The Downstream Regulatory Element of the proU Operon of Salmonella typhimurium Inhibits Open Complex Formation by RNA Polymerase at a Distance*

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The intracellular concentration of K⁺-glutamate, chromatin-associated proteins, and a downstream regulatory element (DRE) overlapping with the coding sequence, have been implicated in the regulation of the proU operon of Salmonella typhimurium. The basal expression of the proU operon is low, but it is rapidly induced when the bacteria are grown in media of high osmolality (e.g. 0.3 M NaCl). It has previously been suggested that increased intracellular concentrations of K⁺-glutamate activate the proU promoter in response to increased extracellular osmolality. We show here that the activation of the proU promoter by K⁺-glutamate in vitro is nonspecific, and the in vivo regulation cannot simply be mimicked in vitro. In vivo specificity requires both the chromatin-associated protein H-NS and the DRE; they are both needed to maintain repression of proU expression at low osmolality. How H-NS and the DRE repress the proU promoter in vivo has so far been unclear. We show that, in vivo, the DRE acts at a distance to inhibit open complex formation at the proU promoter.

Escherichia coli and Salmonella must maintain an intracellular osmolality that is greater than that of their environment, creating a tendency for water to enter the cells and generating an outward directed hydrostatic pressure or turgor. This turgor is essential for growth and division. A sudden increase in the osmolality of the environment must be compensated for by an increase in the intracellular osmolality. This adaptation is essentially a two-step process. First the concentration of K⁺-glutamate is increased by specific uptake systems for K⁺ and the rapid synthesis of glutamate. Subsequently the concentration of "osmoprotectants" such as glycine-betaine, trehalose, proline, or proline-betaine increases either by synthesis or uptake from the environment, followed by an efflux of K⁺-glutamate. High concentrations of osmoprotectants are less toxic to the cell than K⁺-glutamate. This series of events restores the turgor and enables the cell to grow in media with a high osmolality.

The proU operons of E. coli and Salmonella typhimurium encode high affinity glycine-betaine uptake systems that are essential for cell survival in media of high osmolality. The uptake systems for this major osmoprotectant only need to be active in media of high osmolality. The synthesis of the ProU glycine-betaine uptake system is regulated, principally, at the level of transcription, and activity of the proU promoter is increased up to 100-fold by an increase in extracellular osmolality (1–4). The mechanism by which transcription of the proU operon is induced at high osmolality is still unclear. Two mechanisms have been proposed.

In the simplest model, the elevated concentration of K⁺-glutamate is suggested to directly enhance transcription from the proU promoter (5–8). It has been shown that in vitro transcription or transcription-translation systems elevated concentrations of K⁺-glutamate can increase transcription from the proU promoter (5–8). However, the binding of RNA polymerase to promoters that are not activated at high osmolality is also increased dramatically by increased salt concentrations (9–11). We show here that increased open complex formation by RNA polymerase in direct response to intracellular K⁺-glutamate concentrations cannot provide the only signal regulating proU expression.

In the second model, proU expression is regulated by a change in the topology of the DNA, requiring a change in the structure of a complex formed by the downstream regulatory element (DRE), the proU promoter, and the chromatin-associated protein H-NS (12–14). H-NS is an abundant "chromatin-associated" or "histone-like" protein involved in packaging of the DNA into the nucleoid that influences the regulation of the expression of approximately 60 genes (15, 16). Several observations are consistent with the second model: (i) The supercoiling of the DNA changes in response to the osmolality of the environment (13, 17–19). (ii) H-NS, which is the only trans-acting factor substantially influencing the regulation of proU, changes the topology of DNA in vitro (20). (iii) Mutations in the hns gene alter the supercoiling of the DNA isolated form the cell (12, 13, 21). (iv) No sequence-specific regulatory proteins that act at the proU promoter have been identified despite intensive searches (13, 22–24).

DREs are newly described regulatory elements in bacteria, found downstream of promoters. They negatively regulate the activity of these promoters and work in conjunction with the histone-like protein H-NS. The DRE in the proU operon of E. coli and S. typhimurium was the first to be described and is required for repression of the proU promoter at low osmolality and lies within the coding region of the first structural gene proV (25, 26). The DRE contains intrinsically curved DNA, but

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1 The abbreviations used are: DRE, downstream regulatory element; PCR, polymerase chain reaction; β-lac, β-lactamase.

2 R. J. Stephen and J. C. D. Hinton, in preparation.
factors other than curvature are important for its function (27). More recently, DREs have been described in the operon encoding CS1 fimbiae and in the gene encoding the heat-labile enterotoxin (LT) of Enterotoxigenic E. coli (ETEC) strains (28–30). In the bgl operon of E. coli sequences upstream and downstream of the promoter also include operator activity in conjunction with H-N5 (31). Although it has been suggested that the DREs act at the level of transcription initiation at a distance (12, 26, 30), no direct data are available that proof this point. In this paper we demonstrate that, despite its distance from the proU promoter, the DRE of the proU operon in S. typhimurium acts to influence open complex formation by RNA polymerase.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Growth Conditions—Bacterial strains used in this study and their genotypes are listed in Table I. Details of plasmids are given in Table II. Bacteria were grown aerobically at 37 °C in LB broth or on LB plates (32) unless otherwise specified. For assay of luciferase or β-lactamase activity the cells were grown in nutrient broth (Difco) as a low osmolarity medium or in nutrient broth with 0.3 m NaCl added as a high osmolarity medium. Ampicillin (100 μg/ml), kanamycin (50 μg/ml), chloramphenicol (25 μg/ml), or tetracycline (50 μg/ml) were added to the growth media for strains expressing the respective antibiotic resistance genes.

Construction of Plasmid pBJ10—A 952-base pair proU promoter fragment (base 217 to +735, where the start of the transcription (33)) of S. typhimurium was generated by PCR using primers −217 primer (5′-CGGAAATTCCTGCAGGATCGTGGG-3′) and +735 primer (5′-CGGAAATTCCTGCAGGATCGTGGG-3′), which incorporates EcoRI, BamHI, and PstI sites to facilitate cloning. The fragment was digested with EcoRI and cloned into the EcoRI site of vector pBD71 (Table II), upstream the luciferase genes. The resulting plasmid was called pBJ9. The 3-kilobase BamHI fragment of pBJ9 containing the proU promoter and the luciferase reporter genes was cloned into the BamHI site of plasmid pAV1990 (Table II) generating plasmid pBJ10. Sequencing confirmed that no PCR errors had been introduced.

DNA Manipulations—Standard methods were used for gel electrophoresis and the construction of recombinant plasmids (34). Sequencing reactions were performed on plasmid pBJ10 using primer proU−92 (5′-CCGGGAAATTCCTGCAGGATCGTGGG-3′) and an ABI PRISM™ sequencer and ABI PRISM™ software.

Luciferase Assay—Cells were grown in nutrient broth (Difco) as a low osmolarity medium to an A600 of between 0.3 and 0.5. The cell culture was split, and NaCl was added to one half of the culture to a final concentration of 0.3 M to give an osmotic upshock. The other half of the culture provided a low osmolarity control. The cells were grown for a further 20 min, and luciferase activities were assayed as described previously (12); the activities are given as millivolt light output/100 μl of cell culture/A600 nm.

β-Lactamase Assays—Cells were grown in nutrient broth (Difco) as a low osmolarity medium to an A600 of between 0.3 and 0.5. The cell culture was split, and NaCl was added to one half of the culture to a final concentration of 0.3 M to give an osmotic upshock. The other half of the culture provided a low osmolarity control. The cells were grown for a further 20 min, and 400 μl of cells were spun down and resuspended in 70 μl of reagent A (25 mM Tris-HCl, pH 7.6, 50 mM NaCl, 20 mM NaF, 10 mM NaF, 0.2 mM phenylmethylsulfonyl fluoride, 1 mg/ml gelatin) (35). 8 μl of toluene was added, and cells were mixed thoroughly. Next β-lactamase assays were performed essentially as described in Ref. 35. A standard curve using purified β-lactamase (Roche Molecular Biochemicals) was included. The activities were calculated as ng of β-lactamase produced/80 μl of cell culture/A600 nm.

Purification of the H-NS Protein—H-NS protein form S. typhimurium was purified as described (12) and subsequently dialyzed against 25 mM KH2PO4/KHPO4, pH 7.0, 0.5 mM dithiothreitol, 0.5 mM EDTA, and 50% glycerol. The protein was stored at 0.5–2 mg/ml at −20 °C.

KmO4 Footprints in Vitro Using Linear DNA Templates—Linear DNA templates were generated using the PCR. DNA fragments were end-labeled by filling in 5′ overhanging ends using [α-32P]dCTP and Sequenase™ version 2.0. The DNA fragments were generated by using appropriate primers and restriction digestion of the DNA fragments. Primers used to generate the DNA template are the −217 primer (5′-CGGCAATTCGGACTCGATGATACTCAACATGGTCCTACAT-3′) and the +225 primer (5′-CGGCAATTCGGACTCAGGATGTCCTACAAG-3′). The numbers refer to the position of the primer with respect to the proU transcriptional start point (33).

Binding reactions were in 20 μl of H-NS binding buffer (10 mM Tris-HCl, pH 7.6, 1.5 mM KCl, 0.5 mM spermidine, 0.05 mM EDTA and 5 mM MgCl2) with the indicated amount of purified H-NS protein, incubated for 15 min at 37 °C. Next RNA polymerase (Amer sham Pharmacia Biotech) was added as indicated and incubated for another 15 min at 37 °C. RNA polymerase was diluted in RNA polymerase buffer (10 mM Tris-HCl, pH 7.9, 100 mM NaCl, 0.1 mM EDTA, 0, 1 mM dithiothreitol, and 50% glycerol). After the binding reaction, 1 μl of freshly prepared KmO4 (200 mM) was added to the DNA and incubated for 4 min at 37 °C. 50 μl of KmO4 stop solution (3 M ammonium acetate, 0.1 mM EDTA, 1.5 μl β-mercaptoethanol) was added, and samples extracted with phenol-chloroform-isooamyl alcohol (25:24:1) were precipitated with ethanol and dissolved in 100 μl 10% piperidine (freshly diluted). After 30 min of incubation at 95 °C, the samples were quenched on ice, centrifuged for 10 s, and transferred to a new tube. 1 ml of 1-butanol was added, the samples were mixed and centrifuged for 2 min, and the supernatant was removed. The “pellets” were dissolved in 100 μl of 1% SDS and 1 ml of 1-butanol added, mixed, and centrifuged for 2 min, and the supernatant was removed. Dry pellets were dissolved in 10 μl of loading buffer (40% formic acid, 5 μl NaOH, 0.25% bromphenol blue, 0.25% xylene cyanol). Maxam-Gilbert G chemical cleavage sequencing reactions, for use as size markers, were generated as described (36).

In Vitro KmO4 Footprints Using Supercoiled DNA—KmO4 footprints were performed in 20 μl of H-NS binding buffer (see above) containing 0.1 pmol of plasmid DNA. RNA polymerase, H-NS, and KmO4 were added. Samples were dissolved at 37 °C, and the KmO4 footprint was performed as described above. After ethanol precipitation, the samples were dissolved in 10 μl of TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) instead of 100 μl of 10% piperidine. The site of KmO4 attack was detected by primer extension essential as described (37) using primers proU−92 (5′-CCGGGAAATTCCTGCAGGATCGTGGG-3′) or β-lac+95 (5′-GGAGGCAATTCGCTCGACGTCG-3′), and 40 μl of rifampicin (50 μg/ml in methanol) was added to 10 ml of cells, whereas the other 10 ml was left untreated. The cells were incubated for a further 5 min at 37 °C, and freshly prepared 200 mM KmO4 solution (0.25 ml) was added to the cells to a final concentration of 5 mM KmO4, and then incubated for 5 min at 37 °C. Cells were harvested, washed once in 10 ml of minimal medium, and harvested again, and plasmid DNA was purified as described (38). Plasmid DNA was dissolved in 100 μl of TE, and 10 μl of this plasmid solution was used in an extension reaction with radioactive primers as described (see above).

RESULTS

Reconstruction of the proU Promoter Regulation in Vitro—KmO4 footprints were used in an attempt to reconstitute

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| Strain | Relevant genotype | Source |
|--------|------------------|--------|
| S. typhimurium | Wild type | N. Ames |
| | hns101::IS10 | K. Sanderson |
| | zde-171::Tn10316Δ17 | |
| E. coli | DH5α F′ | Life Technologies, Inc. |
| | supE4 thi-1 recA1 gyrA (Nal') relA1 ΔlacZΔYΔargF)K1269 ΔdeoR (Δ805lacZΔM15) | |
regulation of the proU promoter in vitro. KMnO₄ reacts with single-stranded DNA and can therefore be used as a reagent to measure open complex formation by RNA polymerase. The amount of open complex formation is a measure of promoter activity.

First, we determined whether the repressive effect of H-NS can be mimicked in vitro. A linear DNA fragment containing the proU promoter from −217 to +225 (where +1 is the start of the transcription) was incubated with increasing amounts of purified H-NS protein for 15 min at 37 °C. This promoter fragment is shown to be sufficient to confer osmoregulation in vivo (12). Next 2 units of RNA polymerase were added, and the incubation was continued for a further 15 min at 37 °C. KMnO₄ footprints were performed as described for linear DNA fragments (see “Experimental Procedures”). These footprints indicated that 1.5 μg of H-NS was sufficient to inhibit the formation of open complex by RNA polymerase at the proU promoter. Thus, H-NS can repress the activity of the proU promoter in vitro, as it does in vivo at low osmolarity.

To determine whether the repressive effects of H-NS can be overcome by increased concentrations of K⁺-glutamate, different amounts of K⁺-glutamate were added to the reaction mixture to final concentrations of 0, 100, 200, 300, or 400 mM. At 200, 300, and 400 mM K⁺-glutamate increased open complex formation was seen at the proU promoter (Fig. 1). Thus, elevated K⁺-glutamate concentrations can overcome the repressive effect of H-NS at the proU promoter in vitro.

Regulation of the proU and the β-lac Promoters in Vivo—To determine whether the effect was specific, similar KMnO₄ footprints were performed using supercoiled plasmids containing the proU and control promoters (e.g. β-lac promoter). To perform these KMnO₄ footprints, plasmid pBJ10 was constructed containing the proU promoter, and its DRE fused to the luciferase genes in the vector pAV1990 (see “Experimental Procedures”). First, luciferase assays were performed to determine whether the in vivo expression of the proU promoter in pBJ10 was repressed by H-NS and activated at high osmolarity. As expected, the in vivo expression of the proU promoter in pBJ10 was repressed by H-NS and induced 176-fold at high osmolarity (Table III). Next, β-lactamase assays were performed to see whether the in vivo expression of the β-lac promoters in vector pAV1990 and plasmid pBJ10 were repressed by H-NS and activated at high osmolarity. As expected, the β-lac promoter in the vector pAV1990 was not induced at high osmolarity and not repressed by H-NS (Table IV). In contrast, however, the β-lac promoter of pBJ10 was induced 8.7-fold at high osmolarity and repressed 2.58-fold by H-NS (Table IV). Thus, the β-lac promoter is not osmoregulated or repressed by H-NS in the vector pAV1990. The osmoregulation and repression of the β-lac promoter in plasmid pBJ10 must be due to the presence of the proU promoter and/or the DRE on the same plasmid (e.g. because of readthrough from the proU promoter). The β-lac promoter of pAV1990 is therefore a good control to determine whether the in vitro repression by H-NS and derepression by K⁺-glutamate is specific for the proU promoter.

Both the proU and β-lac Promoter Are Activated by High K⁺-Glutamate Concentrations in Vitro—KMnO₄ footprints were performed on both the proU and the β-lac promoters of supercoiled plasmid pBJ10 and on the β-lac promoter of supercoiled vector pAV1990. Increasing amounts of purified H-NS protein were added to the reaction mix and incubated before adding 2 units of RNA polymerase. KMnO₄ footprints were performed as described for circular DNA fragments (see “Experimental Procedures”). These footprints indicated that 1 μg of H-NS was sufficient to inhibit the formation of an open complex by RNA polymerase on the proU promoter of pBJ10 and on the β-lac promoters of pBJ10 and pAV1990. The finding that the proU and β-lac promoter respond similarly is not in agreement with in vivo data showing that the proU promoter is repressed by H-NS (Table III), whereas the β-lac promoter on pAV1990 is not repressed and the β-lac promoter of pBJ10 is

| Plasmid | Vector | Insert/construction | Ref. |
|---------|--------|---------------------|-----|
| pSB71   | pBR322 | Constructed by PCR, containing β-lactamase and ori of pBR322 | 43  |
| pAV1990 | pBR322 | proU fragment (−508 to +308) | 12  |
| pDO178  | pDO182 | proU fragment (−508 to +48) | 26  |
| pDO207  | pDO182 | proU fragment (−217 to +735) | 26  |
| pBJ9    | pSB71  | proU promoter and luciferase genes | This study |
| pBJ10   | pAV1990| proU promoter and luciferase genes | This study |

**Table II**

**Plasmids**

**Table III**

| Strain | Low osmolarity | High osmolarity | Induction ratio (high osmolarity: low osmolarity) |
|--------|----------------|-----------------|-----------------------------------------------|
| LT2 (wild type) | 758 | 16,253 | 216.7 |
| SA4105 (hns−) | 758 | 16,049 | 21.2 |
| Repression ratio (hns−/hns+) | 8.2 | 0.99 |     |

**Fig. 1. KMnO₄ footprints at the proU promoter in vitro.** Open complex formation at the proU promoter was measured using KMnO₄ footprints. The concentration of K⁺-glutamate (K⁺-glut.) added is given in mM. The open complexes at the proU promoter (bases −12 and −13) are indicated by arrows.
Mechanisms of proU Transcription

The Activity of the proU Promoter Is Regulated in Vivo at the Level of Open Complex Formation—In vivo KMnO4 footprints were performed to determine whether or not the in vivo expression of the proU promoter is regulated at the level of open complex formation. The data in Fig. 3 show that the proU promoter is clearly regulated at the level of open complex formation in vivo (compare lanes with the proU promoter at high and low osmolarity). Open complex formation at the control promoter (β-lac) in vivo did not increase at high osmolarity (Fig. 3, compare lanes with the β-lac promoter at high and low osmolarity). Thus, the activity of the proU promoter is regulated at the level of open complex formation in vivo.

The DRE of the proU Operon Inhibits Open Complex Formation in Vivo—Plasmids pDO178 and pDO207 (26) were used in in vivo KMnO4 footprints to study the effect of deleting the DRE on open complex formation at the proU promoter. These plasmids were used because the effect of including the DRE had the greatest effect on osmoregulation in these plasmids (26). Plasmid pDO207 does not contain the DRE, and the proU promoter in this construct is therefore only 5-fold repressed at low osmolarity. In contrast, plasmid pDO178 contains the DRE and is 130-fold repressed at low osmolarity (26). The data in Fig. 4 show that the proU promoter in plasmid pDO207 forms an open complex at low osmolarity, whereas the proU promoter in plasmid pDO178 does not form an open complex at low osmolarity. Thus despite the distance from the proU promoter, the DRE acts to repress open complex formation at this promoter in vivo.

DISCUSSION

In this paper we investigated the mechanism by which K+-glutamate, the DRE, and the chromatin-associated protein H-NS regulate the expression of the proU promoter of S. typhi-
Mechanisms of proU Transcription

Open complex formation at both the proU and the β-lac promoters is similarly inhibited by H-NS and activated by K⁺-glutamate in an in vitro assay. However, unlike the proU promoter, the β-lac promoter is not inhibited by H-NS or induced at high osmolality in vivo. Therefore, the effect of K⁺-glutamate on the proU promoter cannot be a specific osmoregulatory response, and simple binding of H-NS and induction by K⁺-glutamate are not sufficient to explain the specific osmoregulation of proU seen in vivo (27). The fact that K⁺-glutamate generally enhances the binding of RNA polymerase to promoters and facilitates the interaction of proteins with DNA has been described (9, 10, 39). These findings are in agreement with data showing that in vivo accumulation of glutamate is not required for the induction of proU at high osmolality (11). However, because the accumulation of K⁺ is necessary for activation of proU (40), the accumulation of K⁺-glutamate is necessary but not sufficient to explain the induction of proU at high osmolality.

The DRE is located between nucleotide +73 and +274 downstream from the transcription start site (25) of the proU promoter. The DRE is required for full repression by H-NS at low osmolality. However, no effect of the presence or absence of the DRE on open complex formation is seen in vitro, with or without H-NS added (data not shown). So again it is clear that the regulation of the expression of proU is more complex.

The fact that in vivo the expression of proU is regulated at the level of open complex formation further defines the step at which the osmoregulation is achieved. To date it has only been shown that the amount of proU mRNA is increased dramatically at high osmolality (41); this could be explained by a change in transcription-initiation, transcription-elongation, transcription-termination, or RNA decay. Quantifications of the footprints shown in Figs. 3 and 4 show that the proU expression is regulated at the level of open complex formation.

Our data seem to be in contrast with data described by Ueguchi and Mizuno (7). They suggest that the expression of proU can be mimicked in vitro, as measured by single round transcription assays. The differences found between their results and our results might be explained by the fact that different methods are used. Here we use the formation of an open complex by RNA polymerase as a measurement for the expression of proU. We show here that the expression of proU in vivo is regulated at this level. Therefore, we think we are assaying the right step in the transcription of proU.

Recent data indicate that DNA sequences flanking bacterial promoters can repress their activity (28, 28–31, 42). The mechanism by which these flanking DNA regions repress promoter activity has not been resolved yet. In case of the proU promoter of S. typhimurium, the exact distance between the promoter and the DRE is not important for the repression (25, 27). Furthermore the relative orientation of the DRE and the promoter on the “face of the helix” can be changed with little effect on the regulation of the expression (27). Therefore, it seems unlikely that specific interactions between proteins bound at the promoter and the DRE are involved in the regulation of the expression. In this paper we show that the DRE represses the activity of the proU promoter at an early step in the transcription. Either the binding of RNA polymerase or the separation of the two strands (open complex formation) by RNA polymerase is inhibited from a distance by this downstream DNA.

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