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Osmotic Signal Transduction to *proU* Is Independent of DNA Supercoiling in *Escherichia coli*

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***proU* expression has been proposed to form part of a general stress response that is regulated by increased negative DNA supercoiling brought about by environmental signals such as osmotic or anaerobic stress (N. Ni Bhriain, C. J. Dorman, and C. F. Higgins, Mol. Microbiol. 3:933–944, 1989). However, we find that although *proU*-containing plasmids derived from cells grown in media of elevated osmolarity were more supercoiled than plasmids from cells grown in standard media, they did not activate *proU* expression in vitro. The *gyrA96* mutation and anaerobic conditions are known to affect DNA supercoiling but did not alter *proU* expression. Finally, the gyrase inhibitors coumermycin and novobiocin did not reduce in vitro *proU* expression. Therefore, this evidence rules out regulation by changes in DNA superhelicity for *proU* in *Escherichia coli*.**

The ability to adapt to osmotic stress is a fundamentally important process in living systems and occurs with only slight variation across a number of phylogenetic barriers (17). In *Escherichia coli*, the immediate response to hyperosmotic stress is the accumulation of high intracellular levels of K^+ (9) and glutamate (22, 39), which restore positive turgor pressure while maintaining electroneutrality. At the molecular level, osmotic stress also results in a number of other changes within the cell. These include the stimulated expression of a number of uncharacterized genes (13), alteration of the envelope protein and oligosaccharide composition (1, 21), and the expression of genetic functions that lead to either the import or the synthesis of osmotically active compounds such as trehalose, proline, choline, and glycine betaine (for a recent review of adaptation to osmotic stress, see reference 5).

Glycine betaine has been found to be the most effective osmoprotectant for *E. coli* as well as for other eubacteria (19). In *E. coli* and *Salmonella typhimurium*, glycine betaine import is mediated by two transport systems encoded by *proP* and *proU* (2, 30). Whereas *proP* is a low-affinity transport system, the *proU* operon encodes a high-affinity, binding-protein-dependent transport system for glycine betaine (2, 12, 30). In both systems, the rate of glycine betaine transport varies in proportion to medium osmolarity (2, 30). However, while *proP* is constitutively expressed, transcription of *proU* is strongly stimulated by hyperosmotic stress and is repressed by exogenous glycine betaine (1, 2, 30). In cases in which *proU* has been fused to genes encoding reporter enzymes, osmotically inducible *proU* expression increased 60-fold to several hundredfold (1, 2, 13).

A key issue in the regulation of genes that respond to stressful environmental changes is the manner in which the cell transduces an external stimulus into an internal response. In many cases, this process of signal transduction is accomplished via a phosphorylation cascade involving a two-component system of sensor and effector proteins (46). This is certainly the mechanism which operates in the osmotically regulated expression of the *ompC* and *ompF* porin genes by EnvZ and OmpR (18). However, osmotic stimulation of *proU* is not EnvZ or OmpR dependent (30) and appears to proceed through a unique regulatory mechanism. Several genetic studies suggest that osmoregulation of *proU* may occur without the involvement of ancillary regulatory factors, but these conclusions are based on negative results (15, 24). We have shown that K^+ ions provide the molecular signal stimulating *proU* expression in a cell-free, coupled transcription-translation system (S-30) from *E. coli* (37). In addition to confirming this result, other investigators have reported that the *proU* promoter is σ^{70} dependent, but they have suggested the existence of a macromolecular regulatory factor (20).

While these findings identify K^+ as the signal cation, they do not define the mechanism by which *proU* expression is stimulated. One model invokes osmotically mediated increases in the negative supercoiling of DNA as the essential factor (15, 33). In this model, regulation by DNA supercoiling is thought to extend to a variety of other stresses, such as anaerobiosis, heat shock, or extreme pH (33). The level of DNA supercoiling in *E. coli* is affected by the balanced activities of the enzymes gyrase and topoisomerase I (8, 52). Many studies have found a correlation between the level of supercoiling and the expression of various gene systems in procaryotes (6, 43, 51, 54) and have suggested that DNA topology regulates gene expression. However, other investigators argue that topoisomerases do not function in a regulatory capacity but instead act to restore and maintain an average superhelicity required for cellular transcriptional activity (36). Their studies demonstrate that changes in DNA supercoiling result from gene expression, generated by the process of transcription (10, 26, 53); domains of positively and negatively supercoiled DNA are produced by translocation of RNA polymerase along the DNA template and are thought to affect DNA topology to a far greater extent than that produced by gyrase or topoisomerase I (25). Thus, the link between supercoiling and gene expression may not necessarily indicate a cause and effect relationship.

In this study, we examine the relationship between factors which affect DNA supercoiling and expression of *proU*. We conclude that *proU* expression is not supercoiling dependent. Rather, the signal regulating *proU* is a change in the intracellular ion composition.

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TABLE 1. *E. coli* strains and plasmids

Strain or plasmid	Relevant genotype	Reference or source
MC4100	Δ(*arg-lac*)*U169 proU*$^+$ *gyrA*$^+$	3
DH1	*proU*$^+$ *gyrA96*	14
GM50	MC4100 φ(*proU-lacZ*$^+$)3[a]	30
pBP1	*bla proUpproV'*[b] (pTZ18U replicon)	37
pBP4	*bla proUpproV'*-CAT[c] (pKK232-8 replicon)	35
pAT153	*bla tet*	50
pCG311	*bla osmC8115*::Tn*phoA*[d]	C. Gutierrez
pC3101B	*bla lacp*$^+$-CAT[e]	C. Case

[a] Transcriptional fusion of *proU* to *lacZ*.
[b] The plasmid contains the complete *proU* promoter controlling the expression of the truncated, promoter-proximal *proV'* gene.
[c] The plasmid contains a transcriptional fusion where the intact *proU* promoter controls the synthesis of the truncated ProV' and CAT proteins.
[d] *osmC8115*::Tn*phoA* is a translational fusion and results in the synthesis of an OsmC-PhoA hybrid protein.
[e] Synthesis of CAT is under control of the *lacp*$^+$ promoter.

MATERIALS AND METHODS

Strains, growth conditions, and radiolabeling. Strains used in this study are listed in Table 1. Standard media consisted of either Luria broth (LB) or M9 plus 0.2% glucose (32), whereas high-osmolarity media consisted of these media supplemented with the appropriate amount of NaCl (see figure legends). When appropriate, strains were grown in the presence of antibiotics at the following concentrations: ampicillin, 100 μg/ml; nalidixic acid, 50 μg/ml; kanamycin, 30 μg/ml.

Expression of *proU* in response to anaerobiosis was measured with strain GM50 (30) (Table 1). An exponential-phase, aerobic culture of GM50 was harvested by centrifugation, and the cells were washed with oxygen-free M9 medium. Washed cells were injected into O_2-free Hungate tubes (54) with N_2-flushed syringes and an 18-gauge needle. Aerobic cultures were grown in these same tubes without the butyl rubber stoppers. Cultures were then grown over a 19-h period at 37°C, and cells were harvested at intervals for β-galactosidase assays. The Hungate tubes contained either M9 medium or M9 plus 0.3 M NaCl and were prepared by first boiling for 4 min to degas and then cooling on ice while being flushed with N_2 gas which had been deoxygenated by passage through a hot copper column. The tubes were quickly capped with butyl rubber stoppers and autoclaved to boil off any remaining oxygen. Resazurin indicator remained colorless in anaerobic cultures and was pink in aerobic cultures.

Periplasmic fluids were extracted from 2-ml exponential-phase cultures (34) grown either in LB-based medium (Fig. 1) or in M9-based medium (see Fig. 4), labeled in the presence of 100 μCi of Tran^{35}S-label (1 Ci = 37 GBq; ICN Radiochemicals, Irvine, Calif.) for 10 min at 37°C.

Assays. β-Galactosidase activity was assayed according to the method of Miller (32). Protein determinations were assayed according to the method of Lowry et al. (27) with bovine serum albumin as the standard. Chloramphenicol acetyl transferase (CAT) was assayed according to the procedure of Rodriguez and Tait (41).

Plasmids. Plasmids used in this study are listed in Table 1. Plasmids pBP1 and pBP4 (Table 1) (35, 37) bear the same 1,700-bp DNA fragment containing the upstream *proU* promoter region and a truncated portion of the first gene, *proV'*.

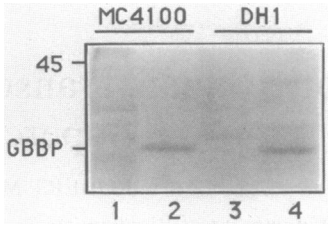

FIG. 1. Periplasmic fluids from osmotically stressed host strains MC4100 (*gyrA*$^+$) and DH1 (*gyrA96*). Periplasmic fluids were extracted from mid-logarithmic-phase cells grown in LB (lanes 1 and 3) or LB plus 0.5 M NaCl (lanes 2 and 4) in the presence of ^{35}S-methionine. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by autoradiography. The left margin indicates the location of the *proU*-encoded glycine betaine-binding protein (GBBP) and the 45-kDa molecular mass marker.

In pBP4, the *proU* segment has been fused to the promoterless CAT gene of plasmid pKK232-8 (Pharmacia LKB Biotechnology, Piscataway, N.J.), resulting in osmotically inducible CAT activity (41). These plasmids were used in both in vivo and in vitro experiments. Plasmids pBP1 and pBP4 were prepared from either overnight or exponential cultures of strain MC4100 grown in standard or high-osmolarity LB media (see figure legends) by the alkali lysis method (28). After overnight digestion with RNase, the DNA was extracted twice with phenol-chloroform and then once with chloroform. Plasmids were ethanol precipitated and resuspended in TE buffer (28). The superhelical distribution of plasmids prepared to this stage was determined electrophoretically in an agarose-chloroquine gel (10, 15). Electrophoresis was carried out with recirculation of the buffer over a 40-h period.

For use in cell extracts, plasmids were further purified by elution through a Bio-Gel A-150m agarose column (Bio-Rad Laboratories, Richmond, Calif.) with TE buffer. Plasmid DNAs were found in fractions that eluted early, as determined with the A_{260}.

Bulk preparations of relaxed pBP1 and pBP4 were prepared by treating approximately 100 μg of column-purified plasmid overnight with 70 U of calf thymus topoisomerase I (BRL, Gaithersburg, Md.) in 400 μl of reaction buffer (50 mM Tris hydrochloride, pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.1 M disodium EDTA).

DNA-directed cell-free protein synthesis. Cell-free coupled transcription-translation was performed as previously reported (37), by using a commercially available kit (Amersham Corp., Arlington Heights, Ill.) with column-purified templates.

RESULTS

In vivo *proU* expression is not reduced by a mutation in the *gyrA* gene. A general model of regulation states that diffuse environmental challenges, such as osmotic stress and anaerobic stress, actively coordinate the expression of many genes through the common mechanism of altering DNA supercoiling. Thus, DNA supercoiling is seen as the primary signal event in the control of a global network of stress response genes (33).

Specifically, increased negative DNA supercoiling associated with high osmolarity has been proposed to cause osmotic induction of *proU* (15). If this were true, we would expect that reduced gyrase activity would interfere with

TABLE 2. Effects of a *gyrA96* mutation on osmotically induced *proU*-CAT expression in vivo

NaCl concn (M) in LB medium	CAT sp act (induction ratio)[a] in:		
	MC4100(pBP4)	DH1(pBP4)	RR5(pBP4)
0	70 (1)	60 (1)	35 (1)
0.15	596 (8.5)	564 (9.4)	335 (9.6)
0.3	962 (13.7)	1,017 (17)	No data
0.4	1,514 (21.6)	1,119 (18.7)	803 (23.1)

[a] Specific activity values were calculated from the differential rates of synthesis of early-log-phase cultures grown over a 2-h period at the indicated osmolarities. Units of CAT specific activity are nanomoles per minute per milligram of protein. Induction ratio values represent the ratio of CAT specific activity at the given osmolarity divided by the CAT activity in standard LB. Relevant genotypes: MC4100, *gyrA*$^+$; DH1 and RR5, *gyrA96*; pBP4, *proU*-CAT.

TABLE 3. Effects of anaerobiosis on *proU* expression in *E. coli*[a]

Growth time (h)	β-Galactosidase sp act[b]			
	Aerobic		Anaerobic	
	M9	M9 + 0.3 M NaCl	M9	M9 + 0.3 M NaCl
0	29	25	29	31
3	36	1,100	20	747
19	20	595	19	584

[a] Strain GM50, containing a *proU-lacZ* transcriptional fusion, was used to monitor *proU* expression. Exponential-phase cells grown in standard M9 medium under aerobic conditions were harvested by centrifugation and used to inoculate Hungate tubes (54) containing M9 medium, aerobically or anaerobically, without or with 0.3 M NaCl as indicated.

[b] Units of specific activity are nanomoles of *o*-nitrophenyl-β-D-galactopyranoside hydrolyzed per minute per milligram of protein.

proU expression. *E. coli* DH1, which carries the *gyrA96* mutation (14), is defective in supercoiling activity and is unable to express the supercoiling-sensitive *nif* genes of *Klebsiella pneumoniae* unless complemented in *trans* by *gyrA*$^+$ (6). Therefore, this strain should be useful in examining the influence of supercoiling on *proU* expression. To measure *proU* induction in the DH1 background, we utilized a *proU*-CAT gene fusion carried on plasmid pBP4 and assayed for *proU*-dependent CAT activity in exponentially growing cells as a function of increasing osmolarity. The presence of the *gyrA96* mutation did not reduce extrachromosomal *proU* expression compared with expression in the *gyrA*$^+$ strain (see Table 2). In both cases, increasing osmolarity resulted in almost identical induction ratios of about 20-fold. To rule out the possibility that the DH1 strain had accumulated suppressors of the *gyrA96* defect, we transduced this mutation into strain MC4100(pBP4) and selected for cells that were resistant to both ampicillin and nalidixic acid. The inducibility of *proU*-CAT in this strain [RR5(pBP4)] was the same as in the other two strains (Table 2), although the amount of CAT activity was slightly reduced under all conditions. Both the MC4100 (*gyrA*$^+$) and DH1 (*gyrA96*) strains also expressed the chromosomal *proU* function normally, since we found that periplasmic fluids extracted from radioactively labeled, osmotically stressed host cells contained equal amounts of the *proU*-encoded glycine betaine-binding protein (Fig. 1). We conclude that in *E. coli* a fully functional gyrase is not required in the response of *proU* to osmotic signals.

***proU* expression is not potentiated by anaerobic conditions in vivo.** *proU* expression in *S. typhimurium* has been shown to be potentiated, though not induced, by anaerobic stress (33). Since anaerobiosis also increased negative DNA supercoiling (7), Dorman et al. suggested that *proU* regulation was part of a global stress response mediated through changes in DNA superhelicity. To determine whether a similar response mechanism operates in *E. coli*, we examined the effects of anaerobic conditions on *proU* expression by using a strain containing a chromosomal *proU-lacZ* transcriptional fusion (strain GM50 [30]). Exponential-phase cells grown aerobically in M9 medium were shifted to four different growth conditions (Table 3). As expected, both short-term (at 3 h) and steady-state (at 19 h) *proU* expression were strongly induced by elevated osmolarity. However, anaerobiosis alone did not induce *proU* expression, nor did it potentiate *proU* expression in combination with high osmolarity. We conclude that anaerobiosis does not provide a positive regulatory signal for *proU* expression in *E. coli*.

Superhelical density of *proU*-bearing DNA templates during osmotic stress. Hyperosmotic stress is reported to increase the negative supercoiling of reporter plasmids and, by extension, of the chromosome (15). We examined the in vivo superhelical transitions of plasmids bearing *proU* sequences during osmotic stress to see whether they behaved in a similar manner. *proU*-containing plasmids used in these studies were pBP1 and pBP4 (Table 1). Plasmid DNAs were isolated from exponential-phase MC4100 cells grown either in LB, in which *proU* was not expressed, or in LB plus 0.3 M NaCl, in which expression was strongly stimulated. Plasmid topoisomers were then electrophoretically separated in an agarose-chloroquine gel system. In this experiment (Fig. 2), osmotic stress led to negligible changes in the superhelical status of pBP1; it became slightly more super-

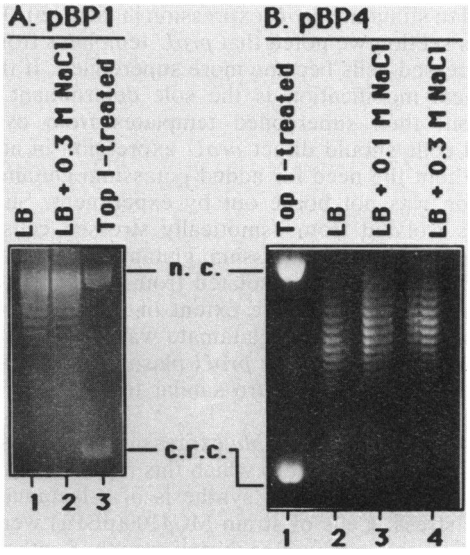

FIG. 2. Effect of medium osmolarity on in vivo supercoiling of *proU*-containing plasmids. Plasmid DNAs were extracted from exponential-phase MC4100 cells grown in LB (lanes 1 and 3) or LB plus NaCl (concentrations as indicated). (A) pBP1 topoisomers separated in a 1% agarose–TPE (28)–chloroquine (25 g/ml) gel; (B) pBP4 topoisomers separated in a 1.2% agarose–TPE–chloroquine (22 g/ml) gel. In both cases, topoisomerase I-relaxed controls were included to show that relaxed circles migrate more quickly than supercoiled forms (panel A, lane 3, and panel B, lane 1). Abbreviations: n.c., nicked circles; c.r.c., closed relaxed circles.

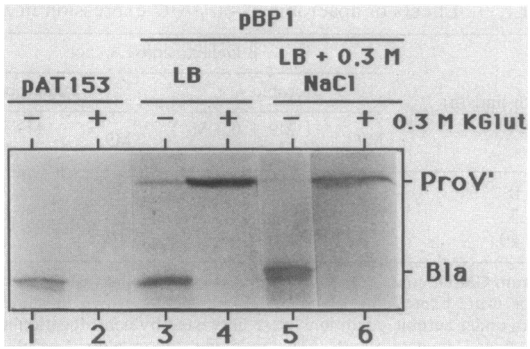

FIG. 3. In vitro expression of *proU* templates derived from cells grown at low and high osmolarities. Plasmid pBP1 was purified from exponential-phase MC4100 cells grown in LB or LB plus 0.3 M NaCl. DNAs were then used to direct in vitro protein synthesis in a standard S-30 mixture (lanes 1, 3, and 5) or S-30 supplemented with 0.3 M potassium glutamate (lanes 2, 4, and 6). The control plasmid pAT153 was used to monitor synthesis of the β-lactamase (Bla) gene product. Proteins synthesized in vitro were separated by SDS-PAGE followed by autoradiography. ProV′ is encoded by pBP1 and is the truncated form of the first gene product specified by the *proU* operon.

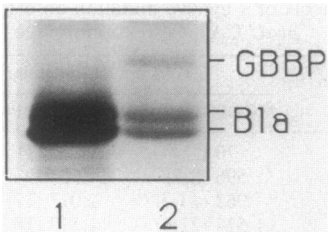

FIG. 4. In vivo osmoregulation of the chromosomal *proU* and extrachromosomal β-lactamase functions of strain MC4100(pBP1). Periplasmic fluids were extracted from exponential-phase cells grown in M9 (lane 1) or M9 plus 0.3 M NaCl (lane 2) in the presence of ^{35}S-methionine. Proteins were separated by SDS-PAGE followed by autoradiography. GBBP, Glycine betaine-binding protein encoded by the chromosomal *proU* operon; Bla, β-lactamase protein encoded by plasmid pBP1.

coiled upon osmotic upshock (panel A). pBP4 displayed the same pattern, an increase of one to five linking numbers in a series of experiments (panel B). We conclude that the increase in superhelicity associated with osmotic stress is small and variable.

In vitro *proU* expression depends on a signal provided by potassium glutamate. Analyzing gene expression in a cell-free system allows more direct manipulation of parameters important in the regulation of *proU*. Previous reports indicated that high concentrations of potassium glutamate were required to stimulate *proU* expression in vitro (20, 37). In the previous section we noted that *proU* templates from osmotically stressed cells became more supercoiled. If this stable topological modification is the sole determinant of *proU* activation, then supercoiled templates from osmotically stressed cells should direct *proU* expression in an in vitro S-30 without the need for added potassium glutamate. This prediction was not borne out by experiment. Supercoiled plasmids isolated from osmotically stressed cells had the same requirement for potassium glutamate to activate *proU* expression as templates isolated from cells grown in standard medium (Fig. 3). The extent of inhibition of the *bla* expression by potassium glutamate was also the same with both templates. The other *proU* plasmid, pBP4, showed a pattern of regulation in vitro similar to that seen for pBP1 (data not shown).

The strong inhibition of *bla* expression in vitro is striking. To determine the extent to which this represents the in vivo pattern, we examined the synthesis of β-lactamase during osmotic stress. Cells of strain MC4100(pBP1) were labeled with radioactive methionine during growth. Synthesis of one periplasmic product of the chromosomal *proU* operon, the glycine betaine-binding protein, was stimulated by hyperosmotic stress, while synthesis of plasmid-encoded β-lactamase was severely decreased (Fig. 4). Therefore, the in vitro effects of potassium glutamate on expression of *bla* and *proU* mimic the in vivo response.

In Fig. 3, it might be argued that potassium glutamate acts by altering the activity of topoisomerases present in the S-30 to promote a transient increase in DNA supercoiling that results in *proU* expression. If this were the case, then in vitro *proU* expression should be eliminated by gyrase inhibitors, such as coumermycin or novobiocin. In vitro *proU* expression was not reduced by the presence of coumermycin A1 or novobiocin in the S-30 (Fig. 5, lanes 4 and 7). The efficacy of these compounds in inhibiting gyrase activity in an S-30 has been demonstrated previously (11). To confirm the inhibition of gyrase activity, we have made use of the difference in mobility between relaxed and supercoiled forms of pBP1 that were reisolated from S-30 mixtures by agarose gel electrophoresis. S-30 extracts without these gyrase inhibitors permitted resupercoiling of relaxed pBP1, even in those mixtures with added 0.3 M potassium glutamate (Fig. 6, lanes 2, 3, and 5). By contrast, the resupercoiling of pBP1 was inhibited in S-30s supplemented with either coumermycin or novobiocin, whether 0.3 M potassium glutamate was added (lanes 4, 5, 7, and 8) or not. These conditions were identical to those used to examine in vitro *proU* expression. These observations support the conclusion from the experiments with the *gyrA96* mutant; namely, that *proU* expression is independent of DNA gyrase activity. Furthermore, the sum of these data indicates that *proU* expression is mediated by a chemical signal provided by potassium glutamate.

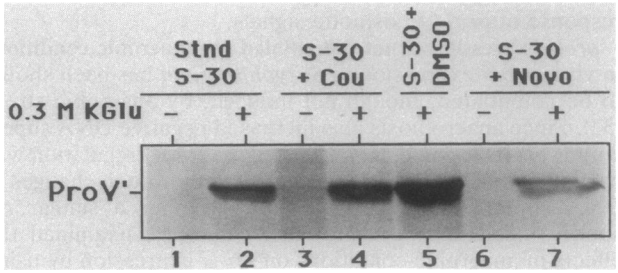

FIG. 5. Effects of gyrase inhibitors on in vitro *proU* expression. Plasmid pBP1 was purified from stationary-phase cells grown in LB. This DNA was then used to direct in vitro protein synthesis in a standard S-30 mixture (lane 1) or in S-30 mixtures with added 0.3 M potassium glutamate (lanes 2, 4, 5, and 7), coumermycin A1 solubilized in dimethyl sulfoxide (final coumermycin concentration in the S-30, 2 μg/ml; lanes 3 and 4), or novobiocin (final concentration in the S-30, 10 μg/ml; lanes 6 and 7). The S-30 supplemented with dimethyl sulfoxide was included as a control for lanes 3 and 4. ProV′ is encoded by pBP1 and is the truncated form of the first gene product specified by the *proU* operon.

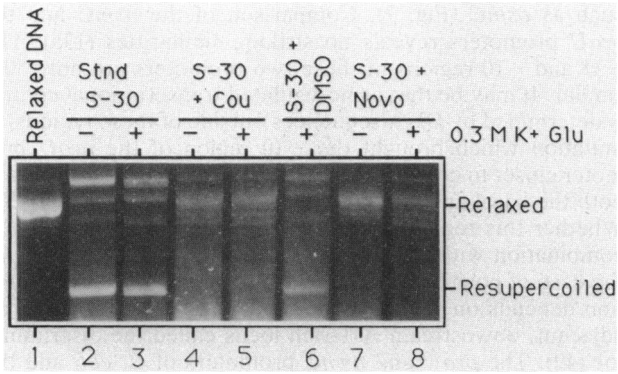

FIG. 6. Effects of gyrase inhibitors on the in vitro resupercoiling of a relaxed *proU* template. Plasmid pBP1 was relaxed by treatment with calf thymus topoisomerase I (lane 1; see Materials and Methods). This DNA was added to a standard S-30 (lane 2) or to S-30 mixtures supplemented with 0.3 M potassium glutamate (lanes 3, 5, 6, and 8), coumermycin A1 solubilized in dimethyl sulfoxide (lanes 4 and 5; final coumermycin concentration in the S-30, 2 μg/ml), or novobiocin (lanes 7 and 8; final concentration in the S-30, 10 μg/ml). The S-30 supplemented with dimethyl sulfoxide was included as a control for lanes 4 and 5. The concentrations of coumermycin and novobiocin used in these experiments were twice the minimum concentration which inhibited resupercoiling of a relaxed template, as determined in a separate titration experiment (data not shown).

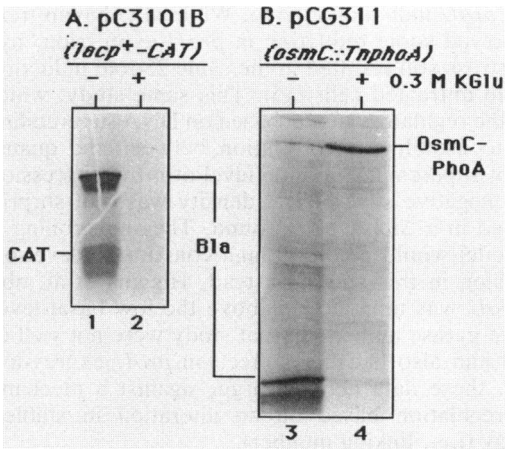

FIG. 7. Effects of potassium glutamate on in vitro expression from *osmC*::Tn*phoA* and the *lacp*$^+$-CAT gene fusion. The *osmC*::Tn*phoA* fusion is encoded on plasmid pCG311, and the wild-type *lac* promoter is fused to the CAT gene in plasmid pC3101B. The plasmids were used to direct protein synthesis in a standard S-30 (lanes 1 and 3) or in an S-30 plus 0.3 M potassium glutamate (lanes 2 and 4) in the presence of ^{35}S-methionine. Proteins were separated in SDS-polyacrylamide gels as follows: panel A, 15% acrylamide; panel B, 10% acrylamide. Abbreviations for gene products: Bla, β-lactamase; OsmC-PhoA, the hybrid protein encoded by the *osmC*::Tn*phoA* fusion gene.

Potassium glutamate signals expression from another osmoregulated promoter: *osmC*. To determine whether potassium glutamate can signal the induction of other osmoregulated genes, we tested *osmC*::Tn*phoA* expression in the S-30. *osmC* encodes an osmotically inducible protein (a molecular mass of 14 kDa) of unknown function (13, 13a). The *osmC8115*::Tn*phoA* translational fusion, found on plasmid pCG311, is predicted to encode a hybrid protein of approximately 63 kDa. As in the case of *proU*, we found that addition of 0.3 M potassium glutamate stimulated in vitro expression of *osmC*::Tn*phoA* (see Fig. 7B). These data suggest that high intracellular levels of potassium glutamate may be a common positive signal for other osmotically inducible genes.

Moderate concentrations of potassium glutamate have been reported to enhance protein-DNA interactions nonspecifically (23). To test the specificity of high concentrations of potassium glutamate as an osmotic stress signal, we examined expression of a *lacp*$^+$-CAT promoter fusion in the S-30. We found that addition of 0.3 M potassium glutamate to the S-30 completely inhibited the expression of both the *lacp*$^+$-CAT and *bla* genes encoded on plasmid pC3101B (Fig. 7A), supporting the specific-signal model.

DISCUSSION

proU expression is strongly induced in cells exposed to media with elevated osmolarity. By analogy to other stress responses, one would expect the involvement of a transcriptional activator or repressor protein. However, the inability of several laboratories to identify genes for *trans*-acting factors specifically involved in *proU* regulation has led to the proposal of alternative models of control. One early study proposed that hyperosmotic conditions increase DNA supercoiling, which in turn stimulates expression of *proU* (15). Alternatively, it is known that K^+ and glutamate are the major osmolytes accumulated simultaneously in vivo during hyperosmotic stress (9, 22, 39). *proU* expression shows a strong correlation with intracellular K^+ levels in vivo (47) and is induced by added K^+ in vitro (20, 37). Thus, *proU* expression may be signalled or mediated by elevated K^+ concentrations.

We find our data to be incompatible with the hypothesis that *proU* expression is controlled by changes in DNA supercoiling in *E. coli*. In vivo *proU* expression was not affected by either the *gyrA96* mutation or anaerobiosis, factors that are known to alter DNA supercoiling. In vitro experiments also preclude regulation by this mechanism. First, we show that plasmids derived from cells grown in media of increased osmolarity showed slightly higher levels of supercoiling (Fig. 2), yet these differences alone did not result in higher levels of in vitro *proU* expression (Fig. 3). The small increases in plasmid linking number may be the result, rather than the cause, of transcription, as demonstrated in studies in which the induction or elimination of plasmid-encoded genes led to an alteration of the superhelical status of the templates (10, 26, 53). Second, one could argue that potassium glutamate in the S-30 stimulates the activity of DNA gyrase, resulting in transient increases in DNA supercoiling. However, despite inhibition of gyrase activity (Fig. 6), in vitro *proU* expression was stimulated by added potassium glutamate (Fig. 5).

Our conclusion that increased DNA supercoiling does not play a role in *proU* osmoregulation is bolstered by two independent observations in vivo. First, the *E. coli* chromosome is divided into approximately 50 topologically distinct domains (44). If domain-specific differences in DNA topology are involved in *proU* expression, placement of the *proU-lacZ* fusion at a site other than its normal map position would be predicted to alter *proU* regulation. Yet recombination of the *proU-lacZ* fusion into the *attB* locus (17 min on the *E. coli* chromosome) results in the same pattern of *proU* expression seen in its native chromosomal site (29). Second, if *proU* expression depends on increased DNA supercoiling mediated by gyrase, antibiotic inhibitors of gyrase should

reduce *proU* induction in vivo. While novobiocin-treated *E. coli* showed some reduction in *proU* expression, hyperosmotic stress still resulted in the same 25-fold induction ratio found in untreated cells (15). This same study, which proposed the regulatory model based on DNA supercoiling, also failed to establish a correlation between the quantitative value of supercoiling and the level of *proU* expression. The largest negative superhelical density was, not surprisingly, observed in a Δ*topA* background. The supercoiling regulation model would predict a high constitutive level of *proU* expression in this strain. Instead, Higgins et al. observed that *proU* was uninducible above the low basal level. The putative gyrase mutants in that study were not well characterized and also had little effect on *proU* expression (15). Hence, these data actually argue against a mechanism of *proU* regulation based on an alteration in stable DNA topology (i.e., linking number).

We have shown that the pattern of gene expression seen in the complex S-30 mixture mimics the pattern seen in living cells under osmotic stress (Fig. 4). Potassium glutamate stimulation of *proU* transcription has now been produced in a simple, purified in vitro system composed of DNA template, RNA polymerase, nucleotides, and buffer with no additional protein factors (35). The implication of these studies is that potassium glutamate controls *proU* expression by acting directly on the transcription complex. Thus, we believe that an elevated concentration of the ionic compound potassium glutamate is the signal that is both necessary and sufficient to activate *proU* expression. A possible analogy is the mechanism of action of the nucleotide ppGpp during amino acid starvation in vivo (reviewed in reference 4) and its documented regulatory effects in vitro (38, 40, 48). ppGpp operates independently of a protein cofactor and is thought to exert its action by directly modulating RNA polymerase specificity at the level of transcription initiation (40, 48). Potassium glutamate might act as a metabolic signal in a similar manner. However, signal molecules are generally present at very low concentrations. Intracellular ppGpp concentration is thought to reach 0.2 mM during the stringent response (4); thus, our requirement of 0.3 M potassium glutamate for maximum stimulation is more than 1,000 times greater than that reported for ppGpp. This difference may reflect the dual role of potassium glutamate in the stressed cell: it must simultaneously contribute to the intracellular osmotic strength and act as a signal to gene expression.

An alternative method of action may be that potassium glutamate causes a transient, localized change in DNA topology that facilitates *proU* expression. Salt effects may also influence the association of other DNA-binding proteins, such as HU (42) or host integration factor (16), that result in small topological changes.

In our previous report, we demonstrated the specificity of *proU* for K^+ as the signal cation (37). However, we are not entirely certain of the role of the anion glutamate. At any osmolarity the intracellular glutamate concentration is significantly less than that of K^+ (38), although a recent study reported that the internal glutamate concentration roughly equaled the free K^+ concentration (31). We found that potassium acetate stimulated some *proU* expression in vitro (36). What we can say is that potassium glutamate best reproduces the signal effect in vitro. The analysis of defects in glutamate biosynthesis should clarify the contribution of this amino acid to the osmotically induced expression of *proU*.

Potassium glutamate may serve as the regulatory signal involved in the activation of other osmoregulated genes, such as *osmC* (Fig. 7). Comparison of the *osmC* and the *proU* promoters reveals no striking similarities (13a). The −35 and −10 regions of these two promoters are only 50% similar. It may be that osmoregulated transcriptional control is determined by DNA sequences outside of these regions. A mutation which brought the −10 region of the *proU* promoter closer to consensus has been found to slightly increase both the basal and inducible levels of *proU* expression (29). Whether this region regulates *proU* expression alone or in combination with another DNA region is undetermined. In the case of positively regulated stringent promoters, regulation depends on sequences found in the −10 region and an adjacent, downstream A-T-rich locus called the discriminator (49). The *proU* and *osmC* promoters of *E. coli* and the *proU* promoter of *S. typhimurium* also share this feature (12, 29, 45). Thus, the isolation and characterization of additional mutations will determine whether this or other regions are involved in *proU* regulation.

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