Transcriptional analysis of the gene encoding peptidyl-tRNA hydrolase in *Escherichia coli*

L. Rogelio Cruz-Vera, José Manuel Galindo and Gabriel Guarneros

Department of Genetics and Molecular Biology, Centro de Investigación y de Estudios Avanzados del IPN, Apartado Postal 14-740, México DF 07000, Mexico

Gene *pth* encodes peptidyl-tRNA hydrolase (Pth), an enzyme that cleaves peptidyl-tRNAs released abortively from ribosomes during protein synthesis. In the *Escherichia coli* chromosome, *pth* is flanked by *ychH* and *ychF*, two genes of unknown function. Pth is essential for cell viability, especially under conditions leading to overproduction of peptidyl-tRNA. In an attempt to unveil the elements that affect *pth* expression, the transcriptional features of the *pth* region were investigated. Northern blot experiments showed that both *pth* and *ychF*, the 3'-proximal gene, are cotranscribed in a bicistronic transcript. However, transcripts containing each of the individual messages were also detected. Accordingly, two transcriptional promoters were identified by primer extension experiments: one located upstream of *pth*, which presumably gives rise to both the mono and bicistronic *pth* transcripts, and the other, preceding *ychF*, which generates its monocistronic message. Deletion analysis indicates that *pth* transcript stability depends on *ychF* integrity. Also, a defect in RNase E activity resulted in Pth overproduction. It is proposed that RNase E processing within *ychF* in the bicistronic message limits *pth* expression.

**Keywords:** bicistronic mRNA, mRNA stability, *pth*, *ychF*, RNase E

INTRODUCTION

Peptidyl-tRNA hydrolase (Pth; EC 3.1.1.29) cleaves peptidyl-tRNAs prematurely released from ribosomes during protein synthesis (Atherly & Menninger, 1972). Pth activity is required to recycle tRNA into cell metabolism, a function essential for *Escherichia coli* viability (Atherly & Menninger, 1972; Garcia-Villegas et al., 1991; Schmitt et al., 1997). The enzyme is encoded in *pth*, a gene located at 26 min in the *E. coli* chromosome. The nucleotide sequence of *pth* has been determined and the predicted 21 kDa polypeptide identified and purified (Cruz-Vera et al., 2000; Galindo et al., 1994; Garcia-Villegas et al., 1991). The three-dimensional structure of crystalline Pth has been solved, and, using site-directed mutagenesis, the active and receptor sites for the substrate have been identified on the molecule (Fromant et al., 1999; Schmitt et al., 1997). A temperature-sensitive mutant (Atherly & Menninger, 1972) harbours a mutation, named *pth*(Ts), which determines an unstable Pth protein (Cruz-Vera et al., 2000). Upon shift-up to non-permissive temperatures, the mutant accumulates peptidyl-tRNAs, protein synthesis is arrested and cells die due to tRNA starvation. Another mutant affected in *pth*, termed *pth*(rap), was selected as non-permissive for phage λ vegetative growth (Garcia-Villegas et al., 1991). Phage mutants able to grow on *pth*(rap) bacteria are affected in minigenes located in loci termed *bar* (Guzmán & Guarneros, 1989). The *bar* minigenes are sequences which encode two-amino-acid peptides (Ontiveros et al., 1997); however, *bar* mRNA is defective in translation termination, releasing peptidyl-tRNA at a high frequency instead of the free peptide (Dinčšas et al., 1999; Hernández et al., 1997; Hernández-Sánchez et al., 1998; Heurgué-Hamard et al., 1998, 2000). Thus, phage growth may be impaired by starvation for specific tRNA due to sequestration as peptidyl-tRNA. Recent results suggest that high-level expression of non-functional proteins inhibits the growth of bacteria defective in Pth activity. The cause of the inhibition may be sequestration of specific tRNA, cognate to the last sense codon of overproduced proteins (Menez et al., 2000). This result may imply that Pth could be central to an alternative translation-termination activity.

*E. coli* *pth* has been the paradigm for a group of homologous ORF sequences conserved in eubacteria.
and eukaryotes (De La Vega et al., 1996; Schmitt et al., 1997). In the E. coli chromosome, pth is flanked by genes of unknown function: ychF (named gtp1 in Galindo et al., 1994) located 3′ to pth, and ychH on the 5′ end (orf2 in Galindo et al., 1994). A polypeptide encoded by ychH has not been shown, but ychF encodes a homologue to a conserved GTP-binding protein (Gtp1 in Galindo et al., 1994). As the concentration of Pth in the cell is critical to prevent peptidyl-tRNA-mediated lethality, we aimed to understand how pth expression is controlled. Our evidence indicates that both 5′ and 3′ sequences flanking pth affect the concentration of pth transcripts and Pth expression. However, except for limiting endonuclease RNaseE activity, Pth concentration is highly stable to changes in the growth conditions of the wild-type and of various mutant strains.

**METHODS**

**Strains and plasmids.** The strains of *Escherichia coli* K-12 used were C600 (F–, hsdR0 thr-1 recA1 lys-3 thi-1 supE44 tonA lacY1, our collection) and C600Δms1. This latter strain was obtained by P1 vac cotransduction of *ms1* with zet229::Tn10. The donor strain was IBPC642 (Hajnsdorf et al., 1994). Bacteria were grown at 32 °C in Luria–Bertani (LB) or LB medium-ampicillin (100 µg ml–1).

**Plasmid constructs.** pRJ5–5 was constructed by cloning the EcoRI (271 min)–KpnI (27 min) chromosomal segment in clone (246) 12A3(–) of Kohara (Kohara et al., 1987; Rudd, 1992) into the EcoRI and KpnI sites of vector pGEM-4 (Promega), a vector convenient to obtain RNA probes through *in vitro* transcription using T7 RNA polymerase. Construct pRJ1·6 was obtained by subcloning the EcoRI–EcoRV 1·6 kb segment from plasmid pRJ5–5 into the EcoRI and Smal sites of pGEM-4. pRJ1·0 was obtained through cloning, into the

**Fig. 1.** Map of the genetic region around pth, showing probes and cloned regions. (A) Arrows showing the origin, size and direction of the transcripts described here. The dotted lines under the RNA3 arrow represent transcripts with a common 5′-end but variable 3′-ends (see Fig. 2, lane 1). The discontinuous line after the RNA4 arrow indicates that the transcript continues, for an undetermined distance, beyond the EcoRI site (see Discussion). (B) Genetic and physical map of the 5·5 kb EcoRI–KpnI chromosome segment containing pth. Open arrows represent size, relative position and direction of the respective ORFs. The position and distances, in kb from the EcoRI end, of some restriction sites are marked above the diagram: A, ApaI; D, DdeI; D′, DraI; C, ClaI; E, EcoRI; E′, EcoRV; K, KpnI; N, NruI. (C) Position, size and direction (represented by leftward arrows labelled P and F) of the RNA probes used in Northern blot assays. The heavy parallel lines represent the location and extension of the DNA probes used in S1 protection assays. Probes were [32P] phosphate labelled (asterisks) at both 5′-ends, a and b; or at the ychF-proximal 3′-end, c. (D) Lengths and positions of the DNA segments cloned in the plasmid constructs used.

**Fig. 2.** Transcripts containing pth and ychF sequences. Northern blot assay of the transcripts isolated from strain C600 transformed with pRJ5–5. The specific transcripts of different sizes (arrows) were revealed by labelled RNA probes P (lane 1) or F (lane 2) (see Fig. 1). The numbers on the left indicate the size, in nt, and position of the in *vivo*-synthesized markers (see Methods). The band positions of the rRNAs (23S and 16S) were visualized with ethidium bromide. The difference in band RNA1 intensities between lanes 1 and 2 was due to the different film exposure times to the blotted membrane. Lane 1 was overexposed to visualize the smeared RNA signal between 800 and 200 nt.
Transcriptional regulation of \( pth \) 

EcoRI and SmaI sites of pGEM-4, of a PCR fragment containing \( pth \) and short flanking segments amplified from the pRSJ5 template. The primers used were 5'-TAGTTATC-TGAATTCGATACGCAGTTT-3', a sequence located upstream from the putative \( pth \) promoter (Dutka et al., 1993) modified to contain an EcoRI site (underlined), and 5'GAATTTTGATATCGCGCTGGT-3', harbouring the EcoRV site (underlined) located within \( ychF \) (Fig. 1B, coordinate 1-6). pRJ0-8 was created by subcloning the 0.8 kb EcoRI-NrI fragment (Fig. 1B, coordinates 0 and 0.8, respectively) into the EcoRI and SmaI sites in pGEM4.

**RNA preparation and Northern blotting.** Total RNA was extracted with hot phenol from a 10 ml culture at 65 °C, as described by Aiba et al. (1981). About 30 μg of RNA was denatured in 40% formamide, 5 μl/mg ethanol solution at 65 °C for 10 min. The RNA species were resolved by electrophoresis through 1.5% denaturing agarose gel containing 2.2 M formaldehyde and transferred to a nylon membrane (Hybond-N, Amersham Pharmacia Biotech) as recommended by the manufacturer (Manson & Williams, 1985). Hybridization was carried out at 55 °C in 5× SSPE [0.9 M NaCl, 0.05 M sodium phosphate and 0.004 M EDTA (pH 7.7)], 0.5% SDS, 100 μM ρ-methyl salmon sperm DNA, 5× Denhardt's solution [0.1% bovine serum albumin BSA, 0.1% Ficoll and 0.1% polyvinyl pyrrolidone], Formamide (40%) was added to hybridization reactions using RNA probes. After 16 h incubation, membranes were rinsed twice at 55 °C with 2× SSPE, 0.1% SDS, once at 65 °C with 1× SSPE, 0.1% SDS and finally once at 65 °C with 0.1× SSPE, 0.1% SDS. The dried membrane was exposed to X-ray film for at least 24 h.

RNA probes for Northern blotting were synthesized by *in vitro* transcription and labelled with [\( ^32 \)P]UTP (see below). For probe P, used in Figs 2 and 3B (upper panel), the template was pRJ0-8 restricted with Ddel (Fig. 1B), whereas for probe F (Fig. 2) the template was pRJ1-6 restricted with Dral (Fig. 1C). The *in vitro* transcription mixtures contained, in 20 μl: 40 mM Tris/HCl (pH 8.0); 10 mM MgCl₂; 5 mM DTT; 50 mM KCl; 300 μM of three NTPs; 10 μCi [\( ^32 \)P]UTP (3000 Ci mmol⁻¹, 111 TBq mmol⁻¹; Amersham Pharmacia Biotech); 50 mg BSA ml⁻¹; 10 units RNasin (Promega); 2 μg template DNA; and 5 units T7 RNA polymerase (New England BioLabs). They were incubated for 30 min at 37 °C. The reaction was stopped with 10 units RNase-free DNase (Boehringer Mannheim, Germany) for 15 min at 37 °C. Finally, 2 μl of 0.5 M EDTA, 80 μl 3 M sodium acetate (pH 5.2) was added to hybridization reactions using RNA probes. After 16 h incubation, membranes were rinsed twice at 55 °C with 2× SSPE, 0.1% SDS, once at 65 °C with 1× SSPE, 0.1% SDS and finally once at 65 °C with 0.1× SSPE, 0.1% SDS. The dried membrane was exposed to X-ray film for at least 24 h.

**Fig. 3.** Characterization of the 5'-ends of \( pth \) and \( ychH \) transcripts. (A) Sequence of the intergenic region between \( ychH \) and \( pth \) showing, underlined, the putative transcriptional promoters (−35 and −10) for \( ychH \), and for \( pth \) (\( pthh \)), appropriately oriented relative to the Shine-Dalgarno (SD) regions and translation starts (also underlined). ∆. Site limiting the DNA insert containing \( pth \) in pRJ10. (B) RNA preparation from strain C600 transformed with pRJ5-5 hybridized to labelled DNA probes (Fig. 1C) and resolved by electrophoresis through a denaturing polyacrylamide gel (see Methods). Untreated self-hybridized probes: probe a, lane 1; probe b, lane 4. RNA hybridized and S1-digested with probe a, lane 2; with probe b, lane 5. S1-digestion controls of the probes a and b mock-hybridized to yeast tRNA, lanes 3 and 6, respectively. Arrows mark the electrophoretic migration distances of the protected fragments I–IV. The different migration, of about 30 bp, of fragment IV in lanes 2 and 5 may be due to limited degradation of probe b during *in vitro* labelling. The numbers on the left-hand side of the autoradiogram are the sizes, in nt, of a set of marker fragments (see Methods). (C) Primer extension assay on the \( pth \) transcripts (rightmost lane) and DNA sequencing ladder of the region. RNAs obtained from strain C600 transformed with pRSJ5-5 DNA were used as reverse-transcription templates. The primer was a designed oligonucleotide, complementary to the sense strand of an internal region in the \( pth \) ORF (see Methods). The signal in the primer extension lane corresponds to a cytidine (arrow +1) between the −10 box and the SD sequences (A).
and 10 µg glycogen (Boehringer Mannheim) were added. The stopped reactions were phenol/chloroform (1:1; v/v) extracted, precipitated with 2.5 vols ethanol at −20 °C for at least 20 min and finally resuspended in 10 µl water. For hybridization, the RNA probe was denatured with 40% formamide at 65 °C for 10 min and used as indicated above. The specific activity of the probes used was about 3 x 10^{6} c.p.m. µg⁻¹.

The RNA size markers used in Fig. 2 were obtained by T7-RNA polymerase run-off transcription of pRJ08 DNA template linearized with Ddel (205 nt), NcoI, (420 nt), EcoRI (800 nt) and PstI (2780 nt); and pRJ1-6 DNA template linearized with BglII (2130 nt).

The DNA probe for β-lactamase (bla) transcripts (Fig. 5, lower panel) was obtained by PCR amplification on a pGEM4 template. Thirty femtomoles of DNA template was added to the amplification mixture [in 50 µl; 40 mM Tris/HCl (pH 8.0), 5 mM MgCl₂, 10 mM DTT, 50 mM NaCl, 150 µM 3 dNTPs,
50 µCi [γ-32P]dCTP (6000 Ci mmol−1; Amersham Pharmacia Biotech), 1 unit Taq DNA polymerase (Applied Biosystems) and 10 pmol of primers. The primers were 5′-GTATTCAACATTCCGGTG-3′, beginning within the second codon of bla, and 5′-CAATGCTTAACTGAG-3′ complementary to the final bla ORF sequence. Amplification was carried out for 50 cycles (95 °C, 30 s; 55 °C, 30 s; 72 °C, 1 min) after an initial denaturation period of 1 min at 95 °C. The amplified product was denatured at 95 °C and added to the hybridization mixture described above.

S1 nuclease analysis. For S1 endonuclease protection analysis, the DNA probes (Fig. 1C, a and b) and φX184 DNA (Fig. 3B, markers) were labelled at the 5′-ends by the phosphate exchange reaction. Ten picomoles of DNA was dissolved in the exchange mixture [in 20 µl: 40 mM Tris/HCl (pH 8.0), 10 mM MgCl2, 5 mM DTT, 50 mM NaCl, 50 µCi γ-32P]ATP (3000 Ci mmol−1, 111 TBq mmol−1; Amersham Pharmacia Biotech) and 10 units T4 polynucleotide kinase (New England Biolabs) and incubated at 37 °C for 30 min. The reaction was stopped at 65 °C for 10 min and extracted with phenol/CHCl3. The solution was precipitated with 2 µl 3 M sodium acetate (pH 5.2) and cold ethanol. Probe c (Fig. 1C) was labelled by 3′-repair at the ApaLI site; 10 pmol of the DNA fragment was dissolved in the repair mixture [in 20 µl: 40 mM Tris/HCl (pH 8.0), 10 mM MgCl2, 5 mM DTT, 50 mM KCl, 50 mg ml−1 BSA, 600 µM of dNTPs except dCTP, 10 µCi [γ-32P]dCTP (6000 Ci mmol−1, Amersham Pharmacia Biotech) and 10 units DNA polymerase Klenow fragment (New England Biolabs)]. The mixture was incubated at 37 °C for 30 min. Finally, the labelled probe c was extracted, precipitated and redissolved in water as described for probes a and b.

For the S1 endonuclease protection assays in Figs 3(B) and 4(C), 3 µg (about 106 c.p.m.) of labelled DNA was hybridized [in 30 µl: 80% formamide, 0.4 M NaCl, 40 mM PIPEs, 1 mM EDTA (pH 6.4)] to 15 µg of total cell RNA, or of yeast tRNA as a control. The mixture was incubated for 10 min at 90 °C, cooled to 55 °C and maintained at this temperature for 8 h. Then, the samples were digested with S1 nuclease (200 units, Boehringer Mannheim) in 300 µl of 50 mM acetate buffer (pH 5), 280 mM NaCl and 4.5 mM ZnSO4 (Regnier & Portier, 1986). After 2 h incubation at 37 °C, the reaction mixture was added to 80 µl stop solution [4 M ammonium acetate, 50 mM EDTA (pH 8.0) and 50 µg yeast tRNA (Boehringer Mannheim)] and precipitated with ethanol. The pellets were rinsed with ethanol (75%), dried and redissolved in loading buffer (80% formamide, 0.1% bromophenol blue, 0.1% xylene cyanol and 2 mM EDTA). The samples were resolved by electrophoresis through a 7 M urea, 4% polyacrylamide gel, and the gels were dried and exposed on to X-ray film for 24 h.

mRNA stability. To measure the decay rate of RNA1 and RNA2, transformants of C600amsl or C600 for pRJ5-5 (Fig. 1D) were grown in LB-ampicillin at 32 °C to late exponential phase (OD600 0.6). The cultures were split in halves and the incubation continued at either 32 °C or 43 °C for 40 min. Rifampicin (500 µg ml−1) was added to stop further RNA initiation, and samples were drawn at different times (see Fig. 1B) to be processed for Northern blot assay as described above. The RNA1 and RNA2 bands were revealed by autoradiography of 32P-labelled probe F bound onto the transferred membranes and the amount of radioactivity estimated in a scintillation counter (Beckman LS6500). The percentage of RNA was calculated from the ratio between the radioactivity measured in an RNA band at a given time and the radioactivity in the band at zero time, after subtraction of background membrane radioactivity.

Fig. 5. Effect of the ychF and ychH deletions on pth expression. (A) Western blot assay of protein extracts obtained from cells transformed with pRJ5-5 (lane 1), pRJ1-0 (lane 2) and pRJ1-6 (lane 3) with anti-Pth (upper panel) and anti-Pnp (lower panel) rabbit antisera (see Methods). The Pth protein concentrations calculated from three independent experiments for each transformant were: pRJ5-5, 20±5 ng; pRJ1-0, 86±3 ng; pRJ1-6, 43±45 ng; per µg total protein. (B) Upper panel, Northern blot assay of the ychF transcripts from RNA preparations obtained from cells transformed with: pRJ5-5, lane 1; pRJ1-0, lane 2; pRJ1-6, lane 3; pRJ0-8, lane 4 revealed with 32P-labelled probe P. The radioactive signal is directly comparable, as the four constructs carry the pth region complementary to the probe. Lower panel, Northern blot assay of bla transcripts from the gene carried in each of the constructs (see Methods).

Primer extension assays. About 10 µg total RNA was assayed, essentially as described by Mateos et al. (1994). For experiments in Fig. 3(C), the same oligonucleotide, 5′-GCGCAAAAGGTGCTTGCC-3′, was used for the extension assay and for sequencing reactions. For experiments in Fig. 4(A), the oligonucleotide used was 5′-GCTTTGATACCCGCT-3′. The oligonucleotides were labelled with [γ-32P]ATP by the phosphate-exchange reaction described above. A sequencing ladder was generated with pRJ5-5 as a template by using the AmpliCycle sequencing kit (Applied Biosystems). A 4 µl sample of the primer extension or sequencing ladder reactions was resolved by electrophoresis through 7 M urea, 8% polyacrylamide gel. The gel was then dried and exposed as indicated above.

Western blotting. The immunodetection assays in Fig. 5(A)
were carried out as described elsewhere (Cruz-Vera et al., 2000). The samples were resolved through an SDS-polyacrylamide gel: 10% for polynucleotide phosphorylase (Pnp), or 15% for Pth. The antisera used were raised by immunization of rabbits with a purified preparation of Pth (Cruz-Vera et al., 2000) or Pnp protein (Garcia-Mena et al., 1999).

RESULTS

Transcripts that contain pth and ychF sequences

Genes pth and ychF are contiguous and oriented in the same direction in the E. coli chromosome (Fig. 1B; Galindo et al., 1994; Garcia-Villegas et al., 1991). We investigated the transcriptional relationships of these genes by Northern blot assays. The concentration of chromosome-directed transcripts from pth is undetectable by Northern blot due to their low concentration. Therefore, we used total RNA extracted from C600 cells transformed with pRJ5-5 (Fig. 1D). pRJ5-5 is a derivative of pGEM-E, a vector which does not interfere with transcription initiated at promoters in the inserts. The RNA was revealed with in vitro-labelled 32P-RNA probes, complementary to the initial segment in pth (P) and in ychF (F) (Fig. 2). Construct pRJ5-5, which directs Pth and Gtp1 proteins (data not shown), yielded two RNA populations revealed by probe P: one, a discrete band of 2000 nt called RNA1, and the other, a smear of RNAs from 800 to 200 nt named RNA3 (Fig. 2, lane 1). Probe F also revealed two types of transcripts: again RNA1, the band of 2000 nt, and a more robust band of 1400 nt (Fig. 2, lane 2 RNA2). It is likely that (1) RNA1 is a bicistronic transcript that harbours both pth and ychF messages, (2) the heterogeneous RNA3 revealed by the probe P contains proximal pth sequences of variable sizes and (3) RNA2, revealed only with the probe F, corresponds to ychF monocistronic transcripts of the predicted size (1400 nt).

Transcriptional initiation of pth and ychH

The reported sequence between ychH and pth in the E. coli chromosome contains three σ70-like promoter sequences (Fig. 3A; Hawley & McClure, 1983; Wosten, 1998). One σ70 promoter presumably controls initiation of pth transcripts and the other two promoters, oriented in the opposite direction, could initiate transcripts containing ychH sequences. To investigate the activity of these promoters, we looked for the predicted transcripts by nuclease S1-protection assays. Total RNA extracted from strain C600 transformed with pRJ5-5 and 5′-end 32P-labelled DNA probes a and b were used (Fig. 1C; see Methods). Probe a, a 2-4 kb EcoRI–ApaI fragment comprising ychH, pth and most of ychF, yielded four protected fragments, visualized as two strong and two weak bands (Fig. 3B, lane 2). However, probe b, 0-5 kb longer than a (Fig. 1C), yielded only one fragment, which migrated nearly as band IV revealed by probe a (Fig. 3B, lane 5). Using the nuclease S1-protection assay, which is about 100-fold more sensitive than Northern blot assay, similar fragments were observed with RNA extracted from untransformed cells (not shown). Therefore, the protecting transcripts were not initiated at vector promoters. These results show that the ends of the RNAs protected by fragments I, II and 750 nt are located within the 0-5 kb segment between the ApaI and Clai sites (Fig. 1C). Fragment IV should be protected by a transcript divergent to the transcripts that protect fragments I, II (Fig. 1C) and should contain ychH sequences (Fig. 3A). The calculated sizes of the protected fragments revealed by probe a indicate that: (1) the faint signal of fragment I, above 1353 bp, is protected by RNA1 (Fig. 1A) the 5′-end of which is located between pth and ychH; (2) the strong band, of about 1000 bp, of fragment II is protected with RNA2 (Fig. 1A), the 5′-end of which is located between pth and ychH; and (3) the fragment of 750 nt, also a weak signal, corresponds to a transcript spanning from a site somewhere within ychF to the end of probe a.

Start sites of pth transcripts

To define the start sites of the pth transcripts, we determined their 5′-ends by primer extension on an RNA preparation of strain C600 transformed with pRJ5-5 (see Methods and Fig. 3C). A unique signal was detected at the cytidine located seven bp downstream from the −10 box of the putative promoter for pth (Fig. 3A, +1). It is very likely that this signal corresponds to a transcriptional initiation site because: (1) in S1 protection assays, only one protected DNA fragment was visualized, implying that the primer extension signal was not due to an unspecific AMV reverse transcriptase drop-off (data not shown); and (2) the 5′-end cytidine was confirmed by primer extension on in vitro transcripts generated by RNA polymerase σ70 (result not shown). These data strongly suggest that the identified Ppth promoter (Fig. 3C) indeed controls pth transcription.

Transcriptional start for ychF transcripts

Evidence for the existence of a transcriptional promoter, specific for ychF, was derived from a plasmid construct that expressed protein Gtp1 even in the absence of a pth promoter region (data not shown). To investigate the presence of a transcriptional promoter in front of ychF, we attempted to identify the 5′-end of the corresponding transcripts through primer extension experiments. The assay, again, was conducted on RNA prepared from cells transformed with pRJ5-5 (see Methods). Results showed that the strongest signal detected corresponded to the adenine located at 6 bp downstream from a consensus −10 box of a putative promoter (Fig. 4A, s1, and Fig. 4B). Other minor signals were identified at one adenine (s2), at 16 bp, and two thymines, at 11 and 17 bp, downstream from the box (Fig. 4B). To find out which of these 5′-ends corresponded to ychF transcription initiation sites, we performed in vitro experiments using RNA polymerase σ70 in the presence of [γ-32P]ATP or [γ-32P]UTP. Results showed the incorporation only of adenine at the 5′-end of the transcripts. Primer extension assays of the in vitro transcripts
revealed only the ends corresponding to the adenines in positions 6 and 16 (not shown). Taken together, these data show the presence of PychF specific for ychF.

3'-ends of transcripts containing ychF sequences

We investigated the termination sites of the transcripts originated at Pthb and PychF. Results in Fig. 3(B) had suggested that the 3'-ends of ychF transcripts were located within the ApaLI–ClaI DNA segment (Fig. 1B). To locate the 3'-ends of those transcripts precisely, we performed S1-nuclease protection using the ApaLI–ClaI DNA fragment 32P-labelled at the ApaLI end (see Fig. 1C, probe c, and Methods). The RNA extracted from strain C600 transformed with pRJ5-5 yielded only one protected DNA fragment (Fig. 4C, lane 2). From this result and the fragment size, it seems that the 3'-end of the transcripts is unique and located about 50 nt past the EcoRV site at coordinate 2-7 (Fig. 1B). The sequence of this region (Oshima et al., 1996) does not show any recognizable rho-independent transcription terminators (Lesnik et al., 2001). However, two pairs of inverted repeats were recognized beyond the ychF termination codon. In addition, we identified a short ORF of 21 codons that overlapped with the second inverted repeat (Fig. 4D).

Effect of ychF sequences on Pth protein concentration

The pthb transcript is found in two types of RNA: a long homogeneous bicistronic pth–ychF message and a smear of transcripts from 200 to 800 nt (Fig. 2). Since pth ORF is 650 nt long, the smear could result from 3'-exonucleolytic degradation of longer pth transcripts. To investigate the efficiency of Pth expression from both types of transcripts, we compared the levels of Pth protein from pRJ5-5, which contains the complete pth–ychF operon, and pRJ1-6, which carries a deletion of most of the ychF 3' region. Results showed that the concentration of Pth protein in extracts from cells transformed with pRJ5-5 was about fivefold higher than that in extracts from cells transformed with pRJ1-6 (Fig. 5A; compare lanes 1 and 3, upper panel). This effect is specific to Pth, since the concentrations of Pnp, an unrelated protein, remained unchanged in both transformants (lower panel). Transcription of ychF from an overproducing construct did not affect Pth concentration (result not shown). This implies that ychF sequences affect pth expression in cis.

Effect of ychF sequences on pth transcripts

To investigate how the ychF region affected Pth levels, we tested the effect of ychF deletions on pth mRNA from pRJ1-6. We carried out Northern blot assays on RNA samples from C600 transformants using probe P. The results showed a 1200 nt RNA in pRJ1-6, instead of the 2000 nt RNA1 observed with pRJ5-5, as expected from the ychF deletion (Fig. 5B, upper panel, compare lanes 3 and 1). Also visible in pRJ1-6 is the smear of partially degraded pth message not present in the pRJ0-8 construct, carrying a pth deletion (lane 4). A quantitative analysis of radioactive probe hybridized in lanes 1 and 3 showed that the pth transcripts from pRJ1-6 are just 20% of those from pRJ5-5. This calculation has taken into account variations in the mean copy number of plasmid constructs per cell, as it has been normalized to the levels of β-lactamase transcripts also encoded in the constructs (Fig. 5B, lower panel). As the constructs used carried the same original pth promoter, it was likely that the variations in pth transcript concentrations in the transformants were due to the effect of distal ychF sequences on pth transcript stability. In fact, the half-life of the pth–ychF complete pRJ5-5 transcripts was 2 min compared with less than 0.5 min for the pRJ1-6 transcripts (data not shown).

Effect of RNase E on the expression of pth from the bicistronic transcript

To investigate whether a particular endoribonuclease affected RNA1 and/or RNA2 stability, we used strains carrying a specific mutation in endoribonuclease E (RNase E) or in RNase III. A strain rnc105, a mutant affected in RNase III activity (Bardwell et al., 1989), carrying pRJ5-5 did not show any differences in concentration or in the 3'-ends of the pth transcripts relative to those in the wild-type strain. The mutation ans1 determines a thermosensitive RNase E at 43°C (McDowell et al., 1993). The half-lives of RNA1 and RNA2, as revealed by probe F, increased dramatically at 43°C in C600ans1, relative to those at 32°C (Fig. 6A, B). pth sequences probed with P also showed an increased stability at 43°C (not shown). Interestingly, the stability of the transcripts from pRJ1-6, carrying pth (RNA3) but deleted for most of ychF, was not affected (not shown). These results suggest that RNase E processes sequences of the ychF transcript, reducing pth mRNA stability. The low stability of pth mRNA is accompanied by a low Pth concentration at 32°C when RNase E is active (Fig. 6C, upper panel). The concentration of Pnp was also higher at 43°C than at 32°C in the ans1 strain, as expected from the fact that the pnp messenger is processed by RNase E (Hajnsdorf et al., 1994). The protein concentrations at 32°C versus 43°C remained constant for both Pth and Pnp in the wild-type strain (Fig. 6, lower panel).

Effect of the PychH region on pth expression

We inquired whether the presence of the ychH region affected pth expression. The levels of Pth protein expressed from two constructs and accumulated in the cells were compared. One of the constructs, pRJ1-0, harbours the Ddel–EcoRV insert comprising pth and the initial sequences of ychF. The other, pRJ1-6, carries a longer segment from site EcoRI before ychH to the same EcoRV site in pRJ1-0 (Fig. 1D). The additional 200 bp segment in pRJ1-0 upstream of Pthb includes the putative ychH promoters (Fig. 3A). The results of a Western blot assay (Fig. 5A upper panel, lanes 2 and 3) showed that the levels of Pth accumulated in trans-
Fig. 6. Expression of *pth* from the *pth–ychF* transcripts in the absence of endoribonuclease E activity. (A) Samples of C600*ams1*, a mutant thermosensitive for RNase E activity, transformed with pRJ5–5 and growing at either 32 or 43 °C were drawn at the indicated times after the inhibition of RNA synthesis with rifampicin. RNA1 and RNA2 were revealed by Northern blotting with 32P-labelled probe *F* (Fig. 1C; see Methods). (B) Time course of RNA decay in (A) measured as a percentage of the radioactive counts of labelled probe bound. ○, RNA1 at 32 °C; ●, RNA1 at 43 °C; □, RNA2 at 32 °C; ■, RNA2 at 43 °C. (C) Western blot assays for Pth and Pnp antigens in C600 (wt) and C600*ams1* (ams1) after 40 min incubation at the indicated temperatures.

**DISCUSSION**

The expression of *pth* in *E. coli* remains tightly controlled under different conditions. Except for a mutation in *rne* (the gene encoding RNase E), and deletions of the regions flanking *pth* described here, we do not know of any other growth conditions or mutations outside of *pth* that affect Pth protein levels. Changes in the medium composition, temperature of incubation and age of the culture did not affect Pth protein levels (data not shown). A mutation affecting Pth protein stability, *pth*(Ts), reduced the levels of the mutant enzyme at non-permissive temperatures, but there is no evidence that Pth is autoregulated (Cruz-Vera et al., 2000; García-Villegas et al., 1991).

The messages of *pth* and the 3'-proximal gene, *ychF*, are found as either bicistronic or monocistronic transcripts (Figs 1 and 2). The *pth*-only transcripts are a smear of RNA-containing proximal *pth* sequences as if they were products of exonucleolytic decay (Figs 2 and 5B). However, we do not know whether the decay substrates
are terminated pth monocistronic transcripts or processed bicistronic messages. If the latter case is correct, processing is not performed by RNase E (see below). It is likely that all pth-containing transcripts originate from Ppth, the only promoter identified upstream from the pth initiation codon (Fig. 3C; Garcia-Villegas et al., 1991). The ychF-only transcript is an abundant message initiated from a previously unidentified promoter, PychF, located between the pth and ychF ORFs.

The complete pth–ychF bicistronic messenger is an efficient source for Pth accumulation; however, in the absence of distal ychF sequences, the pth messenger generates scant Pth protein levels (Fig. 5A). This result indicates that the ychF message stabilizes pth mRNA (Fig. 5B). The effect is in cis, as trans expression of ychF did not affect Pth protein accumulation (not shown). Apparently, there is no evolutionary advantage to keep the pth–ychF pair together in bacterial chromosomes. The gene sequence ychH–pth–ychF observed in E. coli is typical of enterobacteria (McClelland et al., 2001; Parkhill et al., 2001). Other sequenced genomes such as Haemophilus (Fleischmann et al., 1995), Pseudomonas (Stover et al., 2000) and Mesorhizobium (GenBank: XL463) maintain the pair pth–ychF, whereas the rest of the sequenced bacterial genomes keep pth–, ychH- and ychF-homologous genes dispersed in different regions.

In a thermosensitive RNase E mutant, incubated at a non-permissive temperature, the mono- and bicistronic transcripts containing ychF (RNA1 and RNA2) are stabilized, but the heterogeneous pth transcripts are unaffected (RNA3, data not shown). We interpret this result to mean that at least one RNase E processing site is located within ychF. The site may be in the transcript beyond (3′ to) the EcoRV site (coordinate 1-6, Fig. 1B) because the 5′-ends previous to the EcoRV site are the same in the presence or absence of RNase E activity (not shown). Endonucleolytic cleavage of mRNA is usually followed by 3′→5′ exonuclease degradation (Higgins et al., 1993). Thus, RNase E activity may co-ordinately regulate the expression of both pth and ychF from the bicistronic transcript. Negative regulation by RNase E processing of ribonuclease messengers has been described for pnp (Hajnsdorf et al., 1994), rnb (Zilhao et al., 1995) and rne (Jain & Belasco, 1995).

The in vivo concentration of the ychF-only transcript is much higher than that of pth–ychF (Fig. 2). These concentrations probably represent the transcription initiation frequencies at PychF and Ppth because both transcripts are equally stable (Fig. 6A). This notion was reinforced by in vitro σ70 RNA polymerase transcription; PychF is a stronger promoter than Ppth (not presented). Also, the degree of identity to the −10 and −35 consensus promoter boxes and the base composition of the spacer between boxes suggest that PychF may be a better promoter than Ppth (Hawley & McClure, 1983). Although the spacer in Ppth corresponds to the consensus 17 bp, it includes 10 G+C pairs that could reduce the transcriptional efficiency of the promoter (Auble & deHaseth, 1988). In contrast, PychF features an atypical 24 bp spacer but contains 5 bp inverted repeats flanking a thymine tract (Fig. 4B), a sequence described for the promoter Pm of phage Mu. During complex formation of Pm DNA and RNA polymerase, a distortion occurs at the spacing allowing RNA strand separation at the thymine tract. This event favours the transition from a closed to an open complex and the initiation of transcription (Artsimovitch et al., 1996).

The ychH transcript probably originated from either of the two promoters identified by sequence in the ychH–pth intergenic region and oriented divergently from Ppth (Fig. 3A, B). Deletion of a DNA segment containing the ychH promoters in pRJ1-0 results in a stronger expression of pth mRNA and Pth protein (Figs 5A, B). Therefore, these promoters, or other element(s) in the intergenic sequence, may be inhibitory for pth expression.

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