Mol Genet Genomics (2002) 268: 518–524
DOI 10.1007/s00438-002-0764-4

ORIGINAL PAPER

M. Aguena · E. Yagil · B. Spira

Transcriptional analysis of the *pst* operon of *Escherichia coli*

Received: 7 May 2002 / Accepted: 19 September 2002 / Published online: 29 October 2002

Abstract The *pst* operon of *Escherichia coli*, which encodes the phosphate-specific transport system, is composed of five genes, *pstS*, *pstC*, *pstA*, *pstB* and *phoU*, whose transcription is induced by phosphate starvation. A phosphate-regulated promoter located upstream of the most proximal gene (*pstS)* controls the transcription of the entire operon. Though the full-length *pst* mRNA could be detected by an improved RT-PCR protocol, Northern analysis using several *pst*-specific probes failed to reveal this transcript. Instead, smaller but distinct *pst* mRNA species were evident. Primer-extension experiments localized the 5′ ends of *pst* mRNAs within the operon. The data suggest that the full-length mRNA is rapidly processed post-transcriptionally.

Keywords *pst* operon · PHO-regulon · *Escherichia coli*

Introduction

Pst (Phosphate-specific transport) and Pit (Pi transport) are two systems that actively transport inorganic phosphate (Pi) into *Escherichia coli* cells. Pit is a low-affinity, constitutively expressed transporter formed by a single protein, whereas Pst is a complex system composed of four proteins, PstS, PstC, PstA and PstB, that are induced when the cells undergo starvation for Pi (Rao and Torriani 1990). The Pst system belongs to the superfamily of ABC (ATP-binding cassette) transporters (Webb et al. 1992; Boos and Lucht 1996). The Pst proteins are encoded by the *pst* operon located at minute 84 on the *E. coli* chromosome. This operon contains five genes that are transcribed counterclockwise in the following order: *pstS*, *pstC*, *pstA*, *pstB* and *phoU* (Fig. 1). PstS is a periplasmic Pi-binding protein, PstC and PstA are integral membrane proteins that mediate the translocation of Pi through the inner membrane (Webb et al. 1992) and PstB is an ATPase that energizes the transport (Chan and Torriani 1996). The function of the *phoU* product is not yet clear; it does not participate in the transport of Pi and is presumably related to the synthesis of ATP (Steed and Wanner 1993). The *pst* operon is a part of the PHO regulon, a set of genes and operons that are regulated by the concentration of Pi in the medium. Besides its role as a transporter of Pi, the Pst system acts as a repressor of the PHO regulon, because most mutations in *pst* lead to constitutive expression of all PHO genes (Wanner 1996). The genes and operons of the PHO regulon are co-regulated by a two-component system composed of the regulatory proteins PhoB and PhoR. The control region of *pst* consists of a –10 promoter sequence and two PHO-boxes (Fig. 1) that are recognized by PhoB. When the concentration of Pi in the medium falls below 4 μM, the sensor protein PhoR phosphorylates PhoB, and the phosphorylated PhoB binds to the PHO-boxes, interacts with the σ^{70} factor of RNA polymerase and initiates transcription (Makino et al. 1993; Wanner 1993).

Based on the sequence analysis of the *pst* operon a transcription terminator in the form of a stem-loop structure was postulated downstream of *pstS* in the non-coding region between *pstS* and *pstC* (Amemura et al. 1985; Surin et al. 1985). A second stem-loop structure that is followed by a conventional promoter site was proposed to be located in the non-coding region between *pstA* and *pstB* (Amemura et al. 1985) (Fig. 1). However, although the promoter at the beginning of the operon has been well characterized, little is known about the operon's

Communicated by W. Goebel

M. Aguena · B. Spira (✉)
Departamento de Microbiologia
Instituto de Ciências Biomédicas
Universidade de São Paulo,
Av. Prof. Lineu Prestes,
1374 São Paulo-SP CEP 05508-900, Brazil
E-mail: benys@usp.br
Tel.: +55-11-30917347
Fax: +55-11-30917354

E. Yagil
Department of Biochemistry,
Tel-Aviv University, Tel-Aviv 69978, Israel

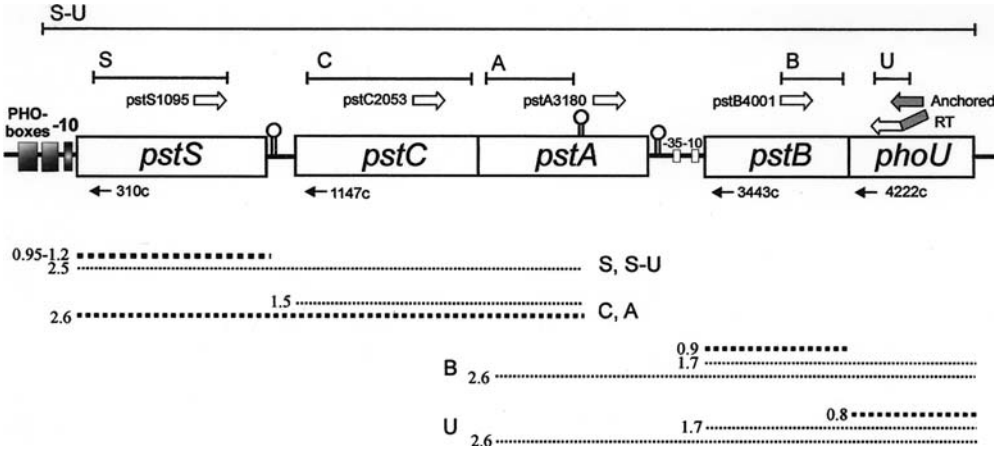

Fig. 1 Schematic representation of the *pst* operon. The *open rectangles* represent the genes of the operon. The two PHO-boxes and the –10 sequence upstream of *pstS*, as well as the proposed –35 and –10 sites upstream of *pstB*, are indicated. The stem-loop *symbols* show the positions of proposed RNA secondary structures. The *bars* at the *top* show the probes used for Northern analysis (see Fig. 2), the *letters* refer to the genes they represent. The *dotted lines* at the *bottom* depict the sections of the mRNA assumed to react with each of the probes (indicated by the letters) in Northern blots. The *thickest dotted lines* represent the major transcripts observed in each blot. The *open arrows* show the forward primers used in the RT-PCR analyses (Fig. 3), the *bent arrow* denotes the RT primer and the *shaded arrow* the anchored primer. The *black arrows* depict the primers used in the primer extension reactions (Fig. 4)

transcription pattern. An earlier study from our laboratory showed that a *pstS* probe, when hybridized with RNA extracted from Pi-starved cells, detected a product of ~1 kb rather than the expected 4.7-kb product that would represents the full-length transcript of the entire *pst* operon (Spira and Yagil 1998). In the present work we show that the *pst* operon is transcribed in its entirety and that the full-length mRNA is cleaved at specific sites, yielding distinct mRNA species. The major transcript of the operon is the product of the most proximal gene, *pstS*.

Materials and methods

Bacterial strains and plasmids

The following strains were used in this study: MG1655 (a wild-type *E. coli* K-12 strain); BS1 (MG1655 *phoB23 proC*::Tn*10*, from our laboratory collection) and BS7 [MG1655 Δ(*pstSCAB-phoU)560::KmR*; transduced from strain BW17335 (Steed and Wanner 1993)]. The plasmid pEY37 is a pBR322 derivative carrying the entire *pst* operon inserted between the *Eco*RI and *Ava*I sites.

Growth conditions

Cells grown exponentially on minimal T-salts medium (Echols et al. 1961) supplemented with 0.4% glucose and 1 mM KH_2PO_4 at 37 C were washed with minimal T-salts, and resuspended to a cell density equivalent to an OD_{540} value of 0.5 in T-salts containing 0.4% glucose and 50 μM KH_2PO_4. Cells were further grown at 37 C and aliquots for alkaline phosphatase (AP) assays and RNA extraction were collected after 10 min (non-starved cells) and 60 min (Pi-starved cells).

Alkaline phosphatase assay

The assays for alkaline phosphatase were done as described previously (Spira et al. 1995). *p*-Nitrophenyl-phosphate was used as the substrate. Data are given as specific activity (enzyme units divided by the OD_{540} value).

RNA extraction and Northern analysis

Aliquots (40 ml) of culture were withdrawn and immediately centrifuged. The cell pellet was homogenized with 4.0 ml of acidic phenol/guanidine thiocyanate (Chomczynski and Sacchi 1987) and incubated at 60 C for 5 min. Following the addition of 1.6 ml of chloroform, samples were centrifuged, and 1 vol of isopropanol was added to the upper phase. The precipitated RNA was centrifuged, washed with 70% ethanol and resuspended in 40 μl of formamide. Aliquots containing equal amounts of RNA were fractionated by electrophoresis in a 1.0% agarose gel containing 7% formaldehyde as described by Sambrook and Russell (2001). RNA was then transferred to a nylon-based membrane (Hybond N+, Amersham-Pharmacia Biotech) and probed with biotinylated DNA fragments as described below. Hybridization and detection of the labeled probes was performed using the North2South Chemiluminescent Nucleic Acid Hybridization and Detection Kit (Pierce) as recommended by the manufacturer. The membranes were then exposed to X-ray films.

Preparation of probes

DNA fragments used as templates for probe synthesis were obtained by PCR amplification using the primers listed in Table 1. Biotinylated probes were obtained by random-primer labeling of the PCR fragments using the North2South Biotin Random Prime Kit (Pierce) as recommended by the manufacturer.

RT-PCR

First-strand cDNA was synthesized by incubating 2 μg of RNA extracted from the Pi-starved MG1655 wild-type strain in the presence of 200 U of SuperScript II reverse transcriptase (Invitrogen Life Technologies) and 2 pmol of primer RT (Fig. 1 and Table 1). Following a 60-min incubation at 42 C, 1/10 of the RT product (2 μl) was used as a template to amplify the specific *pst* fragments in a conventional PCR [2.5 U Taq polymerase (Invitrogen), 0.2 mM dNTPs, 30 pmol of each primer]. In all reactions the reverse primer was the anchored primer and one of the following oligos was used as the forward primer: pstS1095, pstC2053, pstA3180 or pstB4001 (Fig. 1 and Table 1). The PCR consisted of 40 cycles of denaturation at 94 C for 60 s, annealing at 50 C for 60 s and elongation at 72 C for 180 s, except for the *pstB-phoU* cDNA amplification where the

Table 1 List of oligonucleotide primers used

Primer[a]	Sequence (5′→3′)	Designation of PCR product and length of DNA product
Primers used to create probes used in the northern-blots		
pstS(+)	CTTCCCTGCGCCGGTGTATGC	S (608 bp)
pstS(–)	TCAGCGGAGATCAGTTTGGTGT	
pstC(+)	CTGGCGGCGCTGATTGTGCTAT	C (840 bp)
pstC(–)	ATGCGGCGAGGACGATGAAGG	
pstA(+)	CACTGCGGCGCTGGCTGAAT	A (496 bp)
pstA(–)	TCTCGGTGGTGCGGATAACAAT	
pstB(+)	CGGCGCTCGACCCTATCTCTAC	B (196 bp)
pstB(–)	TTGTTTCTTCGCTGGCTTGGTG	
phoU(+)	CCGCGATGCATAACCAGGACAG	U (283 bp)
phoU(–)	GGTATGACGGCCCAGCGACTC	
pst(+)	TCATCTTCGGCTACTTTTTCTC	S-U (4925 bp)
pst(–)	AAGTTATTGGGATTTGTCTGGT	
Primers used in the RT-PCR reactions		
Reverse primers		
RT primer	AGTGGTACACGCAGAGTACTTTC ACCTGCGTGCGGATACTTT	
Anchored primer	AGTGGTACACGCAGAGTACTT	
Forward primers		
pstS1095	AGATGCATGGCCTATTACCTC	
pstC2053	GGCGTATCGTTCTTCCGTTCA	
pstA3180	TATTGATCATTACCCTGTG	
pstB4001	CTACCGGGCGTATTGAAGAGC	
Primers used in the primer extension reactions		
310c	CAGTTGCGACGGTGGTACGC	
1147c	GCAGGCTTGGTTGCAGCCATA	
3443c	CTCGGGGCAGTTTCAACCAT	
4222c	AAGATTGAGACTGTCCATAACGC	

annealing temperature was 58 C and the elongation step lasted 60 s. Negative controls in which 0.2-μg aliquots of RNA were used as templates for PCR were performed for each reaction.

Primer-extension analysis

The reactions were performed essentially as described by Sambrook and Russell (2001). RNA from Pi-starved and non-starved MG1655 cells was extracted as described above. Oligonucleotides complementary to the coding sequence of the genes *pstS*, *pstC*, *pstB* and *phoU*, (oligos 310c, 1147c, 3443c and 4222c, respectively; see Fig. 1 and Table 1) were end-labeled with ^{32}P using the enzyme T4 polynucleotide kinase as described previously (Sambrook and Russell 2001). The labeled oligonucleotides were incubated with 10 μg of total cellular RNA for 90 min at 65 C. The extension reaction was carried out by incubating 10 μg of RNA together with each labeled oligonucleotide in the presence of Mu-MLV reverse transcriptase for 60 min at 42 C. At the end of the incubation, the RT product was treated with 2 vols of RNase reaction mix (100 μg/ml salmon sperm DNA; 20 μg/ml RNase A) for 15 min at 37 C. The cDNA was extracted with one volume of phenol:chlorophorm:isoamyl alcohol (50:49:1) and precipitated with ethanol. The pellet was resuspended in 5 μl of stop/loading dye (Sequenase sequencing kit; USB) and 2–3 μl were loaded onto an 8% acrylamide/7 M urea gel next to the sequencing products. DNA sequencing was done by the dideoxy chain-termination method with a Sequenase (version II) kit (USB) using the same labeled primers as for the primer-extension reaction. The reaction was conducted according to the manual that accompanies the kit. Plasmid pEY37 served as the template.

Results

Northern analysis of the *pst* operon

Exponentially growing cells of strains MG1655 (wild-type), *phoB*23 and Δ*pst* (a strain in which the entire *pst* operon has been deleted) were resuspended in a minimal medium containing 50 μM Pi. Cells harvested after 10 min (non-starved cells) and 60 min (Pi-starved cells) were assayed for alkaline phosphatase (AP) activity. Table 2 shows that in the wild-type AP activity was derepressed ninefold at the 60-min timepoint, indicating that the cells had reached the beginning of the Pi-starvation phase. As expected, the *phoB* mutant exhibited very low levels of AP activity even under Pi-starvation, and the Δ*pst* mutant expressed high levels of AP activity constitutively. Total RNA extracted from these three strains grown under conditions of Pi excess and Pi starvation was hybridized to labeled DNA probes corresponding to internal sequences of each of the *pst* genes and to a probe that covers the entire operon (see Table 1 and Fig. 1). Hybridization was evident in the sample isolated from the wild-type cells that were starved for Pi (Fig. 2) and was absent in the *phoB* and Δ*pst* RNAs (not shown), indicating that all bands observed were RNA/

Table 2 Assay of alkaline phosphatase

Strain[a]	Genotype	High Pi	Low Pi
MG1655	Wild type	0.07	0.65
BS1	*phoB23*	0.04	0.02
BS7	Δ*pst*::Km	5.15	6.03

[a]The indicated strains were assayed for alkaline phosphatase activity 10 min (high Pi) and 60 min (low Pi) after transfer to minimal medium containing an initial concentration of 50 μM Pi. Data are given as specific activities (i.e., enzyme units divided by the OD_{540} of the culture)

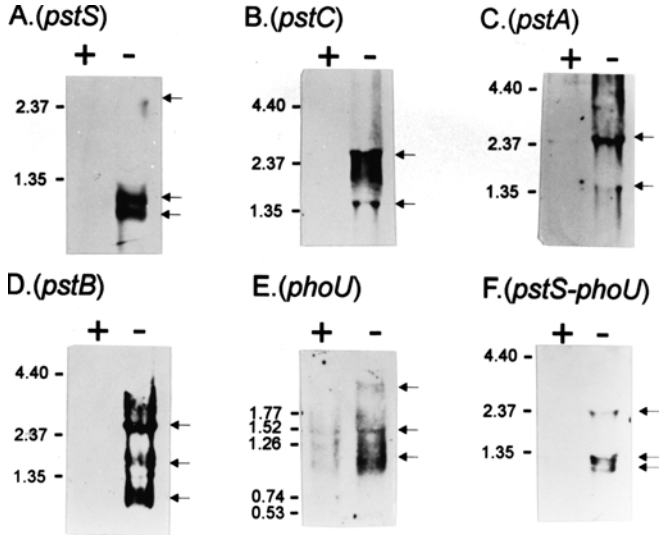

Fig. 2A–F Northern analysis of the *pst* operon**.** The probes used in each case are indicated in *parentheses above* the lanes (see Fig. 1). The *pstS-phoU* probe used in **F** covers the entire operon. RNA was isolated from cells grown in the presence of excess Pi (+) or under conditions of Pi starvation (-). The *numbers* on the *left* indicate the positions and sizes of RNA molecular weight markers. The *arrows* on the *right* indicate the positions of the transcripts

DNA hybrids that were specific for the individual genes of the *pst* operon. No RNA species of the size (~4.7 kb) expected for a full-length transcript of the entire *pst* operon could be detected with any of the probes. The dotted lines in Fig. 1 depict the regions that each hybridization product represents. Hybridization with the *pstS* probe (Fig. 2A) revealed two distinct bands of 0.95 and 1.2 kb. A faint fragment of 2.6 kb became evident when the autoradiogram was exposed for a longer period of time (data not shown). The two smaller bands correspond to two different forms of *pstS* mRNA (whose ORF is 1041 bp long), suggesting either that *pstS* transcription is initiated or terminated at two distinct locations or that a larger *pstS* transcript is cleaved at a specific site shortly after its synthesis. The larger band of 2.6 kb that is much weaker corresponds best to a *pstS-pstC-pstA* transcript. However, it is somewhat smaller than the 2974 nt predicted for a *pstS-pstC-pstA* transcript. A putative inverted repeat sequence ($\Delta G = -35.0$ kJ) that might act as an internal transcription terminator or an RNase recognition site is present in the middle of *pstA* (Fig. 1; nucleotides 2848–2887 of the *pst* operon, Accession No. K01992). If the *pstS-pstC-pstA* mRNA is indeed cleaved or terminated at this site its expected length is reduced to 2.6 kb.

Northern blots hybridized with the *pstC* and *pstA* probes (Fig. 2B and C) show a 2.6-kb band that represents the *pstS-pstC-pstA* transcript, and a weaker band of about 1.5 kb that might be a transcript of the *pstC-pstA* genes (1847 nt, or 1442 nt assuming that the stem-loop sequence in *pstA* is used as a terminator or a processing site).

Hybridization with the *pstB* probe reveals three RNA species (Fig. 2D), a strong one of 2.6 kb, one of 1.7 kb and a third of 0.94 kb. The 2.6-kb band probably represents a *pstA-pstB-phoU* transcript (2573 nt). The 1.7-kb band is of the size expected for *pstB-phoU* (1570 nt) and the 0.94-kb band agrees best with *pstB* alone (829 nt). The *phoU* probe also hybridized with the 2.6-kb *pstA-pstB-phoU* transcript, the 1.7-kb species (*pstB-phoU*), and a 0.9-kb transcript that probably represents *phoU* alone (Fig. 2E). Finally, hybridization with the probe that covers the entire operon (Fig. 2F) revealed two major bands of 0.95 kb and 1.2 kb, and a weaker band of 2.6 kb; the same pattern as seen with the *pstS* probe (Fig. 2A). The fact that these *pstS* bands are the main products detected by the *pstS-phoU* probe suggests that *pstS* is the main transcript of the operon. The stem-loop structure located immediately downstream of the *pstS* coding region shows 73% identity to a repetitive extragenic palindromic (REP) sequence (Bachellier et al. 1996). Such REP structures have been shown to impede the 3′→5′ exonucleolytic degradation of mRNA (Newbury et al. 1987a, 1987b; Higgins et al. 1992), and should thus increase the amount of *pstS* mRNA in comparison to transcripts of the other *pst* genes. The minor 2.6-kb band probably represents the *pstS-pstC-pstA* transcript, which may be stabilized by the stem-loop (95% homology to the REP consensus) found downstream of *pstA* (Fig. 1). However, none of the probes provides any evidence for the existence of a stable mRNA representing the entire operon. Each of the *pst* probes hybridized with more than one RNA species, suggesting that the full-length *pst* transcript is post-transcriptionally processed by cleavage at specific sites or, less likely, that there are transcription initiation points within the operon (see Discussion).

The primary transcript of the *pst* operon

The Northern blot data described above, as well as our attempts to amplify the entire operon by conventional RT-PCR (not shown), failed to reveal a full-length *pstS-phoU* mRNA species, such as would be expected from an operon. We therefore devised a modification of the EXACT RT-PCR procedure (Smith et al. 2001) that prevents amplification of PCR products from contaminating genomic DNA, and thus eliminates the need to treat the RNA template with DNase, preserving the integrity of the RNA. RNA extracted from the Pi-starved wild-type culture (strain MG1655) was reverse transcribed using a hybrid primer of 42 nt (RT primer); the 21 nucleotides at its 5′ end are not homologous to any sequence in the *E. coli* genome, and the 21 nucleotides at its 3′ end correspond to the reverse sequence located 115 bp downstream from the start codon of *phoU* (see Fig. 1 and Table 1). Amplification of the reverse-transcribed DNA/RNA hybrid product was performed in four separate reactions; in all of these, the anchored reverse primer which is composed only of the (5′ half) non-homologous portion of the RT primer was used as the reverse primer. The forward primer in each reaction corresponded to each of the first four coding

regions of the operon (open arrows in Fig. 1). A product that represents an mRNA transcript of the entire operon is expected from the reaction with the *pstS* primer that represents the proximal gene of the operon and the anchored primer that represents its distal gene (*phoU*). Such an RT-PCR product of 3229 bp extending from the 3′ end of the *pstS* coding region through the 5′ end of the *phoU* coding region was clearly evident (Fig. 3, lane 1), indicating that the *pst* operon is fully transcribed as a single mRNA. RT-PCR products of the expected shorter lengths that span *pstC-phoU* (2271 bp), *pstA-phoU* (1144 bp) and *pstB-phoU* (323 bp) were also obtained (Fig. 3, lanes 2–4, respectively). All RT-negative lanes did not show any product (not shown), confirming that the products seen in Fig. 3 were amplified from cDNA. The 3229-bp band is less intense than the others, probably because the synthesis of long PCR products is less efficient than that of shorter ones and because the full-length *pst* mRNA is less abundant.

Primer-extension analysis within the *pst* operon

Primer extension analyses were performed using primers designed to anneal upstream of each of the *pst* operon genes, in order to determine the location of possible endonuclease cleavage sites, transcription initiation sites or of stable RNA secondary structures (Beuzon et al. 1999; Persson et al. 2000). RNA extracted from Pi-starved and non-starved wild-type cells was used as the template for the reactions. The approximate positions of the primers are depicted as black arrows in Fig. 1. Extension of primer 310c (Fig. 4A), whose 3′ end lies 11 bp downstream of the *pstS* start codon, indicated that the transcription start point of *pstS* (and probably the entire operon) is a G-C base pair (position 237 of the *pst* operon, Accession No. K01992) a few bp downstream of the –10 site, confirming previous results (Makino et al. 1988). Primer 1147c, which covers the first six codons of *pstC*, was used to analyze the intergenic region between *pstS* and *pstC*. Four products were observed (Fig. 4B) that extended to the stem-loop structure downstream of *pstS* (Fig. 1), the strongest arrested fragment reaching the most distal base pair at the 3′ end of its double-stranded stem (position 1362). Attempts to extend a primer from *pstA* did not yield any product (not shown). The product obtained with the oligomer 3443c, whose 3′ end reaches 5 bp downstream of the *pstB* start codon, extended to a C-G base pair (position 3380, Fig. 4C) located 27 nt downstream of the proposed –10 site of the internal promoter in the intergenic region between *pstA* and *pstB* (Amemura et al. 1985). Finally, extension of primer 4222c, located at the 5′-end of the *phoU* coding region, yielded a product that ended at a G-C base pair (position 4150, Fig. 4D) 77 bp upstream of *phoU* and 19 codons upstream of the 3′ end of *pstB*. No products were observed when RNA from non-starved cells was used as a template in any of the reactions (data not shown).

In summary, the results of the primer extension analyses confirm the position of the transcription start point of *pstS* (and probably that of the entire operon) (Makino et al. 1988); they also point to the existence of an RNA secondary structure between *pstS* and *pstC*, and they suggest the presence of either transcription start points or ribonuclease cleavage sites within the operon, upstream of *pstB* and *phoU*.

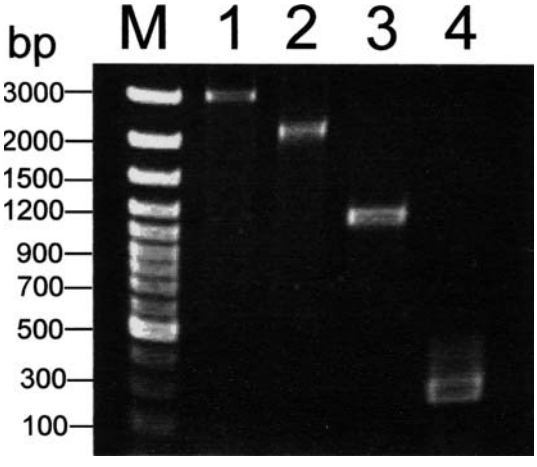

Fig. 3 RT-PCR analysis of the *pst* operon. Lanes 1, *pstS-phoU* product; 2, *pstC-phoU* product; 3, *pstA-phoU* product; 4, *pstB-phoU* product. M, DNA ladder, band sizes are indicated on the *left*

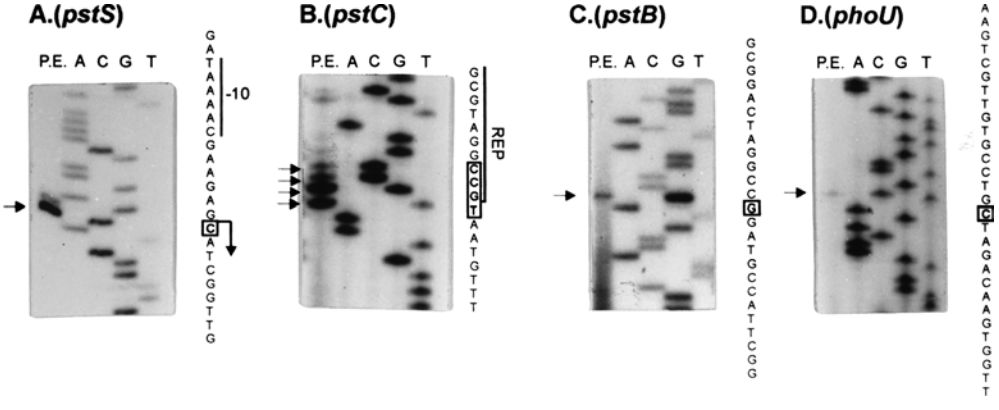

Fig. 4A–D Primer extension analysis of *pst* RNAs. The primers used were 310c (**A**), 1147c (**B**), 3443c (**C**) and 4222c (**D**). The corresponding genes are indicated in *parentheses* at the *top*. The extended products (P.E.) are indicated by the *arrows* to the *left* of the lanes. On the right side are the relevant sequences that correspond to the – strand. The bases representing the extension endpoints are *boxed*

Discussion

Our Northern hybridizations did not detect any RNA species that might correspond to a full-length transcript of the entire *pst* operon. Instead they have revealed much smaller, but discrete, RNA species that were specific to the *pst* operon because they were induced by Pi starvation and were absent in the *phoB* and Δ*pst* mutants. Likewise, the primer-extension experiments revealed the presence of *pst*-specific 5′ RNA ends that correspond to internal positions within the intact *pst* mRNA. Nevertheless our RT-PCR analysis demonstrated that a transcript of the entire *pst* operon is in fact synthesized, albeit in small amounts. This leads to the conclusion that a full-length *pst* mRNA is formed, but is cleaved at specific sites shortly after its formation. The specific nature of these cleavages is inferred from the distinct bands represented by the hybridization products on the Northern blots and from the primer extension results.

The primer extension product obtained with the *pstS* primer (Fig. 4A) confirms the location of the transcription initiation site of the operon (Makino et al. 1988). The extension product upstream of *pstC* (Fig. 4B) extends to the 3′ end of the stem of the REP element located between *pstS* and *pstC*. Therefore, the termination of extension at this point was probably due to the stem-loop structure (Beuzon et al. 1999). The other primer-extension analyses have revealed two internal 5′ ends of *pst*-specific RNA species, one between *pstA* and *pstB* (Fig. 4C) and the other in the coding region of *pstB* (Fig. 4D). Amemura et al. (1985) pointed out the presence of a possible promoter within the *pst* operon, in the coding region between *pstA* and *pstB*, that consists of –10 and –35 consensus sites downstream of a stem-loop structure (Fig. 1). However, it is very unlikely that Pi starvation-induced synthesis of mRNA molecules is initiated within the *pst* operon. This is because all observed transcripts were induced by Pi starvation and were *phoB*-dependent, yet no PHO-box consensus sequences are found inside the *pst* operon. Furthermore, the proposed –10 site (AACACT) does not show much resemblance to the relevant consensus sequences (TATAAT; Harley and Reynolds 1987) and it is actually located at position –27 relative to the 5′-end of the *pstB* primer extension product. We therefore prefer to view this product as being the result of a specific endonuclease cleavage event at a site located between *pstA* and *pstB*. Finally, the primer extension product of *phoU*, which extends into the coding region of *pstB* – where there is no sign of a promoter – strengthens our view that the shorter transcripts are derived from specific endonuclease cleavage of the full-length *pst* transcript. Many studies in other bacterial systems have shown that polycistronic mRNA is post-transcriptionally processed by endonucleolytic cleavage (Faubladier et al. 1990; Murakawa et al. 1991; Gamper and Haas 1993; Naureckiene and Uhlin 1996). The ribonucleases often involved in mRNA processing are RNase III and RNase E, but the specificity of their recognition sites is still controversial (Nicholson 1999; Rauhut and Klug 1999).

The DNA probes that correspond to *pstS* and to the entire operon (*pstS-phoU*) predominantly hybridized with the same 0.95 kb and 1.2 kb species of mRNA (Fig. 2A and F), indicating that these bands, which correspond to *pstS*, are the major *pst* transcripts. The presence of a stable REP secondary structure downstream of *pstS* is probably responsible for the increased amount of *pstS* relative to other *pst* transcripts. Since the primary role of the REP sequences is to stabilize upstream mRNA by protecting it from exonucleolytic attack (Higgins et al. 1992), it is possible that the two smaller and stronger bands that correspond to *pstS* mRNAs are the result of protection by the REP from exonucleolytic degradation of a longer transcript. This conclusion agrees with observations in other ABC-transport operons in which the most proximal gene, usually coding for a periplasmic binding protein, is transcribed in excess relative to the downstream genes of the operon (Higgins et al. 1982; Horazdovsky and Hogg 1987; Hardham et al. 1997). A putative REP sequence that might be involved in the stabilization of the *pstS-pstC-pstA* transcript is also present downstream of *pstA*. However, since the *pstS* mRNA is more abundant than the *pstS-pstC-pstA* transcript, the second REP site is less protective probably because one or more endonuclease cleavage sites within the coding regions of *pstC* and/or *pstA* would expose 3′ ends to exonucleolytic degradation.

In conclusion, this study has shown that the *pst* operon is transcribed as a single mRNA molecule. The full-length *pst* transcript is cleaved at specific points shortly after its synthesis, yielding smaller mRNA products. The REP sequence present in the intergenic region between the first and second genes of the operon ensures that the *pstS* mRNA encoding the periplasmic binding protein is the major transcript of the *pst* operon.

Acknowledgements We thank Barry Wanner for sending us bacterial strains. This research was supported by grants 99/09246-3 and 01/05401-6 from Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP). EY was supported by a grant from the Kurt Lion Foundation.

References

Amemura M, Makino K, Shinagawa H, Kobayashi A, Nakata A (1985) Nucleotide sequence of the genes involved in phosphate transport and regulation of the phosphate regulon in *Escherichia coli*. J Mol Biol 184:241–250

Bachellier S, Gilson E, Hofnung M, Hill CW (1996) Repeated sequences. In: Neidhardt FC, Curtiss R III, Ingraham JL, Lin ECC, Low KB, Magasanik B, Reznikoff WF, Riley M, Schaechter M, Umbarger HE (eds) *Escherichia coli* and *Salmonella*: cellular and molecular biology. ASM Press, Washington D.C., pp 2012–2040

Beuzon CR, Marques S, Casadesus J (1999) Repression of IS*200* transposase synthesis by RNA secondary structures. Nucleic Acids Res 27:3690–3695

Boos W, Lucht JM (1996) Periplasmic binding protein-dependent ABC transporters. In: Neidhardt FC, Curtiss R III, Ingraham JL, Lin ECC, Low KB, Magasanik B, Reznikoff WF, Riley M, Schaechter M, Umbarger HE (eds) *Escherichia coli* and *Salmonella*: cellular and molecular biology. ASM Press, Washington D.C., pp 1175–1235

Chan FY, Torriani A (1996) PstB protein of the phosphate-specific transport system of *Escherichia coli* is an ATPase. J Bacteriol 178:3974–3977

Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159

Echols A, Garen A, Garen S, Torriani A (1961) Genetic control of repression of alkaline phosphatase in *E. coli*. J Mol Biol 3:425–438

Faubladier M, Cam K, Bouche JP (1990) *Escherichia coli* cell division inhibitor DicF-RNA of the *dicB* operon. Evidence for its generation in vivo by transcription termination and by RNase III and RNase E-dependent processing. J Mol Biol 212:461–471

Gamper M, Haas D (1993) Processing of the *Pseudomonas arcDABC* mRNA requires functional RNase E in *Escherichia coli*. Gene 129:119–122

Hardham JM, Stamm LV, Porcella SF, Frye JG, Barnes NY, Howell JK, Mueller SL, Radolf JD, Weinstock GM, Norris SJ (1997) Identification and transcriptional analysis of a *Treponema pallidum* operon encoding a putative ABC transport system, an iron-activated repressor protein homolog, and a glycolytic pathway enzyme homolog. Gene 197:47–64

Harley CB, Reynolds RP (1987) Analysis of *E. coli* promoter sequences. Nucleic Acids Res 15:2343–2361

Higgins CF, Ames GF, Barnes WM, Clement JM, Hofnung M (1982) A novel intercistronic regulatory element of prokaryotic operons. Nature 298:760–762

Higgins CF, Peltz SW, Jacobson A (1992) Turnover of mRNA in prokaryotes and lower eukaryotes. Curr Opin Genet Dev 2:739–747

Horazdovsky BF, Hogg RW (1987) High-affinity L-arabinose transport operon. Gene product expression and mRNAs. J Mol Biol 197:27–35

Makino K, Shinagawa H, Amemura M, Kimura S, Nakata A, Ishihama A (1988) Regulation of the phosphate regulon of *Escherichia coli*. Activation of *pstS* transcription by PhoB protein in vitro. J Mol Biol 203:85–95

Makino K, Amemura M, Kim SK, Nakata A, Shinagawa H (1993) Role of the sigma 70 subunit of RNA polymerase in transcriptional activation by activator protein PhoB in *Escherichia coli*. Genes Dev 7:149–160

Murakawa GJ, Kwan C, Yamashita J, Nierlich DP (1991) Transcription and decay of the *lac* messenger: role of an intergenic terminator. J Bacteriol 173:28–36

Naureckiene S, Uhlin BE (1996) In vitro analysis of mRNA processing by RNase E in the *pap* operon of *Escherichia coli*. Mol Microbiol 21:55–68

Newbury SF, Smith NH, Higgins CF (1987a) Differential mRNA stability controls relative gene expression within a polycistronic operon. Cell 51:1131–1143

Newbury SF, Smith NH, Robinson EC, Hiles ID, Higgins CF (1987b) Stabilization of translationally active mRNA by prokaryotic REP sequences. Cell 48:297–310

Nicholson AW (1999) Function, mechanism and regulation of bacterial ribonucleases. FEMS Microbiol Rev 23:371–390

Persson M, Glatz E, Rutberg B (2000) Different processing of an mRNA species in *Bacillus subtilis* and *Escherichia coli*. J Bacteriol 182:689–695

Rao NN, Torriani A (1990) Molecular aspects of phosphate transport in *Escherichia coli*. Mol Microbiol 4:1083–1090

Rauhut R, Klug G (1999) mRNA degradation in bacteria. FEMS Microbiol Rev 23:353–370

Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual (3rd edn). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Smith RD, Ogden CW, Penny MA (2001) Exclusive amplification of cDNA template (EXACT) RT-PCR to avoid amplifying contaminating genomic pseudogenes. Biotechniques 31:776–782

Spira B, Yagil E (1998) The relation between ppGpp and the PHO regulon in *Escherichia coli*. Mol Gen Genet 257:469–477

Spira B, Silberstein N, Yagil E (1995) Guanosine 3′,5′-bispyrophosphate (ppGpp) synthesis in cells of *Escherichia coli* starved for Pi. J Bacteriol 177:4053–4058

Steed PM, Wanner BL (1993) Use of the rep technique for allele replacement to construct mutants with deletions of the *pstSCAB-phoU* operon: evidence of a new role for the PhoU protein in the phosphate regulon. J Bacteriol 175:6797–6809

Surin BP, Rosenberg H, Cox GB (1985) Phosphate-specific transport system of *Escherichia coli*: nucleotide sequence and gene-polypeptide relationships. J Bacteriol 161:189–198

Wanner BL (1993) Gene regulation by phosphate in enteric bacteria. J Cell Biochem 51:47–54

Wanner BL (1996) Phosphorus assimilation and control of the phosphate regulon. In: Neidhardt FC, Curtiss R III, Ingraham JL, Lin ECC, Low KB, Magasanik B, Reznikoff WF, Riley M, Schaechter M, Umbarger HE (eds) *Escherichia coli* and *Salmonella*: cellular and molecular biology. ASM Press, Washington D.C., pp 1357–1381

Webb DC, Rosenberg H, Cox GB (1992) Mutational analysis of the *Escherichia coli* phosphate-specific transport system, a member of the traffic ATPase (or ABC) family of membrane transporters. A role for proline residues in transmembrane helices. J Biol Chem 267:24661–24668