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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

DAVID G. OVERDIER,[1] ERIC R. OLSON,[2] BRUCE D. ERICKSON,[3] MARTINA M. EDERER,[1] AND LASZLO N. CSONKA[1]*

Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47906[1]; Molecular Biology Research, The Upjohn Company, Kalamazoo, Michigan 49001[2]; and McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, Wisconsin 53706[3]

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Southern blot analysis of 15 *proU* transposon insertions in *Salmonella typhimurium* indicated that this operon is at least 3 kilobase pairs in length. The nucleotide sequence of a 1.5-kilobase-pair fragment that contains the transcriptional control region of the *proU* operon and the coding sequences specifying 290 amino acids of the first structural gene of the operon was determined. The predicted amino acid sequence of the product of this gene shows extensive similarity to the HisP, MalK, and other proteins that are inner membrane-associated components of binding protein-dependent transport systems. S1 mapping and primer extension analysis of the *proU* mRNAs revealed several species with different 5′ ends. Two of these endpoints are sufficiently close to sequences that have weak similarities to the consensus −35 and −10 promoter sequences that they are likely to define two transcription start sites. However, we cannot rule out the possibility that some or all of the 5′ endpoints detected arose as a result of the degradation of a longer mRNA. The expression of *proU-lacZ* operon fusions located on plasmids was normal in *S. typhimurium* regardless of the plasmid copy number. The sequences mediating normal, osmoregulated expression of the *proU* operon were shown by subcloning to be contained on an 815-base-pair fragment. A 350-base-pair subclone of this fragment placed onto a *lacZ* expression vector directed a high-level constitutive expression of β-galactosidase, suggesting that there is a site for negative regulation in the *proU* transcriptional control region which has been deleted in the construction of this plasmid.

The ability to adapt to changes in the osmotic strength of the environment is an important trait for organisms. To withstand fluctuations in the osmolarity of the environment, enteric bacteria accumulate low-molecular-weight solutes such as potassium, glutamate, proline, and glycine betaine (for reviews, see references 12 and 32). These compatible solutes are thought to balance the internal osmolarity with the outside or to protect proteins from the deleterious effects of high ion concentration (23, 48). In *Salmonella typhimurium* and *Escherichia coli*, the uptake of proline and glycine betaine is mediated by the ProP and the ProU systems (4, 8, 9, 11, 17, 24). The activities of these two transport systems are enhanced by exposure of the cells to osmotic stress. In the ProP system, the osmotic control is primarily the result of a posttranscriptional modification of the transport protein(s) (8, 17, 38), whereas in the ProU system, it is brought about by a several-hundred-fold increase in transcription of the *proU* operon (4, 9, 17, 24). The ProU system of enteric bacteria belongs to a family of related transport systems which utilize a periplasmic binding protein (3, 28, 35). Analysis of the protein products (14, 26) and the nucleotide sequence (25) of the *proU* operon of *E. coli* indicated that this locus contains three genes, *proV*, *proW*, and *proX*, arranged in one operon. The predicted amino acid sequence of the *proV* gene showed a high degree of similarity to the inner membrane-associated components of a number of other binding protein-dependent transport systems, and the predicted amino acid sequence of the *proX* gene matched the partial amino acid sequence determined for the glycine betaine-binding protein by Barron et al. (3).

The mechanism for sensing changes in osmolarity of the environment is poorly understood. Epstein (19) and Sutherland et al. (51) suggested that the signal for transcription of *proU* is an increase in intracellular potassium ions. In support of this hypothesis, Ramirez et al. (42) and Jovanovich et al. (33) demonstrated that 0.1 to 0.3 M potassium glutamate stimulated the expression of the *proU* operon in cell-free coupled transcription-translation systems. Druger-Liotta et al. (16) isolated 60 regulatory mutations in *S. typhimurium* which caused the ProU system to be expressed at elevated levels in the absence of hyperosmotic conditions. Each of these mutations proved to be closely linked to the *proU* promoter and to be *cis* dominant in diploid strains, indicating that these mutations are most likely alterations of the promoter-transcriptional control region of the *proU* operon. There are a number of conceivable reasons for the absence of *trans*-acting regulatory mutations for *proU*, such as the possibility that the phenotypes of these mutants would be different from those expected or that the mutations might be lethal. However, an interesting alternative reason for the inability to obtain such mutations might be that the osmotic control of *proU* transcription is mediated entirely by *cis*-acting information contained in the *proU* promoter region (16). Higgins et al. (27) demonstrated that growth in elevated osmolarity increased the in vivo supercoiling of reporter plasmids in *E. coli* and *S. typhimurium*. These researchers isolated *trans*-acting regulatory mutations in *E. coli* which proved to be the *topA* and *osmZ* loci that encode proteins governing the supercoiling of DNA. Because of these ob-

* Corresponding author.

TABLE 1. *S. typhimurium* strains

Strain	Genotype	Source or derivation
CH710	*proU1697*::Tn*10 proP1667*::Tn*5* Δ*putPA230 bio-561 galE503*	9
KS179	*zfi-8*::Tn*10 hisF1009 trpB2 metA22 rpsL201 xyl-1*	50
MS1202	*putA*::Mu dl-8(*lacZ*::Tn*10*)	S. Maloy
TL1	Wild type	*S. typhimurium* LT2 (B. Ames via S. Kustu)
TL154	*recA1 srl-2*::Tn*10 galE496 metA22 metE55 ilv*(?) *xyl-404 rpsL120* H1-*bnml* H2-*enx hsdL6 hsdSA29* Fels$^-$ [Mu dl (Amp^r *lac*) Mu cts62 *h*P1]	This laboratory (13)
TL155	*metA22 metE55 ilv*(?) *xyl-404 rpsL120* H1-*bnml* H2-*enx hsdL6 hsdSA29* Fels$^-$	This laboratory (13)
TL196	*proU1655*::Tn*10 proP1654* Δ*putPA557* Δ*proBA47 zcc-628* ::Tn*5*	This laboratory (11)
TL333, TL334, TL336, TL338 to TL340, TL342, TL343, TL345 to TL347, TL350	*proU1871*, *proU1872*, *proU1874*, *proU1876* to *proU1878*, *proU1880*, *proU1881*, *proU1883* to *proU1885*, *proU1888* ::Mu dl *proP675* Δ*putPA557 galE zcc-678*::Tn*5 zjd-27*:: Tn*10*	This laboratory (17)
TL393	*proU1884*::Mu dl(*B*::Tn*9*)*proP1654* Δ*putPA557 zcc-628*::Tn*5*	This laboratory (17)
TL671	*proU1884*::Mu dI1734	This laboratory (16)
TL1311	*proU1884*::Mu dl-8	P22 (TT7692) → TL671 = Amp^r [His$^+$ Kan^s]
TL1348	*zfi-8*::Tn*10 proU1884*::Mu dI1734	P22 (KS179) → TL671 = Tet^r [Kan^r]
TL1376	*proU1697*::Tn*10 proU1884*::Mu dI1734	P22 (CH710) → TL671 = Tet^r [Kan^r]
TL1402	*proU1872*::Mu dl *proP675* Δ*putPA557 galE zcc-678*::Tn*5* Δ*zjd-27*::Tn*10*	TL334 made Tet^s on Bochner plates (7)
TL1458 to TL1460	*metA22 metE55 ilv*(?) *xyl-404 rpsL120* H1-*bnml* H2-*enx hsdL6 hsdSA29* Fels$^-$(pDO4, pDO5, pDO39)	Transform TL155 with pDO4, pDO5, pDO39 = Amp^r
TL1463	*recA1 srl-2*::Tn*10*	P22 (TL154) → TL1 = Tet^r [UV^s]
TL1467	*proU1884*::Mu dl-8 *recA1 srl-2*::Tn*10*	P22 (TL154) → TL1311 = Tet^r [UV^s]
TL1476 to TL1478	*recA1 srl-2*::Tn*10*(pDO39, pDO5, pDO4)	Transform TL1463 with DNA from TL1460, TL1459, TL1458 = Amp^r
TL1479, TL1480	*metA22 metE55 ilv*(?) *xyl-404 rpsL120* H1-*bnml* H2-*enx hsdL6 hsdSA29* Fels$^-$(pHJS21, pDO57)	Transform TL155 with pHJS21, pDO57 = Spc^r
TL1481, TL1482	*recA1 srl-2*::Tn*10*(pHJS21, pDO57)	Transform TL1463 with DNA from TL1479, TL1480 = Spc^r
TL1483	*hisD9953*::Mu dI1734(*lacZ*::Tn*10*)	P22 (MS1202) → TT10286 = Tet^r [Amp^s Kan^r Lac$^-$ His$^-$]
TL1485	*proU1872*::Mu dI1734(*lacZ*::Tn*10*) *proP675* Δ*putPA557 galE zcc-678*::Tn*5* Δ*zjd-27*::Tn*10*	P22 (TL1483) → TL1402 = Tet^r [Amp^s His$^+$ Lac$^-$]
TL1487	*proU1872*::Mu dI1734(*lacZ*::Tn*10*)	P22 (TL1485) → TL1 = Tet^r [Kan^r]
TL1491	*proU1872*::Mu dl-8	P22 (TT7689) → TL1487 = Amp^r [Tet^s Kan^s His$^+$ Lac$^+$]
TL1497	*proU1872*::Mu dl-8 *recA1 srl-2*::Tn*10*	P22 (TL154) → TL1491 = Tet^r [UV^s]
TL1510	*proU1697*::Tn*10 proP675* Δ*putPA557 galE zcc-678*::Tn*5* Δ*zjd-27*::Tn*10*	P22 (CH710) → TL1402 = Tet^r [Amp^s]
TL1519 to TL1525	*metA22 metE55 ilv*(?) *xyl-404 rpsL120* H1-*bnml* H2-*enx hsdL6 hsdSA29* Fels$^-$(pRS415, pDO10, pDO40, pDO41, pDO70, pDO81, pDO83)	Transform TL155 with pRS415, pDO10, pDO40, pDO41, pDO70, pDO81, pDO83 = Amp^r
TL1526 to TL1532	*recA1 srl-2*::Tn*10*(pRS415, pDO10, pDO40, pDO41, pDO70, pDO81, pDO83)	Transform TL1463 with DNA from TL1519 to TL1525 = Amp^r
TL1533	*metA22 metE55 ilv*(?) *xyl-404 rpsL120* H1-*bnml* H2-*enx hsdL6 hsdSA29* Fels$^-$(pDO101)	Transform TL155 with pDO101 = Amp^r
TL1539	*recA1 srl-2*::Tn*10*(pDO101)	Transform TL1463 with DNA from TL1533 = Amp^r
TT7689	*hisD9953*::Mu dl-8	J. Roth
TT7692	*hisD9950*::Mu dl-8	30
TT10286	*hisD9953*::Mu dI1734	31

served effects of supercoiling on the expression of the *proU* operon, Higgins et al. (27) proposed that the osmotic control of the transcription of *proU* is exerted by changes in the supercoiling of the *proU* promoter region. To gain insights into the mechanism of the transcriptional control of the *proU* operon, we determined the nucleotide sequence of the *S. typhimurium* promoter region.

MATERIALS AND METHODS

Media and growth conditions. Growth conditions for the strains, the composition of the rich medium LB, and minimal medium 63 (M63) have been described previously (16). K medium was employed as the low-osmolarity medium. Antibiotics were used at the following concentrations (milligrams per liter): sodium ampicillin, 25 for Mu dl lysogens and 100 for strains with multiple copies of the *bla*$^+$ gene; chloramphenicol, 12.5; tetracycline, 15; spectinomycin hydrochloride, 1,000 for *S. typhimurium* and 50 for *E. coli*. Strains were grown at 37°C, except for lysogens carrying bacteriophage Mu dl, which were grown at 30°C.

Bacterial strains. The genotypes and derivation of the *S. typhimurium* strains are presented in Table 1. *E. coli* hosts

TABLE 2. Plasmids

Plasmid	Source or derivation
pBW2	Reference 52
pDO1	TL393 DNA + *Pst*I, ligate into pBW2 *Pst*I site, transform HB101 and select Amp^r; see Fig. 1
pDO4	*Pst*I-*Pst*I fragment spanning *proU*-Mu d junction from pDO1 in pBW2; see Fig. 1
pDO5	Δ*Hin*dIII of pDO4; see Fig. 1
pDO10	Δ*Hin*dIII of pDO4; see Fig. 1
pDO39	As pDO1 except use TL334 DNA; see Fig. 1
pDO40	*Eco*RI-*Pvu*II 4.3-kbp fragment from pDO39 in pRS415 *Eco*RI-*Sma*I; see Fig. 1
pDO41	Δ*Sst*II of pDO40; See Fig. 1
pDO42	*Hin*dIII 2.1-kbp fragment from pDO39 in pUC19 *Hin*dIII site such that the orientation of *proU* transcription is the same as that of the *lacZ*α gene
pDO43	As pDO42 except the orientation of the insert is opposite
pDO48	*Hin*cII ~100-bp deletion of pDO43
pDO57	*Hin*dIII 2.1-kbp fragment from pDO39 in pHJS21 *Hin*dIII site; see Fig. 1
pDO66	*Taq*I-*Taq*I 350-bp fragment from pDO42 treated with Klenow fragment and inserted into the *Sma*I site of pUC19 such that the orientation of *proU* transcription is the same as that of the *lacZ*α gene
pDO67	As pDO66 except the orientation of the insert is opposite
pDO70	*Eco*RI-*Eco*RV 1.2-kbp fragment of pDO43 in the *Eco*RI-*Sma*I sites of pRS415; see Fig. 1
pDO81	*Eco*RI-*Bam*HI ~350-bp fragment from pDO66 in the *Eco*RI-*Bam*HI sites of pRS415; see Fig. 1
pDO83	As pDO81 except from pDO67; see Fig. 1
pDO86	pRS415 wtih the *Sma*I site replaced with a *Hin*dIII linker
pDO88	pDO48 digested with *Sst*II and treated with Klenow fragment. A *Hin*dIII linker was attached, and this end was joined to the *Hin*dIII site in the vector
pDO101	*Eco*RI-*Hin*dIII 0.8-kbp fragment of pDO88 in the *Eco*RI-*Hin*dIII sites of pDO86; see Fig. 1
pHJS21	A. Sonenshein, personal communication; see Materials and Methods
pRS415	Reference 49

HB101 and JM103 were used for all in vitro plasmid constructions. Before plasmids were introduced into *S. typhimurium* strains of interest, they were passed through restriction-deficient, modification-proficient *S. typhimurium* TL155. All *S. typhimurium* strains were derivatives of LT2 (Table 1). Phage P22 HT104 *int*3-mediated transductions were done as described by Davis et al. (15).

Plasmids. The construction of plasmids is outlined in Table 2. Plasmid vector pBW2 (52) was used to clone the *proU1884*::Mu d1 and *proU1872*::Mu d1 fusions; the plasmids derived from pBW2 are pDO1, pDO4, pDO5, pDO10, and pDO39 (Fig. 1). Plasmids pDO1 and pDO39 were constructed by digesting with *Pst*I chromosomal DNA from strains TL393 (*proU1884*::Mu d1) and TL334 (*proU1872*::Mu d1), respectively, and ligating the inserts into the *Pst*I site of pBW2. Selection for Amp^r ensured that the 5′ end of the *bla* gene in the Mu d1 DNA was fused to the 3′ end of the *bla* gene in pBW2 (52). These two plasmids contained at least 8 kilobase pairs (kbp) of *S. typhimurium* DNA in addition to the 9 kbp of DNA derived from the end of Mu d1 that carries the *lac* genes. Plasmid pDO1 contained two extra *Pst*I fragments in addition to the fragment carrying the Mu d1 fusion; it was digested with *Pst*I and religated, resulting in pDO4, which had only one *Pst*I insert.

Plasmid pRS415 (49) is derived from pBR322 and contains cloning sites in front of a promoterless *lacZ*$^+$ gene. The plasmids derived from pRS415 are pDO40, pDO41, pDO70, pDO81, pDO83, and pDO101 (Fig. 1).

Plasmid pHJS21 (A. Sonenshein, personal communication) is a low-copy *lacZ* expression vector derived from pSC101 (10) which contains a *Hin*dIII site in front of a promoterless *lacZ*$^+$ gene and a gene conferring spectinomycin resistance (Spc^r). We constructed one derivative of pHJS21, pDO57, by inserting the 2.0-kbp *Hin*dIII fragment from pDO39 into the *Hin*dIII site of pHJS21 (Fig. 1).

Isolation of plasmid DNA, agarose gel electrophoresis, DNA ligations, and restriction enzyme digestions were done as described previously (34). Restriction enzymes were obtained from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.), Boehringer Mannheim Biochemicals (Indianapolis, Ind.), or New England BioLabs, Inc. (Beverly, Mass.). T4 DNA ligase and the Klenow fragment of DNA polymerase I were obtained from Bethesda Research Laboratories.

β-Galactosidase assays. β-galactosidase assays were done with exponentially growing cells cultured in the indicated media, as described by Miller (37).

Southern blotting. The nonradioactive BlueGENE Kit was obtained from Bethesda Research Laboratories, and the directions were followed according to the manufacturer. The probe used was biotin-labeled pDO10.

Sequence analysis. Various fragments of the chromosomal DNA cloned in pDO39 were subcloned into M13mp10, M13mp11, or pUC19 and were used to determine the nucleotide sequence of both strands of the *proU* transcriptional control region. Sequencing of both double-stranded and single-stranded templates was done by the method of Sanger et al. (47) as modified by Zagursky et al. (54) for reverse transcriptase. Oligonucleotides used for sequencing and primer extension were synthesized by the methoxy-phosphoramide triester coupling approach (6) and supplied by N. Thearault (The Upjohn Co.).

Transcript mapping. RNA was prepared by the hot phenol method (46) from strain TL1 grown exponentially at 37°C in K medium (0.07 osM) or in K medium plus 0.5 M NaCl (1.0 osM). For the *proU* probes, *Ava*I- or *Sst*II-digested pDO42 was labeled with [γ-^{32}P]ATP by polynucleotide kinase, cut with *Hin*dIII, and gel purified. A probe carrying the *rpsU-dnaG-rpoD* operon of *S. typhimurium* was used as a control for the expression of a promoter which is not subject to osmotic control. Plasmid pKKW10 carrying this operon (21) was labeled at a *Hin*dIII site in the *rpsU* gene and cut with *Eco*RI. Nuclease S1 analysis was performed by a procedure modified from that of Barry et al. (5). RNA (50 μg) and 0.1

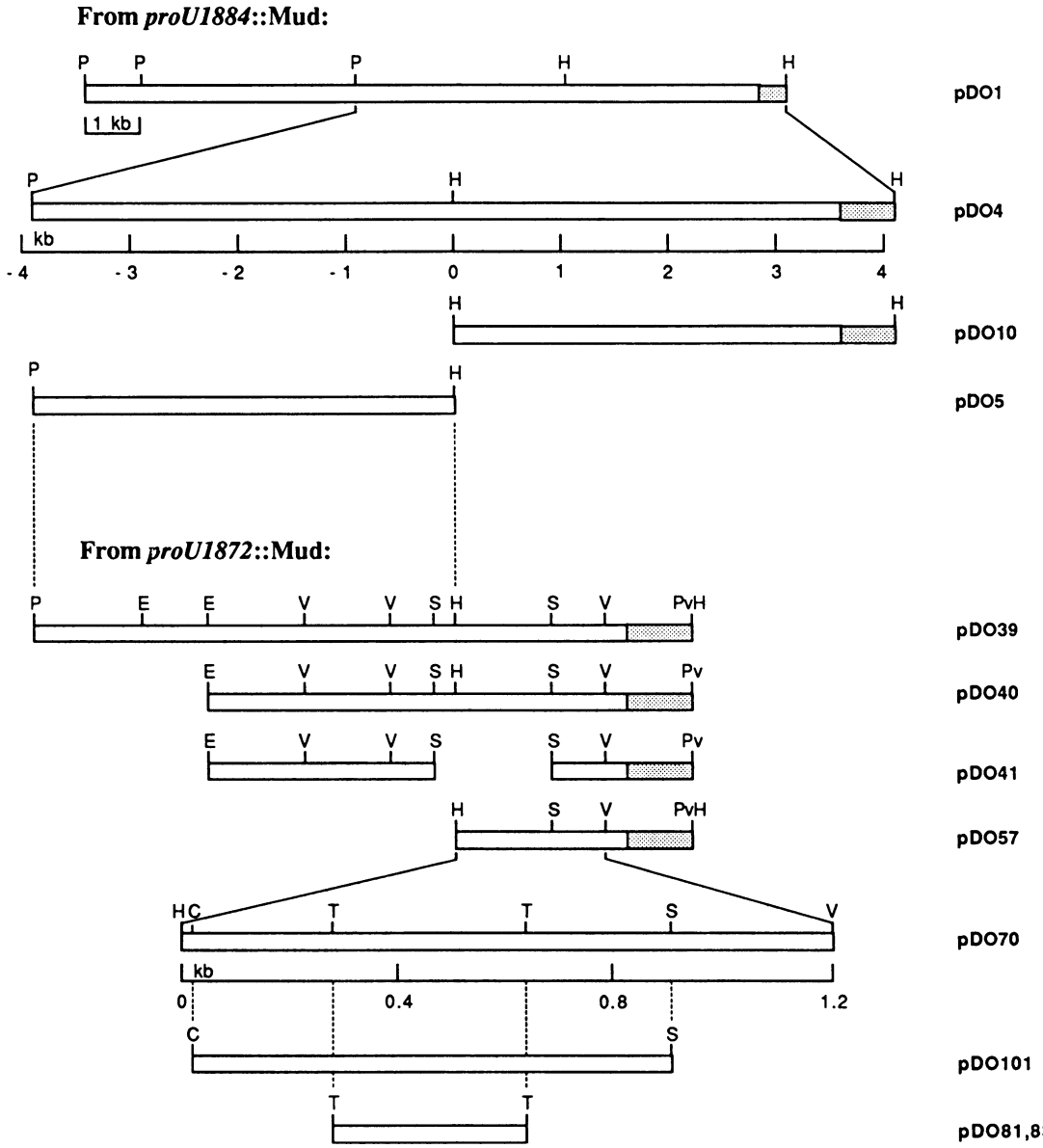


FIG. 1. Restriction nuclease map of DNA fragments containing the transcriptional control region of the *proU* operon of *S. typhimurium*. The names of the plasmids carrying these inserts are indicated in the right-hand column, and their construction is presented in Materials and Methods and Table 2. The open rectangles represent cloned *S. typhimurium* DNA, and the shaded rectangles represent an ~0.5-kbp portion of phage Mu d DNA from the right end to an internal *Hin*dIII site. The origin for the distances in the restriction map is a *Hin*dIII site (0.0 kbp). The order of the two leftmost *Pst*I fragments of pDO1 is not known. Note that the scale has been expanded for the inserts in plasmids pDO70, pDO101, pDO81, and pDO83. Restriction sites are denoted as: C, *Hin*cII; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; Pv, *Pvu*II; S, *Sst*II; T, *Taq*I; and V, *Eco*RV. kb, Kilobases.

μg of DNA probe were hybridized at 45°C for 4 h, followed by digestion with 300 U of S1 nuclease for 30 min at 37°C. The products were electrophoresed on a 5% polyacrylamide sequencing gel. Polynucleotide kinase and S1 nuclease were obtained from New England BioLabs and Boehringer Mannheim Biochemicals, respectively.

Primer extension of RNA was done by following the first-strand cDNA synthesis procedure of Polites and Marotti (41). Total cellular RNA was isolated from strain TL1311 that had been grown at steady state in M63 with 0.3 M NaCl (0.83 osM) and was hybridized to 0.2 μg of an oligonucleotide complementary to nucleotides 919 to 938 (see Fig. 3) that had been end labeled with [γ-^{32}P]ATP. The hybridization mix (final volume, 20 μl) was heated to 65°C and allowed to cool slowly to 37°C. The hybridized products were extended with reverse transcriptase (41) and electrophoresed on a 6% sequencing gel.

Periplasmic proteins. Strains were grown overnight in the indicated media. A sample was removed, and the cells were extracted with $CHCl_3$ (2). The proteins in the resultant extract were precipitated with trichloroacetic acid (10%) at room temperature and then extracted with ether. The pellet was taken up in 10 μl of a buffer containing 8 M urea, 2% sodium dodecyl sulfate, 10 mM Tris (pH 6.8), 10% glycerol,

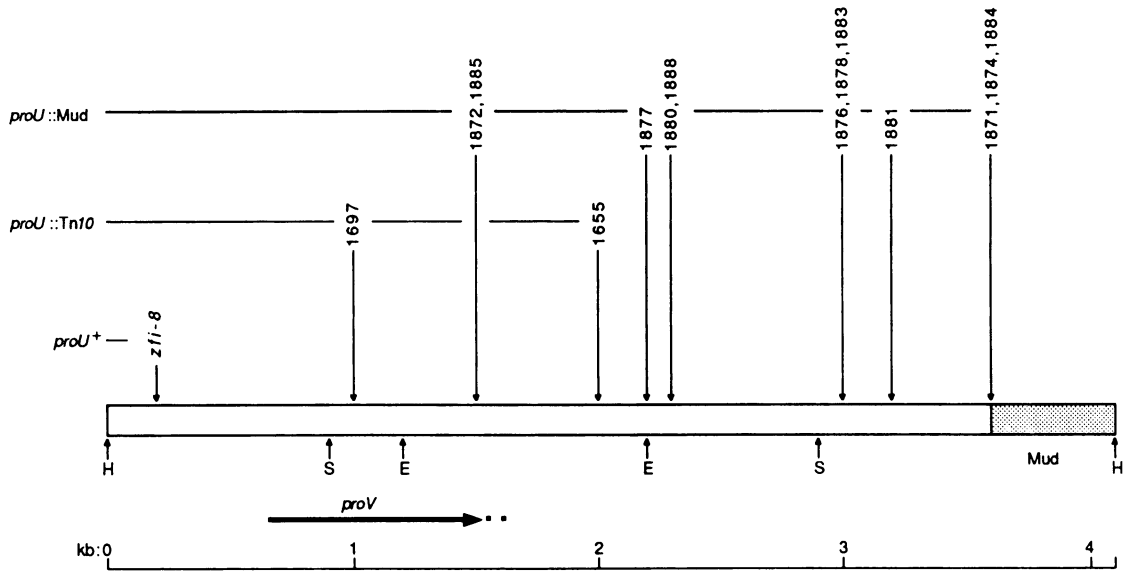


FIG. 2. Location of *proU*::Tn*10* or *proU*::Mu d1 insertions. The locations of these insertions were determined by Southern blot analysis of chromosomal DNA of strains carrying the given *proU* insertion mutations, with plasmid pDO10 as the probe (see Materials and Methods). The restriction map represents the *proU* region from strain TL346, which carries the *proU1884*::Mu d1 insertion. The reference point for the restriction map and the abbreviations for restriction sites are described in the legend to Fig. 1. The top set of numbers above the restriction map are the allele numbers of independent *proU*::Mu d1 insertions, the middle set are the allele numbers of *proU*::Tn*10* insertions, and the lowest number refers to a Tn*10* insertion outside the *proU* locus.

4% β-mercaptoethanol, and bromthymol blue and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis in a 12% polyacrylamide gel.

RESULTS

Physical mapping of *proU* locus. We first cloned the *proU1884*::Mu d1 insertion from strain TL393 into plasmid pBW2 (52), generating pDO1 and its two subclones, pDO4 and pDO10 (Table 2; Fig. 1). Plasmid pDO10, which contains a 4-kbp *Hin*dIII fragment spanning the junction of the Mu d1 and *S. typhimurium* DNA (Fig. 1), was used as a probe in Southern analysis to map the positions of 11 other *proU-lacZ* fusions generated by Mu d1 (17). Chromosomal DNA was prepared from the strains, digested with *Hin*dIII, blotted, and probed as described in Materials and Methods. The results of this analysis are shown in Fig. 2. The *proU*::Mu d1 insertions proved to be located in a 2-kbp region, with the *proU1872* insertion being closest to the *proU* promoter and *proU1884* being the farthest away from the promoter.

Three Tn*10* insertions were also mapped, *proU1655*::Tn*10* and *proU1697*::Tn*10*, which confer a ProU$^-$ phenotype (9, 11), and *zfi-8*::Tn*10* (50), which does not affect the ProU system (M. M. Ederer, unpublished data). The analysis (Fig. 2) indicated that *proU1697*::Tn*10* is located upstream of all the other *proU* insertions tested. When combined with the *proU1884-lacZ* fusion (which is the most distal from the promoter), *proU1697*::Tn*10* conferred a Lac$^-$ phenotype (data not shown), indicating that the 2.5-kbp region between these two insertions is transcribed as a single operon. The Southern analysis also revealed that the *zfi-8*::Tn*10* insertion is located about 0.9 kbp upstream of the site of insertion of *proU1697*::Tn*10*. Since this insertion does not affect the expression of the *proU* operon, the promoter for this operon is between the *zfi-8* and *proU1697*::Tn*10* insertions (Fig. 2).

Deletion analysis of *proU-lacZ* plasmids. Various fragments of plasmids carrying the *proU1884-lacZ* or *proU1872-lacZ* fusion were subcloned into *lac* expression vectors, and the β-galactosidase activities of strains carrying the resultant plasmids and grown in K medium and K medium plus 0.5 M NaCl were determined (Table 3). Strains harboring plasmids which contain the region extending from the *Hin*cII site (0.1 kbp, Fig. 1) up to or beyond the *Sst*II site (0.9 kbp, Fig. 1) (i.e., pDO4, pDO10, pDO39, pDO40, pDO57, pDO70, and pDO101) can express β-galactosidase, with at least a 30-fold increase in the synthesis of this enzyme upon osmotic stress. Plasmid pDO5, which was made from pDO4 by deleting the region between the *Hin*dIII sites at 0.0 and 4.3 kbp, and plasmid pDO41, which was made from pDO40 by deleting

TABLE 3. Expression of β-galactosidase in Φ(*proU-lacZ*) strains

Strain	Plasmid	Fusion	β-Galactosidase (nmol/min per mg of protein)[a] in K medium containing:		Ratio
			No NaCl	0.5 M NaCl	
TL1526	pRS415	None	2	2	1
TL1481	pHJS21	None	1	1	1
TL1467	None	*proU1884*	0.5	160	320
TL1478	pDO4	*proU1884*	24	720	30
TL1527	pDO10	*proU1884*	38	4,960	130
TL1477	pDO5	*proU1884*	28	45	2
TL1497	None	*proU1872*	2	470	235
TL1476	pDO39	*proU1872*	35	7,490	210
TL1528	pDO40	*proU1872*	105	14,000	130
TL1529	pDO41	*proU1872*	4	4	1
TL1533	pDO101	*proU1872*	100	7,700	77
TL1530	pDO70	*proU1872*	375	12,100	32
TL1531	pDO81	*proU1872*	1,490	2,490	1.7
TL1532	pDO83	*proU1872*	3,240	12,900	4
TL1482	pDO57	*proU1872*	4	1,750	440

[a] The β-galactosidase activities of the strains growing exponentially for at least six generations in the indicated media were measured as described in reference 37.

the region between the *Sst*II sites at −0.3 and 0.9 kbp (Fig. 1), can direct only a low-level synthesis of β-galactosidase, which is not inducible by osmotic stress. These results suggest that the *cis*-acting transcriptional regulatory regions of the *proU* operon are contained between the *Hin*cII site at 0.1 kbp and the *Sst*II site at 0.9 kbp (Fig. 1). The expression of the *lacZ* gene on plasmids pDO5 and pDO41 is most likely due to a promoter unrelated to the *proU* operon located in the region upstream of the *Sst*II site at −0.3 kbp or in the vector. These conclusions concerning the position of the *proU* promoter are in agreement with the mapping of the insertions (Fig. 2).

Regulation of *proU-lacZ* fusions on plasmids. The base level of expression of the *lacZ* gene in *S. typhimurium* strains carrying *proU-lacZ* fusions on high-copy derivatives of pBR322 (pDO4, pDO10, pDO39, pDO40, pDO70, pDO101) was approximately 50 times higher than that seen with the fusions carried in single copy on the chromosome (Table 3). Nevertheless, β-galactosidase could be induced quite normally by osmotic stress when the *proU-lacZ* fusions were carried on the high-copy plasmids, such that the ratio of induced to basal levels of β-galactosidase in the plasmid-carrying strains was similar to that of strains carrying the *proU-lacZ* fusions on the chromosome. The strain carrying the *proU-lacZ* fusion on pDO57 (which has the origin of replication of the low-copy plasmid pSC101) had a basal level of β-galactosidase that was about twice as high as that of its parental chromosomal fusion, but its induction ratio was identical to those of the other strains (Table 3).

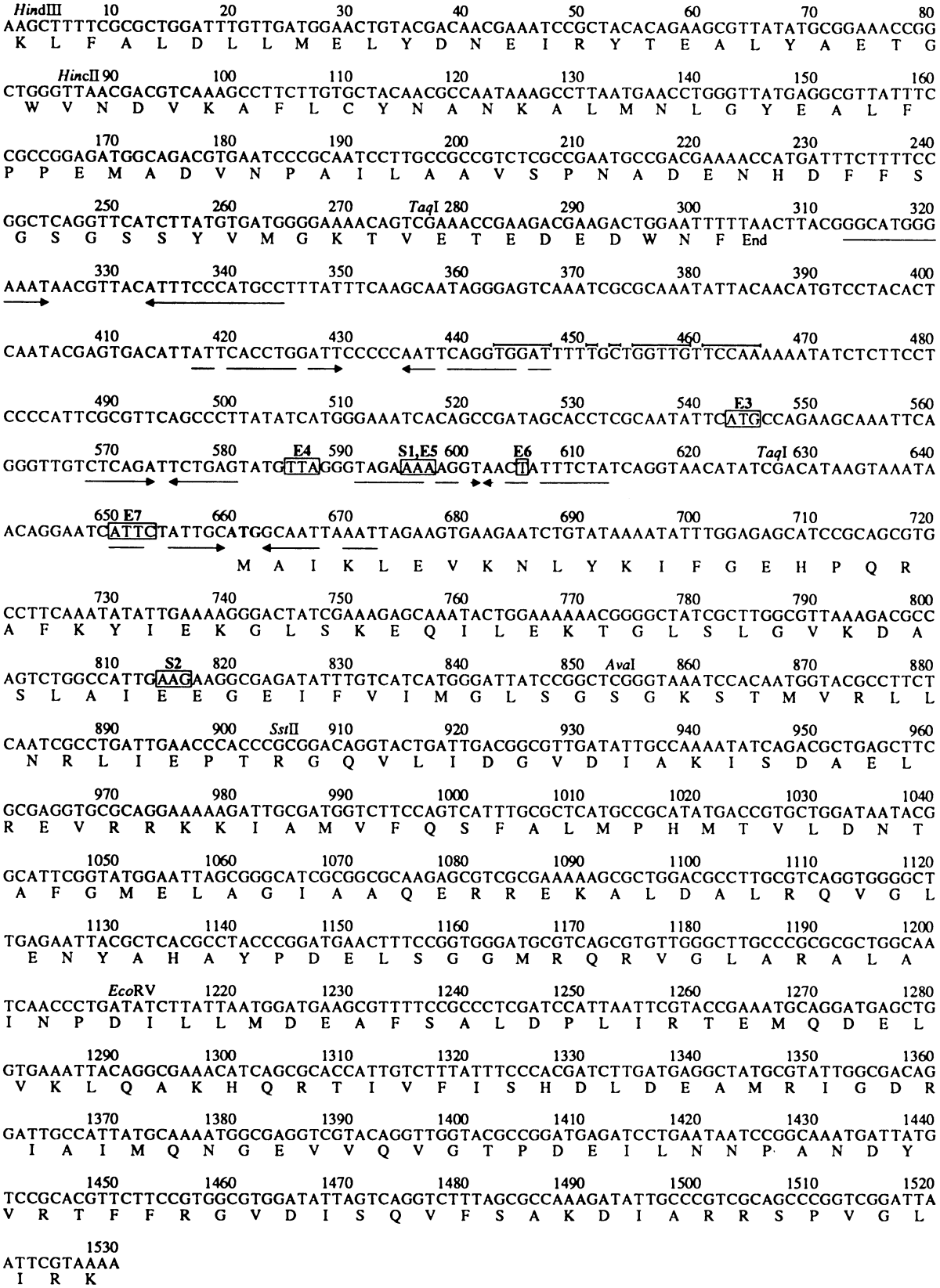
Sequence of *proU* promoter region. The nucleotide sequence analysis of 1.5 kbp of DNA containing the *proU* promoter region is presented in Fig. 3. There is a short open reading frame (ORF1) extending from the *Hin*dIII site (position 1) to position 303, which could encode a protein of at least 101 amino acids. At the 3′ end of ORF1, there is a perfect inverted repeat of 12 nucleotides separated by 8 nucleotides (positions 313 to 344), followed by a run of AT base pairs. This inverted repeat could be the terminator for the transcription of the mRNA for ORF1, but the possibility that it is involved in the transcriptional regulation of the *proU* operon has not been completely ruled out. There is a second long ORF extending from position 660 through the entire known sequence which could encode a protein of at least 290 amino acids. The predicted amino acid sequence encoded by this long ORF has about 50% amino acid homology with the products of the *hisP*, *malK*, *pstB*, and *oppD* genes of *S. typhimurium* and *E. coli* (Fig. 4), with a conservation of sites believed to be involved in ATP binding (1). The observation that this putative gene product has such strong similarity to other inner membrane-associated transport proteins suggests that this gene is the structural gene for an analogous component of the ProU system. In agreement with Dattananda and Gowrishankar (14), we use the symbol *proU* to designate the entire operon that encodes the components of the *S. typhimurium* ProU system, and *proV*, *proW*, and *proX* to designate the genes in the respective order of location on the operon.

Analysis of the intergenic region between ORF1 and the *proV* gene (Fig. 3) from positions 300 to 680 revealed several imperfect direct or inverted repeat sequences at positions 417 to 447, 568 to 580, 591 to 612, and 650 to 672 (Fig. 3). The computer search also identified a region between nucleotides 443 to 465 showing similarity to the DNA gyrase-binding site within the *oriC* locus of *S. typhimurium* and *E. coli* (36). Analysis of this region with a program written to detect sequence-directed bending structures (18) revealed a region centered at position 450 which would be considered a bend. The biological significance of any these above sequences is unknown.

Transcript mapping. S1 nuclease mapping was used to determine the 5′ endpoints of the *proU* mRNA(s) isolated from the wild-type strain TL1 grown in K medium or K medium plus 0.5 M NaCl. The RNA samples were allowed to hybridize with two probes, both having an unlabeled endpoint at the *Hin*dIII site (position 1, Fig. 3) and a labeled endpoint at the *Ava*I site (position 855, Fig. 3) or the *Sst*II site (position 903, Fig. 3), and the S1-resistant hybrid molecules were analyzed as described in Materials and Methods. We detected hybridization to both *proU* probes with RNA isolated from cells grown in K medium plus 0.5 M NaCl but not with RNA from cells grown in K medium (Fig. 5). The osmolarity of the medium did not have notable effects on the expression of the *rpsU-dnaG-rpoD* operon, used as a control. The largest RNA species protected by the *proU* probe labeled at the *Sst*II site (position 903) was 306 to 310 nucleotides long, and the longest RNA species protected by the probe labeled at the *Ava*I site (position 855) was 265 to 266 nucleotides long (Fig. 5). These results are consistent with each other within the limits of resolution of S1 mapping, and they indicate that the 5′ endpoint of a *proU* mRNA is located around positions 594 to 598 (denoted S1 in Fig. 3). Several shorter *proU* mRNA species were resolved by the S1 mapping. The most prominent of these shorter RNA hybrids was one of ~88 nucleotides, detected with the probe labeled at the *Sst*II site (denoted S2 in Fig. 3; see Fig. 5). The 5′ endpoint of this RNA corresponds to position ~815, which is well within the *proV* gene. It is not certain whether this position represents a processing point of the mRNA or a promoter within the *proU* operon. (This short RNA was not detected with the *Ava*I probe, perhaps because the RNA-DNA duplexes formed with this probe were too short to be stable during the hybridization and nuclease S1 treatment.)

The *proU* mRNA was also analyzed by primer extension. In this case, RNA was isolated from strain TL1311 (*proU1884*::Mu d1-8) grown in M63 and M63 with 0.3 M NaCl and hybridized to an oligonucleotide primer complementary to nucleotides 919 to 938 (Fig. 3). We detected five cDNAs (Fig. 6) with apparent endpoints around positions 544, 586, 596, 605, and 651 (denoted E3 through E7 in Fig. 3). Two of these endpoints, namely, at positions 586 and 596, are in excellent agreement with the approximate mRNA endpoint(s) resolved by S1 mapping, so that the composite results of the two techniques are consistent with one transcription start site for the *proU* mRNA being around position 596 and possibly a second one around position 586. However, the possibility that some or perhaps all of the observed 5′ endpoints are due to degradation of a longer mRNA rather than transcription initiation cannot be excluded. The 5′ endpoint of the longest *proU* mRNA detected by primer extension (position 544, Fig. 6) was not seen in the S1 nuclease analysis (Fig. 5), and therefore one (perhaps the only) transcription start site for the *proU* operon may be at or upstream of position 544. However, if this position corresponds to the 5′ endpoint of a species of *proU* mRNA, it is not clear why we did not detect it with the S1 nuclease analysis. An overestimate of the length of an mRNA in primer extension could result from the formation of hairpin structures at the 3′ end of the cDNA serving as primers for the synthesis of a second DNA strand.

Promoters in opposite directions. To examine further the *cis*-acting sequences that are required for osmotic control of transcription of the *proU* operon, we subcloned the 275-


HindIII
HincII
TaqI
TaqI
AvaI
SstII
EcoRV
E3
E4
S1,E5
E6
E7
S2
End

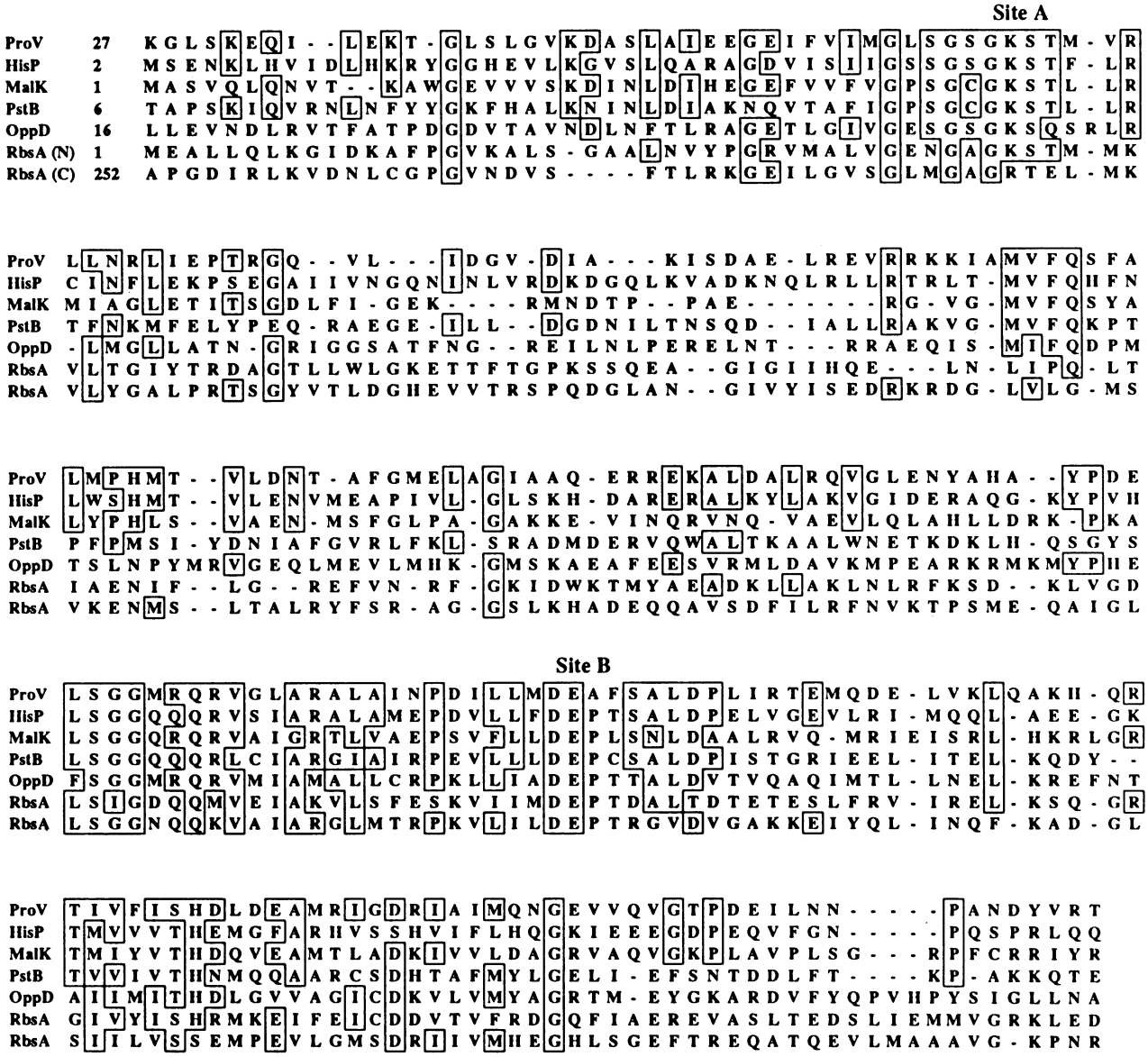


FIG. 4. The *proV* gene product resembles inner membrane-associated components of other binding protein-dependent transport proteins. The predicted sequence of the first 290 amino acids of the *proV* gene product is compared with the amino acid sequences of other transport proteins (1), starting with the residues indicated in the top set of lines. Boxes are drawn if the *proV* gene product is identical at that position to at least two other gene products. The residues at sites A and B have been proposed to be involved in ATP binding (1).

base-pair (bp) *Hin*dIII-*Taq*I fragment (positions 1 to 275, Fig. 3), the 350-bp *Taq*I-*Taq*I fragment (positions 275 to 625, Fig. 3), and the 122-bp *Taq*I-*Taq*I fragment (positions 625 to 747, Fig. 3) into plasmid pRS415. Strains carrying the plasmids with the *Hin*dIII-*Taq*I fragment and the 122-bp *Taq*I-*Taq*I fragment were white on MacConkey-lactose agar containing 0.2 M NaCl, indicating that these two fragments did not have a promoter (data not shown), and they were not studied further. However, the strain carrying the plasmid with the 350-bp *Taq*I-*Taq*I fragment (designated pDO83)

FIG. 3. Nucleotide sequence of the *proU* transcriptional control region. The nucleotide sequence of the strand containing the information corresponding to the putative *proU* mRNA is presented. The origin for the numbering of the nucleotide position is at the *Hin*dIII site (base pair 1), which corresponds to the *Hin*dIII site at 0.0 kbp in Fig. 1 and 2. Perfect or near perfect inverted repeats are highlighted by the inverted arrows below the sequence. The boxes S1 and S2 (positions ~596 and 815, respectively) denote the 5′ endpoints of the two most abundant mRNA species detected by nuclease S1 mapping (see Fig. 5), and the boxes E3 through E7 (positions ~544, ~586, ~596, 605, ~651, respectively) denote the 5′ endpoints of five mRNA species detected by primer extension (see Fig. 6). There is a sequence showing similarities to the proposed gyrase-binding site (36) at positions 443 to 465, highlighted by a line above the nucleotide sequences. The probable translation start site of the *proV* gene is at the ATG at positions 660 to 662. The predicted amino acid sequence of the first 290 amino acids of the *proV* gene product and the last 101 amino acids of a protein of unknown function (ORF1) upstream of the *proU* operon are shown with the single-letter code below the nucleotide sequence.

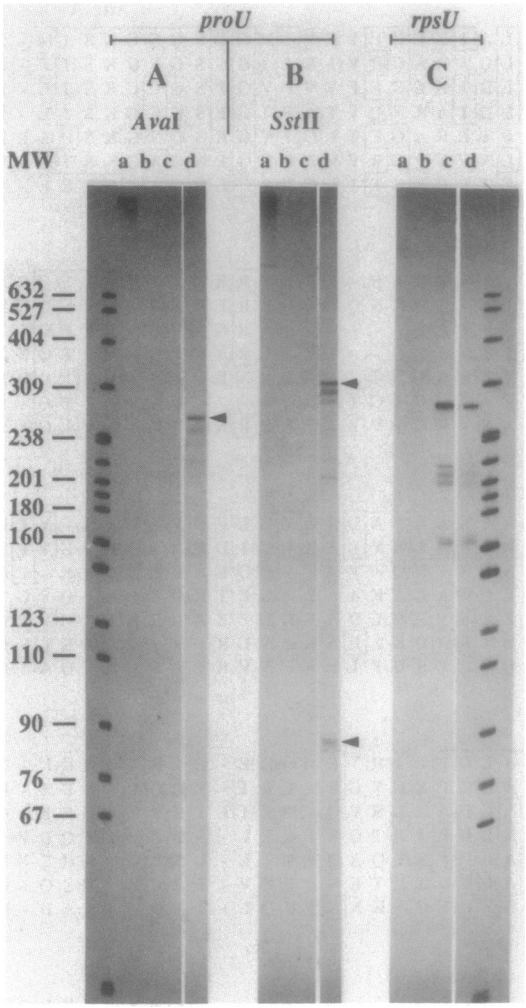


FIG. 5. Nuclease S1 determination of the 5′ endpoints of the *proU* mRNA. RNA was isolated from the wild-type strain TL1, and nuclease S1 protection experiments were done as described in Materials and Methods. (A) Probe was the *Hin*dIII-*Ava*I *proU* fragment (positions 2 to 855) labeled at the *Ava*I site; (B) probe was the *Hin*dIII-*Sst*II *proU* fragment (positions 2 to 903) labeled at the *Sst*I site; (C) control for a promoter not subject to osmoregulation with a probe carrying the *rpsU-dnaG-rpoD* operon of *S. typhimurium* (21). In each panel, the lanes are results with probe only, without RNA or nuclease S1 (lane a), probe without RNA and treated with nuclease S1 (lane b), probe hybridized with RNA isolated from cells grown in K medium and treated with nuclease S1 (lane c), and probe hybridized with RNA isolated from cells grown in K medium containing 0.5 M NaCl and treated with nuclease S1 (lane d). The three most abundant *proU* mRNA species are highlighted with arrowheads. MW, Molecular weight standards; the lengths of fragments in base pairs are indicated.

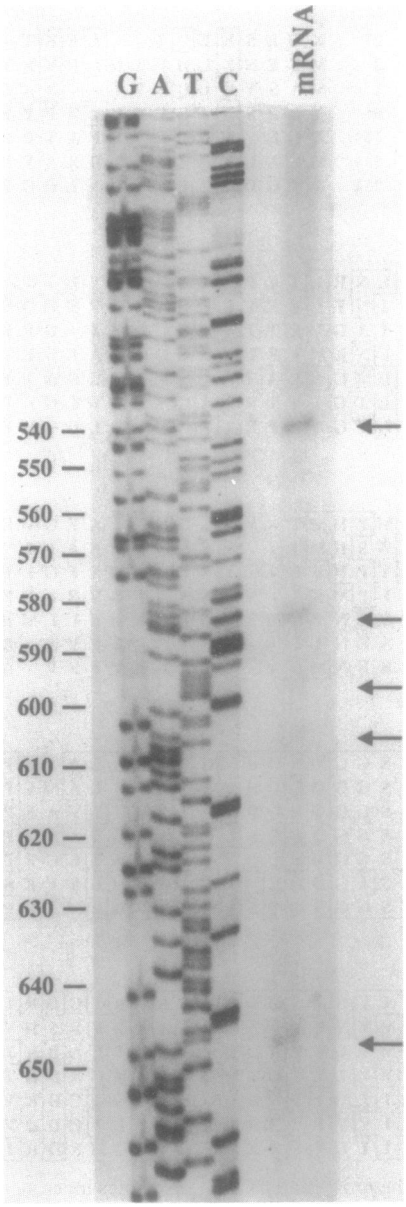


FIG. 6. Primer extension analysis of the 5′ endpoints of *proU* mRNA. RNA was isolated from strain TL1311 (*proU1844*::Mu d1-8) grown in M63 containing 0.3 M NaCl, and primer extension was done as described in Materials and Methods. The primer was an oligonucleotide complementary to nucleotides 919 to 938 (Fig. 3). Five mRNA species that were resolved are highlighted with arrows. The results of the DNA sequence determination are presented in lanes G, A, T, and C; this sequence is for the strand complementary to that given in Fig. 3. The left-hand column of numbers indicates the nucleotide position as shown in Fig. 3.

exhibited a Lac$^+$ phenotype on MacConkey-lactose agar. The β-galactosidase level conferred by this plasmid in cells grown in M63 was at least 10-fold greater than that seen with the parental plasmid pDO101 (Table 3), and it was not increased further by osmotic stress. Interestingly, when the same *Taq*I-*Taq*I fragment was inserted into plasmid pRS415 in the direction opposite to the transcription of *lacZ* from the *proU* promoter (pDO81), it could still direct the synthesis of high and unregulated levels of β-galactosidase (Table 3). These results suggest that there is a promoter in the *Taq*I-*Taq*I fragment (from positions 275 to 625) pointing away from the *proU* operon which can be recognized on a high-copy plasmid.

Analysis of periplasmic proteins. Barron et al. (3) and Faatz et al. (22) reported that in *E. coli*, the structural gene for the glycine betaine-binding protein is the first gene of the *proU* operon. Our DNA sequencing revealed that the first gene of the *proU* operon in *S. typhimurium* encodes an inner membrane-associated protein rather than a periplasmic binding protein and therefore seems to contradict this conclusion. To settle this discrepancy, we analyzed periplasmic protein

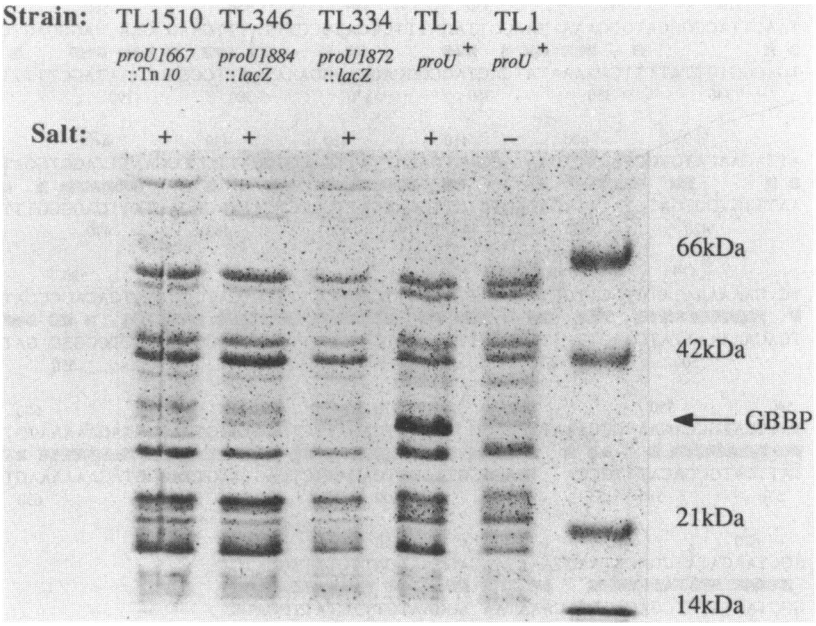


FIG. 7. Periplasmic proteins of *proU* insertion mutants. The periplasmic proteins of the indicated strains were isolated and analyzed as described in Materials and Methods. The strains were grown in M63 (−) or M63 plus 0.3 M NaCl (+). The 33-kilodalton (kDa) glycine betaine-binding protein (GBBP) is present only in extracts of strain TL1 grown in M63 plus 0.3 M NaCl.

extracts from 24 *S. typhimurium* strains carrying various *proU* insertion mutations. Each of them proved to lack the periplasmic glycine betaine-binding protein. The results obtained with representative strains TL334 (*proU1872*::Mu d1), TL346 (*proU1884*::Mu d1), and TL1510 (*proU1697*::Tn*10*) grown in M63 with 0.3 M NaCl are shown in Fig. 7. A 33-kilodalton periplasmic protein was detectable in the wild-type strain TL1 grown in the presence of 0.3 M NaCl but not in any of the other strains. Because the insertions in the strains used are separated by 2.5 kbp, we conclude that the structural gene for the glycine betaine-binding protein is not the first gene of the *proU* operon in *S. typhimurium* but rather is located in a region at least 2.5 kbp downstream from the promoter.

DISCUSSION

There is excellent agreement between our nucleotide sequence results for the *proU* promoter region of *S. typhimurium* and those obtained by Gowrishankar (25) for the *E. coli* counterpart. There is a 79 and an 87% nucleotide sequence identity and an 85 and a 98% amino acid sequence identity for ORF1 and the *proV* gene, respectively, in the regions where the sequences can be compared. Gowrishankar (25) concluded that the gene for the glycine betaine-binding protein, *proX*, is the last gene of the *proU* operon, and he noted, as we have, that the first gene of the operon, *proV*, encodes an inner membrane-associated protein. Last, Gowrishankar (25) placed the 5′ endpoints of two *proU* mRNAs at 52 and 60 nucleotides upstream of the probable translation start of the *proV* gene. These positions correspond to nucleotide positions 607 and 599 of the *S. typhimurium* sequence (Fig. 3), and therefore the endpoints E5 and E6 we determined by primer extension are in close agreement with the results of Gowrishankar (25).

In several respects, there are differences between our results and those of Gowrishankar. Gowrishankar (25) found one set of additional mRNA endpoints, 178 to 180 and 213 to 215 nucleotides upstream of the probable translation start of the *proU* gene, which we did not detect. More important, we found that cloning of the *S. typhimurium proU* operon on high-copy plasmids did not affect the osmotic control of its expression in *S. typhimurium* hosts (Table 3), whereas the *E. coli proU* operon no longer responded to the normal osmotic control when placed on high-copy vectors in *E. coli* strains growing exponentially in media of high osmolarity (14). Last, Dattananda and Gowrishankar (14) reported that in *E. coli*, plasmids which carry the *proV* gene and only portions of the *proW* or *proX* genes resulted in an osmosensitive phenotype. We did not observe any osmosensitivity with any of the *S. typhimurium* clones we tested in either *E. coli* or *S. typhimurium* hosts.

The high degree of similarity between the *E. coli* and *S. typhimurium* nucleotide sequences is not entirely conserved for the intergenic region between ORF1 and the *proV* gene. Figure 8 presents a comparison of the nucleotide sequences of this region from the two organisms. There is only a 68% nucleotide sequence identity in the region corresponding to positions 304 to 662 of the *S. typhimurium* sequence. Interestingly, the sequence conservation between the organisms is stronger in the vicinity of the *proU* promoter region, as there is an 85% nucleotide sequence identity for the nucleotides corresponding to positions 461 to 655 of the *S. typhimurium* sequence, but only a 60% identity for positions 307 to 460. Because the 12-nucleotide inverted repeat structure at positions 313 to 324 and 333 to 344 of the *S. typhimurium* sequence is lacking from *E. coli*, we conclude that this structure is not important for the transcriptional control of the *proU* operon.

A scan of the nucleotide sequences upstream of the first structural gene with the algorithms of Mulligan et al. (40) revealed a number of sequences that may serve as possible promoters for the *proU* operon. Thus, the sequences resembling the *E. coli* consensus promoter (29) are (i) TTGTCT (−35, positions 564 to 569, Fig. 2) and TAGGGT (−10,

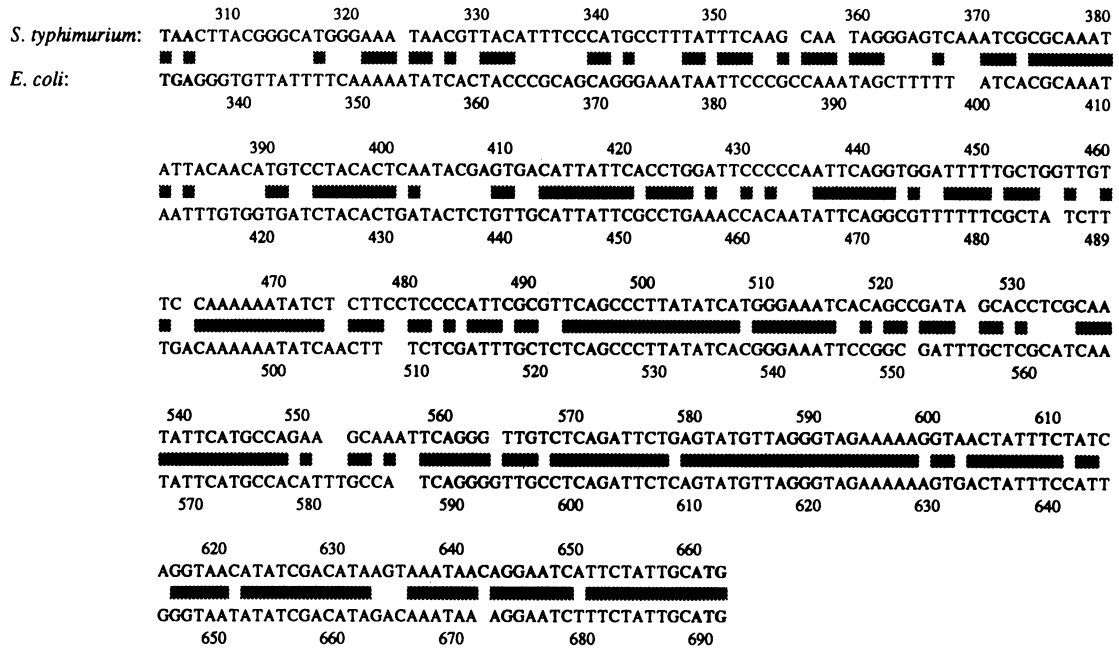


FIG. 8. Comparison of the nucleotide sequence of the promoter region of *S. typhimurium* and *E. coli* K-12. The sequences correspond to nucleotide positions 304 to 662 of the *S. typhimurium* sequence (Fig. 3). The *E. coli* K-12 data are from reference 25.

positions 586 to 591) and (ii) TTCAGG (−35, positions 557 to 562) and TATGTT (−10, positions 581 to 586). These might be possible promoters for the mRNAs detected by S1 mapping and primer extension at positions 597 and 585, respectively. Likewise, the sequences ATCACA (positions 514 to 519) and AATATT (positions 536 to 541) might be the −35 and −10 sequences for an mRNA resolved by primer extension to have a 5′ endpoint at position 544. However, the differences between these sequences and the consensus *E. coli* −35 and −10 sequences are sufficiently great to suggest that they do not constitute strong promoters in the absence of a positive regulatory protein. The *Taq*I-*Taq*I fragment from positions 275 to 625 apparently contains at least one promoter for mRNA transcribed in the orientation opposite that of the *proU* operon. There does not appear to be a strong promoter in this direction either; the computer analysis revealed the hexamers TAGAAA (positions 612 to 607) and TAACAT (positions 587 to 582), which may be the −35 and −10 sequences of a weak promoter.

Both the basal and the induced levels of the expression of the *proU* regulatory sequences fused to the *lacZ* gene on both high- and low-copy plasmids were positively correlated with the copy number of the vectors used. The induction ratio, however, remained nearly constant for all the plasmids that carried the entire *proU* promoter region. The regulation of DNA supercoiling is not understood sufficiently to be able to predict it for a given plasmid, and it is possible that the localized supercoiling of the *proU* insert is not influenced by the various vectors used. However, because supercoiling is determined by a number of factors, including the number of active promoters on a replicon (53), it is unlikely that the supercoiling of the *proU* region on the chromosome is the same as on the low-copy plasmid pSC101 or the high-copy plasmid pBR322. Thus, the nearly invariant induction ratios of the *proU* operon when it is carried on the various replicons do not seem to be readily consistent with the proposal that supercoiling is the main regulatory signal for the transcriptional control of the *proU* operon (27).

The observation that the *proU* operon can be expressed efficiently under conditions of hyperosmotic stress despite the lack of a promoter with strong similarity to consensus −35 and −10 sequences raises some questions concerning the factors involved in the recognition of the *proU* promoter by RNA polymerase. There are no concrete data bearing on this point, but it is possible that some positively acting transcriptional regulatory factor or the degree of supercoiling directs the RNA polymerase to the *proU* promoter under conditions of high osmolarity. The result that the *Taq*I-*Taq*I fragment from positions 275 to 625 can confer a relatively high level of constitutive expression of the *lacZ* gene in pDO83 (Table 3) suggests that there is some negative *cis*-acting site for *proU* transcription (e.g., a target for a repressor protein, a site for transcription attenuation or termination, or a site of mRNA processing) upstream of position 275 or downstream of position 625. However, we have no data to rule out the possibility that an inadvertent promoter had been generated as a result of fortuitous juxtaposition of insert and vector sequences in pDO83. Therefore, more experiments are needed to verify the existence of the proposed negatively acting control site.

Because there is no direct evidence for positive or negative transcriptional control factors for the *proU* operon other than RNA polymerase, it is possible that the transcriptional regulation of the *proU* operon is effected only by the interaction of RNA polymerase with the promoter region. Both the affinity and the rates of binding of proteins to their target sites on DNA are extremely sensitive in vitro to the electrolyte concentration of the buffers used (39, 43–45). Since the intracellular K^+ concentration can vary from 0.2 to 0.9 M depending on the external osmolarity (20, 44), one might expect that fluctuations in the extracellular osmolarity would have dramatic effects on the in vivo transcription of nearly all genes. Different DNA-protein interactions can have very different sensitivities to the electrolyte concentration, and therefore changes in the electrolyte concentration may have very complex effects on proteins that bind to the

same target site. Because there may be opposing promoters in the *Taq*I-*Taq*I fragment from positions 275 to 625, RNA polymerase (alone or together with transcriptional regulatory proteins) might bind preferentially to the promoter read in the direction opposite to the transcription of the *proU* operon and thereby impair the binding of RNA polymerase to the *proU* promoter. Osmotic control of transcription of the *proU* operon might be the result of the preferential weakening of the binding of RNA polymerase to the anti-*proU* promoter in comparison with the *proU* promoter and thereby result in increased transcription of the *proU* operon in media of high osmolarity. There is no concrete experimental proof that the proposed anti-*proU* promoter is recognized or is subject to osmotic control when present at its normal chromosomal location, and until such evidence is available, the above model should be regarded merely as a starting point for the next set of experiments.

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ADDENDUM IN PROOF

The nucleotide sequence data reported in this paper have been submitted to the EMBL, GenBank, and DDBJ nucleotide sequence data bases and have been assigned the accession number M26063.

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