Repression by Binding of H-NS within the Transcription Unit*

V. Nagarajavel†, S. Madhusudan†, Sudhanshu Dole§, A. Rachid Rahmouni†, and Karin Schnetz‡

From the †Institute for Genetics, University of Cologne, 50674 Cologne, Germany, ‡Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115, and §Centre de Biophysique Moleculaire, CNRS, 45071 Orleans, France

H-NS inhibits transcription by forming repressing nucleoprotein complexes next to promoters. We investigated repression by binding of H-NS within the transcription unit using the bgl and proU operons. Repression of both operons requires a downstream regulatory element (DRE) in addition to an upstream element (URE). In bgl, H-NS binds to a region located between 600 to 700 bp downstream of the transcription start site, whereas in proU the DRE extends up to position +270. We show that binding of H-NS to the bgl-DRE inhibits transcription initiation at a step before open complex formation, as shown before for proU. This was shown by determining the occupancy of the bgl transcription unit by RNA polymerases, expression analysis of bgl and proU reporter constructs, and chloroacetaldehyde footprinting of RNA polymerase promoter complexes. The chloroacetaldehyde footprinting also revealed that RNA polymerase is “poised” at the osmoregulated σ70-dependent proU promoter at low osmolarity, whereas at high osmolarity poised of RNA polymerase and repression by H-NS are reduced. Furthermore, repression by H-NS via the URE and DRE is synergistic, and the efficiency of repression by H-NS via the DRE inversely correlates with the promoter activity. Repression is high for a promoter of low activity, whereas it is low for a strong promoter. Inefficient repression of strong promoters by H-NS via a DRE may account for high induction levels of proU at high osmolarity and for bgl upon disruption of the URE.

The bacterial histone-like nucleoid structuring protein H-NS plays an important role as a pleiotropic repressor and as an architectural protein of the chromatin (1, 2). In addition, H-NS is important for silencing of loci acquired by horizontal gene transfer and for bacterial fitness (3–5).

H-NS consists of an N-terminal dimerization/oligomerization domain, a C-terminal DNA binding domain, and a flexible linker in between these domains that is required for oligomerization (6–12). Oligomerization of H-NS is important for repression (13). In solution H-NS is presumably a dimer, which binds to AT-rich and curved DNA sequences with moderate specificity (Ref. 1 and references therein). After binding to such “nucleation sites,” H-NS occupies further low affinity sites resulting in the formation of extended nucleoprotein complexes (1, 13, 14). Thus, when binding close to a promoter, H-NS represses transcription initiation by trapping of RNA polymerase at the promoter or by excluding binding of RNA polymerase (1, 13, 15). Trapping of RNA polymerase has been shown in case of the ribosomal rrnB P1 promoter (16–18) and the hdeAB promoter (19). In both cases binding of H-NS to an AT-rich curved DNA upstream of the promoter allows H-NS to form a bridge to a DNA sequence downstream to the promoter and to zip the two double strands that flank the promoter together. Repression by DNA looping is further supported by a biophysical analysis, in which it was demonstrated that one dimer of H-NS can contact two DNA double strands (20). Repression by H-NS is relieved gene specifically. In several cases a transcription factor binds close to the promoter and acts as an anti-repressor, presumably by disrupting the repressing nucleoprotein complex formed by H-NS (1, 15). Relief of repression can also occur by a temperature-dependent change in the DNA curvature, as shown for virF (1, 21).

For a few loci it has been shown that binding of H-NS to a regulatory element located downstream within the transcription unit is crucial for repression. This was first shown for the proU operon and later for bgl, hilA, and eltAB (22–28). The proU (or proVWX) operon encoding a high affinity uptake system for the osmoprotectants glycine-betaine and proline is induced up to 200-fold by osmotic up-shift, and its level of expression correlates with the osmolarity of the medium (29–34). Highly specific osmoregulation of proU and specific repression by H-NS requires a downstream regulatory element (DRE, also called NRE) (22–24), with H-NS binding up to position +270 relative to the transcription start (6, 25). Additional H-NS binding sites map within the proU promoter and upstream of it (25). Recently two identical sequences mapping at positions +25 and +130 have been identified as high affinity sites for binding of H-NS (14). H-NS selectively inhibits transcription of proU by E. coli RNA polymerase in vitro (35) at a step before open complex formation (36). However, the mechanism of highly specific regulation of proU in vivo remains unsolved (36–38). It was speculated that the modulation of the physical properties of H-NS and changes in DNA supercoiling at high osmolarity are important for repression of proU and for osmoinduction (31, 36, 39). Temperature and DNA supercoiling...
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The bgl operon encoding gene products necessary for the uptake and fermentation of aryl-β-D-glucosides is repressed ~100-fold by H-NS (26, 31, 40). Highly specific repression by H-NS requires a URE and a DRE (26, 27). H-NS binds to the URE located immediately upstream of the CAP receptor protein (CRP)-dependent promoter and within the DRE +600 to +700 bp downstream of the transcription start site (27). Repression of bgl by H-NS is completely relieved by the transcriptional regulators LeuO and BglJ, which both counteract repression via the URE but have no influence on repression via the DRE (41–43). Likewise, mutations that disrupt the URE completely derepress bgl (40, 44, 45). Furthermore, repression by H-NS mediated via the DRE is affected by termination factor Rho and by translation, suggesting that transcription elongation and repression by H-NS influence each other (27). As in the case of proU, highly specific repression of bgl by H-NS could not be mimicked in vitro, where it was merely 4–5-fold (46).

In this study we addressed the role of the DREs in repression of bgl and proU by H-NS. In bgl, as shown before for proU, H-NS inhibits a step of transcription initiation before open complex formation. Further parallels of repression of proU and bgl by H-NS include synergy in repression via the DRE and the URE. In addition, the efficiency of repression via the DREs depends on the promoter activity. Only promoters of low activity are effectively repressed. These and further data led us to propose a model for highly specific repression of bgl and proU by H-NS and for osmo-induction of proU. According to this model, a moderate increase of the promoter activity as it occurs at high osmolarity at the proU promoter or by disruption of the URE in bgl will reduce repression via the DRE. This in turn will result in a further increase in the promoter activity and, thus, efficient induction. Reduced repression via the DRE upon increased transcription may involve remodeling of the repressing H-NS-DNA complex by RNA polymerase.

EXPERIMENTAL PROCEDURES

Strains, Plasmids, and Expression Assays—Escherichia coli K-12 strains used in this study are described in Table 1. Relevant structures of plasmids are given in Table 1 or schematically shown in the figures in which they are used. Details of plasmid construction and their compiled sequences are available upon request. Bacteria were grown in LB (Difco), and antibiotics were added to 12 µg/ml tetracycline, 25 µg/ml kanamycin, 50 µg/ml ampicillin, 15 µg/ml chloramphenicol, and 50 µg/ml spectinomycin, final concentration. The bacterial pellet was resuspended in 500 µl of lysis buffer (10 mM Tris-HCl, pH 7.6, 0.9% NaCl) and once with lysis buffer (10 mM Tris-HCl, pH 8, 20% sucrose, 50 mM NaCl, 10 mM EDTA) (49). The bacterial pellet was resuspended in 500 µl of lysis buffer containing 4 mg/ml lysozyme and incubated at 37°C for 30 min. Further lysis was done by two freeze–thaw cycles. Then 500 µl of FA lysis buffer (50 mM HEPES-KOH pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 2 mM phenylmethylsulfonyl fluoride) was added, and the sample was sonicated to shear the chromatin to an average size of ~500 bp. The cell debris was removed by centrifuging in a microcentrifuge at 12,000 rpm for 15 min, and 100 µl of the supernatant was used. Immunoprecipitation and quantitation of precipitated DNA by quantitative PCR was carried out as described (50). Briefly, 10 µl of mouse monoclonal antibody against the β-subunit of E. coli RNA polymerase (Neoclon) and 5 µl of 50% (v:v, in TBS) Ultralink Protein A/G Silica (Pierce) beads were added and incubated at room temperature for 90 min on an end-over-end rotator. The beads were washed twice with 700 µl of FA lysis buffer and once each with FA lysis buffer containing 500 mM NaCl, ChIP wash buffer (10 mM Tris-HCl pH 8, 250 mM LiCl, 1 mM EDTA, 0.5% Nonidet P-40, 0.5% sodium deoxycholate), and TE. The DNA was then eluted with 100 µl of ChIP elution buffer (50 mM Tris-HCl pH 7.5, 10 mM EDTA, 1% SDS) by incubating at 65°C for 10 min. The immunoprecipitated DNA and an aliquot of the total chromatin was decross-linked by adding 100 µl of TE and ChIP elution buffer, respectively, 8 µl of Pronase (20 mg/ml in TBS) each, and incubating at 45°C for 2 h and at 65°C for 6 h. The decross-linked DNA was phenol-extracted, precipitated with...
TABLE 1

E. coli K-12 strains

| Strain | Relevant genotype or structure* | Construction* | Reference |
|--------|----------------------------------|---------------|-----------|
| PD32   | MC4100 hns::Ap resistance        |               | (66)      |
| S541   | S539 bgl::AC11 ΔlacZ::Y171       |               | (66)      |
| S614   | S541 hns::Ap resistance         | × T4G7/PD32  | (67)      |
| S1193  | S541 attB::Spec* PUV5 bgl ΔlacZ  | × pKESD48     | (27)      |
| S1195  | S541 attB::Spec* PUV5 bgl ΔlacZ  | × pKESD49     | (27)      |
| S1213  | S541 attB::Spec* PUV5 bgl ΔlacZ  | × pKEK30      | (27)      |
| S1816  | S541attB::Spec* PUV5 ΔlacZ       | × pKEK51      |           |
| S1906  | S541 attB::Spec* PUV5 ΔlacZ     | × pKE99       |           |
| S2048  | S541 attB::Spec* proU::Puv5 (−315 to +20) lacZ | × pKEF30 | (27) |
| S2137  | S541 attB::Spec* PUV5 proU::lacZ | × pKEF30   | (27)      |
| S2285  | S541 attB::Spec* Puv5 ΔlacZ      | × pKEN11      |           |
| S2287  | S541 attB::Spec* Puv5 ΔlacZ     | × pKEN13      |           |
| S2501  | S541 attB::Spec* Puv5 (−315 to +303) lacZ | × pKEN14 | (27) |
| S3000  | S541 attB::Spec* Puv5::lacZ      | × pKEN56      |           |
| S3005  | S541 attB::Spec* Puv5::lacZ      | × pKEN58      |           |
| S3100  | S541 Δhns::kan R                  | × S665/S672, pKD4 | (69) |
| S3034  | S541 attB::Spec* Plac proU::lacZ | × pKEN56 | (69) |
| S3058  | S541 attB::Spec* Plac proU::lacZ | × pKEN59 | (69) |
| S3122  | S1906 Δhns::kan R                | × S665/S672, pKD4 | (69) |
| S3124  | S2048 Δhns::kan R                | × S665/S672, pKD4 | (69) |
| S3126  | S2137 Δhns::kan R                | × S665/S672, pKD4 | (69) |
| S3128  | S2501 Δhns::kan R                | × S665/S672, pKD4 | (69) |
| S3134  | S3034 Δhns::kan R                | × S665/S672, pKD4 | (69) |
| S3169  | S2287 Δhns::kan R                | × S665/S672, pKD4 | (69) |
| S3171  | S3005 Δhns::kan R                | × S665/S672, pKD4 | (69) |
| S3175  | S3058 Δhns::kan R                | × S665/S672, pKD4 | (69) |
| S3181  | S541 attB::Spec* bgl ΔlacZ       | × pKEN61      |           |
| S3191  | S541 attB::Spec* bgl ΔlacZ       | × pKEN68      |           |
| S3203  | S3181 Δhns::kan R                | × S665/S672, pKD4 | (69) |
| S3205  | S3191 Δhns::kan R                | × S665/S672, pKD4 | (69) |
| S3207  | S1195 Δhns::kan R                | × S665/S672, pKD4 | (69) |
| S3209  | S1816 Δhns::kan R                | × S665/S672, pKD4 | (69) |
| S3211  | S1193 Δhns::kan R                | × S665/S672, pKD4 | (69) |
| S3212  | S1213 Δhns::kan R                | × S665/S672, pKD4 | (69) |
| S3299  | S2285 Δhns::kan R                | × S665/S672, pKD4 | (69) |
| S3300  | S3000 Δhns::kan R                | × S665/S672, pKD4 | (69) |
| S3346  | S541 Δhns::kan R                | × S665/S672, pKD4 | (69) |
| S3412  | S3181 Δhns::kan R                | × S665/S672, pKD4 | (69) |
| S3420  | S3191 Δhns::kan R                | × S665/S672, pKD4 | (69) |
| S3697  | S541 attB::Spec* Puv5 (−62 to +20) lacZ | × pKEN78 | (27) |
| S3699  | S541 attB::Spec* Puv5 (−62 to +303) lacZ | × pKEN78 | (27) |
| S3740  | S3797 Δhns::kan R                | × T4G7/S3010 | (27) |
| S3742  | S3797 Δhns::kan R                | × T4G7/S3010 | (27) |

* The relevant genotype of the strains (which are all CSH50 derivatives) refers to the bgl, lac, hns, and proU loci. bgl::Ap, refers to a mutation in the start codon and two additional ATG codons at positions 3 and 27 to CGC, thereby rendering the bgl operon non-translatable. t1ΔRAT indicates a mutation in the leader of the bgl operon, which replaces AA at position +67 +68 to T making the construct independent of BglG-mediated antitermination. proU::lacZ refers to the proU fragment from position +1 to +303 relative to the transcription start site.

a) Construction of strains by transduction using T4G7 and by integration of lacZ fusions in attB was performed as described (26, 56, 68). The deletion of hns allele was constructed according to (69) using primers S665, TCTATTATACCTCAACCAAACCCCAACATATGTTGAGATTACTACAgtgtaggctggagctgcttcg, and S672, AAATCCCCGGCGCCGTCGCGGGAGTTAAGCAGTGCAATCTCACAATAGGATTcattatgccattatccgtctctctcctggc. Δhns::kan R refers to the replacement of the chromosomal hns gene by a kanamycin resistance gene cassette, which was amplified from plasmid pKD4 (69).

RESULTS

H-NS Bound to the bgl-DRE and proU-DRE Weakly Affects Transcription Elongation—Repression by binding of H-NS within the transcription unit could be the result of H-NS acting as a roadblock to the transcribing RNA polymerase or of inhibition of transcription initiation. For proU it has been shown that H-NS inhibits transcription initiation before open complex formation (36). For bgl we had found that repression via the downstream site is affected by translation and termination factor Rho, which indicates that the process of transcription elongation is important (27). In addition, in proU H-NS binds close to the promoter, with the DRE extending up to 270 bp downstream, whereas in bgl the binding region is further downstream (600 to 700 bp), as determined by gel mobility shift assays (27).
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To determine whether H-NS represses transcription by acting as a roadblock to the elongating RNA polymerase, transcription through the bgl-DRE was analyzed by ChIP and quantitative PCR. To this end, the RNA polymerase occupancy upstream and downstream of the H-NS binding region was determined in the wild-type and the hns mutant. In this analysis we used a reporter construct that carries the constitutive lacUV5 promoter followed by the bgl-DRE and the lacZ gene. This construct is repressed 7.8-fold by H-NS (Fig. 1A). Occupancy by RNA polymerase was determined for three fragments. Fragment 1 (position +245 to +507) maps upstream of the H-NS binding region, fragment 2 (+494 to +757) encompasses the H-NS binding site, and fragment 3 (+751 to +955) maps downstream to the H-NS binding region. The occupancy of fragment 1 by RNA polymerase was 7-fold higher in the hns mutant than in the wild type (Fig. 1B). This corresponds well with the repression rate determined in the β-galactosidase assay (Fig. 1A). In the hns mutant the occupancy of fragments 2 and 3 was similarly high as that of fragment 1. In the wild type, occupancy by RNA polymerase decreased 2–3-fold from fragment 1 to fragment 3 (Fig. 1B), which is indicative of a moderate reduction of transcription elongation by H-NS. Taken together the data suggest that H-NS when bound downstream represses transcription at an early step in the transcription cycle. Additional support for the absence of strong H-NS-dependent roadblocking events was provided by CAA footprinting experiments in which no H-NS-dependent pausing of RNA polymerase could be detected within the bgl or the proU DRE sequences (data not shown). Indeed, this single strand-specific chemical probe was previously used to reveal in vivo transcription elongation complexes delayed at intrinsic pause sites or artificially halted by a protein roadblock (52).

H-NS Bound to the bgl-DRE Inhibits Transcription Initiation before Open Complex Formation—To analyze whether repression by H-NS via the bgl-DRE affects open complex formation at the promoter, as shown for proU (36), CAA footprinting was performed. For this analysis lacUV5 promoter/bgl-DRE fusions encoded on high copy number plasmids pKENV64 and pKENV67 were used (Fig. 2). Plasmid pKENV64 carries the lacUV5 promoter followed by the bgl-DRE and terminators rrrB-T1T2, whereas plasmid pKENV67 carries in addition the bgl-URE (Fig. 2). In parallel, the expression level of corresponding lacZ fusions was determined in the wild-type and hns mutant (Fig. 3 and see below).

Transformants of the wild-type and hns mutant of plasmids pKENV64 and pKENV67 were grown to the exponential phase and treated with CAA. In addition, a second set of cultures was treated with rifampicin (200 μg/ml) for 5 min before CAA addition. The CAA footprinting of the lacUV5 promoter/bgl-DRE fusion revealed a clear reactivity at the −10 region and the −35 region.
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transcription start site (at positions +1 and +2) (Fig. 2). This reactivity was weaker in the wild-type than in the hns mutant, which correlates well with the 3.5-fold repression of the corresponding lacZ fusion by H-NS (Fig. 3). Open complex formation detected at the lacUV5 promoter flanked by both the URE and DRE was significantly decreased in the wild type as compared with the lacUV5 promoter/bgl-DRE fusion lacking the URE, which is in agreement with the 20-fold repression of the respective lacZ fusion by H-NS (Fig. 3). In the hns mutant, open complex formation at the lacUV5 promoter was high, irrespective of the presence of the URE (Fig. 2). In all cases the reactivities specific for open complexes were similarly enhanced upon the addition of rifampicin (Fig. 2). Rifampicin traps RNA polymerase in the open complex by blocking extension of RNA synthesis beyond +2 or +3 and, thus, allows accumulation of the otherwise transient open complex intermediates (53, 54). Taken together the data suggest that H-NS when binding to the bgl-DRE represses transcription initiation before open complex formation. Repression is more effective in the presence of both the URE and the DRE.

It should be noted that expression of the plasmids used in CAA footprinting experiments (Fig. 2) and of the chromosomal reporters (see below, Fig. 3) was rendered independent of anti-termination by BglG due to a mutation of the terminator bgl-t1 located in the leader. This t1_bgl1 R mutation stabilizes the RNA secondary structure of the BglG binding motif, RAT (55), which prevents folding of the terminator stem-loop structure and, thus, mimics constitutive anti-termination irrespective of the presence or absence of active BglG. This mutant was used because anti-termination by BglG (encoded by the first gene of the bgl operon) is limiting at low expression rates, which results in the amplification of the repression rates by H-NS (56).

Synergistic Repression of bgl and proU by Upstream and Downstream Regulatory Elements—Complete repression of the bgl operon by H-NS requires upstream and downstream regulatory elements to which H-NS binds (26, 27). As shown above by CAA footprinting, open complex formation at the lacUV5 promoter is repressed more efficiently when both the bgl-URE and DRE are present than in the presence of the bgl-DRE alone (Fig. 2). Similarly, expression analysis of corresponding chromosomal lacZ reporter constructs in the wild-type and a hns mutant demonstrated that a fusion carrying the lacUV5 promoter followed by the bgl-DRE and lacZ is repressed 3.5-fold by H-NS (Fig. 3C), whereas repression by H-NS was 20-fold when both the bgl-URE and DRE were present (Fig. 3A). The bgl-URE alone did not cause repression of the lacUV5 promoter by H-NS (Fig. 3B). Likewise, the bgl promoter flanked by the bgl-URE and DRE was repressed 33-fold by H-NS, whereas a bgl-lacZ fusion that carries the URE alone was repressed ~2-fold (Fig. 3D and E), which is in agreement with earlier results (27).

Repression of the bgl promoter by the URE alone may be due to binding of H-NS to the bgl core promoter fragment as determined by mobility gel shift assays (not shown). These data show that repression by H-NS mediated via the bgl-DRE and the bgl-URE is synergistic.

To compare H-NS mediated repression of bgl with that of proU, a similar set of chromosomal proU-lacZ fusions carrying both regulatory elements or the proU-URE or DRE alone was analyzed (Fig. 4). Because of the osmoregulation of the proU operon, the expression of the proU-lacZ fusions was determined from cells grown in LB with low to high osmolarity (containing 0.01, 0.05, 0.1, 0.2, or 0.3 M NaCl) at steady state conditions. At low osmolarity, the proU promoter flanked by the URE and the DRE was repressed 16-fold by H-NS (LB plus 0.01 M NaCl) (Fig. 4A). At high osmolarity (LB plus 0.3 M NaCl) the expression increased 2-fold in the wild type, and the promoter was not repressed by H-NS (Fig. 4A). In the presence of the URE alone the proU promoter was not significantly repressed by H-NS (1.5-fold at low osmolarity and 0.9-fold at high osmolarity), and the expression of the proU-URE promoter-lacZ fusion increased ~3.5-fold from low to high osmolarity (from 1095 to 3760 units) (Fig. 4B). In the presence of the DRE alone, the proU promoter was repressed 5-fold by H-NS at low osmolarity (Fig. 4C). At high osmolarity the expression increased ~3-fold, and repression by H-NS dropped to 2-fold (Fig. 4C). The activity of the proU promoter lacking the URE and the DRE increased 2-fold from low to high osmolarity, and this promoter was not repressed by H-NS (Fig. 4D). For comparison, the repression mediated via the proU-DRE alone was also tested using a fusion of the lacUV5 promoter to the proU-DRE (Fig. 4E). Expression of this lacZ fusion was not osmoregulated. Repression by H-NS was ~4-fold (at low and high osmolarity) (Fig. 4E). These data show that osmoregulation is specific to the proU promoter and not to repression by H-NS via the DRE (Fig. 4, C to E). The data further show that repression by the proU-URE and DRE is synergistic and that in proU as in bgl the DRE is synergistic.

FIGURE 3. Repression by H-NS via the bgl-URE and the bgl-DRE is synergistic. Repression of the lacUV5 and the bgl promoter by H-NS via the bgl-URE and DRE was determined in the wild-type and hns mutant of cells grown in LB to A600 = 0.5. The expression level is given as β-galactosidase units in the wt and hns mutant. The -fold repression by H-NS is given as bars. Strains used: A, wt S3191, hns S3205; B, wt S3412, hns S3420; C, wt S1816, hns S3209; D, wt S3181, hns S3203; E, wt S1213, hns S3296.

because anti-termination by BglG (encoded by the first gene of the bgl operon) is limiting at low expression rates, which results in the amplification of the repression rates by H-NS (56).

Synergistic Repression of bgl and proU by Upstream and Downstream Regulatory Elements—Complete repression of the bgl operon by H-NS requires upstream and downstream regulatory elements to which H-NS binds (26, 27). As shown above by CAA footprinting, open complex formation at the lacUV5 promoter is repressed more efficiently when both the bgl-URE and DRE are present than in the presence of the bgl-DRE alone (Fig. 2). Similarly, expression analysis of corresponding chromosomal lacZ reporter constructs in the wild-type and a hns mutant demonstrated that a fusion carrying the lacUV5 promoter followed by the bgl-DRE and lacZ is repressed 3.5-fold by H-NS (Fig. 3C), whereas repression by H-NS was 20-fold when both the bgl-URE and DRE were present (Fig. 3A). The bgl-URE alone did not cause repression of the lacUV5 promoter by H-NS (Fig. 3B). Likewise, the bgl promoter flanked by the bgl-URE and DRE was repressed 33-fold by H-NS, whereas a bgl-lacZ fusion that carries the URE alone was repressed ~2-fold (Fig. 3D and E), which is in agreement with earlier results (27).

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FIGURE 3. Repression by H-NS via the bgl-URE and the bgl-DRE is synergistic. Repression of the lacUV5 and the bgl promoter by H-NS via the bgl-URE and DRE was determined in the wild-type and hns mutant of cells grown in LB to A600 = 0.5. The expression level is given as β-galactosidase units in the wt and hns mutant. The -fold repression by H-NS is given as bars. Strains used: A, wt S3191, hns S3205; B, wt S3412, hns S3420; C, wt S1816, hns S3209; D, wt S3181, hns S3203; E, wt S1213, hns S3296.
more important than the URE for repression by H-NS (Figs. 3 and 4) (23–25).

**Poising of RNA Polymerase at the proU Promoter at Low Osmolarity**—In the case of proU it was shown that H-NS represses a step of transcription initiation before open complex formation (36). Repression of the σ70-dependent proU promoter by H-NS is less effective when cells are grown at high osmolarity than at low osmolarity (37) (Fig. 4). For σS-dependent osmoregulated promoters it has been shown that RNA polymerase is poised at the promoter at low osmolarity but not at high osmolarity (57, 58). To analyze whether RNA polymerase is likewise poised at the proU promoter and how this correlates with repression by H-NS, open complex formation at the proU promoter was determined by CAA footprinting (Fig. 5). To this end, pKENV73, a high copy number plasmid carrying the proU promoter flanked by the proU-URE and DRE, was used (Fig. 5). Transformants of the wild-type and Δhns mutant with this plasmid were grown in LB of low and high osmolarity (LB and 0.01 and 0.3 M NaCl) to mid-exponential phase, and CAA was added directly or 5 min after rifampicin addition. At all conditions a clear reactivity within the −10

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**Figure 4.** Osmoregulation of the proU promoter and repression by H-NS via the URE and DRE. Expression of proU-lacZ fusions carrying the URE and DRE (A), the URE (B), the DRE alone (C), no RE (D), and a lacUV5 promoter fused to the DRE (E) was determined in the wild-type and Δhns mutant. Cells were grown in LB with the indicated NaCl concentrations. The graphs show the determined expression level (black circle for wild-type and open circle for Δhns mutant). Bars indicated the -fold repression by H-NS. Strains used: A, wt S2501, Δhns S3129; B, wt S2048, Δhns S3124; C, wt S3699, Δhns S3742; D, wt S3697, Δhns S3740; E, wt S2137, Δhns S3126. β-gal, β-galactosidase.

**Figure 5.** RNA polymerase is poised at the proU promoter at low osmolarity. CAA footprinting of RNA polymerase proU promoter complexes was performed in transformants of the wild-type (S541) and Δhns mutant (S3346) with plasmid pKENV73. The position of the reactivity is indicated in the sequence at the bottom. H-NS represses open complex formation. Reduced reactivity in the Δhns mutant grown at high salt (0.3 M NaCl) as compared with low salt (0.01 m NaCl) suggests poising of RNA polymerase. Rifampicin (Rif) was added to 200 μg/ml 5 min before CAA where indicated. M designates a mock reaction without addition of CAA.
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region and from positions −5 to +1 was apparent (Fig. 5). In the wild type, the reactivity specific for open complex formation was rather weak for cells grown at low as well as high osmolarity (Fig. 5). In the hns mutant the reactivity was strong for cells grown at low osmolarity, whereas it was significantly reduced at high osmolarity (Fig. 5, compare wt hns, 0.01 and 0.3 M). This suggests that at low osmolarity RNA polymerase is poised in the open complex at the σ70-dependent proU promoter and that poising is reduced at high osmolarity. The comparison of the signal intensities obtained in the wild-type and the hns mutant confirm that H-NS represses open complex formation at low osmolarity and to a lesser extent also at high osmolarity (Fig. 5, compare wt and hns, 0.01 and 0.3 M) (36). Taken together these data suggest that at low osmolarity RNA polymerase is poised at the proU promoter. At high osmolarity, poising of RNA polymerase at the promoter and repression by H-NS are reduced.

Repression via the bgl-DRE and the proU-DRE Inversely Correlates with the Promoter Activity—To address whether repression via the bgl-DRE and the proU-DRE depends on the activity of the promoter, lacZ reporter fusions were constructed that carry the bgl-DRE and the proU-DRE, respectively, fused to constitutive promoters of low, middle, and high activity (Fig. 6A). Repression of the weak lacI promoter by H-NS via the bgl-DRE was 12.5-fold (Fig. 6B), the lacIUV5 promoter was repressed 2.4-fold, and the strong tac promoter was not repressed by H-NS (1.2-fold) (Fig. 6B). A similar result was obtained when repression of these promoters by H-NS via the proU-DRE was analyzed (Fig. 6C). When cells were grown at low osmolarity (LB plus 0.01 M NaCl), the weak lacI promoter was repressed 11-fold by H-NS via the proU-DRE, the lacIUV5 promoter was repressed 4.4-fold, and the tac promoter was repressed 2-fold (Fig. 6C). Control promoter-lacZ fusions were not repressed by H-NS (Fig. 6D). Thus, repression of promoters via the bgl-DRE and proU-DRE inversely correlates to the promoter activity and/or transcription rate.

**DISCUSSION**

Repression of the proU operon and the bgl operon by H-NS is very specific and in both systems involves upstream and downstream regulatory elements (22–24, 26). The present analysis of the role of the DRE in repression of bgl and proU by H-NS demonstrates that in bgl and as shown before for proU (36), H-NS represses transcription initiation before open complex formation. In addition, repression via the DRE and the URE is synergistic in bgl and in proU. Furthermore, the efficiency of repression by H-NS via the bgl and the proU DRE inversely correlates to the promoter activity; repression is high only for a promoter of low activity. These results suggest that highly specific repression by H-NS via the bgl and proU DREs occurs for promoters with low transcription initiation rates only. Consequently, a disruption of the URE and a moderate enhancement of the promoter activity will result in high levels of induction of bgl and proU.

Repression of the bgl operon by H-NS is ≈100-fold (26, 31). Although repression involves an URE and a DRE, the bgl operon is completely derepressed by mutations that disrupt the functionality of the URE (41–43). The present data suggest that elimination of repression via the URE concomitantly reduces repression by H-NS via the DRE resulting in complete derepression of the bgl operon as follows. First, repression by H-NS via the bgl-URE and DRE is synergistic. H-NS represses the
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lacI V5 promoter flanked by the bgl-URE and DRE 20-fold, whereas repression by the DRE alone is only 3.5-fold, and no repression occurs via the URE alone (Fig. 3). Disruption of the URE thus causes a loss of synergy. Second, repression by H-NS via the DRE depends on the promoter activity (Fig. 6). Upon disruption the URE, the promoter activity increases, and repression via the DRE may further decrease. Third, H-NS bound to the DRE will encounter transcription by RNA polymerase, in contrast to H-NS bound to the URE. It was shown before that co-transcriptional translation and termination factor Rho affect repression of bgl by H-NS via the DRE (27), demonstrating that transcription elongation and repression by H-NS via the DRE influence each other. Initially we speculated that H-NS bound to the DRE may act as a roadblock to RNA polymerase engaged in elongation (27). However, here we have shown that this is not the major mechanism of repression (Fig. 1). Considering that the force needed to disrupt DNA-H-NS-DNA bridges is ~9 piconewtons (20) and that RNA polymerase engaged in elongation exerts a force of ~20 piconewtons (59), it is feasible that RNA polymerase remodels the H-NS complex formed at the DRE. This possibility was already discussed for the proU operon (25). Remodeling of H-NS complexes located within transcription units is further supported by a genome-wide ChiP-on-chip analysis, where it was found that H-NS is excluded from highly transcribed regions (4). Last, the -fold regulation of bgl by H-NS is amplified by the operon-encoded anti-terminator BglG. BglG is limiting at low transcription rates, whereas upon disruption of the URE and concomitantly increased transcription, BglG can anti-terminate, which results in a further increase of transcription (56). This positive feedback loop can promote complete derepression of the operon.

To date the high level of osmoregulation of proU remains unclear. The model proposed in this work may at least in part account for high levels of osmotic induction of proU. The proU promoter is induced ~2–3-fold by high osmolarity (Fig. 4) (23). Osmoregulation of the σ70 proU promoter presumably is the result of “poising” of RNA polymerase at low osmolarity as suggested by CAA footprinting (Fig. 5), similar to poising of RNA polymerase at σ38-dependent osmoregulated promoters (57, 58). Intriguingly, osmoregulated σ38-dependent promoters are activated by an increased potassium glutamate concentration at high osmolarity. This may suggest that potassium glutamate may also be the intracellular signal for induction of proU, although this has been disputed for a long time (60–64). Taken together, it seems plausible that a ~2–3-fold increase of the proU promoter activity from low to high osmolarity will reduce synergy in repression via the URE and the DRE and decrease repression by H-NS via the DRE. In addition, as in bgl, the transcribing RNA polymerase may further hamper repression by H-NS via the DRE by remodeling of the H-NS-DNA complex. Taken together this defines a feedback loop that can explain high induction levels of the proU operon.

The H-NS-mediated repression of proU, the classical model for repression via a DRE, is paralleled in many aspects by repression of bgl (for example Figs. 3, 4, and 6). One parallel is that H-NS represses transcription initiation at a step before complex formation (36) (Fig. 2). Whether RNA polymerase is trapped in the closed complex or whether it is excluded from binding is an open question. A genome scale ChiP-on-ChIP analysis of RNA polymerase and H-NS localization suggests that H-NS excludes RNA polymerase (4), whereas in another study frequent co-localization of H-NS and RNA polymerase was detected (65). For the hdeAB promoter it was shown that DNA bending by the RNA polymerase-σ70 holoenzyme is a prerequisite for repression by H-NS. In this case RNA polymerase is trapped at the promoter in the open complex (19). A second question is how H-NS bound within the transcription unit can repress the promoter. Formation of a nucleoprotein complex by DNA-H-NS-DNA bridging is plausible in the case of the proU operon, in which the downstream binding site is rather close to the promoter (6, 14, 25). However, in bgl H-NS binds 600–700 bp downstream of the transcription start (27). Possibly additional low affinity H-NS binding sites exist, facilitating formation of a nucleoprotein complex that spreads up to and represses the promoter. This relates to the question of the mechanism of synergistic repression via an URE and a DRE. Considering that repression occurs by DNA-H-NS-DNA bridge formation (1, 20), synergy of repression via the URE and DRE may not be the result of classical protein-protein interaction. Rather, H-NS bound to the URE may interact with a downstream DNA segment, resulting in DNA loop formation, and likewise, H-NS bound to the DRE may form a bridge to an upstream DNA segment such that in the presence of both regulatory elements (DRE and URE) a stable nucleoprotein complex may be formed. This possibility was also discussed in respect to the role of the two high affinity H-NS nucleation sites discovered in proU (14). A third question pertains to the -fold factor of repression (and induction) of proU and bgl. The transcriptional lacZ fusions used in this study are repressed 20-fold by H-NS and are, thus, significantly less than the intact bgl and proU operons. In bgl the posttranscriptional amplification by BglG presumably accounts for this difference (56). In proU so far no posttranscriptional level of osmoregulation has been characterized. In conclusion, the findings presented clarify several aspects of highly specific repression of bgl and proU by H-NS and of osmoregulation of proU.

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