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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

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Transcription of the *proU* operon in *Escherichia coli* is induced several hundredfold upon growth of cells in media of elevated osmolarity. A low-copy-number promoter-cloning plasmid vector, with *lacZ* as the reporter gene, was used for assaying the osmoresponsive promoter activity of each of various lengths of *proU* DNA, generated by cloning of discrete restriction fragments and by an exonuclease III-mediated deletion approach. The results indicate that expression of *proU* in *E. coli* is directed from two promoters, one (P2) characterized earlier by other workers with the start site of transcription 60 nucleotides upstream of the initiation codon of the first structural gene (*proV*), and the other (P1) situated 250 nucleotides upstream of *proV*. Furthermore, a region of DNA within *proV* was shown to be involved in negative regulation of *proU* transcription; phage Mu dII1681-generated *lac* fusions in the early region of *proV* also exhibited partial derepression of *proU* regulation, in comparison with fusions further downstream in the operon. Sequences around promoter P1, sequences around P2, and the promoter-downstream negative regulatory element, respectively, conferred approximately 5-, 8-, and 25-fold osmoresponsivity on *proU* expression. Within the region genetically defined to encode the negative regulatory element, there is a 116-nucleotide stretch that is absolutely conserved between the *proU* operons of *E. coli* and *Salmonella typhimurium* and has the capability of exhibiting alternative secondary structure. Insertion of this region of DNA into each of two different plasmid vectors was associated with a marked reduction in the mean topological linking number in plasmid molecules isolated from cultures grown in high-osmolarity medium. We propose that this region of DNA undergoes reversible transition to an underwound DNA conformation under high-osmolarity growth conditions and that this transition mediates its regulatory effect on *proU* expression.

The growth rate of *Escherichia coli* and *Salmonella typhimurium* in high-osmolarity media is promoted by the addition of small concentrations of L-proline or glycine betaine to the culture medium. The osmoprotective effect of both of these compounds is presumed to be consequent upon their intracellular accumulation under conditions of water stress and is in part dependent on the presence of a functional ProU transporter in these cells, encoded by genes of the *proU* locus (for a review, see reference 6).

Complementation studies using a number of *proU* mutants, in combination with nucleotide sequence analysis of the locus, have shown that *proU* is an operon composed of three structural genes, *proV*, *proW*, and *proX* (7, 13). The product of *proX* is a periplasmic protein which has been purified and shown to be a glycine betaine-binding protein in vitro; furthermore, the deduced amino acid sequences of the products of *proV* and *proW* each show similarities to components of other well-characterized transport systems such as those for histidine, maltose, or arabinose, thus permitting the inference that ProU is a member of the broad family of multicomponent ATP-driven binding-protein-dependent substrate transporters in the enterobacteria (6, 13).

Transcription of *proU* is increased several hundredfold in high-osmolarity growth media (6); such a magnitude of induction is the highest known for an osmoresponsive locus in any biological system. The mechanism of osmotic induction of *proU* is still not completely understood. No mutant has been isolated in which osmoresponsivity of *proU* expression is completely abolished by a *trans*-acting mutation. Higgins and coworkers have shown (i) that the expression of *proU* during growth at elevated osmolarity is correlated with increased supercoiling of reporter plasmid DNA (18) and (ii) that each of a variety of environmental perturbations or mutations simultaneously affects both *proU* osmoresponsivity and supercoiling of reporter plasmid DNA (17, 18). On the basis of these data, they have suggested that changes in DNA supercoiling play a primary, and perhaps direct, role in the osmotic induction of *proU*. Villarejo and colleagues, however, have disputed this conclusion (37); they instead postulate, on the basis of in vitro expression experiments (35–37), a key role for potassium glutamate in directly activating the transcription of *proU* by σ^{70}-RNA polymerase holoenzyme in cells grown at elevated osmolarity. Their proposal is supported by results of earlier work indicating that intracellular accumulation of potassium glutamate occurs upon growth in high-osmolarity media (10, 38, 42) and that *proU* induction in vivo is abrogated under K^+-limiting conditions (42). Jovanovich et al. (24) have also reported stimulation by potassium glutamate of *proU* expression in vitro, but their conclusions differ from those of Villarejo et al. in that they additionally implicate a nondialyzable *trans*-acting factor in mediating osmotic induction.

In this study, we looked at the role of sequences in the vicinity of the *E. coli proU* promoter on osmotic regulation of transcription. We showed earlier that all of the information needed for normal regulation of *proU* is contained

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TABLE 1. *E. coli* K-12 strains

Strain	Genotype[a]	Reference
W3110	F⁻ IN(*rrnD-rrnE*)*1*	Laboratory stock
DH5α	F⁻ Δ(*argF-lac*)*U169 supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1* (φ80d*lacZ*Δ*M15*)	39
POII1681	F⁻ *araD ara-3*::Mu cts *rpsL* (Mu dII1681)	4
MC4100	F⁻ Δ(*argF-lac*)*U169 rpsL150 relA araD139 flbB5301 deoC1 ptsF25*	12
GJ314	MC4100 Δ*putPA101 proP221 proX224*::*lac recA* Δ(*pyr-76*::Tn*10*)*462* Δ(*srl*::Tn*10*)*461*	14
GJ316	MC4100 Δ*putPA101 proP222* Δ*proU233* Δ(*pyr-76*::Tn*10*) *recA srl*::Tn*10*	14
GJ1330	MC4100 *osmZ zch-900*::Tn*10*dKan	23

[a] The nomenclature for genetic symbols follows that described by Bachmann (3).

downstream of an *Eco*RV site in the locus, within the region whose nucleotide sequence was determined (7, 13). One osmotically regulated *proU* promoter, with a start site of transcription 60 nucleotides upstream of the initiation codon of *proV*, has been identified in both *E. coli* and *S. typhimurium* (13, 26, 29, 33, 41). Primer extension analysis of *proU* mRNA had also indicated the existence of osmotically regulated transcripts extending as much as 250 nucleotides upstream of the *proV* gene (13), but no substantiating evidence for a promoter so far upstream has been reported. The results of the present study indicate that there indeed are two independent osmotically regulated promoters in this region, whose relative positions are consistent with earlier data from 5′-end mapping of mRNA molecules (13), and furthermore that sequences downstream of the two promoters, overlapping the early part of the *proV* structural gene, are involved in the negative control of osmoresponsive expression of the *proU* operon.

MATERIALS AND METHODS

Media and chemicals. The growth media used included LB (32), minimal A (32), and the low-osmolarity K medium described earlier (12). LBON medium was prepared by the omission of NaCl from LB medium.

All chemicals, antibiotics, and reagents for recombinant DNA work were obtained from commercial sources. The final concentrations of antibiotics used in growth medium were (in micrograms per milliliter): ampicillin, 50; kanamycin, 10 in LBON; chloramphenicol, 25; and trimethoprim, 20 in minimal A and 75 in rich media. Isopropyl-β-D-thiogalactoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) were used at final concentrations of 0.5 mM and 25 μg/ml, respectively.

Bacterial strains. All strains used in this study are derivatives of *E. coli* K-12 and are listed in Table 1.

Nucleotide number designations in the *proU* locus. The frame of reference for all nucleotide position numbers at the *proU* locus in this report is the first base of the *Eco*RV site used to determine the sequence of a 4-kb stretch of the *E. coli proU* operon (13). In this numbering scheme, the start site of translation of the first structural gene, *proU*, is at position 688. Instances in which positions have been determined only by restriction endonuclease analysis are indicated by an approximation symbol (~) preceding the number designation.

Plasmids. The multicopy plasmid vectors pBR322, pACYC184, pUC18, pBluescript SK, and pBluescript KS have been described previously (5, 39). Plasmid pMU575, obtained from A. Andrews, is a low-copy-number IncW-derived promoter-cloning vector that encodes trimethoprim resistance and employs *lacZ* as the reporter gene (47). Also used in this study were the following plasmids carrying cloned regions of *proU* that had been constructed earlier: pHYD58, a pBR322-derived plasmid carrying *proU* DNA from a *Bgl*II site at position approximately −550 at one end to position 4361, downstream of the last gene in the operon, at the other (13, 14); pHYD94, a pACYC184-derived plasmid carrying *proU* DNA between positions 1804 and 4361 and also the temperature-sensitive repressor gene of phage Mu on a 1-kb fragment derived from the Mu *c* end (14); and the M13 clones E5.8 and RE14.3, obtained by exonuclease III deletion in the project on *proU* sequencing, which carry *proU* DNA from positions 658 to 3777 and from positions 1 to 577, respectively (13). Other plasmids were constructed in this study as follows.

(i) pHYD215 and pHYD230. The region of *proU* DNA between position 658 and the *Hin*cII site at 1198 was introduced from the M13 clone E5.8 into the polycloning site of each of the vectors pUC18 and pBluescript SK to generate plasmids pHYD215 and pHYD230, respectively (details of construction steps not shown). The orientations of the insert DNA in pHYD215 and pHYD230 are such that the end proximal to the *lac* promoter of the vector corresponds to *proU* position 658 in the former and to position 1198 in the latter.

(ii) pHYD220. Plasmid pHYD220 was constructed in two steps from the pBR322-derived plasmid pEA305 obtained from M. Ptashne (1). In the first step, the *proU* 1198–658 region from E5.8 was cloned into the *Hin*dIII-*Eco*RV sites downstream of the *tac* promoter in pEA305 (such that the *proU* end at 1198 is proximal to *tac*). Into the *Eco*RI site of the resultant plasmid (pHYD219) was subsequently cloned the *lacI* gene, isolated on an *Eco*RI fragment from plasmid ptac-85 (27); derivatives of pHYD219 carrying the *lacI* gene were identified in the cloning experiment after transformation into W3110 by their ability to prevent repressor titration and constitutive overexpression of the chromosomal *lac* operon in this strain. One such plasmid derivative was designated pHYD220.

Recombinant DNA techniques. The procedures described by Sambrook et al. (39) were followed in work relating to preparation of plasmid DNA, electrophoresis on agarose and polyacrylamide gels, in vitro DNA manipulations, and transformation.

Mu dII1681 transpositions in pHYD58. Transpositions into pHYD58 of Mu dII1681, a mini-Mu phage derivative which encodes Kan^r and generates *lac* gene fusions upon insertion, were generated by temperature induction of POII1681/pHYD58 as described previously (4). The resulting Mu phage lysate was used to infect MC4100/pHYD94, and selection was made for Amp^r Kan^r clones on LBON–X-Gal medium at 30°C. All pHYD58 molecules transferred by this procedure would be expected to carry Mu dII1681 transpositions; the resident Mu cts gene on pHYD94 in the recipient strain enabled a higher frequency of recovery of transductant clones (4).

***proU* promoter cloning in pMU575 and deletion mutagenesis.** The ends of the *Sal*I fragment of pHYD58 containing the region of *proU* from ~−550 to 1809 (along with a small region of vector pBR322 DNA upstream of the *proU* segment [Fig. 1]) were converted to *Hin*dIII ends by linker addition according to standard procedures (39); the fragment was then cloned into the *Hin*dIII site in the polycloning site upstream of the *lacZ* reporter gene in pMU575 to generate plasmid pHYD251. Progressive deletions into the *proU* locus were then generated from the upstream *proU* end in pHYD251 by the exonuclease III method of Henikoff (16); the enzymes *Bam*HI and *Pst*I were used respectively to open the upstream *proU* end for and to protect the vector end from exonuclease III attack. One plasmid obtained from this procedure with the *proU* upstream end at position 376, pHYD270, was subjected to a second round of similar deletion mutagenesis, this time from the *proU* downstream end at 1809; the enzymes *Xba*I and *Kpn*I were used for opening and for protection, respectively. By this procedure, plasmids pHYD272 to -275 were obtained. In all deletion clones, the size of the *proU* insert region remaining in pMU575 was determined by *Sst*I digestion, since recognition sites for this enzyme exist in the vector immediately flanking either end of the insert.

Other pMU575 derivatives carrying various regions of *proU* were constructed as follows. Plasmid pHYD276, which carries the region of *proU* between position 376 and the *Hin*cII site at 1198, was constructed by digestion of pHYD270 with *Sst*I (which cuts the plasmid in the vector region flanking the *proU* deletion endpoint at 376) and *Hin*cII, purifying the relevant fragment, and cloning it after *Hin*dIII linker addition into the *Hin*dIII site of pMU575. The *proU* promoter region from each of the *proV*::Mu dII1681 insertion derivatives of pHYD58 was cloned after digestion of the latter with *Sal*I (which cuts in pBR322 beyond the upstream end of *proU* at ~−550) and *Bam*HI (which cuts immediately upstream of the lacZ reporter gene within the transposon [4]) and inserting the fragment so released into the *Sal*I-*Bam*HI sites of pMU575.

Nucleotide sequence determination. All nucleotide sequences were determined on double-stranded plasmid DNA substrates by the method of primer-directed [^{32}P]DNA synthesis and chain termination with dideoxynucleotides (39). The sequence across each of the *proV*::Mu insertion junctions was determined by using the commercial 15-mer M13 sequencing primer, which primes DNA synthesis from within the *lac* sequence in Mu dII1681 across 116 bases of the Mu *S* end into the *proV* gene (4). The precise endpoints for various deletions generated in the pMU575 derivatives were determined after cloning the *Sst*I fragment carrying the insert from each of them into the high-copy-number plasmid vector pBluescript KS and by using commercially available primers for the sequencing of fragments cloned into this vector.

Primer extension analysis. To map the promoter in an upstream *proU* fragment, primer extension analysis on total cellular RNA was done as described earlier, making use of a radiolabelled single-stranded DNA primer with its 3′ end corresponding to the *Taq*I site at position 513 in the bottom strand of the *proU* sequence, which had been prepared from the M13 phage clone RE14.3 (13). After hybridization with RNA and extension with reverse transcriptase, the primer and products were separated on a sequencing gel and sized by comparison against the sequence ladder of RE14.3 itself.

Plasmid DNA supercoiling measurements. Plasmid-bearing derivatives of DH5α were grown to mid-exponential phase in media with appropriate antibiotic selection. Plasmid DNA was obtained from cultures by the method of alkaline lysis followed by phenol-chloroform extraction, as described for small-scale preparations by Sambrook et al. (39). Treatment with lysozyme was omitted in the lysis protocol to avoid the problem of topoisomer relaxation induced in spheroplasts (15, 40). Plasmid topoisomers were separated by electrophoresis on chloroquine-agarose gels (18); the gels were then washed free of chloroquine by soaking in water, and DNA bands were visualized with UV transillumination after staining with ethidium bromide. The concentration of DNA in each band was determined by densitometric analysis of photographic negative film of the stained gel.

Other methods. The possibility of occurrence of alternative secondary structure in a given nucleotide sequence was examined with the aid of the program of Jacobson et al. (21) for prediction of secondary structure in RNA.

The specific activity of β-galactosidase in exponential-phase cultures grown at 30°C was measured by the method of Miller (32), and the values were calculated in terms of Miller units described therein. Unless otherwise specified, expression of β-galactosidase from various plasmids was assayed in strain DH5α, and each of the values reported represents the average of at least five independent determinations. It was found that plasmid pMU575 itself expresses β-galactosidase to an activity of approximately 3 U, and all values for promoter constructs in this vector have been corrected for this background level of expression.

RESULTS

Mu dII1681 insertions in the early region of *proV*. In an earlier study on complementation analysis of the *proU* locus wherein a large number of *proU*::*lac* mutants had been isolated and characterized (7), relatively few insertions were obtained in the first structural gene, *proV*, compared with the numbers in *proW* or *proX*. We therefore undertook another attempt at generating *proU*::*lac* insertions, with the aim of identifying some more that had occurred in *proV*.

The method made use of the mini-Mu *lac* transposon Mu dII1681, which generates gene fusions upon insertion in the correct orientation and reading frame into a target gene. Transpositions were generated on the *proU*$^+$ plasmid pHYD58, and those that were Lac$^+$ were screened by restriction mapping to identify clones that had insertions in the *proV* gene.

Four *proV*::*lac* alleles were characterized in detail. Nucleotide sequence determination across each of the fusion sites indicated that the insertions had occurred at positions 751, 883, 1000, and 1582, respectively, in the *proU* sequence (the fusions were assigned *proV* allele numbers *242* to *245*, respectively); in each case, the orientation and reading frame of the insertion were such that an authentic ProV–β-galactosidase hybrid protein would be produced from the gene fusion. Indeed, a protein band of the size expected for the fusion protein was seen in the extracts from each of these strains after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown).

The expression of *lac* from each of the fusion plasmids was assayed after growth of the corresponding DH5α derivatives in LBON medium and in LBON supplemented with 0.4 M NaCl (Table 2). The fusion at 1582 showed normal regulation of expression (similar to the pattern seen with fusions in *proW* and *proX* studied earlier [7]), whereas the fusion at 1000 exhibited a 3-fold increase and those at positions 883 and 751 exhibited a 50-fold increase in expres-

TABLE 2. β-Galactosidase levels from *proV*::*lac* fusions on multicopy plasmids

proV::*lac* allele no.	Fusion site[a]	β-Galactosidase sp act (U)[b] for fusion on:		
		pHYD58 (LBON)	pHYD58 (LBON + NaCl)	pACYC184 (LBON)
242	751	2,080	11,960	1,902
243	883	1,725	10,830	1,780
244	1,000	82	11,540	162
245	1,582	33	11,760	62

[a] Nucleotide position in *proU* of the Mu dII1681 insertion.
[b] Derivatives of DH5α carrying the various *proV*::*lac* derivatives of pHYD58 or pACYC184 were grown at 30°C to early log phase in ampicillin- or chloramphenicol-supplemented LBON medium as appropriate, and β-galactosidase activities were measured immediately before (LBON) or 3 h after addition of NaCl to 0.4 M and continued incubation at 30°C (LBON + NaCl).

sion in the low-osmolarity medium, although they all retained at least some degree of osmoresponsivity. The exact magnitude of osmotic inducibility could not be ascertained because of problems of growth inhibition and plasmid instability associated with *proU-lac* overexpression from multicopy plasmids (7, 29).

Each of the four *proV-lac* fusions was also cloned on a *Sal*I fragment (which included DNA from the upstream *proU* end at ~−550 to a site downstream of the *lacA* gene in Mu dII1681) into the *Sal*I site of the vector pACYC184, in the orientation such that the direction of transcription of *lac* on the fragment is opposite to that of *tet* in the vector. DH5α transformants of these pACYC184 derivatives exhibited a pattern of *lac* expression in low-osmolarity medium similar to that observed with the corresponding pHYD58 *proV*::*lac* derivatives (Table 2). These results provided the first suggestion that sequences within the region of *proU* that is both transcribed and translated might be involved in negative regulation of expression of the operon.

Does the downstream regulatory sequence encode a repressor for *proU*? In addition to serving as part of the *proV* structural gene, the region implicated above in the negative control of *proU* expression carries a short open reading frame on the opposite (that is, bottom) DNA strand which could conceivably encode a 70-residue peptide (beginning with a GTG codon complementary to base positions 929 to 927 on the top strand and terminating at a TAA stretch complementary to bases 719 to 717 [13]); this putative peptide has five S(T)PXX stretches in its sequence, a motif that has earlier been described to be especially prevalent in DNA-binding proteins (43). We considered the possibility that this region of DNA encodes a repressor protein involved in osmotic regulation of *proU* expression, because such an explanation would also account for (i) the failure of earlier attempts to identify a regulatory gene unlinked to *proU* (6, 26) and (ii) the observation that the presence of *proU* on a multicopy plasmid does not lead to titration effects on regulation of the operon (14, 41).

The following experiments, however, exclude the hypothesis that this regulatory region encodes a *trans*-acting repressor product. (i) Digestion of the *proV242*::Mu dII1681 derivative of pHYD58 with *Bam*HI and *Hin*dIII and recircularization (following filling in of the ends) yielded a plasmid that carries only the *proU* promoter region without any of the downstream *proU* sequences beyond position 751 or any of the *lacZYA* genes. When the latter plasmid was introduced into the chromosomal *proX-lac* fusion strain, GJ314, there was no derepression of *lac* expression, indicating that there is no titration effect even when the putative regulatory gene is present only in a single chromosomal copy in the strain (data not shown). (ii) Each of plasmids pHYD215 and pHYD230, bearing the cloned region of *proU* between 658 and 1198 (encompassing the regulatory region but excluding the promoter), was introduced into a strain with the pACYC184-derived plasmid derivative that carried the *proV242*::*lac* insertion at position 751; no effect in *trans* of pHYD215 or of pHYD230 on the derepressed level of *proV-lac* expression was observed (data not shown). In another experiment, plasmid pHYD58 was shown not to affect *lac* expression from *proV242*::*lac* on pACYC184, excluding the possibility that it is the N-terminal part of ProV which negatively regulates *proU* transcription.

We also tested the possibility that the downstream regulatory region encodes a repressor protein that is preferentially *cis* acting, as has been described in other systems (31). Several plasmids were constructed in which the *proV242*::*lac* fusion was cloned in *cis* to the region encoding the potential regulatory peptide, but there was little effect on *lac* regulation in any of them (data not shown).

In a definitive test of the repressor gene hypothesis, plasmid pHYD220, carrying both the *proU* 1198–658 region downstream of the *tac* promoter and also the *lacI* gene, was constructed as described above. IPTG-mediated induction of transcription from *tac* of the putative repressor gene on pHYD220 was shown to have no effect in *trans* on the derepressed expression of *proV242*::*lac* carried on a pACYC184 vector in the same strain; even when the *lac* fusion allele was cloned in *cis* into the *Bam*HI site on pHYD220 (0.2 kb downstream of the *proU* 1198–658 region), no effect of IPTG on the regulation of *proV*::*lac* expression was observed (data not shown).

Deletion analysis of *proU* regulatory region cloned in pMU575. We also undertook a study of the transcriptional regulatory region of *proU* after cloning it into the plasmid vector pMU575. This plasmid is a promoter-cloning vector containing *lacZ* as the assayable reporter gene (47); it was expected that the low copy number (one to two per cell) in which this plasmid is maintained would both avoid possible artifactual alterations of regulation associated with multicopy plasmid studies and also permit steady-state β-galactosidase measurements in media of low and of high osmolarity.

The *proU* transcriptional regulatory region extending from ~−550 to 1809 was cloned into pMU575 to generate plasmid pHYD251. DH5α derivatives carrying pHYD251 exhibited a 230-fold inducibility of β-galactosidase expression after growth in medium supplemented with 0.3 M NaCl (Fig. 1) comparable with that observed earlier for chromosomal *proU-lac* strains. Plasmid derivatives with different extents of deletions in the *proU* insert region of pHYD251 were then obtained as described above; these included plasmids pHYD270 and pHYD272 through -276 (Fig. 1).

In a related set of experiments, the series of fragments with left endpoints at ~−550 and right endpoints at 1582, 1000, 883, and 751, respectively, were cloned into pMU575 from the corresponding pHYD58 *proV*::Mu dII1681 insertion derivatives, to generate plasmids pHYD280 to -283. Furthermore, a *Sau*3A1 digest of the insert from the last of these was used to clone the *proU* fragment extending from positions 420 to 751 into pMU575 (pHYD284).

The extent of *proU* locus carried in each of these promoter constructs is depicted in Fig. 1. Also shown for each of the plasmids are the steady-state β-galactosidase activities after growth of the corresponding strains for several generations

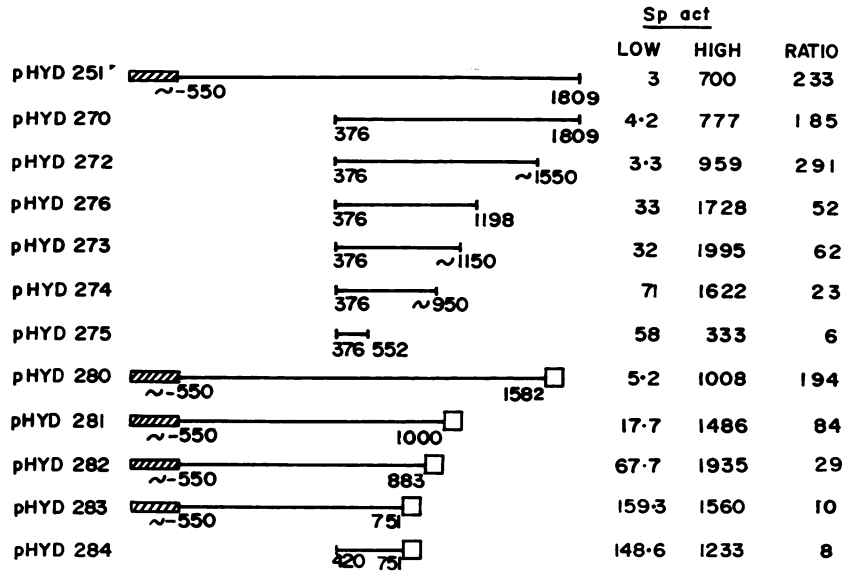

FIG. 1. Extent of *proU* DNA carried on various pMU575 derivatives and correlation with magnitude of osmoresponsivity of *lac* expression. Plasmid designations are given on the left, and for each the extent and composition of DNA inserted upstream of *lac* is shown to scale, oriented such that transcription proceeds from left to right. Representation of DNA from various sources is as follows: *proU*, thin lines; pBR322 (between its *SalI* and *Bam*HI sites), hatched bar; and Mu *S* end, open box. The nucleotide positions for the ends of *proU* DNA on each plasmid are marked. The columns on the right indicate the β-galactosidase activity for each plasmid after growth of the corresponding DH5α derivative at 30°C for several generations in K medium (LOW) or in K medium supplemented with 0.3 M NaCl (HIGH) and the calculated magnitude of osmotic inducibility (RATIO).

in low-osmolarity K medium and in K medium supplemented with 0.3 M NaCl. The results are somewhat complex to interpret, but the following conclusions may be drawn:

(i) Plasmid pHYD275, carrying the region of *proU* from 376 to 552, does express the gene for β-galactosidase, and enzyme specific activity is increased sixfold at high osmolarity. Therefore, there is an osmotically regulated promoter (designated P1) in this segment, which is different from the one (designated P2) with the transcription start site at 628 characterized earlier (13, 26, 29).

(ii) The smallest insert obtained in this study that shows completely normal osmotic inducibility is that in plasmid pHYD272, extending from positions 376 to approximately 1550. Sequences upstream of position 376 do not appear to influence *proU* regulation.

(iii) As the position of the right end of the insert is progressively reduced below 1500, there is an increasing degree of abnormality of *proU* regulation, manifesting itself mainly as an elevation of β-galactosidase activity in low-osmolarity growth medium; this finding is consistent with evidence for downstream sequence-mediated negative regulation obtained from the experiments described above with *proV*::Mu dII1681 fusions on multicopy plasmids. Three categories of abnormal expression, which correlated roughly with the positions of deletion endpoints at the right end, could be distinguished: there was a 4-fold derepression in plasmids with right endpoints between approximately 1000 and 1200 (pHYD276, pHYD273, and pHYD281), 10-fold derepression in those with endpoints around 900 (pHYD274 and pHYD282), and 25-fold derepression in those with right endpoints at position 751 (pHYD283 and pHYD284).

(iv) Plasmid pHYD284, as discussed below, is expected to carry only promoter P2 and adjacent sequences (without either P1 or the downstream regulatory element) and shows a residual eightfold osmotic inducibility of *lac* expression.

Identification of the start site of promoter P1 transcription. To determine the start site of transcripts initiated from the *proU* 376–552 fragment in pHYD275, total RNA was isolated from a pHYD275 derivative of GJ316 grown in LB medium supplemented with 0.2 M NaCl, and the 5′ ends of *proU* mRNA present in the preparation were mapped by primer extension analysis. GJ316 is a Δ*proU* mutant, and therefore only transcripts expressed from the P1 promoter on the plasmid were expected to be identified in the experiment. The method followed was the same as that reported earlier by us in mapping 5′ ends of mRNA from this upstream segment of the intact *proU* regulatory region (13). The most prominent, as also the longest, extension product observed after hybridization with RNA from GJ316/pHYD275 was that corresponding to a 5′ end at position 438 (Fig. 2); we conclude that this represents the transcription initiation site of the P1 promoter. The present result is in agreement with that from our earlier primer extension experiments (13), in which we had also shown that this population of mRNA molecules is more abundant in cultures grown at elevated osmolarity than at low osmolarity.

Effect of an *osmZ* mutation on *lac* expression from different *proU* deletion constructs. Mutations in the *osmZ* locus of *E. coli* and *S. typhimurium* have pleiotropic effects, including an increase in the expression of *proU* at low osmolarity (18, 26); *osmZ* has subsequently been shown to encode the histonelike DNA-binding protein H1 or H-NS (11, 20, 25, 28). The purified H-NS protein has also been shown to bind bent DNA sequences with very high affinity in vitro (44), including the bent-DNA motif previously identified in the region between nucleotide positions 390 and 510 in *proU* (13, 44). To test the effect of *osmZ* on *lac* expression from the various *proU* deletion constructs in pMU575, we transformed each of the plasmids into MC4100 and into its isogenic *osmZ* derivative, GJ1330, and assayed β-galactosi-

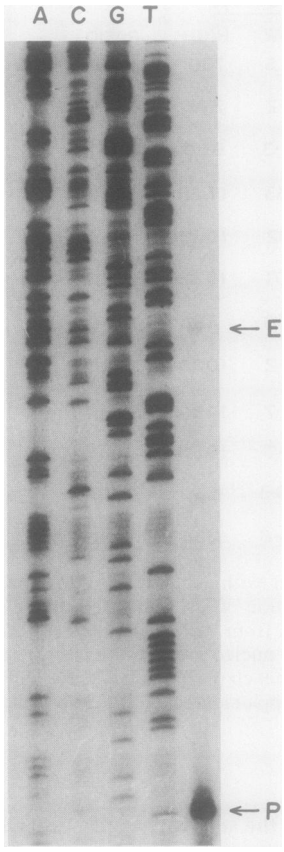

FIG. 2. Results of primer extension analysis on RNA from GJ316/pHYD275, using primer prepared from RE14.3. Lanes A, C, G, and T represent the sequence ladder of RE14.3, run as markers on the gel. On the test lane, the bands corresponding to the primer (P) and its extension product (E) are indicated; these comigrate with the RE14.3 bands, T at position 513 and C at position 438, respectively, on the bottom strand of the *proU* sequence.

dase activity after growth in K medium and in K medium supplemented with 0.3 M NaCl (Table 3). The data for the assays done in low-osmolarity-grown cultures indicate that (i) there was the expected 8-fold derepression of expression for the wild-type *proU-lac* plasmid, pHYD270, in the *osmZ*

TABLE 3. β-Galactosidase activity expressed from pHYD270 and deletion derivatives in *osmZ*$^+$ and *osmZ* strains[a]

Plasmid	Extent of *proU* sequence	β-Galactosidase sp act (U) in K medium supplemented with:			
		No NaCl		0.3 M NaCl	
		osmZ$^+$	*osmZ*	*osmZ*$^+$	*osmZ*
pHYD270	376 to 1809	8.9	70.4	495	652
pHYD273	376 to ~1150	84.0	253	572	ND[b]
pHYD274	376 to ~950	81.2	221	627	714
pHYD283	~−550 to 751	172	275	877	2,080
pHYD284	420 to 751	356	469	587	1,071
pHYD275	376 to 552	134	30.6	639	195

[a] Derivatives of MC4100 and GJ1330 carrying each of the various plasmids were grown at 30°C to mid-log phase in K medium without and with 0.3 M NaCl supplementation before assay.
[b] ND, not determined.

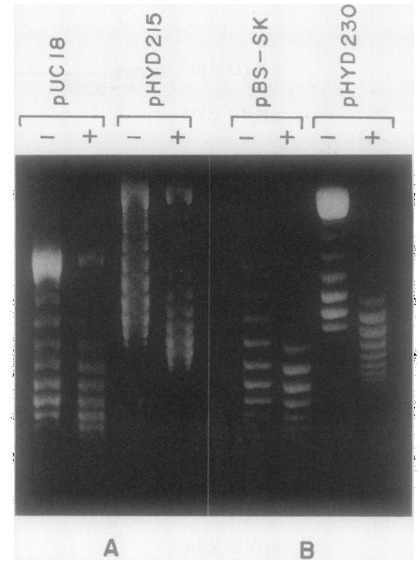

FIG. 3. Comparison of topoisomer distribution patterns of plasmids carrying the *proU* 658–1198 region with their corresponding parental vectors, after isolation from cultures grown at low (−) and at high (+) osmolarity. The low- and high-osmolarity growth media used were glucose-half-strength minimal A medium without and with supplementation with 0.3 M NaCl, respectively. (A) pUC18 and its derivative, pHYD215; (B) pBluescript SK (pBS-SK) and its derivative, pHYD230. The plasmid preparations were electrophoresed on 1% agarose gels in the presence of 2 μg (A) or 1.5 μg (B) of chloroquine per ml; under either of these conditions, the more negatively supercoiled topoisomers migrate faster (that is, further downward) through the gel (18).

mutant compared with that in the *osmZ*$^+$ strain; (ii) for the various deletion plasmid derivatives, the extent of derepression in the *osmZ*$^+$ strain (compared with the expression from pHYD270) was more or less similar to that described above in DH5α; and (iii) in the *osmZ* mutant, most of these deletion derivatives showed a further 1.5- to 3-fold increase in expression, but plasmid pHYD275 (carrying only the P1 promoter) exhibited a consistent 4-fold-lower level of expression compared with the value in the *osmZ*$^+$ strain. In all instances, growth in the high-osmolarity medium was associated with induction of *lac* expression; consistent with earlier results (18, 28), the wild-type plasmid (pHYD270) exhibited an induced level of activity in GJ1330 which was only marginally higher than that in MC4100, whereas with respect to each of the deletion plasmids pHYD283, pHYD284, and pHYD275, the magnitude of osmotic induction was approximately the same in both the *osmZ*$^+$ and *osmZ* strains (Table 3).

Effect of the *proU* 658–1198 region on plasmid supercoiling. The region of *proU* DNA between positions 658 and 1198, carrying a major part of the negative regulatory element (defined by the genetic studies above) without the *proU* promoters, was cloned into the plasmid vectors pUC18 and pBluescript SK. The supercoiling status of each of the resulting plasmids, pHYD215 and pHYD230, was compared with that of the vectors themselves by chloroquine-agarose gel electrophoresis after the plasmids had been isolated from cultures of the corresponding DH5α derivatives grown in low- and high-osmolarity media (Fig. 3). The distribution pattern of topoisomers in each of the plasmid preparations was also quantitated by densitometric analysis (Fig. 4), from

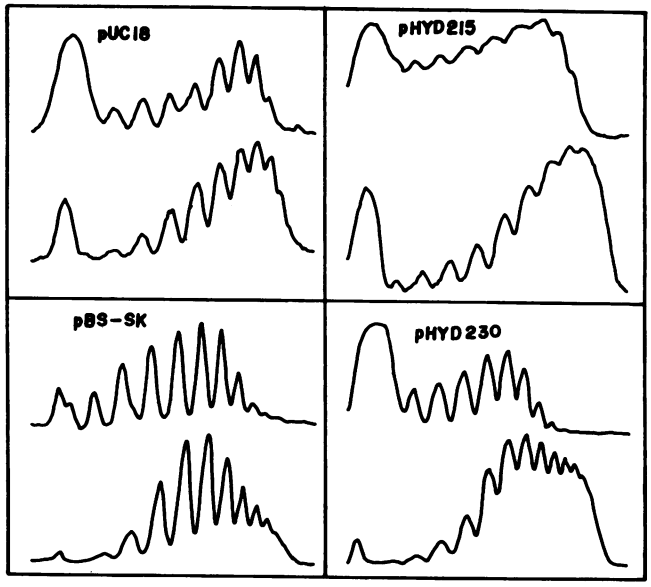

FIG. 4. Densitometric analysis of the plasmid topoisomer bands visualized in Fig. 3. The top and bottom tracings in each panel represent the results of scanning of the lanes of the low- and high-osmolarity-grown culture preparations, respectively, of the particular plasmid-bearing strain. Direction of electrophoresis was from left to right; the pair of tracings in each panel was aligned with respect to both the origin (not shown) and the peak corresponding to the nicked plasmid band (the first peak from the left in each trace).

which the mean for each distribution was calculated. The data indicate that a shift-up in osmolarity of the growth medium was associated with a marginal decrease in the mean linking number (that is, an increase in the degree of negative supercoiling of DNA in its isolated state) of the two vectors (pUC18 and pBluescript SK) by 0.5 to 1 link, whereas plasmids pHYD215 and pHYD230 exhibited a much more pronounced reduction in mean linking number of around 3 to 4 under the same conditions. A model to explain this property of the *proU* 658–1198 region, and to place it in context of the mechanism of action of the negative regulatory element, is discussed below.

Other properties of the promoter-downstream negative regulatory element. To check for the presence of any promoter in the *proU* 658–1198 region, this fragment was cloned in either orientation into the polycloning site of pMU575. In agreement with the data of Lucht and Bremer (26), no promoter activity was detected in the 658-to-1198 orientation of the cloned fragment. Clones carrying the fragment in the opposite orientation (1198 to 658) exhibited weak promoter activity (β-galactosidase specific activity in DH5α transformants, 15 U) that was unaffected by the osmolarity of the growth medium.

The *proU* 658–1198 region was also cloned immediately downstream of the λp_{RM} promoter in a plasmid, pS(wt), carrying the λp_{RM}-*lac* fusion (2). The orientation of the *proU* fragment in the resulting plasmid, pHYD225, is such that the end at position 658 is adjacent to the λp_{RM} promoter in pS(wt). Derivatives of DH5α transformed with pHYD225 showed no alteration compared with DH5α/pS(wt) in the expression of β-galactosidase in both low- and high-osmolarity growth media (data not shown), indicating that this region of *proU* is by itself unable to confer osmotic inducibility of transcription on a heterologous promoter.

DISCUSSION

The results described above indicate that there exist at least three mechanisms which together operate to confer 200- to 400-fold osmotic inducibility on expression of the *proU* operon: (i) one, which confers 6-fold inducibility on a promoter (P1) with the transcriptional start site at position 438, in plasmid pHYD275; (ii) another, acting upon a second promoter (P2) with the start site of transcription at position 628, which contributes another 8-fold osmoresponsivity in plasmid pHYD284; and (iii) a negative regulatory element downstream of both P1 and P2, whose disruption is associated with a 25-fold reduction in the magnitude of osmoinducibility.

Osmoresponsivity mediated by sequences around P1 and around P2. Promoter P1 was unambiguously identified in this study, first by the demonstration in vivo of β-galactosidase expression driven by the *proU* 376–552 fragment cloned into pMU575 and subsequently by 5′-end mapping of the transcripts produced from this plasmid. The sequences in and around P1 which contribute to the observed five- to sixfold osmotic inducibility of expression in pHYD275 are not known. A comparison of the β-galactosidase activities of isogenic derivatives carrying pHYD275 or pHYD272 indicates that the sequences downstream of P1 exert a negative effect on transcription initiated from this promoter during growth in low-osmolarity medium; whether this effect is mediated by the negative regulatory element discussed below is not clear. It is noteworthy that there is a near-perfect sequence identity between *E. coli* and *S. typhimurium* at the +1, −10, and −35 regions of P1 (but not in the intervening spacer regions), suggesting that this promoter might also be functional in the latter organism (13, 33, 41).

The P2 promoter, with its start site of transcription at position 628, is the one that had earlier clearly been identified in *E. coli* and *S. typhimurium* by both mRNA mapping and mutational studies (13, 26, 29, 33, 41). Our results with plasmid pHYD284, which has an insert from nucleotide positions 420 to 751 of *proU* (and which therefore carries P2 but not P1) are consistent with the data of Park et al. (34) and of Lucht and Bremer (26) that transcription from P2 is osmotically inducible; our findings indicate that in the absence of the downstream regulatory element, the magnitude of such induction is approximately eightfold. Once again, neither the operative mechanism nor the sequence around P2 that is required for osmoresponsivity is known.

Negative regulatory element downstream of P2. The existence of a transcriptional negative regulatory element downstream of P2 was first inferred from the results with *proV*::*lac* gene fusions on the multicopy plasmids pHYD58 and pACYC184; it was subsequently confirmed by promoter expression studies in the low-copy-number vector pMU575. That this downstream element plays a role in regulation of *proU* expression even in its native chromosomal location is suggested by the fact that of the various chromosomal operon or gene fusions in *proU* (presumably identified by using the criterion of osmoresponsivity of *lac* expression) that have been reported, the most promoter-proximal ones are all beyond nucleotide position 1500, that is, approximately three-quarters down the length of the *proV* structural gene (7, 29, 33, 41). Our results are at variance with the conclusions of Park et al. (34), who found no difference between the magnitude of osmotic inducibility of an *E. coli* *proU* promoter fragment extending from positions 420 to 1360 and that of another from positions 420 to 674; it is possible that strain differences, or the fact that they had been

FIG. 5. Possible alternative secondary structure in the region of *proU* between positions 831 and 919, identified by the program of Jacobson et al. (21). A · T and G · C base pairing possibilities (that would occur in both arms of an extruded cruciform) are marked.

using multicopy plasmids for steady-state assays of osmotic induction, might have contributed to the discrepant results. On the other hand, Overdier et al. (33) found that a 0.8-kb fragment from the *S. typhimurium proU* promoter region (extending approximately from nucleotide positions 115 to 935 in the corresponding *E. coli* sequence) conferred normal osmotic inducibility of expression, whereas a smaller fragment between (corresponding) positions 305 to 656 exhibited derepressed expression in low-osmolarity medium. However, it was not determined whether the negative regulatory element resided in the upstream or in the downstream region that had been deleted in the latter construct.

Mechanism of downstream negative regulation. Although our results suggest that promoter-downstream negative regulation requires a stretch of DNA several hundred base pairs in length, it is likely that there is a core element within it mediating such regulation, with the adjacent sequence(s) modulating in some way the action of the core element. Such an explanation would be consistent with the increasing degree of derepression observed above with progressive deletions into this region. The 116-bp *proU* region between positions 820 and 935 is likely to be part of this core element, because this sequence is absolutely conserved between *E. coli* and *S. typhimurium*; in comparison, there are 111 substitutions (97 of which are synonymous codon changes) between the two organisms in the remaining 754 bp of the *proV* structural gene (for which sequence information is available [13, 33].)

The presence of an open reading frame that is potentially capable of encoding an S(T)PXX-rich polypeptide on the complementary DNA strand of this region made us test the hypothesis that this element is a repressor gene for *proU*. We were unable, however, to obtain any evidence in support of this model.

The alternative possibility is that this region is acting in *cis* to reduce *proU-lac* expression in low-osmolarity medium, functioning either as an operator site for binding of an unidentified repressor protein or as an attenuator site to effect premature termination of transcripts initiated at P1 and P2. Yet another model is that the negative regulatory region acts at the level of controlling mRNA stability in an osmolarity-dependent manner, but our finding that it is unable to confer osmoresponsivity on a heterologous promoter (λp_{RM}) argues against this possibility.

A secondary-structure prediction algorithm (21) identified the region of *proU* nucleotide sequence between positions 831 and 919 (that is, precisely within the segment of absolute identity between *E. coli* and *S. typhimurium*) as one capable of exhibiting alternative secondary structure (Fig. 5). It is possible that this motif, occurring either in DNA or in the nascent mRNA transcript, is in some way involved in negatively regulating expression of the *proU* operon. Such an explanation might also be consistent with the observed need for a long stretch of DNA for achieving negative regulation.

A model for downstream negative regulation of *proU*. Plasmids pHYD215 and pHYD230, in which the *proU* 658–1198 region had been cloned into the pUC18 (2.7-kb) and pBluescript SK (3.0-kb) vectors, respectively, exhibited considerably more pronounced changes in linking number as a function of osmolarity of the growth medium than did the parental vectors themselves. Within each pair, the magnitude of the observed difference between test and the control plasmid is quite significant, even after correcting for the fact that the former is approximately 20% larger than the latter. It was also shown that the *proU* 658–1198 region did not possess significant promoter activity in either orientation; hence, the possibility is excluded that the supercoiling results might have been artifactually affected by the activity of strong promoters on pHYD215 or pHYD230 that were not present on the control plasmids (46).

In light of the observations presented above, we propose the following mechanism of action for the promoter-downstream negative regulatory element. We suggest that at low osmolarity, this region of DNA is maintained in a normal right-handed double-helical conformation, and that under these conditions transcription of *proU* is actively suppressed. We further suggest that growth of the cells in high-osmolarity medium is associated with the transition of this DNA segment into an alternative underwound (that is, supercoil-absorbing) conformation, and that the latter is unable to participate in active inhibition of *proU* transcription. The conformational change may be mediated by changes in the specific binding of one or more proteins to this segment at low and at high osmolarity and could involve, for example, the extrusion of a cruciform structure (45), predicated on part of the stem-and-loop possibility depicted in Fig. 5; alternatively, it could involve the formation of a DNA triple helix (45), in view of the occurrence of several polypurine and polypyrimidine stretches in this region. The model would account for (i) osmotic control of *proU* transcription by a negative regulatory mechanism and (ii) the observation that pHYD215 and pHYD230 exhibit a more pronounced decrease in linking number than the corresponding controls upon growth at elevated osmolarity, because transition of the insert region in the test plasmids to a supercoil-absorbing conformation in vivo would signal the cellular homeostatic system to compensate by unwinding the plasmids so that their torsional stress is restored (9). Indeed, similar alterations in topoisomer profile have been used previously as assays to determine the extent of occurrence of underwound DNA conformations (cruciforms or Z-DNA) in cloned synthetic DNA fragments in vivo (8, 15, 22). The topoisomer distribution patterns seen for pHYD215 and pHYD230 are also reminiscent of the bimodal patterns described for plasmids bearing the synthetic DNA fragments discussed above (8, 22), suggesting perhaps the existence of two plasmid subpopulations (with the *proU* insert fragment in the underwound conformation in one population and in the B-DNA conformation in the other) whose relative abundance is altered by changes in osmolarity of the growth medium.

Our observations with linking number change in the control plasmids would support earlier suggestions (8, 18, 19, 30) that the overall negative superhelical density in vivo under high-osmolarity growth conditions is itself reset to a value that is marginally higher than that prevailing at low osmolarity, a feature which might contribute to the *proU* conformational alteration proposed in the model (8, 30). Our model would also explain the earlier findings of Higgins and coworkers that perturbations (for example, mutations in

topA or exposure to DNA gyrase inhibitors) which directly increased or decreased the supercoiling status within the cell caused concomitant activation or inhibition of *proU* transcription (17, 18), because such perturbations would be expected to shift the equilibrium state of the negative regulatory element respectively to its underwound or wound conformation.

***proU* expression in *osmZ* mutants.** We examined the effect of *osmZ* on the various promoter-deletion plasmids of *proU* in an attempt to delineate which, if any, of the three mechanisms for *proU* regulation described above might be mediated by the action of the H1 (H-NS) protein. Our studies indicate that the effect of *osmZ* on *proU* expression cannot be ascribed to any one single mechanism and suggest instead that the *osmZ* mutation might affect each of the mechanisms differently. Particularly striking is the observation that whereas the wild-type *proU-lac* fusion in pHYD270 is derepressed eightfold at low osmolarity in the *osmZ* mutant, the isolated P1 promoter in pHYD275 is downregulated under the same conditions. Mutations in *osmZ* thus appear to have pleiotropic effects even within the restricted context of *proU* regulation in *E. coli*.

Concluding remarks. Two themes have dominated studies and discussion of the mechanism of osmoresponsivity of *proU* transcription. According to one, increase in osmolarity of the growth medium leads to increased supercoiling of DNA within the cells, and this in turn leads to *proU* induction (17–20, 30). According to the other, growth in high-osmolarity media is associated with intracellular accumulation of potassium glutamate, which then directly activates *proU* expression (24, 35–38). However, neither mechanism by itself has so far been shown capable of accounting quantitatively for the several-hundredfold magnitude of *proU* induction observed in vivo, and evidence presented in this report for the existence of multiple controls on *proU* transcription suggests that the two themes discussed above might be complementary to one another rather than mutually exclusive. The results on the effects of DNA supercoiling can be explained by our model for the mechanism of action of the promoter-downstream negative regulatory element, and it is possible that the effects of potassium glutamate are mediated through the P1 or P2 promoter.

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