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

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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; García-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; García-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 (García-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 (Dinçbas *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

Abbreviations: Pnp, polynucleotide phosphorylase; Pth, peptidyl-tRNA hydrolase.

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, *Clal*; 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 [32 P] 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 migration position of the *in vitro*-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.

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⁻ *hsdR thr-1 leu-6 thi-1 supE44 tonA lacY1*, our collection) and C600*ams1*. This latter strain was obtained by P1_{vir} cotransduction of *ams1* with *zct 229::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* (27.1 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 *SmaI* sites of pGEM-4. pRJ1.0 was obtained through cloning, into the

A

ATGAGCACGTTACCGAGTAACGAAGCGTTTTTGC GTT

CATATGTCACCTCCGAACTTCTGGGTTGTAACAGGG
ychH start SD

AATACCCCTCTTCCTTATGTGTAAAGTATAGACAACACC
 -10 -35

CAGTGGGTTATGTGCGGGCGTGATCACAATTACAACCTT
 -10 -35

TATTTCAACAAACTTTACAAATAAACGCCTGAACACC

TTGACTTTCTAATAGTCCACTCAGGCAATTATTACGTT

ATCTGCATTTGATACGCAGTTTTTTTGTCAGCGGGGCC
 Δ pRJ1.0 -35

GCAACCACTAAACTACGCGCCAGTTATGTACACACTC
 -10 +1

AGGACAAAAAACGTGACGATTAAATTGATTGTCGGCC
 SD *pth* start

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* (*P_{pth}*), appropriately oriented relative to the Shine–Dalgarno (SD) regions and translation starts (also underlined). Δ , Site limiting the DNA insert containing *pth* in pRJ1.0. (B) RNA preparation from strain C600 transformed with pRJ5.5 hybridized to

EcoRI and *SmaI* sites of pGEM-4, of a PCR fragment containing *pth* and short flanking segments amplified from the pRJ5.5 template. The primers used were 5'-TACGTTATC-TGAATTCGATACGCAGTTT-3', a sequence located upstream from the putative *pth* promoter (Dutka *et al.*, 1993) modified to contain an *EcoRI* site (underlined), and 5'-GAATTTGTCGATATCGCCGGTCTGG-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*–*NruI* 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 µg ml^{−1} ethidium bromide 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 µg ml^{−1} 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 [α -³²P]UTP (see below). For probe P, used in Figs 2 and 5B (upper panel), the template was pRJ0.8 restricted with *DdeI* (Fig. 1B), whereas for probe F (Fig. 2) the template was pRJ1.6 restricted with *DraI* (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 [α -³²P]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)

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 20 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 pRJ5.5 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).

Fig. 4. The 5'- and 3'-ends of the *ychF* transcripts. (A) Primer extension assay on the *ychF* transcripts (rightmost lane) and sequencing ladder of the corresponding DNA region. On the right-hand edge of the panel are the interpreted nucleotide sequence of the region and four identified signals corresponding to two adenines (arrows). The adenines marked s1 and s2 correspond with *in vitro* transcriptional starts (see text). (B) Nucleotide sequence of the *pth-ychF* intergenic region. The -35 and -10 regions of the putative transcriptional promoter and SD region for *ychF* are underlined, and inverted repeats are underlined by convergent pairs of arrows. $\wedge$, Thymine identified in (A). (C) S1 endonuclease protection analysis as in Fig. 3(B) but using 3'-labelled probe c (Fig. 1C); lane 1, mock hybridization with yeast tRNA, showing a signal of the self-hybridized probe; lane 2, hybridization with RNA from the C600-pRJ5-5 transformant. (D) DNA sequence of the 3'-region beyond *ychF* showing, underlined, two short ORFs and the one-letter code for the amino acids encoded above. The convergent arrows indicate complementary inverted repeats. The 3'-end of the *ychF* transcript is located close before the underlined *EcoRV* site.

and 10 μ g 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 μ l 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 3×10^6 c.p.m. ng^{-1} .

The RNA size markers used in Fig. 2 were obtained by T7-

RNA polymerase run-off transcription of pRJ0-8 DNA template linearized with *DdeI* (205 nt), *NciI*, (420 nt), *EcoRI* (800 nt) and *PvuII* (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 μ l; 40 mM Tris/HCl (pH 8.0), 5 mM MgCl_2 , 10 mM DTT, 50 mM NaCl, 150 μ M 3 dNTPs,

50 μCi [α - ^{32}P]dCTP (6000 Ci mmol $^{-1}$, 222 TBq mmol $^{-1}$; Amersham Pharmacia Biotech), 1 unit *Taq* DNA polymerase (Applied Biosystems) and 10 pmol of primers]. The primers were 5'-GTATTCAACATTTCCGTG-3', beginning within the second codon of *bla*, and 5'-CAATGCTTAATCAGT-GAG-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 MgCl $_2$, 5 mM DTT, 50 mM NaCl, 50 μCi [γ - ^{32}P]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/CHCl $_3$. 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 *Apa*LI 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 MgCl $_2$, 5 mM DTT, 50 mM KCl, 50 mg ml $^{-1}$ BSA, 600 μM of dNTPs except dCTP, 10 μCi [α - ^{32}P]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 10^6 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 4.5), 280 mM NaCl and 4.5 nM ZnSO $_4$ (Régner & 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 C600*ams1* or C600 for pRJ5.5 (Fig. 1D) were grown in LB-ampicillin at 32 °C to late exponential phase (OD $_{600}$ 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. 6B) to be processed for Northern blot assay as described above. The RNA1 and RNA2 bands were revealed by autoradiography of ^{32}P -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 ± 5.0 ng; pRJ1.0, 8.6 ± 3.5 ng; pRJ1.6, 4.3 ± 4.5 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 ^{32}P -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'-GCGCAAAC-GCTCTGCC-3', was used for the extension assay and for sequencing reactions. For experiments in Fig. 4(A), the oligonucleotide used was 5'-GCTTCAATACCGGC-3'. The oligonucleotides were labelled with [γ - ^{32}P]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 (García-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; García-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-4, a vector which does not interfere with transcription initiated at promoters in the inserts. The RNA was revealed with *in vitro*-labelled ^{32}P -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 σ^{70} -like promoter sequences (Fig. 3A; Hawley & McClure, 1983; Wosten, 1998). One σ^{70} 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 ^{32}P -labelled DNA probes a and b were used (Fig. 1C; see Methods). Probe a, a 2.4 kb *EcoRI*-*Apa*LI 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 *Apa*LI and *Cl*AI 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 *ychF*; 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 σ^{70} (result not shown). These data strongly suggest that the identified P_{pth} 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 σ^{70} in the presence of [γ - ^{32}P]ATP or [γ - ^{32}P]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 *Ppth* and *PychF*. Results in Fig. 3(B) had suggested that the 3'-ends of *ychF* transcripts were located within the *ApaLI*–*Clal* DNA segment (Fig. 1B). To locate the 3'-ends of those transcripts precisely, we performed S1-nuclease protection using the *ApaLI*–*Clal* 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 *pth* 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). *Trans*-expression 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 con-

struct, 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 carry 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 5'-ends of the *pth* transcripts relative to those in the wild-type strain. The mutation *ams1* determines a thermosensitive RNase E at 43 °C (McDowall *et al.*, 1993). The half-lives of RNA1 and RNA2, as revealed by probe F, increased dramatically at 43 °C in C600*ams1*, 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 *ams1* 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 *DdeI*–*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 *Ppth* 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.

formants for pRJ1·0 are, at least, twice those for pRJ1·6. This difference in Pth protein concentration is paralleled by the *pth* transcript levels (Fig. 5B upper panel, compare lanes 2 and 3). Since the stability of the *pth* transcripts from both pRJ1·0 and pRJ1·6 is the same, and the *ychH* expression from a plasmid did not affect Pth expression (data not shown), we conclude that *ychH-pth* intercistronic sequences affect *pth* transcription capacity. These sequences may be the putative *ych* promoters (Fig. 3A) or part of the RNA polymerase binding site for *PptH* (Nickerson & Achberger, 1995).

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; García-Villegas *et al.*, 1991). The *ychF*-only transcript is an abundant message initiated from a previously unidentified promoter, *PsychF*, 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: XL465) 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' exonucleolytic 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 *PsychF* and *Ppth* because both transcripts are equally stable (Fig. 6A). This notion was reinforced by *in vitro* σ^{70} RNA polymerase transcription; *PsychF* 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 *PsychF* 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, *PsychF* 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 spacer allowing DNA 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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