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A transcriptional silencer downstream of the promoter in the osmotically controlled *proU* operon of *Salmonella typhimurium*

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ABSTRACT **The *proU* operon of *Salmonella typhimurium* is induced by conditions of high osmolality. The cis-acting sequences that mediate osmotic control of transcription were characterized by deletion analysis. The nucleotide sequence between −60 and +274 (relative to the transcription start point) is sufficient for normal osmotic control. Deletions that removed sequences upstream of position +274 but left the promoter intact resulted in greatly increased expression from the *proU* promoter in the absence of osmotic stress. Thus, the transcription control region of the *proU* operon consists of two discrete components: (*i*) the promoter and (*ii*) a negatively acting site that overlaps the coding sequence of the first structural gene of the operon, *proV*. That this negative regulatory element is a transcriptional terminator or mRNA processing site was ruled out. Our results suggest that the negative regulatory element behaves as a transcriptional silencer that inhibits transcription initiation at the *proU* promoter in medium of low osmolality by some action at a distance. We propose several possible mechanisms for the function of this regulatory site.**

Organisms generally respond to increases in the osmolality of their environment by elevating the intracellular concentrations of a few species of low-molecular-weight compounds, which regulate osmolality of the cells (1). In bacteria, proline and glycine betaine are two of the prominent solutes at high concentrations under conditions of osmotic stress. In *Salmonella typhimurium* and *Escherichia coli*, these two compounds are accumulated in medium of high osmolality via two permeases, the constitutive ProP system and the osmotically inducible ProU system (1). The proteins of the latter system are encoded in the *proV*, *proW*, and *proX* genes, which make up the *proU* operon (2, 3). Transcription of this operon is increased >100-fold by conditions of high osmolality (1).

Previously, we found that the *proU* promoter of *S. typhimurium* was expressed at a very high, unregulated level from a fragment that contained 320 nucleotides upstream and 32 nucleotides downstream of the transcription start site (4). This result indicated that the *proU* operon is under negative control that operates at site(s) located outside of this region. Here we show that an important transcriptional regulatory element is located downstream from the *proU* promoter, within the first structural gene of the operon.

MATERIALS AND METHODS

Recombinant DNA Manipulations. Recombinant DNA procedures and nuclease S1 analysis of mRNAs were done as described (4). The low-copy *lac* expression vector pDO182 (Fig. 1) consists of a 4.0-kilobase-pair (kbp) *Pst* I–*Eco*RI fragment from pHJS21 (6), carrying a gene for spectinomycin resistance and the pSC101 origin of replication, an *Eco*RI–*Hin*dIII–*Bam*HI linker (5′-GAATTCCCGAAGCTTCGGGGATCC-3′), and a 6.3-kbp *Bam*HI–*Sal* I fragment from pRS415 (7), which carries the promoterless *lacZYA* operon; the *Pst* I site derived from pHJS21 and the *Sal* I site from pRS415 were converted to blunt ends and ligated together. Plasmid pTAT13 34-1 contains, in the following order: the *tacII* promoter, the *phoA*$^+$ gene, *Xba* I, *Sac* I, *Stu* I, and *Bgl* II cloning sites, and the *lacZ* gene; it is similar to pTAT7 (8), except for the cloning sites between the *phoA* and *lacZ* genes (N. Franklin, personal communication).

According to the usual convention, the nucleotide of the *proU* operon that specifies the start site of the major species of proU mRNA is designated as +1, with nucleotides downstream of this position being denoted by positive numbers and nucleotides upstream denoted by negative numbers. This notation represents a change from our previous numbering (4): the nucleotide position previously designated as 596 is now referred to as +1. This start point for transcription of the *proU* operon agrees well with that determined by others (3, 9, 10). The translation start site of the *proV* gene is at position +65. The cloned *proU* promoter region we used had been derived from an *S. typhimurium* strain carrying the *proU1872*::Mud-*1* insertion (4). Because of the long inverted repeat within the right (*lac*) end of the phage (5), we were unable to unequivocally determine the exact site of the *proU1872*::Mud-*1* insertion. According to our best estimate, this insertion is approximately at position +1170 of the *proU* operon and will be denoted position +1.2 kbp hereafter. All of the *proU* promoter fragments were analyzed on both the pSC101-derived pDO182 and the ColE1-derived pRS415 *lac* expression vectors. We obtained consistent results with the two sets of plasmids and therefore, we present only the data for the low-copy, pDO182-based plasmids.

Details of the construction of the plasmids used for deletion analysis of the *proU* transcriptional control region are as described (11); here we present only the main features of the plasmids. Deletions upstream (5′) or downstream (3′) of the *proU* promoter were generated by exonuclease Bal-31 digestion. The upstream deletions originated from a *Hin*dIII site located at position −595; the ends generated were ligated to *Hin*dIII or *Eco*RI linkers, and the *proU* fragments were placed upstream of the *lacZ* gene in pDO182 (Fig. 1). The downstream deletions originated from an *Sst* II site at position +308; the ends were ligated to *Hin*dIII or *Bam*HI linkers, and the *proU* promoter fragments were inserted into pDO182 (Fig. 2). The precise endpoints of the 5′ and 3′ deletions were established by sequence determination. Plasmid pDO244 carries *proU* sequences from position −320 to position +274, but with a 17-bp *Bam*HI linker (5′-GGGGGATCCGGCGCCCC-3′) inserted into a *Taq* I site, between positions +31 and +32 (Fig. 2).

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Abbreviations: IPTG, isopropyl β-D-thiogalactoside; kilobase pairs (kbp).

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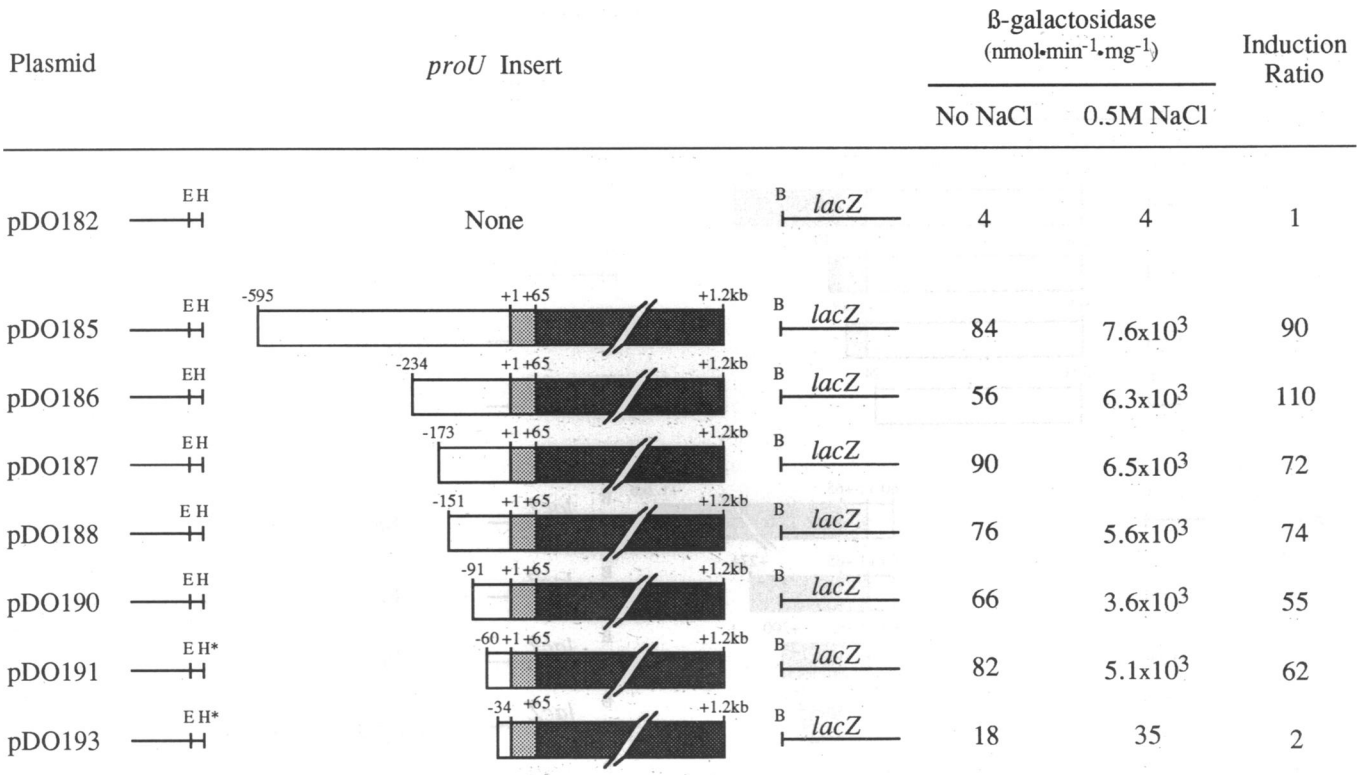

FIG. 1. 5′-Deletion analysis of the *proU* promoter region. Inserts were placed between the *Hin*dIII (H) and *Bam*HI (B) or the *Eco*RI (E) and *Bam*HI (B) sites of pSC101-derived plasmid pDO182. Numbers above boxes refer to nucleotide positions within the *proU* inserts, where +1 and +65 are start sites for transcription and translation, respectively. Except for pDO182, each plasmid carries along with the *proU* insert a 0.18-kbp region from phage Mu*d-1*, fused to the 3′ end of the *proU* insert; this Mu*d-1* fragment contains sequences from the right end of the phage to the first *Pvu* II site (5). The plasmids were transformed into *S. typhimurium* strain TL1463 (*recA1 srl-1*::Tn*10 proU*$^+$), and the β-galactosidase activities were measured in extracts of cells growing exponentially in the indicated media. There is a 4-bp difference between the sequence at the 5′ end of the inserts in plasmids pDO191 and pDO193 and that of the others, indicated by H*. In these two plasmids, the sequence is *Eco*RI-GGAGCTTC-*proU* insert, whereas in the others it is *Eco*RI-CCGAAGCTTC-*proU* insert (see text).

Strains, Media, and Enzyme Assays. Media and growth conditions were described (4). *S. typhimurium* strains carrying pDO182 or pTAT13 34-1-derived plasmids were grown with spectinomycin at 1 mg/ml or ampicillin at 0.1 mg/ml, respectively. For determination of β-galactosidase and alkaline phosphatase activities, the cells were grown overnight in LB medium (4), subcultured at a dilution of 1:100 into the low-osmolal K medium (4) or the high-osmolal K medium containing 0.5 M NaCl and grown overnight with vigorous aeration. The cells were diluted 20-fold into the same respective media and grown to mid-exponential phase (1–3 × 10^8 cells per ml). Assays of β-galactosidase and alkaline phosphatase were done as in refs. 4 and 12, respectively.

RESULTS

The *proU* Transcriptional Control Region Is Between Nucleotide Positions −60 and +274. On plasmid pDO185, which carries *proU* sequences from −595 to +1.2 kbp, the *proU* promoter was induced 90-fold by 0.5 M NaCl (Fig. 1). Nucleotides upstream of position −60 are dispensable for the transcriptional control of the *proU* operon because on plasmids pDO186, pDO187, pDO188, pDO190, and pDO191 the deletion of the sequences between positions −595 and −60, did not substantially affect osmotic induction (Fig. 1). There was a marked decrease in expression of the reporter *lacZ* gene upon deletion of the region normally present upstream of position −34 (plasmid pDO193); this deletion could have removed part of the −35 sequence of the promoter or some other positively acting control site.

The *proU* promoter on a fragment that carries sequences from positions −508 to +308 (pDO178) was induced 130-fold by 0.5 M NaCl (Fig. 2). The main sequences mediating osmotic control are probably within a shorter region because a 62-fold osmotic induction of the *proU* promoter was manifested on a fragment that carries sequences from −60 to +274 (pDO195; Fig. 2), but subordinate negative regulatory site(s) could occur between positions −508 and −61 or between positions +275 and +308.

A Negative Regulatory Site Is Downstream from the *proU* Promoter. Deletion of sequences between positions +90 and +308 (pDO206) or between positions +48 and +308 (pDO207) resulted in a respective 16- and 34-fold increase in transcription of the *proU* promoter in low osmolality, as compared with that seen with control plasmid pDO178 (Fig. 2). A similar trend can be seen with plasmids pDO197 (*proU* positions: −60 to +200), pDO199 (*proU* positions: −60 to +161), pDO201 (*proU* positions: −60 to +117), and pDO202 (*proU* positions: −60 to +95), as compared with pDO195 (*proU* positions: −60 to +274). Furthermore, the basal level of expression of the *proU* promoter on plasmid pDO208 (*proU* insert: positions −320 to +32) was 43-fold higher than on plasmid pDO178 (Fig. 2). These results indicate an important control element for the *proU* operon downstream of its promoter.

The level of transcription from the *proU* promoter in low osmolality increased gradually with the deletion of increased lengths of sequences between positions +274 and +33 (Fig. 2). Thus, the downstream regulatory element is unusual in that it seems to be made up of a number of sequences spread over 100–200 bp, having cumulative effects, rather than of a short, discrete element. Consequently, position +274 may have been chosen somewhat arbitrarily as the upper limit for the 3′ endpoint of the regulatory site. On all of the shortened

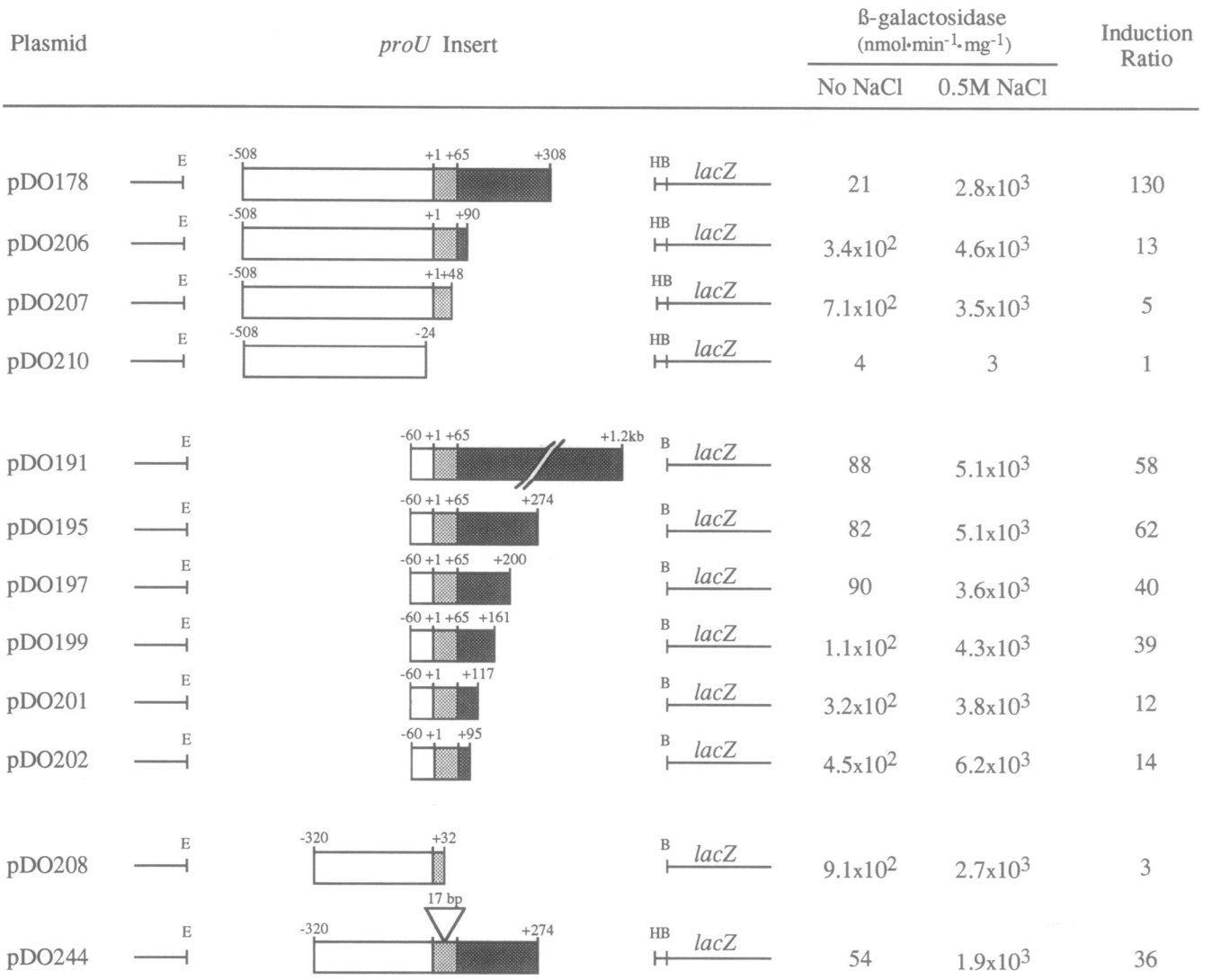

FIG. 2. A negative regulatory site is located downstream of the *proU* promoter. Indicated fragments of the *proU* transcriptional control region were placed between the *Eco*RI (E) and *Hin*dIII (H) or the *Eco*RI and *Bam*HI (B) sites of plasmid pDO182. Numbers above boxes refer to nucleotide positions within the *proU* inserts. Plasmids were introduced into strain TL1463 (*recA1 srl-1*::Tn*10 proU*$^+$), and the β-galactosidase activities were determined for cells grown exponentially in the indicated media.

fragments, including the one carrying positions −320 to +32 (pDO208), the *proU* promoter exhibited a residual induction by high osmolality (Fig. 2). Therefore, other sequences near or upstream of the promoter may participate in the osmotic control.

Conceivably, the deletions that resulted in increased constitutive expression of the *lacZ* gene may have generated or unmasked a new promoter. To test this, we determined by nuclease S1 mapping the transcription start sites of proU mRNAs synthesized *in vivo* from three plasmids carrying *proU* inserts from −508 to +48, from −60 to +117, and from −320 to +32. With all three plasmids, the constitutively synthesized proU mRNAs were initiated at the correct transcription start point of the *proU* operon (data not shown). To test whether the downstream regulatory site in the *proU* operon has a trans-acting function, we introduced plasmids carrying various combinations of the *proU* promoter and the downstream regulatory element into a strain that harbored a chromosomal *proU*::Tn*phoA* fusion. The presence or absence of the downstream regulatory site on the plasmids had no effect on the osmotic control of expression of the chromosomal *proU*::Tn*phoA* fusion (data not shown), indicating that the regulatory element acts only in cis to modulate expression of the *proU* promoter.

A 17-bp Insertion Between the Promoter and the Downstream Regulatory Site Does Not Abolish Osmotic Regulation. Plasmid pDO244 carries a 17-bp insertion between *proU* positions +31 and +32, but nevertheless the *proU* promoter on this plasmid exhibited nearly normal osmotic control (Fig. 2). Therefore, a 17-bp increase in the distance and an ≈220° change in the phasing angle between the promoter and the regulatory site can be tolerated for the osmotic control of *proU* operon expression.

The Downstream Regulatory Site Is Not a General Transcription Terminator. Transcriptional control at sites downstream of the promoter could entail environmentally regulated termination or pausing of transcription or degradation of mRNA. To ascertain whether such mechanisms function in the *proU* operon, we used nuclease S1 to determine whether a promoter-proximal part of the proU mRNA is synthesized constitutively in cells grown in medium of low osmolality. The probe carried *proU* sequences from −57 to +137 and was labeled at the 5′ end of the strand complementary to the proU mRNA; although we could detect a protected fragment of 137 nucleotides in length with RNA isolated from wild-type cells grown under high-osmolality conditions, we were unable to resolve a protected fragment with RNA isolated from cells grown in low osmolality (data not shown). This result, which

agrees with the primer-extension analysis of Stirling *et al.* (10), indicates that not even a 5′ portion of the proU mRNA is synthesized in the low-osmolality medium, and therefore the *proU* operon is probably regulated at the level of transcription initiation. However, it is possible that we were unable to detect a short, terminated proU mRNA because of its rapid turnover.

We conducted a second experiment to test the possibility that the downstream regulatory element in the *proU* operon is an osmotically regulated transcriptional polarity site. We placed this element between the *tac* promoter and the *lacZ* gene on the transcription termination assay vector pTAT13 34-1 and determined whether it would confer osmotic regulation on transcription originating at the *tac* promoter (Fig. 3). In low osmolality without isopropyl β-D-thiogalactoside (IPTG), the β-galactosidase specific activity obtained with plasmid pDO223 (*proU* insert: −60 to +274) was 19-fold lower than with plasmid pDO225 (*proU* insert: −60 to +95). This result, which was obtained with high-copy, ColE1-based plasmids, corroborates our conclusion that there is a negative regulatory element downstream of the *proU* promoter. This regulatory element, however, had <2.5-fold effect on the IPTG-inducible transcription of the *lacZ* gene from the *tac* promoter (Fig. 3; compare the β-galactosidase activity obtained with plasmid pDO225 with that obtained with plasmids pDO223, pDO229, and pDO227 in the medium of low osmolality plus IPTG). Thus, the negative regulatory element does not function as a general transcriptional polarity site.

DISCUSSION

We found a negative regulatory element in the *proU* operon of *S. typhimurium*, downstream of the promoter, between positions +33 and +274. As the translation start site of the *proV* gene is at position +65, osmotic regulation of *proU* operon expression could entail some form of translational control. This hypothesis can be ruled out because increased basal expression of the *proU* operon occurred with deletions that did not excise the translation start site of the *proV* gene and with deletions that removed it. Moreover, the *lacZ* gene on plasmids pDO182 and pTAT13 34-1 has its own translation start site (7, 8) and, at least for pDO182, is preceded by nonsense codons in all three reading frames (7).

Lucht and Bremer (9) reported the isolation of an IS1 insertion into the *proU* promoter of *E. coli* that generated a new promoter with a −35 sequence from the IS1 and the −10 sequence of *proU*. Although the hybrid promoter was expressed at a much lower level than the *proU* promoter, it still responded to osmotic control with almost normal induction ratio. This result supports our inference that the osmotic control of the *proU* operon is mediated at sites downstream of the promoter and, combined with our observations, suggests that the −35 element of the *proU* promoter and sequences upstream from it are not crucial for proper osmotic regulation.

Genetic control at the mRNA level could be exerted at four points: (*i*) initiation, (*ii*) elongation, (*iii*) termination of transcription, or (*iv*) mRNA degradation. That the downstream regulatory element did not diminish transcriptional efficiency of the *tac* promoter (Fig. 3) indicates that the downstream regulatory element is not a generalized transcription termination or pausing site or the target for mRNA degradation. However, sequences adjacent to promoters can modulate the ability of RNA polymerase to read-through various terminators (13–15), and therefore the downstream regulatory element might be an osmotically controlled transcriptional terminator that is recognized only by transcription complexes emanating from the *proU* promoter. Although we cannot completely rule out this possibility, the failure to detect any synthesis of the 5′ end of the proU mRNA in cells grown in low osmolality by S1 nuclease mapping (see above) and primer extension (10) provides some evidence against it. Furthermore, there is no apparent terminator structure in the +1 to +274 region of the *proU* operon. For these reasons, it

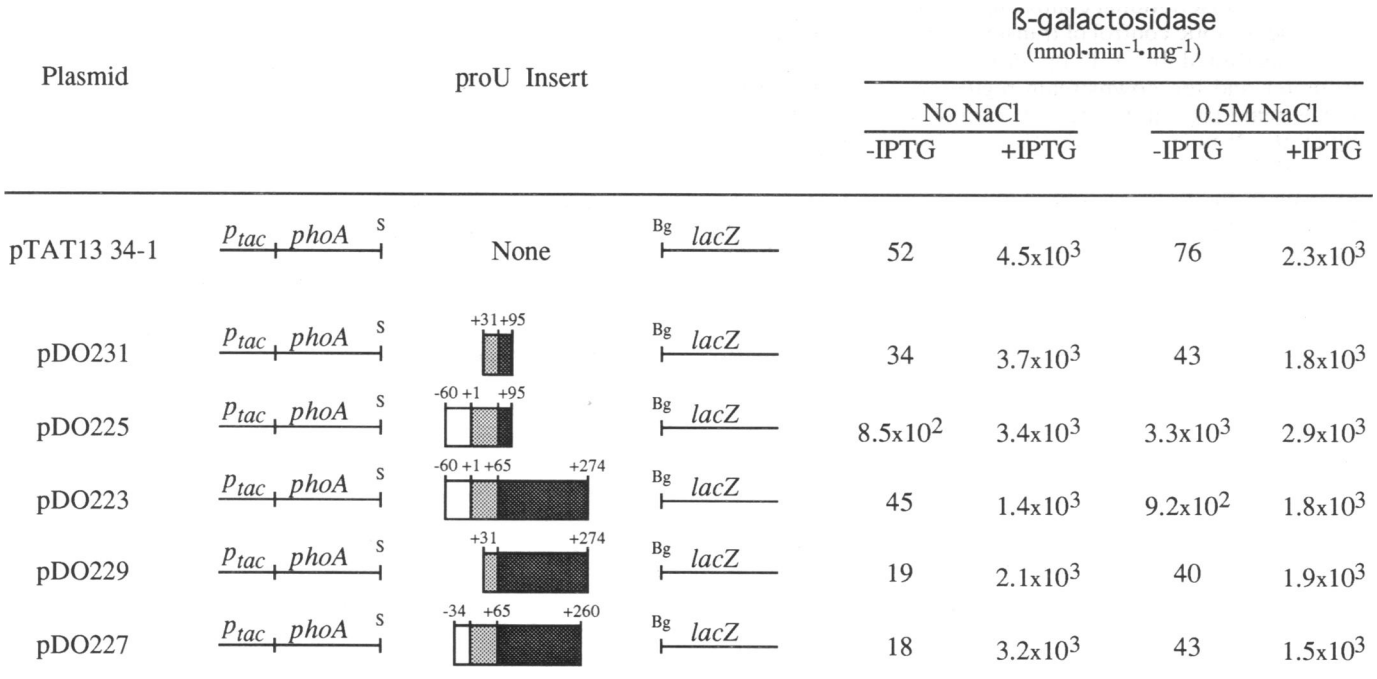

FIG. 3. The downstream regulatory region is not a generalized transcription terminator. Fragments carrying the indicated sequences of the *proU* operon were cloned between the *Sst* I (S) and *Bgl* II (Bg) sites of the transcription-terminator assay vector pTAT13 34-1. The plasmids were introduced into *S. typhimurium* strain TL1596 (F′$_{128}$*zzf*::Tn*10 lacZ ΔM15 lacI*^{q}*/recA1 proU*$^{+}$), and the β-galactosidase and alkaline phosphatase activities were determined in cells growing exponentially in the indicated media. When used, IPTG was 1 mM. Alkaline phosphatase activities (nmol·min^{-1}·mg of protein^{-1}) for strains carrying plasmids pTAT13 34-1, pDO231, pDO225, pDO223, pDO229, and pDO227 were, respectively, in K medium: 10, 11, 11, 10, 10, and 9; in K medium plus IPTG: 490, 760, 790, 760, 790, and 680; in K medium plus 0.5 M NaCl: 12, 11, 16, 13, 11, and 11; and in K medium plus IPTG and 0.5 M NaCl: 350, 440, 490, 440, 410, and 350.

is more likely that the downstream regulatory site is a transcriptional silencer that controls transcription initiation by some action at a distance. Examples for this mode of transcriptional regulation exist in both eukaryotes (16–18) and prokaryotes (19–21). The *gal* operon of *E. coli* is transcribed from two promoters which are flanked by a pair of binding sites for the repressor proteins (19). Repressor protein complexes bound to these sites also bind each other and form a DNA loop, which blocks productive transcription by some unknown mechanism. Every bacterial operon that has a negatively acting site downstream of the promoter thus far described also contains a second binding site upstream of the promoter, with the repression being effected by a DNA loop formed by multimers of a single species of protein (21). However, the −60 to +274 nucleotide region of the *proU* operon does not contain an obvious tandemly repeated sequence of sufficient length to suggest this type of mechanism for the *proU* operon. It is possible though, that a DNA loop could be held together by heterologous proteins bound to two different sequences or by a single protein that can bind to two different sequences on the DNA, as seen with the Int protein of phage λ (22).

However, other repression mechanisms could be envisioned in which the regulatory protein binds to only one site on the DNA and transmits the regulatory signal to the promoter by some means other than a DNA loop. The regulatory site downstream of the promoter could be the binding site for a protein that influences some three-dimensional feature of the promoter, such as supercoiling or conversion to the Z-DNA structure. In this model, the *proU* promoter would be inherently a strong promoter, but in medium of low osmolality it would be incapacitated because of the action of a protein at the downstream regulatory site. It is also conceivable that the downstream regulatory site could be the binding site for a scaffold protein that under conditions of low osmolality wraps neighboring DNA into a nucleosome-like structure and buries the promoter, as proposed for transcriptional control by chromatin in eukaryotes (23). The osmotic control of transcription of the *proU* operon is not disrupted by a 17-bp insertion between the *proU* promoter and the downstream regulatory element, but the downstream regulatory element cannot act on the *tac* promoter at a distance of 1.8 kbp (Fig. 3). The pleiotropic OsmZ (Hns) DNA-binding protein, which is required for full repression of the *proU* operon in low osmolality (24), may be part of the regulatory apparatus that acts at the downstream regulatory site. To distinguish among possible models, it will be important to determine whether there are any constraints on the relative order, distance, or phasing of the *proU* promoter and the downstream element for proper osmotic control of transcription. Also, it will be important to identify the regulatory protein(s) acting at the downstream regulatory site.

Note. During the review of this manuscript, Dattanada *et al.* (25) also reported a negative regulatory site within the transcribed region of the *proU* operon of *E. coli*.

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