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In Vivo Expression from the RpoS-Dependent P1 Promoter of the Osmotically Regulated *proU* Operon in *Escherichia coli* and *Salmonella enterica* Serovar Typhimurium: Activation by *rho* and *hns* Mutations and by Cold Stress

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Unlike the σ^{70}-controlled P2 promoter for the osmotically regulated *proU* operon of *Escherichia coli* and *Salmonella enterica* serovar Typhimurium, the σ^{s}-controlled P1 promoter situated further upstream appears not to contribute to expression of the *proU* structural genes under ordinary growth conditions. For *S. enterica proU* P1, there is evidence that promoter crypticity is the result of a transcription attenuation phenomenon which is relieved by the deletion of a 22-base C-rich segment in the transcript. In this study, we have sought to identify growth conditions and *trans*-acting mutations which activate in vivo expression from *proU* P1. The cryptic *S. enterica proU* P1 promoter was activated, individually and additively, in a *rho* mutant (which is defective in the transcription termination factor Rho) as well as by growth at 10°C. The *E. coli proU* P1 promoter was also cryptic in constructs that carried 1.2 kb of downstream *proU* sequence, and in these cases activation of in vivo expression was achieved either by a *rho* mutation during growth at 10°C or by an *hns* null mutation (affecting the nucleoid protein H-NS) at 30°C. The *rho* mutation had no effect at either 10 or 30°C on in vivo expression from two other σ^{s}-controlled promoters tested, those for *osmY* and *csiD*. In cells lacking the RNA-binding regulator protein Hfq, induction of *E. coli proU* P1 at 10°C and by *hns* mutation at 30°C was still observed, although the *hfq* mutation was associated with a reduction in the absolute levels of P1 expression. Our results suggest that expression from *proU* P1 is modulated both by nucleoid structure and by Rho-mediated transcription attenuation and that this promoter may be physiologically important for *proU* operon expression during low-temperature growth.

The ProU transporter in *Escherichia coli* and *Salmonella enterica* serovar Typhimurium is a binding-protein-dependent transport system that mediates the cytoplasmic accumulation of compatible solutes such as glycine betaine, L-proline, and related compounds during growth of cells in media of elevated osmolarity (9, 10). The subunit polypeptides of the transporter are encoded by three genes, *proV*, *proW*, and *proX*, which together constitute (in that order) the *proU* operon (16).

Transcription of *proU* in both *E. coli* and *S. enterica* is activated several-hundredfold in cultures grown in high-osmolarity media, but the mechanism of osmotic induction of the operon is not fully understood (reviewed in references 10, 19, and 29). Two *cis* regulatory elements that have been identified (see Fig. 1) include a σ^{70}-driven promoter whose transcription start site is situated 60 bases upstream of *proV* (16, 24, 28, 54) and a negative regulatory element (NRE) approximately 500 bp long, which is situated downstream of the promoter (overlapping the *proV* coding region) and whose deletion results in partial derepression of *proU* at low osmolarity (11, 24, 37, 38). Mutations in *hns*, the gene encoding an abundant nucleoid protein, H-NS, also result in partial derepression of *proU* expression (for a review of H-NS, see reference 58); two regions of curved DNA exist in the *proU cis* regulatory region, one falling within the *proU* NRE and the other located about 200 bp upstream of the promoter (16, 38, 50, 51), to both of which H-NS exhibits preferential binding (30, 38, 50).

Also situated upstream is a second promoter, whose role (if any) in *proU* regulation is still enigmatic (17). For convenience, the upstream and downstream promoters are designated P1 and P2, respectively (Fig. 1). The two promoters are 190 bp apart and are oriented to transcribe in the same direction, that is, toward the *proU* structural genes. Data from in vivo and in vitro studies have shown that P1 transcription is moderately osmoresponsive and absolutely dependent on the stationary-phase sigma factor RpoS or σ^{s} (11, 16, 31, 42, 43). However, the physiological function of P1 is unclear for the reasons that (i) *cis* constructs that carry P2 with the NRE but have P1 deleted continue to exhibit normal osmotic regulation of reporter gene expression (28), (ii) *cis*- or *trans*-acting mutations that affect P2 activity abolish all expression from constructs that carry both P1 and P2 (34, 60, 61), and (iii) *proU* osmotic regulation is unaffected in *rpoS* mutants that lack RpoS (31).

One clue to the paradox of the P1 promoter has been the finding that, at least in *S. enterica*, the promoter is rendered cryptic in vivo because of transcription attenuation occurring in the leader region between P1 and P2. Attenuation was not observed in a defined in vitro transcription system, leading to the suggestions that the phenomenon is factor dependent and therefore that, under particular culture conditions (hitherto unidentified), attenuation is relieved and P1 may be able to transcribe the *proU* structural genes (42).

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TABLE 1. List of *E. coli* K-12 strains[a]

Strain	Mutation(s)
GJ11	*proU224*::*lac*
GJ862	Nil
GJ863	*rho*
GJ866	*hns*
GJ867	*rho hns*
GJ870	*rpoS*
GJ871	*rho rpoS*
GJ872	*hns rpoS*
GJ873	*rho hns rpoS*
GJ884	*csiD*::*lac*
GJ885	*csiD*::*lac rho*
GJ887	*csiD*::*lac rho rpoS*
GJ888	*osmY*::*lac*
GJ889	*osmY*::*lac rho*
GJ891	*osmY*::*lac rho rpoS*
GJ2733	*csiD*::*lac rpoS*
GJ2734	*osmY*::*lac rpoS*
GJ2739	*csiD*::*lac hns*
GJ2741	*osmY*::*lac hns*
GJ2743	*proU224*::*lac rpoS*
GJ2745	*hfq-2*
GJ2746	*hfq-1*
GJ2748	*hfq-1 hns*
GJ2750	*csiD*::*lac hns rpoS*
GJ2751	*osmY*::*lac hns rpoS*
GJ2752	*hfq-1 hns rpoS*

[a] All listed strains are F$^-$ and, with the exception of GJ11 (15), were constructed in this study. All strains are derivatives of MC4100 (15), and mutations for each in addition to those present in this strain are listed. Genotype designations are as in the work of Berlyn (6). The complete genotype of MC4100 is Δ(*argF-lac*)*U169 rpsL 150 relA1 araD139 flbB5301 deoC1 ptsF25*. Allele numbers of the listed mutations and the strains (sources) from which they were transferred by P1 transduction are as follows: *rho*, *rho-4*(Am) from CGSC5072 (*E. coli* Genetic Stock Center); *hns*, *hns-206*::Ap from PD32 (12); *rpoS*, *rpoS359*::Tn*10* from RH90 (5); *csiD*::*lac*, *csiD*::λp*lac*Mu15 from DW12 (5); *osmY*::*lac* (previously called *csi-5*::*lac*), *osmY*::λp*lac*Mu55 from RO151-a (5); *hfq-1*, *hfq-1*::Ω from TX2822 (M. E. Winkler strain derived from TX2808 [53]); and *hfq-2*, *hfq-2*::Ω from TX2765 (M. E. Winkler strain derived from TX2758 [53]).

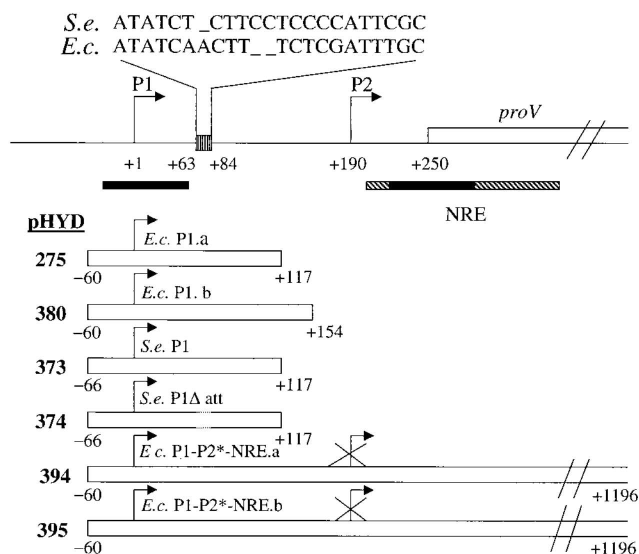

FIG. 1. Description of plasmids used for studies of in vivo expression from the *proU* P1 promoter. The line on top depicts the proximal region of the *proU* operon, and the positions of the *cis* regulatory elements (namely, P1, P2, and the NRE) are marked relative to that of its first structural gene, *proV*. Nucleotide number designations are given, with the start site of P1 transcription taken as +1. Solid bars, in the vicinity of P1 and within the NRE, mark the positions of bent-DNA motifs in this region. The box with vertical stripes depicts the location of a 22-base C-rich segment on the P1-initiated transcript of *S. enterica* (*S.e.*). The sequence of this segment from *S. enterica* and from *E. coli* (*E.c.*) is shown above the line. Beneath are represented (by the individual open bars) the extents of DNA from the *proU* regulatory region carried upstream of the *lacZ* reporter gene in the single-copy-number plasmids (whose pHYD number designations, and abbreviated descriptions, are given alongside) that were used for the in vivo expression studies. Deletion of the 22-base region in pHYD374 is denoted by the interrupted line segment. Note that the P2 promoter in each of the plasmids pHYD394 and pHYD395 has been mutationally inactivated.

In this study, we have sought to identify growth conditions as well as *trans*-acting mutations that relieve in vivo crypticity of the *proU* P1 promoter. Reporter gene expression from constructs carrying P1 was shown to be increased in an RpoS-specific manner by mutations in *rho* (the gene for transcription termination factor Rho [reviewed in references 21, 44, and 56]) and *hns* and by the growth of cultures at 10°C. Our results suggest that the P1 promoter is involved in expression of the *proU* operon during cold stress and provide additional support for the hypothesis that transcription initiated from the promoter is regulated by transcription attenuation.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* K-12 strains that were used are listed in Table 1. The high-copy-number plasmid vector used was pBluescript II KS (Stratagene, La Jolla, Calif.). Derivatives of the IncW-based single-copy-number plasmid vectors pMU575 and pMU2385 (both of which encode trimethoprim resistance and carry the *lacZ* reporter gene downstream of a multiple cloning site [MCS] region) (2, 18) were used to measure in vivo expression of *lacZ* from various *proU* P1-carrying fragments that had been cloned into the MCS region of the vectors. The extent of *proU* DNA (relative to the start site of P1 transcription, taken as +1) carried on each of the plasmids is shown in Fig. 1.

Two derivatives of the vector pMU2385 and four derivatives of pMU575 were employed in this study. The *proU* insert in each of the pMU2385-derived plasmids pHYD394 and pHYD395 is a modification of that in pMU6441 (18), which carries the entire wild-type *E. coli proU cis* regulatory region from −60 to +1196 (relative to P1), including the P1 and P2 promoters and the downstream NRE. The insert of pMU6441 (subcloned into an M13 phage vector) was modified at the P2 promoter by site-specific mutagenesis using either of two different mismatched primers corresponding to the bottom-strand sequence, 5′-ACTTTTTT CTACCC*GG*ACATACTGAGAATC-3′ or 5′-TAGTCACTTTTTTC*GG*CCCT AACATACTGA-3′ (mismatches italicized), so that the −10 region of P2 was changed from TAGGGTA to *CC*GGGTA or TAGGG*CC*, respectively. The modified *proU* inserts were then cloned into the MCS region of vector pMU2385 to generate plasmids pHYD394 and pHYD395, respectively. Therefore, plasmids pHYD394 and pHYD395 each carry the entire *proU cis* regulatory region (from −60 to +1196) with a site-specific inactivation of promoter P2 and are referred to as *E. coli* P1-P2*-NRE.a and *E. coli* P1-P2*-NRE.b, respectively (Fig. 1).

Of the four pMU575-derived plasmids that were used, three (pHYD275, pHYD373, and pHYD374 [Fig. 1]) have been described earlier (11, 42). Plasmids pHYD275 (*E. coli* P1.a) and pHYD373 (*S. enterica* P1) carry the isolated P1 promoters from *E. coli* (−60 to +117) and *S. enterica* (−66 to +117), respectively. Plasmid pHYD374 (*S. enterica* P1Δ*att*) is a derivative of pHYD373 with a 22-bp deletion from +63 to +84 which results in relief of attenuation of the transcripts initiated from P1. Plasmid pHYD380, which is a pMU575 derivative carrying *E. coli proU* P1 from −60 to +154 (*E. coli* P1.b), was constructed as follows. A pBluescript II KS derivative carrying the wild-type *proU* insert (−60 to +1196) of pMU6441 was used as a template in PCR with a pair of primers, 5′-TGTAG*AGATCT*GATGGCAAATGTGG-3′ and 5′-TGTAG*AGATCT*TTT CTATTGCATGGC-3′. The primers were designed to read outwards from within the *proU* insert such that the entire plasmid except the region between +154 and +240 (indicated by the bases marked in boldface for the bottom strand on the first primer and the top strand on the second primer, respectively) was amplified by PCR. Digestion of the PCR product with *Bgl*II (recognition sites in

TABLE 2. *proU* P1-*lac* expression at 30 and 10°C[a]

Temp and GJ *rpoS*$^+$ strain no.[d]	Mutation(s)	β-Galactosidase sp act for P1-*lac*-bearing plasmid (description) on *rpoS*$^+$ strain[b]											
		pHYD373 (*S. enterica* P1)		pHYD374 (*S. enterica* P1Δ*att*)		pHYD275 (*E. coli* P1.a)		pHYD380 (*E. coli* P1.b)		pHYD394 (*E. coli* P1-P2*-NRE.a)		pHYD395 (*E. coli* P1-P2*-NRE.b)	
		−NaCl	+NaCl	−NaCl	+NaCl	−NaCl	+NaCl	−NaCl	+NaCl	−NaCl	+NaCl	−NaCl	+NaCl
30°C													
862 (870)	Nil	4 (<1)	4 (<1)	60 (2)	111 (<1)	63 (4)	275 (8)	51 (5)	277 (8)	3 (2)	6 (3)	2 (1)	6 (3)
863 (871)	*rho*	51 (<1)	69 (3)	136 (5)	159 (4)	126 (15)	322 (18)	73 (13)	291 (16)	4 (3)	11 (4)	3 (2)	8 (6)
866 (872)	*hns*	5 (3)	7 (2)	391 (3)	301 (4)	407 (16)	620 (22)	349 (35)	ND[c]	75 (8)	112 (14)	58 (10)	168 (15)
867 (873)	*rho hns*	58 (5)	82 (2)	472 (7)	331 (5)	393 (27)	500 (38)	ND	ND	106 (27)	207 (41)	80 (23)	251 (35)
10°C													
862 (870)	Nil	54 (2)	22 (2)	728 (6)	288 (4)	962 (16)	665 (16)	ND	ND	5 (4)	8 (7)	5 (1)	10 (1)
863 (871)	*rho*	463 (35)	277 (20)	917 (67)	427 (38)	885 (69)	667 (47)	ND	ND	114 (17)	136 (13)	136 (22)	175 (13)

[a] Isogenic *rpoS*$^+$ and *rpoS* cultures were grown in trimethoprim-supplemented K-tryptone medium without NaCl (−NaCl) or with 0.3 M NaCl (+NaCl) at 30 or 10°C for β-galactosidase assays. Enzyme specific activity values are reported in Miller units (35). Values in parentheses are for *rpoS* derivatives.
[b] Plasmid descriptions are as explained in Fig. 1 and its legend.
[c] ND, not determined.
[d] Strain numbers outside and within parentheses designate isogenic *rpoS*$^+$ and *rpoS* derivatives, respectively.

the two primer sequences italicized) and its circularization by ligation yielded a plasmid derivative (pHYD401) with a *proU* insert carrying P1 and the NRE and bearing a unique *Bgl*II site at the site of P2 deletion between nucleotides +154 and +240. The presence of the new *Bgl*II site was exploited to subclone a *proU* fragment, extending from −60 to +154, from pHYD401 into the MCS region of pMU575 so as to generate pHYD380.

Media and growth conditions. For routine experiments, Luria-Bertani (LB) medium (35) and glucose-minimal A medium (35) were used as the nutrient and defined media, respectively, and the incubation temperature for growth was 37°C. Unless otherwise indicated, cultures for β-galactosidase assays were grown with shaking at 10 or 30°C (as specified) in media that were based on either LBON (which is LB medium with NaCl omitted [11]) or a modified version of low-osmolarity K medium (15) in which 0.5% Casamino Acids had been replaced by 1% Bacto Tryptone (Difco) (K-tryptone); in either growth medium, typical culture doubling times at 10°C were around 24 h for *rho*$^+$ strains and 48 h for *rho* mutant strains. When required, K-tryptone medium was supplemented with NaCl to 0.3 M. Concentrations of antibiotics used were as earlier described (31).

The procedure for growth of biofilm cultures was essentially as described previously (41). The bacterial strain was inoculated in 2 ml of K-tryptone medium in a sterile 35-mm-diameter polystyrene petri plate and incubated without shaking for 40 h at 37°C. The liquid medium containing the free-living bacterial forms was carefully removed, and the sessile biofilm bacteria growing on the inner surface of the plate were then harvested in a small volume of fresh medium by repeated and vigorous pipetting.

Experimental techniques. The procedures for phage P1 transduction (15) and recombinant DNA manipulations (45) were as described previously. Mutations in *hns*, *hfq*, and *rpoS* were introduced by P1 transduction, with the aid of antibiotic resistance markers (to ampicillin, kanamycin, and tetracycline, respectively) that were 100% linked to them. The chromosomal *osmY*::*lac* and *csiD*::*lac* fusions were also transduced by selecting for the kanamycin resistance marker situated adjacent to each of them. The *rho-4* mutation was introduced by cotransduction with the *ilv-3164*::Tn*10*Kan marker from the collection of Singer et al. (46), following which the *ilv* marker was crossed out in a second transduction to prototrophy. Site-directed mutagenesis was performed with the aid of a kit from United States Biochemical Corp. and was based on the method of Vandeyar et al. (55).

β-Galactosidase assays were performed by the method of Miller (35), and enzyme specific activity values are reported in Miller units. The component of σ^s-specific expression for any particular combination of promoter-*lacZ* fusion, *trans*-acting chromosomal mutations, and culture growth conditions was calculated as the difference between the β-galactosidase specific activity value for the *rpoS*$^+$ strain and that for an isogenic *rpoS* mutant.

RESULTS

Reporter gene expression from *S. enterica proU* P1: effects of *rho* and *hns* mutations. In an earlier study (42), we had shown that the in vivo expression from an *S. enterica proU* P1 promoter fragment extending from −66 to +117 (*S. enterica* P1) of the *lacZ* reporter gene borne on a very low-copy-number plasmid (pHYD373) is prevented because of transcription attenuation occurring some distance downstream of the site of transcription initiation. Attenuation was relieved, and *lacZ* expression was consequently observed, in strains carrying a mutant plasmid derivative (pHYD374) that had suffered a 22-bp deletion between nucleotides +63 and +84 relative to the transcription start site (*S. enterica* P1Δ*att*). The deleted stretch of nucleotides is C rich on the strand corresponding to the mRNA transcript (Fig. 1), a feature which suggested that it may be the site for loading on mRNA of the transcription termination factor Rho (21, 44). Furthermore, such a C-rich stretch is absent at the corresponding site downstream of the *E. coli* P1 promoter which (when present on a similar fragment extending from −60 to +117) is active for reporter gene expression in vivo (*E. coli* P1.a [Fig. 1]).

Based on these considerations, we examined the effect of a *rho* mutation on expression from *proU* P1 of *S. enterica*. As explained above, all *lac* expression values were obtained in, and are reported for, pairs of isogenic *rpoS*$^+$ and *rpoS* derivatives, whose difference has been taken to represent the in vivo activity of the RpoS-dependent *proU* P1 promoter under the particular test conditions; by these criteria, the plasmid vectors pMU575 and pMU2385 carrying the promoterless *lacZ* reporter gene displayed insignificant RpoS-dependent *lac* expression under any of the conditions tested in this study (data not shown). It may also be noted that the in vivo *proU* P1 activation studies were not amenable to analysis by mRNA primer-extension experiments, in light of our earlier findings (16, 42) that even the cryptic promoter in both *S. enterica* and *E. coli* exhibits normal transcription initiation.

With plasmid pHYD373 (*S. enterica* P1), *lac* expression was absent in the wild-type strain, as expected; in the *rho* mutant, however, there was a marked (at least 12-fold-induced) level of RpoS-dependent *lac* expression which was further elevated moderately in cultures grown with 0.3 M NaCl supplementation (Table 2). With plasmid pHYD374 (*S. enterica* P1Δ*att*), we

observed a 15-fold increase in expression (over pHYD373) even in the wild-type strain and there was only an additional 2-fold effect of the *rho* mutation under these conditions. These results suggest that the Rho factor is involved, directly or indirectly, in rendering the *S. enterica* P1 promoter cryptic and that the nucleotide stretch downstream of the promoter that is identified by the deletion in pHYD374 mediates this effect of Rho.

As described above, the nucleoid protein H-NS binds with high affinity to two regions in *proU*, one of which overlaps the P1 promoter region (30, 38, 50). Furthermore, cells lacking H-NS have elevated σ^s levels (5, 59). We therefore examined the effect of *hns* mutations, alone or in combination with *rho*, on *S. enterica* P1 expression. The wild-type P1 promoter on plasmid pHYD373 was unaffected by *hns* in either the *rho*$^+$ or *rho* mutant strains (Table 2). On the other hand, the deletion derivative pHYD374 displayed a sixfold increase in P1 expression in strains lacking H-NS, and there was a marginal additivity with the *rho* mutation (Table 2). These results are further discussed below.

Environmental stimulus for P1 activation in *S. enterica*: low-temperature growth. In order to determine whether any environmental stimuli could activate expression from the cryptic P1 promoter on plasmid pHYD373, we tested two candidate culture conditions, namely, growth in biofilms and growth at low temperature (10°C). The rationale for undertaking these tests was (i) implication of a role for RpoS in biofilm physiology (1) and a recent report which had suggested that *E. coli proU* is induced in biofilms (41) and (ii) evidence that an untranslated RNA, DsrA, acts to increase RpoS levels during exponential growth at low temperature (48).

No activation of *lac* expression from pHYD373 was observed when cells were grown as biofilms by the protocol described in the earlier report (41); the *lac* expression values for strain GJ862/pHYD373 were 3 and 4 Miller units after growth as free-living cells and as biofilms, respectively. On the other hand, a 12-fold induction was obtained when the strain carrying pHYD373 was cultivated at 10°C (Table 2). This low-temperature induction appeared to be mediated by a mechanism different from that leading to the 12-fold induction at 30°C in the *rho* mutant described above, because the same *rho* mutation also conferred an additional 8-fold activation of P1 expression at 10°C (Table 2).

With the deletion-bearing plasmid pHYD374, growth at 10°C resulted in a remarkably high level of RpoS-dependent *lac* expression (nearly 200-fold more than that for the cryptic wild-type promoter at 30°C) in the *rho*$^+$ strain, which was again only marginally elevated by introduction of the *rho* mutation (Table 2). The *hns* effect on promoter P1 activity at low temperature could not be tested because *hns* mutants are inviable at 10°C (12).

Wild-type *E. coli* P1 behaves like deletion-bearing *S. enterica* P1 in vivo. As noted above, the *E. coli proU* regulatory sequence lacks the C-rich segment whose presence downstream of P1 in *S. enterica* is correlated with transcription attenuation in the latter. Two different plasmids, pHYD275 and pHYD380, which carry the *E. coli proU* P1 promoter sequences from −60 to +117 (*E. coli* P1.a) and from −60 to +154 (*E. coli* P1.b) upstream of the vector-borne *lacZ* reporter gene, respectively, behaved virtually identically to the *S. enterica* deletion plasmid derivative pHYD374 in experiments testing the effects of various conditions on P1 expression in vivo. Thus, at 30°C, each of the two plasmids with *E. coli* P1 showed modest expression in the wild-type strain that was induced around sevenfold in the *hns* mutant and around twofold in the *rho* derivative (Table 2). We had earlier found that expression from pHYD275 (*E. coli* P1.a) is in fact reduced in another *hns* mutant (11, 43), but the latter had carried an uncharacterized missense mutation in *hns* whereas the present results have been obtained with a true null *hns* allele.

Cultures of *rho*$^+$ and *rho* strains with plasmid pHYD275 were also tested after growth at 10°C, and very pronounced *lac* expression was observed (as for pHYD374) even in the *rho*$^+$ *hns*$^+$ background (Table 2).

Expression from *E. coli* P1 in the presence of other *proU cis* regulatory elements. The studies above had been done with the isolated P1 promoters of either *S. enterica* or *E. coli*, in the absence of the other *proU cis* regulatory sequences downstream of around +120 (relative to P1). In order to study how *E. coli proU* P1 expression in vivo might be affected by the presence of additional downstream elements such as the NRE (which is also known to bind H-NS with high affinity [30, 38]), we constructed two *lac* expression derivatives, pHYD394 (*E. coli* P1-P2*-NRE.a) and pHYD395 (*E. coli* P1-P2*-NRE.b), each of which carried the *proU* sequences from −60 to +1196 (that is, encompassing P1, P2, and the NRE) with site-specific mutations in the −10 region of P2 that knocked out the activity of this promoter.

At 30°C, both plasmids pHYD394 and pHYD395 conferred nil *lac* expression in wild-type or *rho* strains; RpoS-dependent expression was induced around 50-fold in the *hns* mutant, and there was a further marginal elevation in the *hns rho* double mutant (Table 2). At 10°C, there was a 30- to100-fold induction of RpoS-dependent expression in the *rho* mutant but none in the wild-type strain (Table 2). Once again, the *hns* effect could not be studied at 10°C because of the problem of inviability of the mutant strains (12).

Effects of *rho* and *hns* mutations and low-temperature growth on other σ^s-dependent promoters. In order to determine whether the conditions activating *proU* P1 were specific for this promoter or common to other σ^s-controlled promoters, we tested the effects of *rho* and *hns* mutations, and of growth at 10°C, on activity in vivo of the promoters for *csiD* and *osmY* (both of which are known to be σ^s dependent).

RpoS-dependent expression of *osmY-lac* in LBON medium was unaffected by *rho* but was increased around 6-fold by *hns* (comparable to that reported earlier [5]) and around 25-fold by growth at 10°C (Table 3). The magnitude of *osmY* expression was growth medium dependent, and the activating effects of *hns* and low temperature were not as pronounced in K-tryptone medium that had been used by us for the *proU* P1 studies (Table 3). On the other hand, the described effects of *rho*, *hns*, and low temperature on *proU* P1 were largely independent of the growth medium used (data not shown). It may also be noted from the data in Table 3 that there is an unexpectedly high component of σ^s-independent *lac* expression in the *osmY-lac rho* strain in K-tryptone medium at 10°C, but the basis for this is not known.

RpoS-dependent expression of *csiD-lac* was not affected by any of the conditions, that is, by *rho* or *hns* mutations or growth at low temperature (Table 3). Once again, we observed a

TABLE 3. *lac* expression from control promoters *osmY* and *csiD*[a]

lac fusion	β-Galactosidase sp act for *rpoS*+ and *rpoS* strains in medium at temp[b]:									
	K-tryptone					LBON				
	30°C			10°C		30°C			10°C	
	w.t.	*rho*	*hns*	w.t.	*rho*	w.t.	*rho*	*hns*	w.t.	*rho*
osmY::*lac*	376 (14)	171 (30)	287 (ND[c])	587 (20)	334 (216)	43 (12)	78 (ND)	257 (12)	835 (36)	489 (ND)
csiD::*lac*	522 (64)	195 (12)	372 (ND)	251 (33)	258 (18)	110 (10)	80 (4)	96 (4)	37 (10)	152 (39)

[a] The following GJ strains were used in the experiment, given in the order wild type (w.t.), *rho* mutant, and *hns* mutant for each with the isogenic *rpoS* derivatives indicated in parentheses: *osmY*::*lac*, 888 (2734), 889 (891), and 2741 (2751); *csiD*::*lac*, 884 (2733), 885 (887), and 2739 (2750).
[b] Cultures of the various *rpoS*+ and isogenic *rpoS* strains were grown in trimethoprim-supplemented K-tryptone or LBON medium at 30 or 10°C for β-galactosidase assays. Enzyme specific activity values are reported in Miller units (35). Values in parentheses are for *rpoS* derivatives.
[c] ND, not determined.

medium dependence in the absolute values of *lac* expression (including a reduction in expression in the wild-type background for LBON medium at 10°C), but the conclusion concerning the absence of effect was valid for both media tested.

In a related context, we also tested the effect of low-temperature growth on activity of the σ^{70}-dependent P2 promoter of *proU*, by measuring *lac* expression from the chromosomal *proU-lac* fusion strain GJ11 and its *rpoS* derivative GJ2743. For both strains, β-galactosidase specific activity values were around 3 and 11 Miller units after growth at 30 and 10°C, respectively, in the low-osmolarity K-tryptone medium, suggesting that the P2 promoter is also not significantly stimulated by cold stress.

Effect of *hfq* on *proU* P1. The RNA-binding protein Hfq has earlier been shown to be a positive regulator of σ^s synthesis at the level of translation (reviewed in references 14, 20, and 23). Mutations in *hfq* have been reported previously to be epistatic to *hns* (36) and *dsrA* (49) with reference to *rpoS* regulation, suggesting that the regulatory effects of both H-NS and DsrA are mediated indirectly via Hfq. In light of these reports, we tested the effects of *hfq* insertion mutations in our *proU* P1 assay systems.

The chromosomal *hfq* gene is part of a complex operon, and one needs to distinguish between a true *hfq* mutant effect and that caused by polarity of the *hfq* insertion on downstream genes in the operon. For this purpose, we employed the strategy of comparing the phenotypic effects of two different insertion mutations, one near the 5′ end of *hfq* (*hfq-1*::Ω) and the other just beyond its 3′ end (*hfq-2*::Ω), as suggested earlier by Tsui et al. (53). When *lac* expression from the isolated *E. coli proU* P1 promoter on plasmid pHYD275 was examined, the *hfq-2*::Ω mutation had little effect at either 30 or 10°C, whereas the *hfq-1*::Ω mutation was associated with a three- to fivefold reduction in expression at both growth temperatures (Table 4). Importantly, however, the magnitude of cold induction of the P1 promoter was roughly similar in all the three strains. We conclude that Hfq (i) affects the absolute level of *proU* P1 promoter activity in vivo but (ii) is not required to mediate its induction during low-temperature growth.

We also tested the epistatic interaction, if any, of the *hfq-1*::Ω insertion on activation by *hns* of *E. coli proU* P1 borne on plasmid pHYD275. In LB medium-grown cultures, the specific activity values of β-galactosidase for the pHYD275 derivatives of GJ862 (wild type), GJ866 (*hns*), GJ2746 (*hfq-1*), and GJ2748 (*hns hfq-1*) were 70, 301, 21, and 153 Miller units, respectively, and in all four instances the measured expression was shown to be predominantly σ^s dependent (data not shown). Similar results were also obtained for cultures grown in LBON or glucose-minimal A medium (data not shown). Our results indicate that *hns*-mediated derepression of an RpoS-controlled promoter occurs even in an *hfq-1* mutant and therefore are at apparent variance with the conclusion from an earlier report (36) that the latter is epistatic to the former with regard to regulation of σ^s synthesis.

DISCUSSION

In this study, we have identified several conditions in which expression in *trans* from the cryptic σ^s-controlled P1 promoter of *proU* in *E. coli* and *S. enterica* is activated or enhanced. In general, such enhancement could be envisaged as occurring at either the level of σ^s synthesis itself (whose regulation is known to be extremely complex [14, 20, 23]) or the more local level of the *proU* P1 *cis* regulatory region. In order to distinguish between these alternatives, we have examined whether each of the conditions that activates reporter gene expression from *proU* P1 also does so from other σ^s-controlled promoters such as those for *osmY* or *csiD*. Based on the results with the *osmY-lac* strain (Table 3), it appears that the activating effect of the *rho* mutation on *proU* P1 occurs at the local level, whereas that of low-temperature growth occurs at the more upstream level of σ^s synthesis. The latter conclusion is consistent with the findings of an earlier report (48). The absence of effect of low-temperature growth (or of *hns* [see below]) on *csiD* expression may perhaps be explained on the grounds that this promoter requires another transcriptional activator (Crp) for

TABLE 4. *lac* expression from pHYD275 (*E. coli* P1.a) in *hfq* mutants[a]

Strain	Mutation	β-Galactosidase sp act at temp:	
		30°C	10°C
GJ862	Nil	123	839
GJ2746	*hfq-1*	24	243
GJ2745	*hfq-2*	85	750

[a] Cultures of the indicated strains carrying plasmid pHYD275 were grown in trimethoprim-supplemented K-tryptone medium at 30 or 10°C for β-galactosidase assays. Enzyme specific activity values are reported in Miller units (35).

its expression (32) and therefore that an increase in σ^s levels alone may not be sufficient for its activation.

H-NS and *proU* P1. As discussed below, our data suggest that the *hns* mutant effect on *proU* P1 is exerted at both the local and the upstream levels. It is known that σ^s synthesis is derepressed about sixfold in strains lacking H-NS (5, 59), and this could explain the moderate increase in *proU* P1-*lac* expression from plasmids pHYD275 (*E. coli* P1.a) and pHYD374 (*S. enterica* P1Δ*att*) as well as the increase in *osmY-lac* expression. On the other hand, the *hns* mutant effect on *proU* P1-*lac* expression from plasmids pHYD394 and pHYD395 (which carry more than 1 kb of DNA downstream of P1 including the NRE and inactivated P2) is around 50-fold, which is very much more pronounced than can be accounted for by the upstream effect on σ^s synthesis alone; it is likely, therefore, that in this case H-NS is also acting locally, perhaps by binding to the high-affinity binding sites at the NRE and around P1 (30, 38, 50), to repress reporter gene expression.

A null mutation in *stpA*, the gene encoding the H-NS-like protein StpA, which is believed to represent a molecular backup of H-NS (58), had no effect on *proU* P1 expression either by itself or in combination with other mutations such as *rho* and *hns* (data not shown).

Rho and *proU* P1. The activating effect of the *rho* mutation on *proU* P1 is most prominent in two situations (where the promoter is otherwise cryptic), namely, on *S. enterica* P1 (plasmid pHYD373) at 30°C and on *E. coli* P1-P2*-NRE (plasmids pHYD394 and pHYD395) at 10°C. The magnitude of *rho*-mediated P1 activation on pHYD373 at 10°C is also quite significant. Although the mechanism by which P1 activation occurs in the *rho* mutants is not known, two lines of evidence suggest that it may be related to the relief of attenuation of transcripts initiated from P1. First, such a mechanism will be consistent with the previously characterized activities and functions of the Rho protein (21, 44, 56). Second, the *rho* mutant effect is considerably diminished for *S. enterica* P1Δ*att* (plasmid pHYD374), which bears the deletion of a 22-base C-rich segment (on the coding strand) and which has previously been shown (42) to be defective in attenuation. It is reasonable, therefore, to postulate that the C-rich target segment on the *S. enterica* P1-initiated transcript serves as a site for Rho factor loading and consequent termination of transcription. The precise site of occurrence of the latter remains to be determined. This scheme is reminiscent of the mechanism by which Rho factor autoregulates its own synthesis by transcription attenuation (4, 25, 33). Our results would also suggest, by analogy, that Rho-dependent attenuation occurs for native *E. coli* P1-associated expression (that is, in the presence of the long downstream sequence), at least during growth at 10°C, but once again the *cis* site of action is not known.

Low-temperature growth and *proU* P1. As argued above, at least one component of the activation of *proU* P1 during low-temperature growth may be accounted for by an upstream effect at the level of σ^s synthesis. At the same time, a synergism is apparent between low-temperature growth and loss of Rho activity with respect to the ability of each to activate *lac* expression from P1 of both *S. enterica* (on pHYD373) and *E. coli* (on pHYD394 and pHYD395). It is possible that transcription termination events in the cell generally become more critically Rho dependent at the low temperature; for example, Bae et al. (3) have reported that transcriptional readthrough at Rho-independent terminator sites is increased in cultures grown at low temperature. Alternatively, Rho might influence temperature-responsive changes in DNA topology, as suggested by Tobe et al. (52).

Sledjeski et al. (48, 49) have reported that the induction of σ^s synthesis during low-temperature growth is mediated by an untranslated RNA, DsrA, and that Hfq is required for DsrA to stimulate the translation of RpoS. On the other hand, our own data demonstrating that *hfq* mutants are not defective in cold induction of *proU* P1 do not readily fit into the model proposed by Sledjeski et al. We were unable to obtain the *dsrA*-null strain to continue these studies.

Physiological role of *proU* P1 promoter? The role of the *proU* P1 promoter in enterobacterial physiology has so far remained obscure (17). This study has identified at least three factors which in an interactive manner may be involved in stimulating expression from this promoter, namely, growth at low temperature and inactivation of the Rho and H-NS proteins. Thus, the in vivo activity of the isolated wild-type *E. coli* P1 promoter in cells grown at 10°C (Table 2) marks it as one of the very strong and substantially regulated promoters under these conditions. However, the mechanisms of interaction among the three identified factors, and the physiological relevance of such interactions, remain to be determined.

It is possible that under certain growth conditions the activity of Rho or of H-NS is reduced or antagonized even in a *rho*$^+$ *hns*$^+$ strain. For example, (i) other factors involved in transcription elongation and termination such as NusA and NusG are known to modulate Rho function (7, 8, 21, 39, 44); (ii) phage-encoded proteins Psu (from P4) and gp5.5 (from T7) are physiological antagonists of Rho and H-NS, respectively (26, 27); and (iii) the cellular functions of Rho and H-NS are antagonized by overexpression of the chromosomally encoded *yaeO* (40) and *dsrA* (47) genes, respectively. There is also in vitro evidence that the activities of H-NS (43, 54) and of Rho (39, 57) are sensitive to the potassium salt concentration, which is known to vary within the cell with changes in osmolarity of the growth medium (10).

Finally, an overlap between adaptation to osmotic stress and to cold stress has been demonstrated earlier in plants and other bacteria. In *Listeria monocytogenes*, intracellular accumulation of glycine betaine is necessary for growth both at low temperature and in media of elevated osmolarity and occurs via an active uptake mechanism (13). Holmstrom et al. (22) have reported that synthesis of glycine betaine in transgenic tobacco lines is associated with improved tolerance to both salinity and low temperature. It is, therefore, possible that the *proU* operon in *E. coli* or *S. enterica* mediates adaptive accumulation of glycine betaine in response to both osmotic stress and cold stress and that the P2 and P1 promoters are primarily responsible for transcription of the operon under the respective conditions.

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