

## Effects of H-NS and Potassium Glutamate on $\sigma^S$ - and $\sigma^{70}$ -Directed Transcription In Vitro from Osmotically Regulated P1 and P2 Promoters of *proU* in *Escherichia coli*

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**We have used supercoiled DNA templates in this study to demonstrate that transcription in vitro from the P1 and P2 promoters of the osmoreponsive *proU* operon of *Escherichia coli* is preferentially mediated by the  $\sigma^S$ - and  $\sigma^{70}$ -bearing RNA polymerase holoenzymes, respectively. Addition of potassium glutamate resulted in the activation of transcription from both P1 and P2 and also led to a pronounced enhancement of  $\sigma^S$  selectivity at the P1 promoter. Transcription from P2, and to a lesser extent from P1, was inhibited by the nucleoid protein H-NS but only in the absence of potassium glutamate. This study validates the existence of dual promoters with dual specificities for *proU* transcription. Our results also support the proposals that potassium, which is known to accumulate in cells grown at high osmolarity, is at least partially responsible for effecting the in vivo induction of *proU* transcription and that it does so through two mechanisms, directly by the activation of RNA polymerase and indirectly by the relief of repression imposed by H-NS.**

The *proU* operon in *Escherichia coli* and *Salmonella typhimurium* encodes a binding-protein-dependent transporter that mediates the osmoprotective effects of exogenous glycine betaine and L-proline when these organisms are grown in media of elevated osmolarity. *proU* transcription is markedly induced (more than 100-fold) in high-osmolarity media, and the mechanism by which this is brought about has been the subject of intensive, but as yet inconclusive, genetic and biochemical studies (for reviews, see references 6, 16, and 29).

With regard to the *cis* elements mediating *proU* osmoreponsivity, there is a consensus on the existence of (i) a promoter whose transcription start site is approximately 60 nucleotides upstream of the initiation codon of the first structural gene (*proV*) (10, 15, 41, 46, 55) and (ii) a negative regulatory element (NRE) situated in a region overlapping the proximal (5') end of *proV* whose deletion leads to a 25-fold derepression of *proU* expression at low osmolarity (8, 13, 30, 40, 42). This promoter is recognized in vitro by the  $\sigma^{70}$ -RNA polymerase holoenzyme ( $E\sigma^{70}$ ) (10, 55).

Our group has also identified, by in vivo studies, another promoter located 250 nucleotides upstream of *proV* in *E. coli* (8, 15) and has shown recently that this promoter is both RpoS ( $\sigma^S$ ) dependent and stationary-phase inducible (31). We have designated the two (*proV*-proximal and *proV*-distal) promoters P2 and P1, respectively. The role of the upstream P1 promoter and  $\sigma^S$ -RNA polymerase ( $E\sigma^S$ ) in *proU* regulation is still uncertain, however, for the following reasons: (i) *proU* expression in vivo is not affected by deletion of P1 or by mutations in *rpoS* (28, 31), whereas a mutation in *rpoD* (encoding  $\sigma^{70}$ ) results in nearly complete abolition of *proU* expression (58); (ii) *proU* expression in vivo is not significantly induced in stationary-phase cultures (31); (iii) Overdier et al. (41) have failed to

identify an equivalent promoter during subcloning experiments with *S. typhimurium proU*; and (iv) in vitro transcription from P1 was not detected from a linear *E. coli proU* template by using  $E\sigma^S$  (10).

With regard to *trans*-acting factors that affect *proU* transcription, mutations in the *hns* gene (previously called *osmZ*), which encodes the nucleoid protein H-NS, have been shown to result in partial derepression of *proU* (19, 21, 28, 45). H-NS exhibits preferential binding to the 5' end of *proV* that comprises the NRE (30, 42, 54), and there is genetic evidence that such binding is required for the inhibition by H-NS of *proU* transcription at low osmolarity (8, 13, 30, 42; reviewed in reference 16). Nevertheless, deletion of the NRE has a more pronounced effect on *proU* regulation than have mutations in *hns*, and Fletcher and Csonka (13) have speculated that the StpA protein, which is very similar to H-NS, may be capable of substituting for the latter in mediating partial repression of *proU* in the *hns* mutants. There also is a component of P2 osmotic regulation which is both H-NS and NRE independent (8, 13, 30, 34, 40, 42), and this component appears to be affected in mutants defective in either of two other nucleoid proteins, HU and IHF (31).

Growth at high osmolarity is associated with changes in DNA superhelicity (9, 19, 33, 38) and with the intracellular accumulation of potassium glutamate (5, 11, 47). Both DNA supercoiling (19, 20, 38) and potassium glutamate (23, 43, 47) have also been implicated in osmotic regulation of *proU* expression in vivo.

Ueguchi and Mizuno (55) used  $E\sigma^{70}$  and *proU* DNA templates which included a major portion of the NRE to show that transcription from P2 is (i) supercoiling sensitive, (ii) activated by potassium glutamate, and (iii) inhibited by H-NS. Furthermore, H-NS-mediated inhibition of P2 transcription was not observed in the presence of potassium glutamate, suggesting that potassium glutamate relieves the P2 repression by H-NS. Ding et al. (10) have shown that potassium glutamate directly

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activates in vitro transcription from P2 on a linear template in the absence of H-NS.

In this study, we have examined  $E\sigma^S$ - and  $E\sigma^{70}$ -directed transcription in vitro from supercoiled DNA templates carrying the *E. coli proU* P1 and P2 promoters and the effects of nucleoid proteins and potassium glutamate thereon. Our results identify P1 promoter activity for the first time in vitro and confirm that it is  $E\sigma^S$  dependent. The data also provide insights into the possible roles of potassium glutamate and H-NS in *proU* regulation.

#### MATERIALS AND METHODS

***E. coli* strains and plasmids.** Strain DH5 $\alpha$  (44) was used in the cloning experiments and for the preparation of DNA templates for in vitro transcription. MC4100 (8) and GJ1330 (8), used in the experiments with promoter-*lac* fusions, are isogenic  $\Delta lac hns^+$  and *hns-200* derivatives, respectively.

The plasmid vectors included (i) the low-copy-number *lacZ* operon fusion vector pMU575 (2), (ii) plasmid pSelect (Promega Corp., Madison, Wis.) for use in site-directed mutagenesis experiments, and (iii) pCU22 (55), the vector used in the in vitro transcription experiments (see Fig. 1). Plasmids pHYD272 and pHYD275 have been described earlier (8) and are derivatives of pMU575 that carry the *proU* regions from nucleotides 376 to 1631 and from 376 to 552, respectively. (Nucleotide number designations in *proU* are as described by Gowrishankar [15], according to which the start sites for transcription of P1 and P2 and for translation of *proV* are at positions 438, 628, and 688, respectively.) Other plasmids described below were constructed in this study by following the general procedures for manipulation of recombinant DNA described by Sambrook et al. (44).

(i) **pHYD351.** A *SacI* fragment derived from pHYD275, bearing the *proU* region from 376 to 552, was cloned into the *SacI* site of the multiple-cloning-site region (MCS) in pCU22 to generate pHYD351 (see Fig. 1).

(ii) **pHYD298 and pHYD355.** The *proU* region from 376 to 1631 in pHYD272 was transferred (via several intermediate vectors) into pSelect, and a site-directed T-to-G mutation was introduced at position 676 in the *proU* sequence by using the oligonucleotide 5'-CAATAGAAAGCTTCCTTTATT-3' as the mutagenic primer, and the kit and protocol for site-directed mutagenesis on pSelect were provided by Promega Corp. This mutation creates a *HindIII* site at positions 674 to 679 in *proU*, and the *proU* region from 376 to 675 was thus introduced on a *PstI-HindIII* fragment into the appropriate sites of the MCS in pMU575 to generate pHYD298. The same region was subcloned on a *PstI-KpnI* fragment from pHYD298 into the MCS of pCU22 to generate pHYD355 (see Fig. 1).

**Nucleoid proteins.** H-NS was purified by the method of Tanaka et al. (52). Purified HU and IHF preparations were a kind gift from K. Imamoto.

**In vitro transcription reactions.** For the preparation of naturally supercoiled plasmid DNA templates, pHYD351 or pHYD355 transformants of strain DH5 $\alpha$  were grown in Luria-Bertani medium (36) supplemented with ampicillin at 50  $\mu$ g/ml, and plasmids were isolated with the aid of a commercially available plasmid purification kit (Qiagen GmbH, Hilden, Germany) and associated protocols.

RNA polymerase core enzyme, devoid of  $\sigma^{70}$  and  $\sigma^S$ , was purified following three cycles of phosphocellulose chromatography (27, 48).  $E\sigma^{70}$  and  $E\sigma^S$  were reconstituted as described previously (10, 27, 48) by mixing (in a molar ratio of 1:4) the highly purified core RNA polymerase with purified  $\sigma^{70}$  and purified  $\sigma^S$ , respectively, and incubating the mixtures at 37°C for 10 min.

Single-round transcription reactions were carried out as described previously (22, 59). A total of 0.15 pmol of supercoiled plasmid DNA template and 1 pmol (core enzyme concentration) of either  $E\sigma^{70}$  or  $E\sigma^S$  were mixed in a total volume of 35  $\mu$ l of transcription buffer (50 mM Tris-HCl [pH 7.8 at 37°C], 3 mM magnesium acetate, 0.1 mM EDTA, 0.1 mM dithiothreitol, 50 mM NaCl, and 25  $\mu$ g of bovine serum albumin [nuclease free; Boehringer-Mannheim] per ml) and incubated at 37°C for 20 min to permit the formation of open complexes (preincubation mixture). A substrate-heparin mixture dissolved in 15  $\mu$ l of the same transcription buffer was then added to achieve the following final concentrations: 160  $\mu$ M (each) ATP, GTP, and CTP; 50  $\mu$ M UTP; 2  $\mu$ Ci of [ $\alpha$ - $^{32}$ P]UTP (Amersham); and 200  $\mu$ g of heparin per ml. When required, potassium glutamate, glycine betaine, or L-proline was added to both the preincubation mixture and the substrate-heparin mixture; on the other hand, the proteins H-NS, HU, and IHF were added to the DNA template in transcription buffer (i.e., preincubation mixture without enzyme) and incubated for 20 min at 37°C, and then  $E\sigma^{70}$  or  $E\sigma^S$  was added so as to constitute the preincubation mixture in a 35- $\mu$ l volume (55). After 5 to 8 min of incubation of the 50- $\mu$ l transcription mixture at 37°C, the reaction was terminated by the addition of 50  $\mu$ l of ice-cold stop solution (40 mM EDTA, 300  $\mu$ g of *E. coli* tRNA per ml). After precipitation with ethanol, the nucleic acids were dissolved in 15  $\mu$ l of sample buffer (80% [vol/vol] deionized formamide, 8 mM EDTA, and 0.01% each bromophenol blue and xylene cyanol), denatured by heating to 90°C and quick chilling, and analyzed by electrophoresis on 5% denaturing polyacrylamide gels with 8 M urea (44). Radiolabelled transcripts that had been separated on the gels were detected by

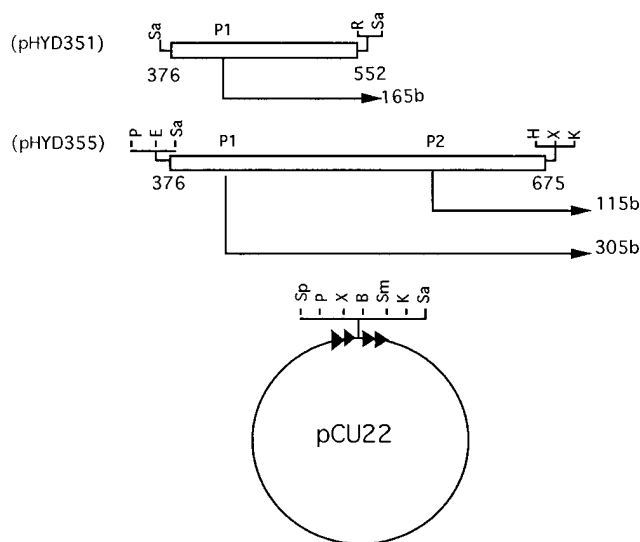


FIG. 1. Structures of and *proU* transcripts from plasmids pHYD351 and pHYD355. The circular map of vector pCU22 (55) is shown at the bottom, and the unique restriction sites in the MCS are marked. Double arrowheads represent tandem pairs of phage *fd terS* terminator sequences situated upstream (left) and downstream (right) of the MCS. At the top are the structures of the inserts cloned into pCU22 to construct plasmids pHYD351 and pHYD355. Open bars represent *proU* DNA (the nucleotide coordinates of whose two ends are indicated), while the thin lines represent flanking sequences from the MCSs of other plasmid vectors. The start sites of transcription from the P1 (position 438) and P2 (position 628) promoters are marked to scale in the *proU* DNA segments, and arrows indicate the direction and extent of transcription from the *proU* promoters; the lengths of transcripts obtained following termination at the proximal *fd terS* terminator downstream of the MCS in pCU22 are indicated in bases (b). Abbreviations for restriction sites: B, *Bam*HI; E, *Eco*RI; H, *Hind*III; K, *Kpn*I; P, *Pst*I; R, *Eco*RV; Sa, *Sac*I; Sm, *Sma*I; Sp, *Sph*I; X, *Xba*I.

autoradiography and densitometrically quantitated on a Molecular Dynamics computing densitometer. To obtain size markers for the gels, a 485-bp *Bam*HI fragment bearing the *dnaQ* P1 (310 bases), *dnaQ* P2 (224 bases), and *mh* (281 bases) promoters (39) was used as a template for  $E\sigma^{70}$ -directed transcription as described above (transcript sizes are in parentheses); the 108-base RNA I transcript (53) from pHYD351 and pHYD355 DNA also served as a size marker on the gels.

**$\beta$ -Galactosidase assays.** Cultures of chromosomal  $\Delta lac$  strains carrying the pMU575-derived plasmids were grown to mid-exponential phase at 30°C in low-osmolarity medium (K medium [14]) supplemented with 40  $\mu$ g of trimethoprim per ml and optionally with 0.3 M NaCl.  $\beta$ -Galactosidase assays were done on these cultures by the method of Miller (36) after treatment with sodium dodecyl sulfate-chloroform, and the specific enzyme activities are specified in the units defined in reference 36.

#### RESULTS AND DISCUSSION

**$E\sigma^S$ - and  $E\sigma^{70}$ -directed transcription from supercoiled templates bearing P1 and P2.** A recent study (10) performed in one of our laboratories was unable to demonstrate  $E\sigma^S$ -directed transcription in vitro from the P1 promoter of *proU*. However, that study had employed standard linear DNA templates in runoff transcription experiments. In light of both in vivo and in vitro evidence that *proU* transcription is supercoiling sensitive (19, 43, 55), we decided to reexamine the in vitro activities of the P1 and P2 promoters, borne this time on supercoiled templates.

Plasmids pHYD351 and pHYD355 were constructed from the vector pCU22 (55) as described above and carry, respectively, the P1 promoter and both the P1 and P2 promoters upstream of a tandem pair of strong transcription terminator sequences (Fig. 1). Neither plasmid carries the DNA sequence (from the 5' end of *proV*) which functions as the NRE in vivo. Single-round transcription from supercoiled pHYD351 and

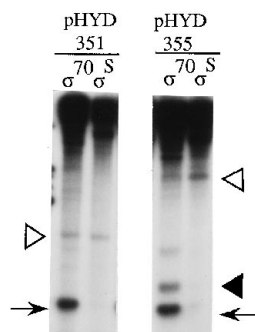


FIG. 2.  $E\sigma^{70}$ - and  $E\sigma^S$ -directed transcription in vitro from supercoiled pHYD351 and pHYD355. Symbols:  $\rightarrow$ , RNA-I;  $\blacktriangleright$ , P2 transcript;  $\triangleright$ , P1 transcript.

pHYD355 DNA templates was carried out as described above by using holoenzymes that had been reconstituted with a highly purified preparation of *E. coli* RNA polymerase core enzyme and either  $\sigma^S$  or  $\sigma^{70}$ ; the 108-base RNA I transcript (53) from the vector DNA served as a convenient size marker and internal control in these experiments.

The results shown in Fig. 2 established that, as expected, transcription from the P2 promoter is achieved nearly exclusively by  $E\sigma^{70}$ . On the other hand, transcription from P1 (in both pHYD351 and pHYD355) was observed with both  $E\sigma^{70}$  and  $E\sigma^S$ , somewhat more so with the latter (see also Fig. 3 and 4). The RNA I transcript was prominent only in transcription reactions carried out with  $E\sigma^{70}$ . The evidence that had been obtained by us earlier for the existence of the P1 promoter, which is  $\sigma^S$  dependent in vivo (8, 15, 31), therefore was corroborated by the in vitro experiments. It may be noted that a majority of the promoters that are *rpoS* dependent in vivo are also transcribed in vitro by both  $E\sigma^S$  and  $E\sigma^{70}$  under the standard assay conditions (10, 25, 27, 37, 48, 50). Our data are also consistent with the proposal by Espinosa-Urgel and Tormo (12) that  $E\sigma^S$ -dependent promoters are located in regions of intrinsic DNA curvature, since the P1 promoter region of *proU* is associated with just such a bend (15, 30, 49, 51).

**Potassium glutamate effects on transcription from P1 and P2.** We examined the effects of potassium glutamate (up to 0.3 M) on  $E\sigma^S$ - and  $E\sigma^{70}$ -directed transcription from pHYD351 and pHYD355 DNA templates (Fig. 3).  $E\sigma^{70}$ -mediated transcription from P2 in pHYD355 was increased in the presence of potassium glutamate (Fig. 3A, lanes 10 to 12, and Fig. 3B, lanes 5 and 6), which is consistent with the earlier in vitro data (10, 43, 55). Another  $E\sigma^{70}$ -generated transcript band from the pHYD355 template, of a size intermediate between those marked for the P2 and P1 messages and substantially stimulated by potassium glutamate (most prominently seen in Fig. 3A, lane 12), also represents transcripts initiated from the P2 promoter itself which have terminated at the second transcription terminator situated 40 bp downstream from the first (55).  $E\sigma^S$ -mediated transcription of P1 was also directly stimulated by potassium glutamate (Fig. 3A, lanes 4 to 6 and 7 to 9, and Fig. 3B, lanes 7 and 8).

Csonka et al. (7) have recently suggested that the activation by potassium glutamate of transcription in vitro might be non-specific, and we too observed that synthesis of the vector RNA I transcript by both  $E\sigma^{70}$  (Fig. 3A, lanes 1 to 3 and 10 to 12, and Fig. 3B, lanes 3 to 6) and  $E\sigma^S$  (Fig. 3A, lanes 7 to 9, and Fig. 3B, lanes 7 and 8) was stimulated by the salt. On the other hand, potassium glutamate has been shown to inhibit transcription in vitro from the *lacUV5* (10, 27) and *alaS* (26) promoters

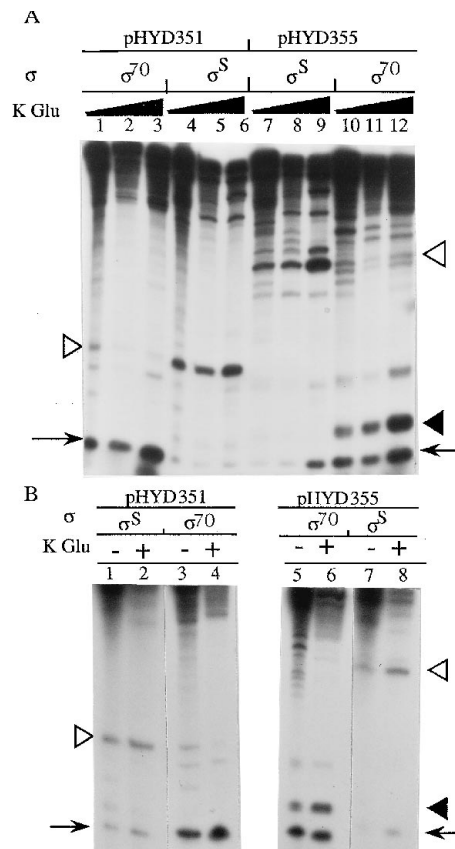


FIG. 3. Potassium glutamate effects on transcription in vitro from supercoiled pHYD351 and pHYD355. Panels A and B show results from two independent experiments. Transcript symbols are defined in the legend to Fig. 2. K Glu, potassium glutamate. Each wedge in panel A represents increasing concentrations of added potassium glutamate (0, 0.15, and 0.3 M) from left to right. - and +, 0 and 0.3 M potassium glutamate, respectively.

under conditions identical to those used in this study, and Prince and Villarejo (43) have also shown that the salt inhibits transcription from the *lac*, *pepN*, and *bla* promoters. Thus, the question of whether the direct effect of potassium glutamate on *proU* P1 and P2 transcription is specific or nonspecific remains an open one, although the hypothesis for a specific role is attractive (11, 16, 43, 47).

The other interesting finding from this set of experiments was that of an enhancement of  $E\sigma^S$  selectivity at the P1 promoter in the presence of potassium glutamate. The enhancement was a consequence of both an increase in  $E\sigma^S$ -directed transcription (see above) and a decrease in  $E\sigma^{70}$ -directed transcription (Fig. 3A, lanes 1 to 3 and 10 to 12, and Fig. 3B, lanes 3 to 6) from this promoter. A similar increase, in the presence of potassium glutamate, of  $E\sigma^S$  selectivity at promoters for two other osmoreponsive genes, *osmB* and *osmY* (which are *rpoS* controlled in vivo [17, 18, 58]), was observed earlier (10, 27). Our findings therefore confirm that ionic strength is an important determinant of  $E\sigma^S$  selectivity at particular promoters. Other mechanisms that have also been postulated to influence such selectivity include promoter sequence determinants (25, 48), DNA superhelicity (27), and the binding of particular protein factors (1, 3, 32).

**Effects of H-NS on P1 and P2 in vitro.** In vitro transcription from the pHYD351 and pHYD355 templates was studied in the presence of increasing concentrations of H-NS (Fig. 4).

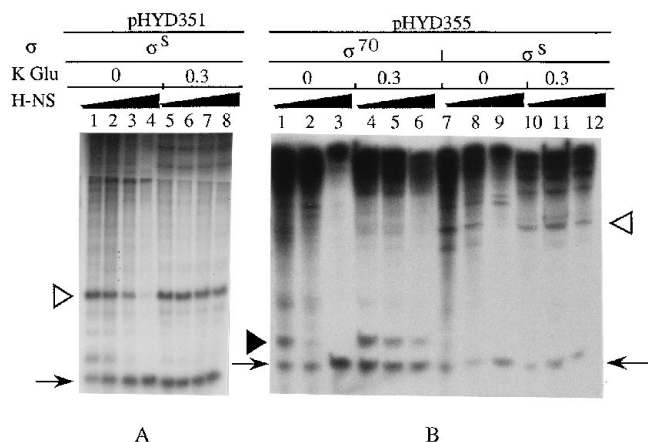


FIG. 4. Effects of H-NS and potassium glutamate on in vitro transcription from supercoiled pHYD351 (A) and pHYD355 (B). Transcript symbols are defined in the legend to Fig. 2. K Glu, potassium glutamate (whose molar concentrations are indicated). Each of the wedges represents increasing amounts of added H-NS (0, 10, 20, and 50 pmol in panel A; 0, 10, and 50 pmol in panel B) from left to right.

Both  $E\sigma^{70}$ -directed transcription from P2 on pHYD355 (Fig. 4B, lanes 1 to 3) and  $E\sigma^S$ -directed transcription from P1 on pHYD351 (Fig. 4A, lanes 1 to 4) or pHYD355 (Fig. 4B, lanes 7 to 9) were inhibited by H-NS, and P2 was more sensitive to such inhibition than was P1. Inhibition of P1 transcription is probably a consequence of high-affinity H-NS binding to the bent-DNA motif in this region (30, 42, 49). Synthesis of the control RNA-I transcript was not inhibited, and was in fact stimulated two- to threefold, by the addition of H-NS (compare lanes 1 and 3 in Fig. 4B or lanes 1 and 4 in Fig. 4A). Inhibition of P1 and P2 transcription by H-NS was greatly reduced in the presence of potassium glutamate (Fig. 4A, lanes 5 to 8, and Fig. 4B, lanes 4 to 6 and 10 to 12), and there was some evidence that this relief of inhibition might be specific for *proU* (compare, for example, the degree of general inhibition of background radiolabel incorporation in lanes 3 and 6 in Fig. 4B with the intensities of the P2 transcript in these two lanes). Densitometric analysis of bands on the autoradiograms depicted in Fig. 4 indicated that the residual extent of P2 transcription following addition of 10 pmol of H-NS (compared with no H-NS addition) was 13 and 56%, respectively, in the absence and presence of potassium glutamate. The corresponding values for residual P1 transcription following the addition of 20 pmol of H-NS were 55 and 94%, respectively. The relief of H-NS inhibition at P2 by potassium glutamate had been observed by Ueguchi and Mizuno (55), and we sug-

gest that this result is of physiological relevance in *proU* osmotic regulation.

It may be noted that the *proU* region from 376 to 675 carried by pHYD355 is expected not to include the NRE (8, 13, 30, 40, 42), which had earlier been identified as the site for high-affinity binding of H-NS (30, 42, 54). In vivo-expression experiments using plasmid pHYD298, which bears a *lacZ* operon fusion to the *proU* region from 376 to 675, yielded results which were in conformity with these expectations. Thus,  $\beta$ -galactosidase expression from pHYD298 in the *hms*<sup>+</sup> strain MC4100 was substantially derepressed at low osmolarity in comparison with that from plasmid pHYD272, which carries the full-length *cis*-regulatory region of *proU*, including the NRE (Table 1). Furthermore, in the *hms* mutant GJ1330, expression from pHYD272 was partially derepressed at low osmolarity whereas that from pHYD298 was not significantly higher than in the *hms*<sup>+</sup> strain (Table 1). Similar results have been described by other workers, with the reported change in the expression of *proU* at low osmolarity (and, consequently, the magnitude of osmotic inducibility) in the *hms* mutants varying from 6- to 40-fold (8, 13, 19, 30, 34, 45).

Thus, whereas the in vivo results indicate the need for NRE in mediating *proU* repression by H-NS, the data from this study demonstrate that H-NS represses P2 transcription in vitro even from templates lacking the NRE. Nevertheless, this inhibition is not nonspecific, because control RNA-I synthesis is not inhibited, and Ueguchi and Mizuno (55) have shown that *tac* promoter transcription is also not inhibited under identical reaction conditions. To explain the observed difference between the in vivo and in vitro results, we suggest that H-NS represses *proU* ultimately by binding in the vicinity of P1 and P2 but that its effective concentration in vivo is sufficiently low as to need the high-affinity binding site offered by the NRE to initiate the formation of a local nucleoprotein structure (30, 54), whereas in vitro the concentration of H-NS employed may have been higher. Because the full-length NRE resides on a 600-bp-long segment downstream of P2 (8), it is technically not feasible to undertake in vitro transcription studies with H-NS using templates bearing the entire NRE. Another potential complicating factor in the in vitro experiments may have been the existence of a high-affinity H-NS-binding site in the vicinity of the  $\beta$ -lactamase gene in the pCU22 plasmid vector (30, 60).

The inhibitory effect of H-NS on P1 transcription in vitro is also in contrast to the observations in vivo, where *hms* mutations lead to reduced P1 expression (8) (see also Table 1 for results with plasmid pHYD275, which carries a P1-*lacZ* fusion). The in vivo results therefore probably reflect an indirect action of H-NS, which may involve changes in either DNA topology or the concentration of other regulatory protein factors (19–21). Mutations in *hms* actually lead to increased RpoS

TABLE 1. *hms* mutant effect on *proU* expression in vivo<sup>a</sup>

Plasmid	<i>proU</i> extent <sup>b</sup> (regulatory element[s])	$\beta$ -Galactosidase sp act <sup>c</sup>			
		MC4100		GJ1330	
		Without NaCl	With NaCl	Without NaCl	With NaCl
pHYD272	376–1631 (P1 + P2 + NRE)	6	518	46	1,848
pHYD275	376–552 (P1)	204	677	73	370
pHYD298	376–675 (P1 + P2)	190	712	216	1,775

<sup>a</sup> Derivatives of MC4100 (wild type) and GJ1330 (*hms*-200) carrying the indicated pMU575-derived plasmids were grown in K medium without or with supplementation with 0.3 M NaCl for  $\beta$ -galactosidase assays.

<sup>b</sup> The nucleotide positions of the left and right ends of the *proU* sequence carried on each plasmid (and the associated *cis* elements in parentheses) are marked.

<sup>c</sup> Expressed in Miller units.

synthesis in vivo (4, 57), but some *rpoS*-controlled genes, such as *csiD*, appear to behave like *proU* P1 in exhibiting reduced expression in *hns* mutants (4).

**Effects of other nucleoid proteins and compatible solutes on *proU* in vitro.** In view of the finding that potassium glutamate affects *proU* transcription in vitro, we also examined the effects on such transcription of two other compatible solutes, L-proline and glycine betaine, which are known to accumulate in cells grown at elevated osmolarity (6). Neither solute, at concentrations up to 0.3 M, had any effect (activating or inhibitory) on  $E\sigma^S$ - and  $E\sigma^{70}$ -directed transcription from the P1 and P2 promoters on pHYD355 (data not shown).

Manna and Gowrishankar (31) had found that mutants defective in HU or IHF displayed reduced *proU-lac* expression. At amounts up to 21 pmol (HU) and 6.6 pmol (IHF) tested in vitro, these proteins did not affect P1 or P2 transcription, either in the presence (0.3 M) or absence of potassium glutamate (data not shown), suggesting that these proteins also act indirectly to affect *proU* expression in vivo.

**Concluding remarks.** We have demonstrated in this in vitro study the existence of two promoters, P1 and P2, for *proU*. We have also confirmed the participation of  $E\sigma^S$  in *proU* transcription, which has been documented for a variety of other osmoreponsive operons in *E. coli* (17, 18, 24, 35, 58). Three effects of potassium glutamate on *proU* transcription in vitro were identified, namely, (i) direct activation of P1 transcription by  $E\sigma^S$  (and P2 transcription by  $E\sigma^{70}$ ), (ii) inhibition of P1 transcription by  $E\sigma^{70}$  (both together leading to the increase in selectivity for  $E\sigma^S$  at P1), and (iii) antagonism of inhibition of H-NS. Direct activation of P1 and P2 transcription might be mediated through increased DNA twist (56). Although, as discussed above, questions as to the specificity of the potassium glutamate effects and the physiological relevance of the P1 promoter in *proU* regulation persist, we suggest that the various in vitro effects observed in this study are relevant to *proU* regulation in vivo and that the P2 and P1 promoters contribute to osmolarity-dependent expression of the operon in the exponential and stationary phases of growth, respectively. Csonka et al. (7) have suggested that glutamate itself is not involved in *proU* transcriptional regulation, and it is therefore possible that the physiological correlates of our in vitro observations are effected by  $K^+$  ions alone (47).

There is evidence from in vivo studies that factors such as the higher-order chromatin organization at the *proU* locus, as influenced by the curvature and supercoiling of DNA and by the binding of nucleoid proteins (20, 42, 51), may be important in its transcriptional regulation from both the P1 and P2 promoters. The future challenge will be to devise novel methods for testing such hypotheses by genetic and in vitro experiments.

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