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 $\sigma^{70}$ 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 different selectivities 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 (Er$^{70}$) (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 also RpoS ($\sigma^S$)-dependent and stationary-phase inducible (31). We have designated the two (proV-proximal and proV-distal) promoters P1 and P2, respectively. The role of the upstream P1 promoter and $\sigma^S$-RNA polymerase (Er$^{70}$) 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 Er$^{70}$ (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 Er$^{70}$ 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
activates in vitro transcription from P2 on a linear template in the absence of H-NS.

In this study, we have examined Er<sup>S</sup>- and Er<sup>70</sup>-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 Er<sup>S</sup> dependent. The data also provide insights into the possible roles of potassium glutamate and H-NS in regulation.

MATERIALS AND METHODS

E. coli strains and plasmids. Strain DH5α (44) was used in the cloning experiments and for the preparation of DNA templates for in vitro transcription. MC14100 (8) and GJH330 (8), used in the experiments with promoter-lac fusions, are isogenic Δlac his<sup>R</sup> and bnu-200 derivatives, respectively.

The plasmid vectors included (i) the low-copy-number lacZ operon fusion vector pMUS575 (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 pMUS575 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 et al. (15), according to which the start sites for transcription of P1 and P2 and for translation of proU′ 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) to pSelect, and a site-directed T-to-G mutation was introduced at position 675 in the proU sequence by using the oligonucleotide 5′-CATAAGAAAAGCCTCCTTAT-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 pCUlHindIII fragment into the appropriate sites of the MCS in pMUS575 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 DH5α were grown to mid-exponential phase at 30°C in low-osmolarity medium (K medium (14)) supplemented with 40 μM of tri-methoprime per ml and optionally with 0.3 M NaCl. β-Galactosidase assays were done on these cultures by the method of Miller (36) after treatment with sodium dodecyl sulfate-lysozyme, and the specific enzyme activities are specified in the units defined in reference 36.

RESULTS AND DISCUSSION

Er<sup>S</sup>- and Er<sup>70</sup>-directed transcription from supercoiled templates bearing P1 and P2. A recent study (10) performed in one of our laboratories was unable to demonstrate Er<sup>S</sup>-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
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 $\sigma^70$. On the other hand, transcription from P1 (in both pHYD351 and pHYD355) was observed with both $\sigma^70$ and $\sigma^S$, 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 $\sigma^70$. 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 $\sigma^S$ and $\sigma^70$ under the standard assay conditions (10, 25, 37, 48, 50). Our data are also consistent with the proposal by Espinosa-Urgel and Tormo (12) that $\sigma^S$-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 $\sigma^S$- and $\sigma^70$-directed transcription from pHYD351 and pHYD355 DNA templates (Fig. 3). $\sigma^70$-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 $\sigma^70$-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).

Eor$^S$-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 nonspecific, and we too observed that synthesis of the vector RNA I transcript by both Eor$^70$ (Fig. 3A, lanes 1 to 3 and 10 to 12, and Fig. 3B, lanes 3 to 6) and Eor$^S$ (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 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 Eor$^S$ selectivity at the P1 promoter in the presence of potassium glutamate. The enhancement was a consequence of both an increase in Eor$^S$-directed transcription (see above) and a decrease in Eor$^70$-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 Eor$^S$ 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 Eor$^S$ 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).
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 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 suggest 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, β-galactosidase expression from pHYD298 in the hns strain 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 hns mutant GJ1330, expression from pHYD272 was partially derepressed at low osmolarity whereas that from pHYD298 was not significantly higher than in the hns 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 hns 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 β-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 hns 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 hns actually lead to increased RpoS

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**TABLE 1. hns mutant effect on proU expression in vivo**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>proU extent(^a)</th>
<th>β-Galactosidase sp act(^c)</th>
<th>MC4100</th>
<th>GJ1330</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(regulatory element[s])</td>
<td></td>
<td>Without NaCl</td>
<td>With NaCl</td>
</tr>
<tr>
<td>pHYD272</td>
<td>376–1631 (P1 + P2 + NRE)</td>
<td>6</td>
<td>518</td>
<td>46</td>
</tr>
<tr>
<td>pHYD275</td>
<td>376–552 (P1)</td>
<td>204</td>
<td>677</td>
<td>73</td>
</tr>
<tr>
<td>pHYD298</td>
<td>376–675 (P1 + P2)</td>
<td>190</td>
<td>712</td>
<td>216</td>
</tr>
</tbody>
</table>

\(^a\) Derivatives of MC4100 (wild type) and GJ1330 (hns-200) carrying the indicated pM5757-derived plasmids were grown in K medium without or with supplementation with 0.3 M NaCl for β-galactosidase assays.

\(^b\) 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.

\(^c\) Expressed in Miller units.
synthesis in vivo (4, 57), but some ptsS-controlled genes, such as csiD, appear to behave like proU P1 in exhibiting reduced expression in hms 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 Eos and Eos-directed transcription from the P1 and P2 promoters on phypD555 (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 Eos in proU transcription, which has been documented for a variety of other osmoregulatory 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 Eos (and P2 transcription by Eos), (ii) inhibition of P1 transcription by Eos (both together leading to the increase in selectivity for Eos 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 P1 and P2 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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