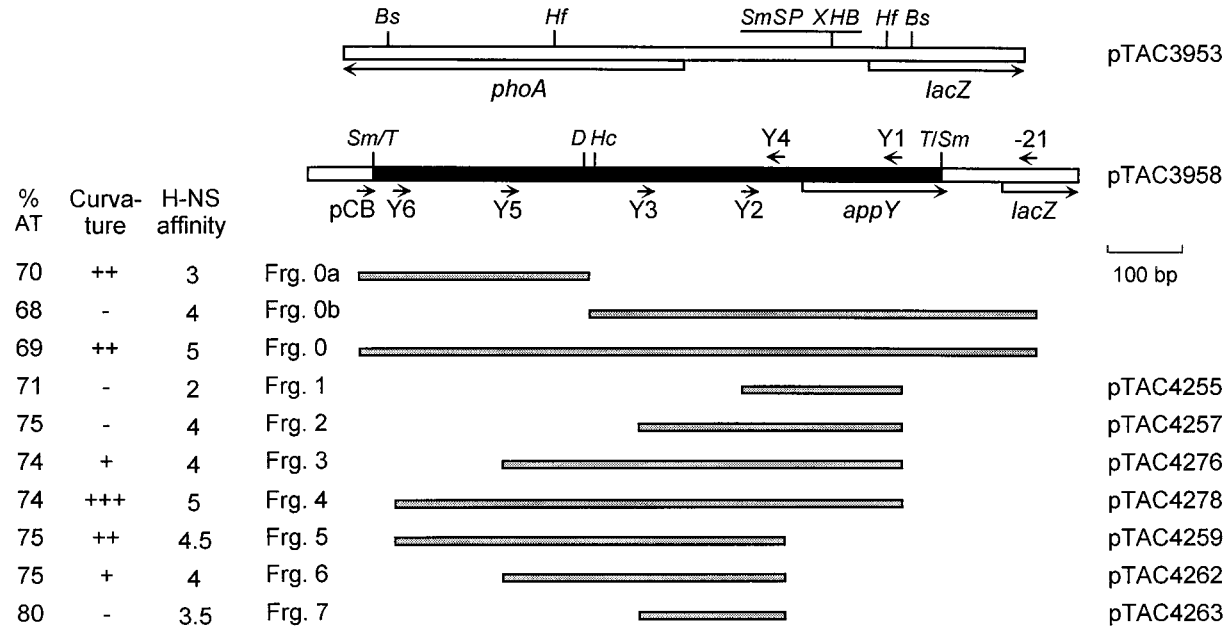

FIG. 6. Physical structures of the *appY* promoter region and the PCR fragments used for analysis of curved DNA. (Top) Part of the *lacZ* promoter probe vector pTAC3953 (8) with relevant restriction enzyme sites indicated. *B*, *Bst*BI; *Bs*, *Bst*NI; *D*, *Dra*I; *H*, *Hin*dIII; *Hc*, *Hin*cII; *Hf*, *Hin*fI; *P*, *Pst*I; *S*, *Sal*I; *Sm*, *Sma*I; T, *Taq*I; X, *Xba*I. (Bottom) Part of plasmid pTAC3958 carrying the *appY Taq*I promoter fragment cloned into the *Sma*I site of pTAC3953. Small arrows labelled Y1, etc., indicate the positions of the PCR primers. The sequences of the primers are shown in Materials and Methods. The extents of DNA in the different PCR fragments (fragments [Frg.] 0 to 7) are indicated. After digestion with *Sal*I-*Xba*I, PCR fragments 1 to 7 were cloned into *Sal*I-*Xba*I-digested pTAC3953, giving rise to the plasmids indicated to the right. % AT, base composition of the PCR fragment; curvature, degree of curvature judged from two-dimensional gel electrophoretic analysis of the PCR fragment; H-NS affinity, relative H-NS affinity on a scale of 0 to 5; 0, no specific binding; 1, affinity of the *phoA* fragment; 2, affinity of the *ori* fragment; 3, affinity of the *bla* promoter fragment; 5, affinity of the entire *appY* promoter fragment. The affinities were assigned from gel retardation experiments with restriction enzyme-digested plasmid DNA and with mixtures of PCR fragments (Fig. 7 and 8 and data not shown).

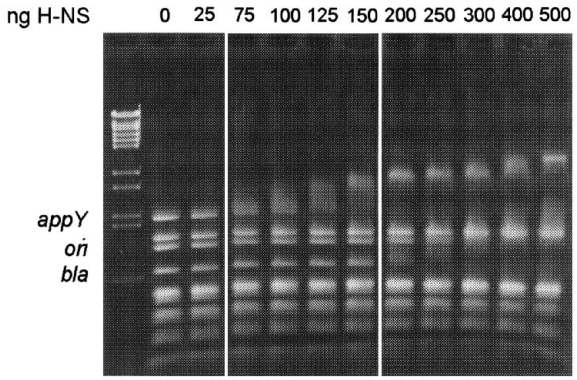

FIG. 7. Specific binding of H-NS protein to the *appY* promoter region. Purified DNA from a restriction enzyme digest of plasmid pTAC3958 with *Bst*NI-*Pst*I-*Xho*I was incubated with the indicated amounts of purified H-NS protein and was separated by electrophoresis on a 1% agarose gel. A molecular weight standard of λ DNA digested with *Bst*EII was run in the first lane. The identities of the three specifically retarded fragments are indicated to the left.

stimulated by overproduction of AppY (1) and *appY* transcription is increased fivefold under the growth conditions used (compare Table 2), we also tested the effect of the *hns* mutation in an *appY* background. In both cases, the increase in the level of expression caused by *hns* inactivation was eliminated by the *appY* mutation, suggesting that the effect of H-NS on the *hya* and *cyx* promoters might be indirect.

Although the *cyx* and *hya* promoter fragments do not exhibit the feature of curved DNA, (data not shown), we performed H-NS gel retardation assays with restriction enzyme digests of the two promoter *lacZ* fusion plasmids pTAC3956 and pTAC3960 carrying the *cyx* and *hya* promoters. In accordance with the noncurved nature of these fragments, there was no specific binding of H-NS protein to these promoters, whereas the *bla* promoter fragment was bound specifically at the usual H-NS concentration (data not shown).

DISCUSSION

In this paper, we have shown that the nucleoid-associated protein H-NS negatively regulates the expression of the transcriptional anaerobic activator AppY. Unlike most other systems in which H-NS represses transcription, *appY* gene expression in the wild-type strain was not affected by changes in osmolarity or by changes in temperature in the 30 to 42°C range. However, the effect of inactivation of the *hns* gene was consistently stronger at 30 than at 37°C, suggesting that H-NS plays a role in thermoregulation of the *appY* gene like it does for the *cfa*, *pap*, and *S. flexneri vir* genes (18, 22, 34). We used two different *hns* insertion mutations in the work presented here, the *205* allele that carries an insertion in codon 93 and the *206* allele in which the insertion is located in codon 37 (12). The effects on *appY* expression of the two mutations were qualitatively similar.

TABLE 3. Effect of the *hns-205* mutation on expression from the *cyx* and *hya* promoter regions[a]

Relevant genotype	β-Galactosidase activity (U ml^{-1} A_{450}^{-1})[b]	
	$p_{cyx'}$-*lacZ*	$p_{hya'}$-*lacZ*
hns$^+$ *appY*$^+$	0.10	0.08
hns-205 *appY*$^+$	1.8	0.21
hns-205 Δ(*appY-ent*)	0.1	0.11

[a] Strains carrying the indicated fusions (see Table 1 for full genotypes) were grown exponentially at 37°C in AB minimal medium supplemented with glucose leucine, isoleucine, and methionine.
[b] β-Galactosidase activities were determined as described in Materials and Methods.

Two-dimensional gel electrophoretic analysis of the *appY* promoter region demonstrated that, as expected from the sequence characteristics, it contains curved DNA, and analysis with overlapping PCR fragments showed that the major region of bent DNA is located approximately 350 bp upstream of the *appY* start codon. Gel retardation experiments showed that purified H-NS protein bound specifically to the *appY* promoter, strongly suggesting that H-NS acts directly as a repressor for *appY* gene expression. Comparison of our data with those from the very similar experiments described by Lucht et al. (24) indicates that H-NS has at least as high an affinity for the *appY* promoter region as it has for the *proV* promoter region. In contrast to the experiments with the *hns* and *proV* promoters (13, 24), we observed the appearance of several quite well-defined retarded species of the entire *appY* promoter fragment at concentrations of H-NS at which there was

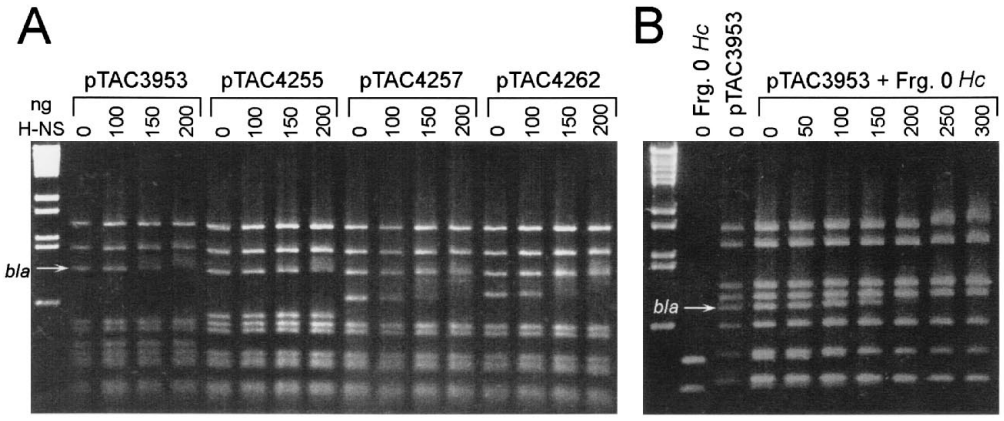

FIG. 8. Localization of high-affinity H-NS-binding sites in the *appY* promoter region. Purified DNAs from restriction enzyme digests were incubated with the indicated amounts of purified H-NS protein and were separated by electrophoresis on 1.3% agarose gels. A molecular weight standard of λ DNA digested with *Bst*EII was run in the first lane of each gel. (A) *Hin*fI-*Xho*I digests of the indicated plasmids (see Fig. 6 for structures of the plasmids); (B) *Hin*cII digest of PCR fragment (Frg) 0 mixed with a *Bst*EII-*Dra*I-*Eco*RV-*Hin*dIII-*Pvu*II digest of pTAC3953. *bla*, fragment carrying the *bla* promoter.

no binding to noncurved fragments, suggesting that there might be more than one H-NS binding site in the *appY* promoter. In agreement with this hypothesis, both parts of *Hin*cII-digested PCR fragment 0 bound H-NS with high affinity. H-NS might repress *appY* transcription by interacting with the curved DNA sequence upstream of the promoter, similarly to the way it represses its own synthesis (13). Alternatively, the negative effect of H-NS on *appY* expression might be mediated by binding to the noncurved DNA close to the translational start, similarly to the situation in the *proV* promoter region (24). We are presently testing these possibilities using integrated copies of the different PCR fragments fused to *lacZ*.

With the aim of identifying the physiological role of the H-NS repression, we tested the effect of inactivation of the *hns* gene on the induction of *appY* by different environmental stimuli. Induction of *appY* by entry into the stationary phase under well-aerated conditions and growth rate regulation were virtually normal in the *hns-206* mutant. However, anaerobic induction was strongly reduced by inactivation of *hns*. Most of the anaerobic induction in the wild type is mediated by the ArcA response regulator (9), which is activated via phosphorylation by the ArcB sensor under conditions of reduced respiration (21). Inactivation of *arcA* in the *hns* mutant had very little effect on the *appY* expression under oxygen-limiting conditions. Therefore, we propose that the activated ArcA protein might act as an anti-repressor for H-NS in the wild-type strain, similarly to the role of the cAMP/CRP complex in *papB* expression (16) and the CfaD protein in *cfaB* expression (22).

Although we demonstrated that H-NS protein binds with very high affinity to the *appY* promoter, we cannot exclude that the increased *appY* expression during exponential growth might be indirect. Growth phase induction of *appY* is slightly reduced in an *rpoS* mutant (9), indicating that σ^S participates in *appY* transcription. Therefore, it is conceivable that the increase in the concentration of σ^S found in *hns* mutants (4, 39) contributes to the increase in the level of *appY* expression. We are presently investigating this possibility.

We found that *hns* inactivation led to an increase in the levels of expression of the two AppY target promoters *hya* and *cyx*. However, the effect was dependent on an intact *appY* gene, and there was no specific binding of H-NS to these promoters, indicating that the effect is indirect, being mediated by increased concentrations of AppY protein. The stronger effect on the *cyx* promoter is in agreement with the higher responsiveness of this promoter to increased synthesis of AppY from a *lacP*-controlled plasmid (7).

Like CfaD and VirF—its close relatives in the AraC family—AppY is a link in a regulatory cascade which involves the H-NS protein. However, closer inspection of these three regulatory systems reveals that there are significant differences (Fig. 9). H-NS plays a dual role in the CfaD system (Fig. 9B), acting both as a repressor of CfaD transcription and as a CfaD antagonist for expression of the structural genes of *Cfa*I fimbriae (22). In the virulence regulatory system in *S. flexneri*, H-NS probably acts only as a repressor for the VirF-activated transcription of VirB, which in turn activates expression of several operons encoding functions required for virulence (33, 34). The AppY regulatory cascade is rather complex and might function to integrate different signals concerning the metabolic state of the cell. Synthesis of the AppY protein is regulated by the ArcA regulator, which responds to changes in respiration (21), and the AppY protein is itself activated in response to anaerobiosis (7), probably by association with a low-molecular-weight effector like some of the other members of the AraC family of activators. In this case, the H-NS protein plays a role only in expression of the AppY activator but does not participate directly in the regulation of the target operons.

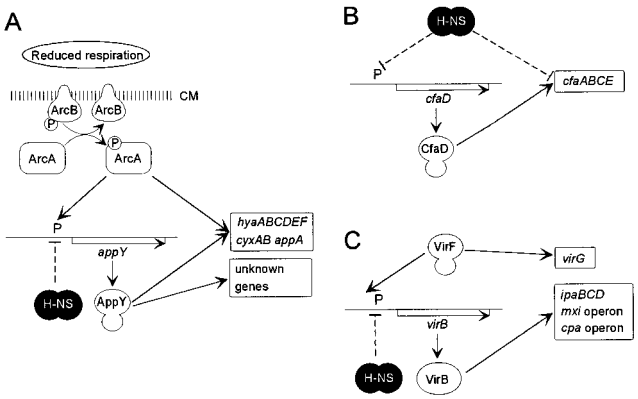

FIG. 9. Interplay between H-NS and three different members of the AraC family of transcriptional activators. Black straight arrows, activation of transcription; stippled straight arrows, repression of transcription; curved thin arrows, activation of regulatory proteins by environmental signals; CM, cytoplasmic membrane; P, promoter. (A) The ArcA-AppY regulatory cascade based on the results presented here and previously (1, 7, 8, 9); (B) the H-NS CfaD regulatory cascade based on results described by Jordi et al. (22); (C) the VirF VirB regulatory cascade based on results from the Sasakawa group (33, 34).

ACKNOWLEDGEMENTS

We thank Erhard Bremer for generously providing strain PD32 (*hns-206*) and plasmid pPD3 for overproduction of H-NS. We are grateful to Erhard Bremer and Ole Michelsen for discussions and critical reading of the manuscript. We thank Anne Nielsen for her expert technical assistance.

This work was supported by the Danish Center of Microbiology.

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