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Evidence for Transcription Attenuation Rendering Cryptic a σ^S-Dependent Promoter of the Osmotically Regulated *proU* Operon of *Salmonella typhimurium*

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The osmotically regulated *proU* locus in *Escherichia coli* has two promoters, P1 and P2, that are recognized, respectively, by the σ^S- and σ^{70}-bearing RNA polymerase holoenzymes. However, the equivalent of the P1 promoter does not appear to exist in *Salmonella typhimurium*. We demonstrate in this study that wild-type *S. typhimurium* has a cryptic P1 promoter that is recognized by σ^S RNA polymerase in vitro and that a 22-bp deletion from +63 to +84 (relative to the start site of transcription) confers σ^S-dependent in vivo expression of a reporter gene fusion to P1. Primer extension analysis of RNA isolated from cells carrying the wild-type and mutant *S. typhimurium proU* constructs indicated that a primer which hybridizes proximal to +60 is able to detect P1-initiated transcripts from both constructs but a primer which hybridizes distal to +85 is able to do so only from the latter. Our results suggest that the σ^S-controlled *proU* P1 promoter in *S. typhimurium* may be rendered cryptic because of factor-dependent transcription attenuation within a short distance downstream of the promoter start site.

Adaptation of microorganisms to growth in media of elevated osmolarity (osmoregulation) is associated with the cytoplasmic accumulation of nontoxic low-molecular-weight organic compounds that are collectively designated compatible solutes. In the enterobacteria, the list of identified compatible solutes includes trehalose, glutamate, L-proline, and glycine betaine (reviewed in reference 4). The *proU* operon in *Escherichia coli* and *Salmonella typhimurium* encodes a binding-protein-dependent transporter that mediates the accumulation of glycine betaine and L-proline in response to hyperosmotic stress. Transcription of *proU* in both organisms is induced several-hundred-fold under these conditions (reviewed in references 4, 9, and 16).

In *E. coli*, two promoters, P1 and P2, have been identified for *proU*, with start sites of transcription approximately 250 and 60 bp, respectively, upstream of the first structural gene, *proV*. A negative regulatory element, situated downstream of P2 and overlapping the proximal end of *proV*, is also required in *cis* to repress transcription of *proU* in low-osmolarity media (reviewed in references 9 and 16). Data from both genetic and in vitro transcription experiments indicate that the P1 and P2 promoters are transcribed by RNA polymerase holoenzymes bearing, respectively, the σ^S (that is, stationary-phase sigma factor) and the σ^{70} polypeptides (17, 23, 28, 32). The finding that *proU* has a σ^S-dependent promoter that is induced in stationary-phase cultures (17) is in keeping with the overlap that has been observed between osmotic and stationary-phase-specific gene regulation at several loci in *E. coli* (14).

The mechanism of *proU* regulation in *S. typhimurium* is very similar to that in *E. coli*, with one exception. Overdier and Csonka (21) were unable to identify promoter activity equivalent to that of the σ^S-regulated *E. coli* P1 during subcloning experiments with *S. typhimurium proU*, despite the fact that sequence residues in both the −10 (10, 17, 26) and the −35 (17, 29) regions postulated to be important for σ^S-directed transcription are conserved between the two organisms (Fig. 1). We demonstrate in this study that *S. typhimurium* does have a P1 promoter which serves for the initiation of transcription both in vivo and in vitro in a σ^S-specific manner and, furthermore, that expression from this promoter in vivo is rendered cryptic most probably because of attenuation of transcription at a site around 60 bp downstream from the promoter.

***S. typhimurium proU* P1 is inactive in vivo and active in vitro.** Earlier studies from our laboratory had established that a subcloned DNA segment from *E. coli proU* extending from −60 to +116 (with respect to the start site of P1 transcription defined as +1; Fig. 1) was sufficient to direct the expression from P1 both of a *lacZ* reporter gene in vivo (in plasmid pHYD275 [5]) and of discrete transcripts from a supercoiled template in vitro (23). In both cases, σ^S specificity of transcription was also demonstrated (17, 23).

Using standard PCR techniques (24), we amplified the segment of *proU* equivalent to the region between −66 and +117 from *S. typhimurium* LT2 (Fig. 1). This segment was cloned as a *Pst*I-*Bam*HI fragment at a site upstream of the *lacZ* reporter gene in the single-copy-number plasmid vector pMU575 (2) and into the vector pCU22 (28) for in vitro transcription studies. (The flanking restriction sites were derived from the primers used for PCR.) The resultant plasmids were designated pHYD373 and pHYD370, respectively. As a control, we also amplified by PCR the −68 to +115 region from *E. coli proU* (Fig. 1) and cloned it into the same two vectors to generate plasmids pHYD371 and pHYD368, respectively.

E. coli Δ*lac* strain MC4100 (17) transformed with plasmid pHYD371 (carrying the *E. coli* PCR product cloned upstream of *lacZ*) exhibited β-galactosidase expression comparable to the level observed for the P1 promoter in the earlier-studied P1-bearing plasmid pHYD275 (5); this expression was moderately osmotically inducible and was also *rpoS* dependent (in that *lac* expression from pHYD371 was drastically reduced in

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      -69         -60                         -40                 -20
S.t.   C CA[TGCCTTTATTTCAAG_CAA_TAGGGAGTCAAATCGCGCAAATATTACAA
E.c.   G[.. G.GAAA..A...CC.C...A...CTTT.T__...A........A.TTGT
      -69         -60                     -40                     -20

                         +1                   +20
S.t.   CATGTCCTACACTCAATACGAGTGACATTATTCACCTGGATTCCCCCAATTCAG
E.c.   GG..AT.......G.TACTCT..TG........G....A.AC.A.AAT......
                         +1                   +20

          ______Primer P______      __________Δ__________
        +40                   +60                   +80
S.t.   GTGGATTTTGCTGGTTGTTC_CAAAAAATATCT_CTTCCTCCCCATTCGCGTT
E.c.   .C.TT....C...A_.CT..GA...........AA...__..T.G...T..TC.
        +40                   +60                     +80

        ______Primer D______
              +100              +120
S.t.   CAGCCCTTATATCATGGGAAATCACAGCCG]ATA
E.c.   ..............C.......TC.G.._.]..T
               +100             +118
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FIG. 1. Features of the *proU* P1 promoter region of *S. typhimurium* and *E. coli*. The *S. typhimurium* (*S.t.*) sequence (22, 25) is shown, below which is aligned the *E. coli* (*E.c.*) sequence (7); each position of identity in the latter is indicated by a period. Gaps introduced in either sequence to improve the alignment (22) are represented by underscore symbols. Nucleotide number is with respect to the start site of P1 transcription identified in this study, denoted as +1. The ends of the PCR-amplified fragments from the two organisms (see the text) are delimited by brackets. The 22-bp deletion in pHYD374 is identified by the overline and triangle. The *S. typhimurium* sequences to which primers P and D (employed for the primer extension experiments) are complementary are overlined.

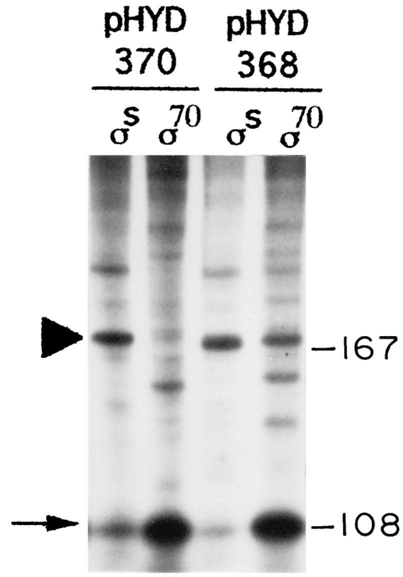

FIG. 2. $E\sigma^S$- and $E\sigma^{70}$-directed transcription in vitro from supercoiled templates of plasmids pHYD368 and pHYD370 carrying the *proU* P1 regions of *E. coli* and *S. typhimurium*, respectively. Bands corresponding to RNA I and P1-initiated transcripts are identified by the arrow and filled arrowhead, respectively. The positions of migration of two RNA size markers are depicted on the right: a 167-base runoff transcript from *alaSp* obtained as described previously (11) and the 108-base RNA I transcript (27) from vector pCU22.

the *rpoS*::Tn*10* mutant RH90 [17]) (Table 1). On the other hand, and consistent with the earlier results of Overdier and Csonka (21), derivatives of MC4100 and RH90 transformed with the corresponding plasmid pHYD373 carrying the *S. typhimurium* PCR product displayed negligible *lac* expression (Table 1).

In vitro transcription experiments with supercoiled DNA templates of plasmids pHYD368 (with *E. coli* P1) and pHYD370 (with *S. typhimurium* P1), using highly purified *E. coli* RNA polymerase core enzyme reconstituted with either σ^{70} or σ^S polypeptides, were done as described earlier (23), and the results are presented in Fig. 2. Both plasmids carry a tandem pair of transcription-terminator sequences immediately downstream of the insert fragment in the pCU22 vector (28), so that transcripts of discrete size are generated in vitro from any promoter within the insert. Plasmid pHYD368 carrying the *E. coli* fragment yielded a σ^S-specified RNA product whose size (approximately 170 bases) corresponded to that expected for transcription initiated at position +1 of the P1 promoter; consistent with our earlier data (23), this promoter was also recognized, to a lesser extent, by the σ^{70}-bearing RNA polymerase holoenzyme, whereas the vector-specified RNA I transcript (for both plasmids pHYD368 and pHYD370) was predominantly observed only in the σ^{70} lanes. Most interestingly, plasmid pHYD370 carrying the *S. typhimurium proU* fragment also directed synthesis, catalyzed by σ^S RNA polymerase, of a transcript whose size was very similar to that from pHYD368; in fact, the *proU* promoter in pHYD370 was more specific for σ^S than was that in pHYD368. These results indicated that the equivalent region of *proU* in *S. typhimurium* does have a P1 promoter that is functional in a defined in vitro transcription system, although this promoter is unable to express a downstream reporter gene in vivo.

TABLE 1. *proU* P1-directed *lacZ* expression in vivo[a]

Plasmid	*proU* P1 promoter[b]	β-Galactosidase sp act		
		MC4100		RH90 (High)
		Low	High	
pHYD275	*E.c.* WT	21	139	2
pHYD371	*E.c.* WT	15	115	4
pHYD373	*S.t.* WT	<1	<1	<1
pHYD374	*S.t.* mutant	21	94	2

[a] Derivatives of MC4100 ($rpoS^+$) and RH90 (*rpoS*) carrying the indicated pMU575-derived plasmids were grown at 30°C to mid-exponential phase ($A_{600} \approx 0.75$) in half-strength minimal A medium (19) with 0.2% glucose and without (Low) or with (High) additional supplementation with 0.2 M NaCl for β-galactosidase assays (19). Enzyme specific activities are reported as Miller units (19), determined after correction for the background level of β-galactosidase expression from the pMU575 vector (5).

[b] *E.c.*, *E. coli*; *S.t.*, *S. typhimurium*; WT, wild type.

In vivo activation of P1 promoter of *S. typhimurium* by mutation. Given the finding that the *S. typhimurium* P1 promoter is active at least in vitro, we next attempted to obtain mutations in this region of *proU* that would also allow expression from the promoter in vivo. Spontaneous Lac^+ mutants of strain MC4100/pHYD373 were selected on minimal-lactose agar plates supplemented with 0.15 M NaCl. One of eleven Lac^+ mutants examined was shown to carry a plasmid (designated pHYD374) that conferred (i) osmotically modulated β-galactosidase activity upon transformation into MC4100 and (ii) negligible *lac* expression in the isogenic *rpoS* strain RH90 (Table 1). In other words, plasmid pHYD374 carries an alteration that confers on the *S. typhimurium* P1 promoter in vivo properties of osmotic inducibility and σ^S dependence that are similar to those of the *E. coli* P1 promoter. Nucleotide sequence analysis of the *proU* insert region in pHYD374 indicated that the mutation was a 22-bp deletion of the sequence from +63 to +84 inclusive (Fig. 1); no other sequence changes were present in this region. Thus, a downstream deletion appears to be responsible for activating in vivo expression from the *proU* P1 promoter of *S. typhimurium*.

Primer extension mapping of *S. typhimurium* wild-type and mutant P1 promoters. To determine whether the deletion in pHYD374 was facilitating reporter gene expression by acting

at the step of initiation of transcription from P1 or at some postinitiation step, we undertook primer extension experiments on RNA obtained from cells carrying the wild-type and mutant P1 promoters. Total cellular RNA was prepared as described previously (1) from transformant derivatives of *E. coli* GJ146 (Δ*proUp*) carrying plasmid pHYD373 or pHYD374 grown to late logarithmic phase ($A_{600} \approx 1.1$) in LB medium (19) supplemented with 0.2 M NaCl. The host strain, GJ146, was chosen because it carries a chromosomal deletion of the *proU* regulatory region (8), so there would be no interference from *E. coli proU* transcripts in the experiments. Two 5′-end-labelled oligonucleotide primers, P and D, designed to be complementary to the transcript regions proximal to +63 and distal to +84, respectively (Fig. 1), were used for hybridization to 10 μg each of the RNA preparations and for primer extension with avian myeloblastosis virus reverse transcriptase, as described previously (3). The products of the extension reactions were electrophoresed on a urea-polyacrylamide gel (along with a sequence ladder generated by standard methods [24] with labelled primer P on pHYD370 as a template to serve as size markers) and visualized by autoradiography (Fig. 3).

The proximal primer P yielded identical extension products of comparable intensities with RNA preparations from derivatives carrying either the wild-type (pHYD373) or mutant (pHYD374) *S. typhimurium proU* construct (Fig. 3, compare lanes 2 and 4), and the size of the extension product with each preparation corresponded to that expected for transcription initiated from the +1 site marked in Fig. 1. The distal primer D yielded an extension product only with RNA prepared from the mutant *proU*-bearing strain (Fig. 3, lane 3), and its size (30 bases longer than that with primer P) again matched that expected for transcripts initiated from P1 (after taking into account the distance between the hybridization sites for the two primers on the mutant *proU* transcript as the template). No primer extension product corresponding to the P1 start site was observed after hybridization with RNA prepared from *rpoS*::Tn*10* derivatives of GJ146 that had been transformed with pHYD373 or pHYD374 (data not shown).

These results permitted the following conclusions: (i) the P1 promoter of wild-type *S. typhimurium proU* is recognized in vivo by σ^S RNA polymerase; (ii) the deletion mutation in pHYD374 does not create a new promoter; (iii) transcripts initiated from the wild-type P1 promoter are apparently truncated in vivo in the region around +60 to +85; and (iv) the deletion in pHYD374 serves to relieve this truncation. Thus, the reason why the *lacZ* reporter gene is not expressed from pHYD373 in vivo is not because of lack of activity of the P1 promoter on the plasmid but probably because of transcription attenuation (12) that occurs before the transcripts extend into the structural gene.

A comparison between lanes 3 and 4 in Fig. 3 indicated that the intensity of the extension product signal obtained with primer D on the mutant template was much weaker than that with primer P. It is possible that this difference reflects an incomplete relief of attenuation in the deletion mutant; the in vivo P1-*lacZ* expression data (Table 1), on the other hand, suggest that attenuation is probably substantially relieved by the deletion mutation. An alternative possibility is that the difference in intensities may have been caused by variations in the efficiency of hybridization of the two different primers to the P1-initiated mutant transcripts under the annealing conditions that were used in the experiments.

Implications and concluding remarks. To our knowledge, ours is the first report of gene expression being rendered cryptic by a mechanism that acts downstream of transcription initiation and also the first wherein crypticity has involved a σ^S-controlled promoter. Although some possible explanations are discussed below, the precise relevance of a cryptic promoter for *proU* remains unclear. In this context, it may be noted that there is as yet no definitive answer even to the more general question of why cryptic genes and operons occur at all in bacteria (20).

All the *cis* elements from the *proU* region that are necessary and sufficient for the postulated in vivo attenuation of P1-initiated transcription in plasmid pHYD373 are also present in plasmid pHYD370 used for in vitro transcription, in which,

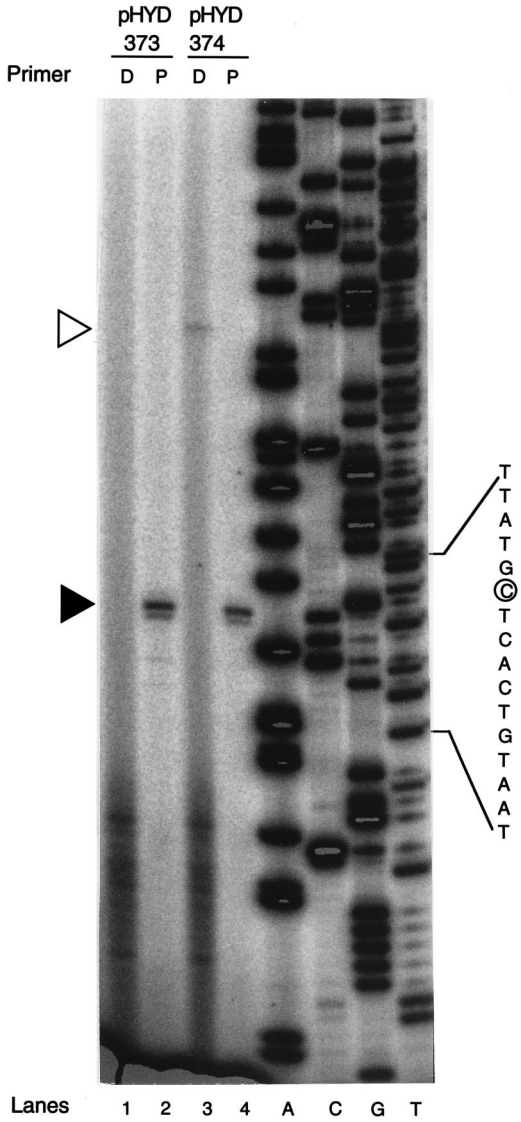

FIG. 3. Identification by primer extension of transcripts initiated in vivo from *proU* P1 of *S. typhimurium*. Primers P and D were used in primer extension experiments with RNA prepared from the Δ*proUp* strain GJ146 carrying either of two plasmids, pHYD373 (cryptic P1, wild type) or pHYD374 (activated P1, mutant), as marked (lanes 1 to 4). Bands corresponding to the P1 start site obtained with primers P and D are identified by the filled and open arrowheads, respectively. Lanes A, C, G, and T represent the sequence ladder generated with primer P on pHYD370 template DNA (*S. typhimurium* wild-type *proU*), and the sequence of the relevant region (complementary to that in Fig. 1) is given on the right. The encircled nucleotide corresponds to the band which comigrates with the major primer extension band in lanes 2 and 4 and identifies the +1 nucleotide in Fig. 1.

however, no attenuation was observed. These results may be contrasted with that reported earlier for attenuation in each of the amino acid biosynthetic operons wherein termination is the default option (12, 30) and suggest that attenuation within *proU* may be a factor-dependent phenomenon (as it is, for example, in the *E. coli tna* [12, 31] and *Bacillus subtilis trp* [6, 31] and *pyr* [15] operons). The identity of the concerned factor(s) remains to be established, as well as whether the efficiency of transcription termination at this site is governed by environmental conditions. Such an arrangement may represent a novel mechanism of stationary-phase gene regulation in bacteria. An alternative but less likely possibility is that the sequence between +60 and +85 serves as a site not for attenuation but for endonucleolytic processing of the mRNA followed by rapid degradation of the downstream transcript in a 5′-to-3′ direction, as has been postulated to occur for CsrA-regulated expression of the *glg* genes in *E. coli* (13).

The role of the P1 promoter in *proU* regulation even in *E. coli* is enigmatic. It has not been possible (17) to demonstrate an effect either of growth phase or of *rpoS* mutations on transcription directed by any of the *E. coli proU* constructs that carry both the P1 and P2 promoters (as opposed to the very marked effects elicited by these perturbations on the isolated P1 promoter). Mutations in the −10 or −35 region of P2 in these constructs (or in equivalent constructs of *S. typhimurium proU*) abolish all expression (18, 33). Finally, an *E. coli proU* construct carrying both P1 and P2 was inactive for reporter gene expression in an *rpoD*(Ts) mutant at the restrictive temperature (32).

The possibility therefore needs to be considered that, under the laboratory culture conditions tested, transcription from P1 in *E. coli* is also terminated at a site upstream of *proV* (perhaps in the vicinity of P2); even so, the *cis* signals mediating such termination may not be identical to that identified in *S. typhimurium* (see above) given that (i) *E. coli proU* inserts extending up to +116 do exhibit P1-directed reporter gene expression in vivo, and (ii) one of us had shown in an earlier study (7) that P1-initiated transcripts from the chromosomal *proU* locus in *E. coli* are detectable by extension with a primer whose 3′ end is complementary to +74 in the transcript. Another intriguing possibility is that P1 promoter function is not so much to transcribe the structural genes encoding the ProU transporter as to regulate transcription initiation from the downstream P2 promoter.

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