JOURNAL OF BACTERIOLOGY, Aug. 1995, p. 4508–4513 Vol. 177, No. 15
0021-9193/95/$04.00+0

Fine-Structure Deletion Analysis of the Transcriptional Silencer of the *proU* Operon of *Salmonella typhimurium*

SUSANNE A. FLETCHER AND LASZLO N. CSONKA*

Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907-1392

Received 23 December 1994/Accepted 18 May 1995

Transcriptional control of the osmotically regulated *proU* operon of *Salmonella typhimurium* is mediated in part by a transcriptional silencer downstream from the promoter (D. G. Overdier and L. N. Csonka, Proc. Natl. Acad. Sci. USA 89:3140–3144, 1992). We carried out a fine-structure deletion analysis to determine the structure and the position of the silencer, which demonstrated that this regulatory element is located between nucleotide positions +73 to +274 downstream from the transcription start site. The silencer appears to be made up of a number of components which have cumulative negative regulatory effects. Deletions or insertions of short nucleotide sequences (<40 bp) between the *proU* promoter and the silencer do not disrupt repression exerted by the silencer, but long insertions (≥0.8 kbp) result in a high level of expression from the *proU* promoter, similar to that imparted by deletion of the entire silencer. The general DNA-binding protein H-NS is required for the full range of repression of the *proU* operon in media of low osmolality. Although in the presence of the silencer *hns* mutations increased basal expression from the *proU* promoter three- to sixfold, in the absence of the silencer they did not result in a substantial increase in basal expression from the *proU* promoter. Furthermore, deletion of the silencer in *hns*$^+$ background was up to 10-fold more effective in increasing basal expression from the *proU* promoter than the *hns* mutations. These results indicate that osmotic control of the *proU* operon is dependent of some factor besides H-NS. We propose that the transcriptional regulation of this operon is effected in media of low osmolality by a protein which makes the promoter inaccessible to RNA polymerase by forming a complex containing the *proU* promoter and silencer.

The *proU* operon, which specifies the three constituent proteins of a transport system for osmoprotectants such as glycinebetaine and proline (5), is induced >100-fold by high osmolality in *Salmonella typhimurium* and *Escherichia coli*. Transcriptional control of the *proU* operon is mediated at least in part by a *cis*-acting negative element, or silencer, that is located ~100 to 200 bp downstream of the promoter (7, 18). In *S. typhimurium*, deletion of this element resulted in up to a 40-fold increase in expression from the *proU* promoter in media of low osmolality (18).

Thus far, attempts to isolate mutations in regulatory genes that are unique to the *proU* operon have not been successful. This impasse has led to the formulation of two models which propose that the transcriptional control mechanism of the *proU* operon is unusual in that it does not involve any specific regulatory proteins. Both models are based on the assumption that the *proU* promoter, which does not have a good match to the consensus optimal promoter (Fig. 1), is inherently weak and needs some activating factor for high-level expression in media of high osmolality. According to one model, potassium glutamate, which accumulates to high concentrations in media of high osmolality (10), modulates the affinity of RNA polymerase for the *proU* promoter such that RNA polymerase can bind efficiently to the otherwise weak *proU* promoter in the presence of high concentrations of this solute (22). In the other model, osmotic stress, which is known to result in increased negative supercoiling of DNA, brings about a change in the conformation of the *proU* promoter which enables RNA polymerase to bind to it (11).

Mutations in the *hns* (*osmZ*) gene, which encodes a pleiotropic DNA-binding protein, confer a partially constitutive expression on the *proU* operon in media of low osmolality (7, 11, 14). Upstream and downstream of the *proU* promoter, there are several AT-rich runs of nucleotides (Fig. 1). DNA fragments containing these AT-rich sequences were found to exhibit aberrant mobilities in electrophoretic gels (7, 14, 20, 30). This result led to the suggestion that DNA in these regions is bent. The H-NS protein, which has a relatively high binding affinity for bent DNA (30), has been shown to bind the AT-rich DNA fragments flanking the *proU* promoter. It was proposed that the regulatory function of the silencer is imparted by the bending in the DNA rather than any specific nucleotide sequence (20).

The existence of the silencer in the *proU* operon was originally demonstrated by the analysis of the effects of deletions which removed long blocks of sequences 3′ to the *proU* promoter (7, 18, 20). However, these studies did not pinpoint the exact location or structure of this regulatory element. In particular, they did not resolve the 5′ boundary of the silencer or determine whether there are any constraints on its distance from the promoter for its ability to exert osmotic control of transcription. In this work, we present results of a deletion and insertion analysis that was carried out to define the structure of the silencer more precisely.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. Bacterial strains used are listed in Table 1. The compositions of LB medium and of low-osmolal K medium have been described previously (9). Strains were grown aerobically at 37°C. Antibiotics were used at the following concentrations: sodium ampicillin, 25 mg/liter for *E. coli* and 100 mg/liter for *S. typhimurium*; kanamycin sulfate, 75 mg/liter; tetracycline, 15 mg/liter; and spectinomycin hydrochloride, 50 mg/liter for *E. coli* and 1 g/liter for *S. typhimurium*.

Plasmids. Plasmids pRS415 (*bla*$^+$) and pRS551 (*bla*$^+$ *kan*$^+$), which are useful for transferring *lacZ* transcriptional fusions to derivatives of phage λ (28), were obtained from R. W. Simons. These two closely related pBR322-based plasmids contain a promoterless *lac* operon and unique *Eco*RI, *Sma*I, and *Bam*HI sites upstream of the *lacZ* gene. Plasmid pΔ2 (*bla*$^+$) was obtained from T. Silhavy; it

* Corresponding author. Phone: (317) 494-4969. Fax: (317) 496-1496.

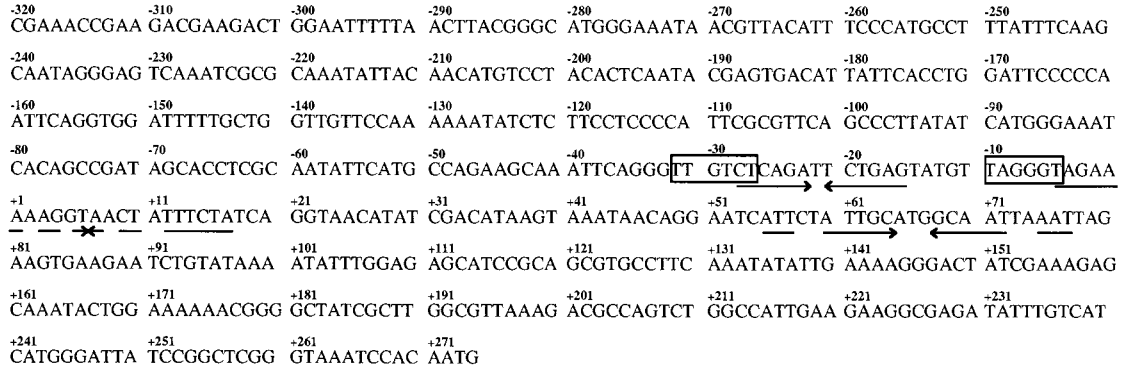

FIG. 1. Sequence of the promoter region of the *proU* operon of *S. typhimurium* (19, 29). The transcription start site is designated +1 (18); the translation start site of the first gene of the *proU* operon is at +65. The −10 and −35 elements of the promoter are highlighted by the rectangles. Palindromic sequences are indicated by arrows.

contains the *lamB*$^+$ gene of *E. coli* (designated Eco *lamB*$^+$ in genotypes) expressed from the pBR322 *tet* promoter. Plasmid pCMKM4, obtained from C. I. Kado, carries the *kan* gene of Tn*5* between the *Sal*I sites on pUC4; the *kan* gene can be excised from pCMKM4 as a ~0.7-kbp *Bgl*II-*Bam*HI fragment (12). Plasmid pTAT-13 *Xba*, obtained from N. Franklin, is similar to pTAT13 (8) except that downstream of the *phoA* gene there is a polylinker consisting of *Xba*I, *Nsi*I, *Sna*BI, *Bcl*I, *Bgl*II, *Stu*I, *Sac*I, *Nde*I, *Nsi*I, *Sna*BI, *Nsi*I, and *Xba*I sites; the *phoA* gene can be excised from this vector on a 1.5-kbp *Bam*HI-*Bgl*II fragment. Plasmid pDO182 is a low-copy-number *lac* expression vector derived from the pSC101-based plasmid pHJS21 (26); it confers resistance to spectinomycin and streptomycin and contains *Eco*RI, *Hin*dIII, and *Bam*HI sites upstream of a promoterless *lac* operon (18). Plasmid pSF002 was constructed by cleaving pDO182 with *Bam*HI, filling in the ends to blunt by the Klenow reaction, and religating. The analysis of the transcriptional control of the *proU* operon was carried out with plasmid pSF002 as the *lac* expression vector, with the *proU* promoter fragments cloned between the *Eco*RI and *Hin*dIII sites. In this plasmid, there are nonsense codons in all three reading frames between the *Hin*dIII site and the *lacZ* gene. The *lacZ* gene on this plasmid has its own translation start site (28), and consequently each promoter-*lacZ* fusion directs the synthesis of an identical β-galactosidase product. Plasmid pSF003 carries *proU* nucleotides −320 to +32 joined by a 17-bp *Bam*HI linker to *proU* sequences +33 to +274; it is identical to pDO244 (18) except that the vector backbone of pSF003 is pSF002. Plasmid pSF044 carries the *Eco*RI-*Bam*HI fragment (*proU* positions −320 to +32) of pSF003 followed by the 9-bp linker GGGGATCTG, cloned between the *Eco*RI and *Hin*dIII sites of pSF002.

Recombinant DNA constructions involved standard procedures (3). Deletion derivatives carrying portions of the transcriptional control region of the *proU* operon of *S. typhimurium* were generated by exonuclease III digestion or PCR amplification. The endpoints of the exonuclease III- and PCR-generated deletions were determined by DNA sequence analysis of double-stranded templates by the method of Sanger et al. (24). The structures of the *proU* promoter fragments that we generated are shown in schematic form in Fig. 2 and 3. *E. coli* DH5α was the host for plasmids during their construction; plasmids were introduced into this strain by $CaCl_2$-heat shock transformation (3). Analysis of the transcriptional control of the *proU* promoter was carried out in *S. typhimurium* TL1463 (*proU*$^+$ *recA1*); the plasmids were brought into this strain by electroporation (21) and selection for spectinomycin-resistant derivatives.

Introduction of *proU-lacZ* fusions into the chromosome of *S. typhimurium*. Two *proU-lacZ* fusions were crossed by in vivo recombination into phage λRS45 (*imm*^{21} *nin5*) by the technique of Simons et al. (28). Phage λRS45 has two regions of homology to plasmids derived from pRS551: within the 5′ end of the *bla* gene and throughout the *lacZYA* genes. It is possible to transfer desired promoter-*lacZ* fusions from pRS551-based plasmids to phage λRS45 by growing the phage on *rec*$^+$ strains harboring the plasmids and selecting progeny phage that acquired the *kan*$^+$ gene together with flanking regions from the plasmids by homologous recombination (28).

Plasmids pDO101 and pDO109 carry inserts of *proU* nucleotides from −508 to +308 and from −508 to +48, respectively, cloned between the *Eco*RI and *Bam*HI sites of plasmid pRS415 (6, 17). The *Pst*I-*Eco*RI fragment of plasmid pRS551, which contains the *kan* gene, was used to replace the *Pst*I-*Eco*RI fragments of plasmids pDO101 and pDO109. The resultant plasmids, pSF063 and pSF064, which carry *proU* sequences −508 to +308 and −508 to +48,

TABLE 1. Bacterial strains used[a]

Strain	Genotype	Source or construction
S. typhimurium		
CH1816	*hns-101* (*osmZ1*)::IS*10 zde-1710*::Tn*10*	N. P. Higgins
NH685	*hns-1*::*kan*	N. P. Higgins
TL549	*hsdL6 hsdSA29 galE496 metA22 metE55 proB9 ilv zah*::Tn*10 xyl-404 rpsL120 H1-b nml H2-enx* (Fels2)$^-$/pΔ2 (Eco *lamB*$^+$)	This laboratory
TL1463	*recA1 srl-2*::Tn*10*	This laboratory (18)
TL1491	*proU1872*::Mu d1-8	This laboratory (18)
TL1911	*hns-1*::*kan proU1872* Mu d1-8	P22 NH685→TL1491
TL2643	*galE2712*/pΔ2 (Eco *lamB*$^+$)	This laboratory
TL2647	*hns-1*::*kan galE2712*/pΔ2 (Eco *lamB*$^+$)	P22 TL1911→TL2643 = Km^r [Ap^r]
TL2703	λ(*proU p*$^+$*s*$^+$) *galE2712*/pΔ2	λ(*proU p*$^+$*s*$^+$)→TL2643 = Km^r
TL2705	λ(*proU p*$^+$Δ*s*) *galE2712*/pΔ2	λ(*proU p*$^+$Δ*s*)→TL2643 = Km^r
TL2714	*hns-101*::IS*10 galE2712 zde-1710*::Tn*10*/pΔ2 (Eco *lamB*$^+$)	P22 CH1816→TL2647 = Tc^r [Km^s]
TL2718	λ(*proU p*$^+$*s*$^+$) *hns-101*::IS*10 galE2712 zde-1710*::Tn*10*/pΔ2 (Eco *lamB*$^+$)	λ(*proU p*$^+$*s*$^+$)→TL2714 = Km^r
TL2720	λ(*proU p*$^+$Δ*s*) *hns-101*::IS*10 galE2712 zde-1710*::Tn*10*/pΔ2 (Eco *lamB*$^+$)	λ(*proU p*$^+$Δ*s*)→TL2714 = Km^r
TL2825	λ(*proU p*$^+$*s*$^+$) *hns-1*::*kan galE2712*/pΔ2 (Eco *lamB*$^+$)	λ(*proU p*$^+$*s*$^+$)→TL2647 = Lac$^+$
TL2828	λ(*proU p*$^+$Δ*s*) *hns-1*::*kan galE2712*/pΔ2 (Eco *lamB*$^+$)	λ(*proU p*$^+$Δ*s*)→TL2647 = Lac$^+$
E. coli		
DH5α	Δ(*lac-argF*)*-169 hsdR17 thi-1 recA1 gyrA96 relA1 supE44*/F′ ϕ80d*lac*Δ*M15*	From J. Hamer
MC4100	Δ(*lac-argF*)*-205 thi araD139 flbB5301 ptsF25 deoC1 relA1 rpsL150*	From B. Wanner

[a] Genetic nomenclature is according to reference 23. The notation P22 or λ(*M*)→*N* = *A* [*B*] denotes P22 or λ lysates, respectively, of strain *M* transduced into strain *N*, selecting phenotype *A* and scoring phenotype(s) *B*. Km^r, kanamycin resistant; Ap^r, ampicillin resistant.

respectively, are identical to pRS551 in the vector backbone, including the *kan*$^+$ gene that is between the *bla*$^+$ gene and the *Eco*RI site. Plasmids pSF063 and pSF064 were transformed into strain MC4100, and the resultant strains were infected with phage λRS45 (obtained from R. W. Simons). Phage lysates were used to infect MC4100 again, and kanamycin-resistant, λ lysogenic transductants were selected. Phage obtained from these lysogens by spontaneous induction were plaque purified on MC4100. One phage line obtained from the strain carrying pSF063 (*proU* sequences −508 to +308) was called λ(*proU p*$^+$*s*$^+$), and one obtained from the strain carrying pSF064 (*proU* sequences −508 to +48) was designated λ(*proU p*$^+$Δ*s*).

Wild-type phage λ cannot infect and replicate in *S. typhimurium*. However, because phage λRS45 carries the immunity region of phage 21, it can both grow and form lysogens in *galE* strains provided that the hosts express the λ receptor (*lamB*$^+$) gene of *E. coli* (23). Phage from single plaques of λ(*proU p*$^+$*s*$^+$) and λ(*proU p*$^+$Δ*s*) obtained from MC4100 were used to infect *S. typhimurium* TL549 (*galE hsdL hsdSA*/pΔ2 [Eco *lamB*$^+$]), generating lysates with titers of 1×10^9 to 5×10^9 PFU/ml on the latter host. These lysates were used to infect *S. typhimurium* TL2643 (*hns*$^+$ *galE*/pΔ2 [Eco *lamB*$^+$]), TL2717 (*hns-101*::IS*10 galE*/pΔ2 [Eco *lamB*$^+$]), and TL2647 (*hns-1*::*kan galE*/pΔ2 [Eco *lamB*$^+$]). Stable lysogens were obtained with strains TL2643 and TL2714 by selecting kanamycin-resistant transductants and with strain TL2647 by screening for Lac$^+$ transductants on LB plates containing 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) per ml.

β-Galactosidase assays. Bacteria were grown from a single colony in LB, inoculated at a 1:100 dilution into K medium and K medium containing 0.3 M NaCl, and grown overnight. The cells were subcultured again at 1:40 dilution into K medium containing the same NaCl concentrations as used during the previous growth, and after two doublings, β-galactosidase was assayed as described previously (9). For strains carrying *proU-lacZ* fusions on plasmids derived from pSF002, spectinomycin was included at 1 g/liter throughout all cycles of growth.

RESULTS AND DISCUSSION

Deletion analysis of the *proU* silencer. The *proU* promoter on a fragment which contains the entire *proU* sequences from −320 to +274 (pSF068) exhibited a 70-fold osmotic induction (Fig. 2A). Upon the introduction of a 17-bp *Bam*HI linker between positions +32 and +33 (pSF003), there was a slight (2.4-fold) increase in the basal level of expression from the *proU* promoter, but the promoter still responded with a 42-fold osmotic induction. Deletion of nucleotides from positions +33 to +46 (pSF010), +33 to +48 (pSF012), and +33 to +73 (pSF009) did not have any substantial effects on the range of osmotic control. However, when longer stretches of nucleotides were deleted, from positions +33 to +106 (pSF013), from positions +33 to +162 (pSF014), and the entire block of sequences downstream of position +32 (pSF044), there was a progressive increase in expression from the *proU* promoter in the medium of low osmolality.

These results indicate that the silencer is located downstream of *proU* position +48. Short changes in the distance between the promoter and silencer or in the phasing angle that are associated with the changes in the distance can be tolerated without disrupting the functioning of the silencer, as indicated by the observation that the insertion of 17 bp (pSF003) or subsequent deletion of a total of 25 bp (pSF010) or 27 bp (pSF012) of linker and *proU* sequences between the promoter and silencer did not have substantial effects on the expression of the *proU* promoter. The result that the basal level of transcription from the *proU* promoter increased gradually upon the progressive deletion of sequences downstream of position +32 suggests that the silencer may not consist of a single discrete sequence but rather could be made up of a number of components that have cumulative effects. Owing to the composite structure of the silencer, it may not be possible to determine its boundaries precisely, but its main components appear to be between positions +73 and +274. We constructed two other families of nested deletions which removed sequences from *proU* position +48 or from position +90 to points downstream; the results obtained with these deletions were completely consistent with those in Fig. 2A (data not shown).

To test whether sequences upstream of nucleotide +33 contribute to the functioning of the silencer, we constructed two deletions which removed sequences between this position and the promoter (Fig. 2B; pSF023 [deleted from +1 to +32] and pSF024 [deleted from −4 to +32]). Although these deletions altered slightly both the basal and induced levels of expression from the *proU* promoter, they had only minor effects on the induction ratio, indicating that the region between positions −4 and +32, which includes a run of five A T base pairs and a portion of an inverted repeat sequence (Fig. 1), is not important for osmotic control (at least in the presence of other components of the silencer downstream of position +32).

There is a particularly AT-rich sequence between positions +95 and +107 (Fig. 1). Because of the suggestion that AT-rich sequences or the bending of DNA imparted by them may be important for the negative regulatory function of the silencer (20), we targeted two mutations to this region. One was the replacement of the TAAAA (positions +97 to +101) with CGCGT (Fig. 2C, pSF086), and the other was the deletion of the entire block of nucleotides from positions +96 to +106 (Fig. 2C, pSF085). Although both the 5-bp replacement (pSF086) and the 11-bp deletion (pSF085) increased expression from the *proU* promoter in the medium of low osmolality compared with that seen with the most closely related control plasmid pSF009 (Fig. 2A), their effects were only slight (<4-fold). This analysis does not bear out any unique significance for the AT-rich sequences between positions +95 and +107.

Osmotic control is disrupted by long insertions between the *proU* promoter and silencer. The results presented above demonstrated that the regulatory mechanism acting at the silencer can tolerate short changes in the distance or in the phasing angle between the *proU* promoter and silencer. We tested the effects of longer insertions by placing a 0.8-kbp fragment that specifies the *kan* gene or a 1.5-kbp fragment that specifies the *phoA* gene between the *proU* promoter and silencer. The introduction of these longer insertions resulted in almost the same high level of constitutive expression from the *proU* promoter in medium of low osmolality as did the deletion of the silencer (Fig. 3). These results demonstrate that the interaction between the silencer and the *proU* promoter, which is responsible for repression of transcription in media of low osmolality, is almost completely destroyed when the silencer and promoter are moved far apart.

Effects of *hns* mutations. Two conflicting mechanisms have been advanced for the role of the H-NS protein in the regulation of the *proU* operon. It was suggested (31) that the H-NS protein binds to the *proU* promoter and acts as a transcriptional repressor by interfering with the binding of RNA polymerase. In contradiction to this model, *hns* mutations were almost without effect on expression from the *proU* promoter on constructs that carried partial deletions of the silencer (15, 16, 20); this observation led to the suggestion that the H-NS protein acts at the silencer rather than the promoter. However, interpretation of the latter experiments is complicated by the fact that they were carried out with deletion derivatives that retained the intact *proU* sequences for at least 100 bp downstream of the promoter, which on the basis of our results (Fig. 2) are likely to contain remnants of the silencer. Therefore, we tested the effects of *hns* mutations on *proU* expression with a deletion derivative that lacks the entire silencer. Because *hns* mutations affect plasmid supercoiling (11), it is possible that they can alter expression from plasmid-borne copies of the *proU* promoter by indirect effects through plasmid topology, in addition to effects that they might exert directly at the promoter. To avoid this complication, we introduced the *proU-lacZ* fusions into phage λ*imm*^{21} and analyzed the effects of *hns* mutations with strains carrying these phage integrated into the

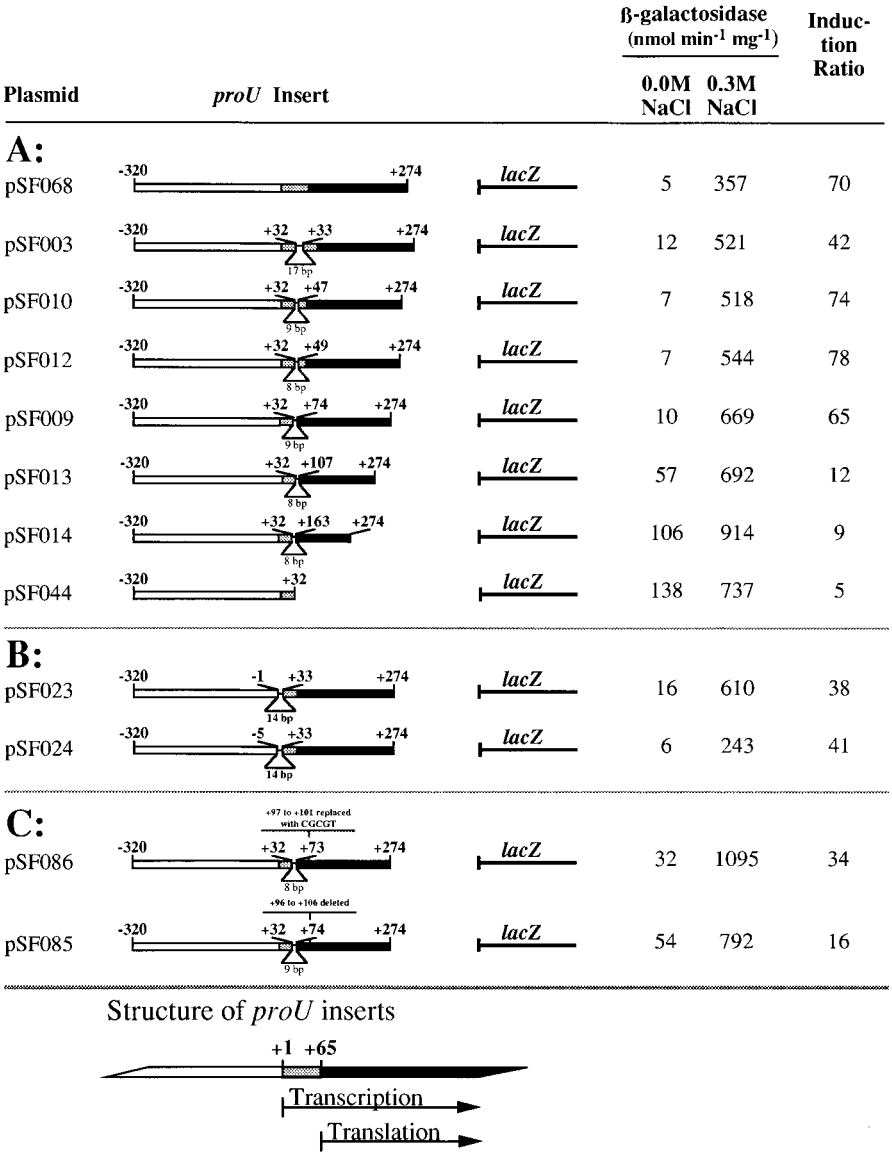

FIG. 2. Deletion analysis of the *proU* promoter region. Fragments carrying the indicated sequences from the *proU* promoter region of *S. typhimurium* were placed upstream of the *lacZ* gene on the pSC101-based *lac* expression vector pSF002 (Materials and Methods). Linkers inserted into *proU* sequences downstream of position +32 have the following sequences: in pSF003, GGGGATCCGGCGCCCCG (17 bp); in pSF009, pSF010, and pSF085, GGGGATCCG (9 bp); and in pSF012, pSF013, pSF014, and pSF086, GGGGATCC (8 bp). The linker downstream of positions −1 and −5 in pSF023 and pSF024, respectively, is GGATCCGGCGCCCC (14 bp). For all plasmids except pSF044, the indicated *proU* sequences were cloned between the *Eco*RI and *Hin*dIII sites of the vector; in pSF044, the *Hin*dIII site (AAGCTT) had been converted to AGCTT. Expression from the *proU* promoter was analyzed as β-galactosidase specific activity in *S. typhimurium* TL1463 (*proU*$^+$ *recA*) in exponential growth in the indicated media as described in Materials and Methods.

chromosome in single copy (Materials and Methods). We generated two λ*imm*^{21} phage harboring *proU-lacZ* transcriptional fusions: λ(*proU* p^+s^+), which carries *proU* positions −508 to +308, and λ(*proU* $p^+\Delta s$), which carries *proU* positions −508 to +48.

Although the *proU* promoter was expressed at an ~7-fold-lower level from the single-copy constructs than on the corresponding pSC101-based plasmids, on the single-copy vector λ(*proU* p^+s^+), the *proU* promoter exhibited >120-fold induction by osmotic stress in the *hns*$^+$ background (Table 2). Deletion of the silencer on the single-copy vector led to a 29-fold increase in the basal expression of the *proU-lacZ* fusion [Table 2; compare λ(*proU* p^+s^+) and λ(*proU* $p^+\Delta s$) in the *hns*$^+$ background]. These results demonstrate that the *proU* promoter is regulated very similarly on the single-copy vector and on plasmids (compare Table 2 with Fig. 2 and 3).

The *hns* mutations resulted in a three- to sixfold increase in the basal level of expression from the *proU* promoter in the presence of the intact silencer [Table 2, λ(*proU* p^+s^+)]. This slight increase in *proU* expression was similar to that reported by other groups (7, 11, 15). The *hns* mutations had an even less pronounced effect in the absence of the silencer: expression from the *proU* promoter on λ(*proU* $p^+\Delta s$) was increased only ~1.3-fold by the *hns-1*::*kan* allele, and it was actually decreased 3-fold by the *hns-101*::IS*10* allele, compared with the level seen in the *hns*$^+$ background (Table 2). These data do not support the proposal that the H-NS protein acts directly at the *proU* promoter (31), but they are consistent with the suggestion that

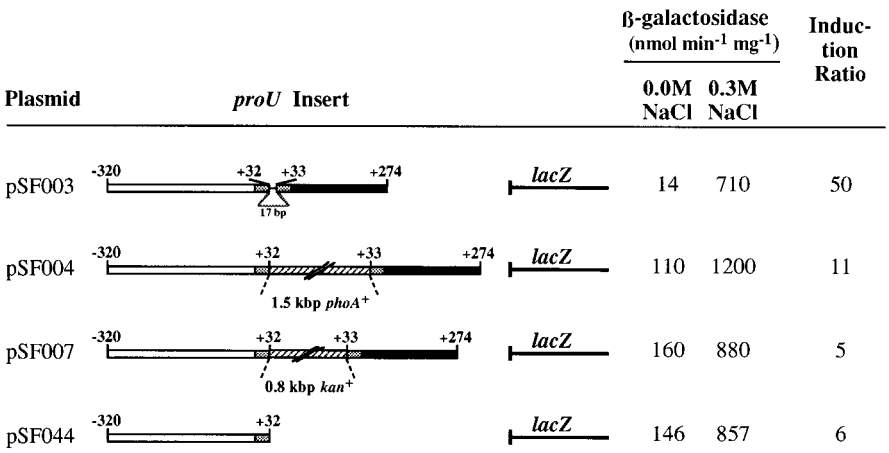

FIG. 3. Effects of long insertions between the *proU* silencer and promoter on transcriptional control of the *proU* operon. For details, see the legend to Fig. 2.

the H-NS protein acts at the silencer (7, 11, 15). The location of the strong H-NS binding site between positions +60 and +110, which was demonstrated by DNase I protection studies of the *E. coli proU* transcriptional control region (15), is in good agreement with the position of the silencer that we deduced by the in vivo deletion analysis in *S. typhimurium*.

In previous analyses, partial deletions of the silencer in the *hns*$^+$ background were found to have effects similar to those of *hns* mutations in increasing the basal level of expression from the *proU* promoter (7, 11, 15). In contrast, we found that in the *hns*$^+$ background, the increase in the basal level of expression from the *proU* promoter conferred by deletion of the entire silencer was 5- to 10-fold higher than that caused by the *hns* mutations when the silencer was present (Table 2). While our results are consistent with the possibility that the H-NS protein acts at the silencer, they indicate that the H-NS protein does not account for the entire range of osmotic control that is exerted through the silencer. Thus, these observations suggest that the H-NS protein plays a role secondary to that of a hitherto unidentified osmoregulatory protein that also acts at the silencer. This conclusion is corroborated by the observation that in the presence of the silencer, the *proU* promoter exhibited >20-fold residual osmotic induction even in *hns* mutants (Table 2 and references 7, 11, and 15). One possibility worth investigating is that the partial repression of the *proU* operon in media of low osmolality in *hns* mutants is mediated by the StpA protein, which has 60% amino acid sequence similarity to H-NS (27).

Model for the mechanism of action of the silencer. The negative regulatory element downstream of the *proU* promoter is unlike classical operators, such as those in the *lac*, *gal*, and *trp* operons, because it appears to be made up of a number of components which have a less rigidly defined nucleotide sequence than the typical operators. Furthermore, classical operator sites are almost invariably located very close to the promoters, and thus they affect transcriptional repression by bringing their respective repressor proteins in tight proximity to the promoters so that the repressor proteins interfere with the binding of RNA polymerase (4). Not only is the distance between the promoter and the downstream regulatory element of the *proU* operon considerably greater than seen typically in other negatively regulated operons, but also there is much greater flexibility in the distance from the promoter over which this element can exert transcriptional repression than seen in other negatively regulated operons. For these reasons, the downstream regulatory element is similar to eukaryotic transcriptional silencers, and we refer to it by that designation (18).

Although deletion of sequences downstream of the *proU* promoter could result in greatly elevated transcription from the *proU* promoter in the absence of osmotic stress, there was a residual osmotic induction with each deletion derivative. For example, the greatest increase in the basal level of transcription was generated by deletion of the entire region downstream of position +32, but nevertheless the promoter on this deletion derivative could still be induced five- to sixfold by osmotic stress (Fig. 2 and 3, pSF044). There are two possible explana-

TABLE 2. Effects of *hns* mutations on expression from the *proU* promoter in the presence and absence of the silencer[a]

Phage [*proU* nucleotides]	*hns*$^+$			*hns-101*::IS*10*			*hns-1*::*kan*		
	β-Galactosidase sp act		Induction ratio	β-Galactosidase sp act		Induction ratio	β-Galactosidase sp act		Induction ratio
	No NaCl	0.3 M NaCl		No NaCl	0.3 M NaCl		No NaCl	0.3 M NaCl	
λ(*proU p*$^+$*s*$^+$) [−320 to +308]	0.3	37	122	1.8	39	22	0.9	69	76
λ(*proU p*$^+$Δ*s*) [−320 to +48]	8.6	87	10	3.2	28	9	12	54	4.5

[a] Fragments carrying the indicated *proU* sequences were placed upstream of a promoterless *lacZ* gene on phage λRS45 and then introduced into the chromosome of *S. typhimurium* TL2643 (*hns*$^+$), TL2714 (*hns-101*::IS*10*), and TL2647 (*hns-1*::*kan*) as described in Materials and Methods. Resultant strains were grown in K medium containing the indicated NaCl concentrations, and their β-galactosidase specific activities were determined in early-exponential-phase cells as described in the legend to Fig. 2. The β-galactosidase specific activities are expressed as nanomoles of *o*-nitrophenol formed per minute per milligram of protein.

tions for this result. It could be that the silencer has vestigial components upstream of position +32 that are responsible for the residual osmotic regulation. Alternatively, it is possible that the *proU* operon is regulated by two independent mechanisms (16): one which acts at the silencer that is downstream of the promoter, and a second one which acts at the promoter or at sites further upstream.

Our data do not support the models which proposed that the increase in the affinity of RNA polymerase for the *proU* promoter is brought about by the effect of high concentrations of K^+ (or potassium glutamate) on RNA polymerase (22) or by changes in the topology of the *proU* promoter as a result of increased negative supercoiling (11). Both models are based on the assumption that the *proU* promoter is inherently a weak promoter, and they predict that all information necessary for osmotic regulation is specified by the promoter itself. Our data demonstrate that there is an important regulatory element outside the promoter. The finding that deletion of this element results in greatly elevated transcription in the absence of osmotic stress indicates that the *proU* promoter itself is a strong promoter and that it is regulated by a negatively acting mechanism that turns it off in media of low osmolality.

Despite the fact that the *proU* silencer is located at some distance downstream of the promoter, it nevertheless can regulate transcription initiation. It was proposed (18) that the silencer is the recognition target for some protein which in media of low osmolality folds nearby DNA into some higher-order structure in which the *proU* promoter is inaccessible to RNA polymerase. This complex might entail a DNA loop, similar to that proposed for the *deo* (2), *gal* (1), and *ara* (25) operons, or a more convoluted structure, as in eukaryotic nucleosomes (13). The protein which forms the silencer complex is sensitive to an osmotic signal such that it disengages from the *proU* promoter in media of high osmolality. Because the silencer complex entails a higher-order DNA structure, it could be sensitive to the level of DNA supercoiling, which could explain the effect of changes in DNA supercoiling on *proU* expression (11).

ACKNOWLEDGMENTS

We thank Vic DiRita for constructive comments on the manuscript and Naomi Franklin for the generous gift of plasmid pTAT13-13 *Xba* prior to its publication.

This work was supported by Public Health Service grant GM3194401.

REFERENCES

1. **Adhya, S.** 1987. The galactose operon, p. 1505–1512. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
2. **Amouyal, M., L. Mortensen, H. Buc, and K. Hammer.** 1988. Single and double loop formation when *deoR* repressor binds to its natural operator sites. Cell **58:**545–551.
3. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. R. Smith, and K. Struhl (ed.).** 1989. Current protocols in molecular biology. Greene Associates/Wiley Interscience, New York.
4. **Collado-Vides, J., B. Magasanik, and J. D. Gralla.** 1993. Control site location and transcriptional regulation in *Escherichia coli*. Microbiol. Rev. **55:** 371–394.
5. **Csonka, L. N., and A. D. Hanson.** 1991. Prokaryotic osmoregulation: genetics and physiology. Annu. Rev. Microbiol. **45:**569–606.
6. **Csonka, L. N., T. P. Ikeda, S. A. Fletcher, and S. Kustu.** 1994. The accumulation of glutamate is necessary for optimal growth of *Salmonella typhimurium* in media of high osmolality but not induction of the *proU* operon. J. Bacteriol. **176:**6324–6333.
7. **Dattananda, C. S., K. Rajkumari, and J. Gowrishankar.** 1991. Multiple mechanisms contribute to osmotic inducibility of *proU* operon expression in *Escherichia coli*: demonstration of two osmoresponsive promoters and of a negative regulatory element within the first structural gene. J. Bacteriol. **173:**7481–7490.
8. **Doelling, J. H., and N. C. Franklin.** 1989. Effects of all single base substitutions in the loop of *boxB* on antitermination of transcription by bacteriophage λ's N protein. Nucleic Acids Res. **17:**5565–5577.
9. **Druger-Liotta, J., V. J. Prange, D. G. Overdier, and L. N. Csonka.** 1986. Selection of mutations that alter the osmotic control of transcription of the *Salmonella typhimurium proU* operon. J. Bacteriol. **169:**2449–2459.
10. **Epstein, W.** 1986. Osmoregulation by potassium transport in *Escherichia coli*. FEMS Microbiol. Rev. **39:**73–78.
11. **Higgins, C. F., C. J. Dorman, D. A. Stirling, L. Waddell, I. R. Booth, G. May, and E. Bremer.** 1988. A physiological role for DNA supercoiling in the osmotic regulation of gene expression in *S. typhimurium* and *E. coli*. Cell **52:**569–584.
12. **Hirooka, T., and C. I. Kado.** 1986. Location of the right boundary of the virulence region on *Agrobacterium tumefaciens* plasmid pTiC58 and a host-specifying gene next to the boundary. J. Bacteriol. **168:**237–243.
13. **Kornberg, R. D., and Y. Lorch.** 1992. Chromatin structure and transcription. Annu. Rev. Cell Biol. **8:**563–587.
14. **Lucht, J. M., and E. Bremer.** 1991. Characterization of mutations affecting the osmoregulated *proU* promoter of *Escherichia coli* and identification of 5′ sequences required for high-level expression. J. Bacteriol. **173:**801–809.
15. **Lucht, J. M., P. Dersch, B. Kempf, and E. Bremer.** 1994. Interactions of the nucleoid-associated DNA-binding protein H-NS with the regulatory region of the osmotically controlled *proU* operon of *Escherichia coli*. J. Biol. Chem. **269:**6578–6586.
16. **Manna, D., and J. Gowrishankar.** 1994. Evidence for involvement of proteins HU and RpoS in transcription of the osmosensitive *proU* operon in *Escherichia coli*. J. Bacteriol. **176:**5378–5384.
17. **Overdier, D. G.** 1990. Genetic and molecular analysis of the osmotically regulated *proU* operon of *Salmonella typhimurium*. Ph.D. thesis. Purdue University, West Lafayette, Ind.
18. **Overdier, D. G., and L. N. Csonka.** 1992. A transcriptional silencer downstream of the promoter in the osmotically controlled *proU* operon of *Salmonella typhimurium*. Proc. Natl. Acad. Sci. USA **89:**3140–3144.
19. **Overdier, D. G., E. R. Olson, B. D. Erickson, M. M. Ederer, and L. N. Csonka.** 1989. Nucleotide sequence of the transcriptional control region of the osmotically regulated *proU* operon of *Salmonella typhimurium* and identification of the 5′ endpoint of the *proU* mRNA. J. Bacteriol. **171:**4694–4706.
20. **Owen-Hughes, T. A., G. D. Pavitt, D. S. Santos, J. M. Sidebotham, C. S. J. Hulton, J. C. D. Hinton, and C. F. Higgins.** 1992. The chromatin-associated protein H-NS interacts with curved DNA to influence DNA topology and gene expression. Cell **71:**255–265.
21. **Pfau, J., and P. Youderian.** 1990. Transferring plasmid DNA between different bacterial species with electroporation. Nucleic Acids Res. **18:**6165.
22. **Prince, W. S., and M. R. Villarejo.** 1990. Osmotic control of *proU* transcription is mediated through direct action of potassium glutamate on the transcription complex. J. Biol. Chem. **265:**17673–17679.
23. **Sanderson, K. E., and J. R. Roth.** 1988. Linkage map of *Salmonella typhimurium*, edition VII. Microbiol. Rev. **52:**485–532.
24. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
25. **Schleif, R.** 1987. The L-arabinose operon, p. 1473–1481. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
26. **Schreier, H. J., S. W. Brown, H. D. Hirshi, J. F. Nomellini, and A. L. Sonenshein.** 1989. Regulation of *Bacillus subtilis* glutamine synthetase gene expression by the product of the *glnR* gene. J. Mol. Biol. **210:**51–63.
27. **Shi, X., and G. N. Bennett.** 1994. Plasmids bearing *hfq* and the *hns*-like gene *stpA* complement *hns* mutants in modulating arginine decarboxylase gene expression in *Escherichia coli*. J. Bacteriol. **176:**6769–6775.
28. **Simons, R. W., F. Houman, and N. Kleckner.** 1987. Improved single and multicopy *lac*-based cloning vectors for protein and operon fusions. Gene **53:**85–96.
29. **Stirling, D. A., C. S. J. Hulton, L. Waddell, S. F. Park, G. S. A. B. Stewart, I. R. Booth, and C. F. Higgins.** 1989. Molecular characterization of the *proU* loci of *Salmonella typhimurium* and *Escherichia coli* encoding osmoregulated glycine betaine transport systems. Mol. Microbiol. **3:**1025–1038.
30. **Tanaka, K., S. Muramatsu, H. Yamada, and T. Mizuno.** 1991. Systematic characterization of curved DNA segments randomly cloned from *Escherichia coli* and their functional significance. Mol. Gen. Genet. **226:**367–376.
31. **Ueguchi, C., and T. Mizuno.** 1993. The *Escherichia coli* nucleoid protein H-NS functions directly as a transcriptional repressor. EMBO J. **12:**1039–1046.