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Enteroinvasive *Escherichia coli* virulence-plasmid-carried apyrase (*apy*) and *ospB* genes are organized as a bicistronic operon and are subject to differential expression

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In *Shigella flexneri* and enteroinvasive *Escherichia coli* (EIEC) the expression of the virulence-plasmid(pINV)-carried potential pathogenesis-associated *apy* gene, which encodes apyrase (ATP diphosphohydrolase), is regulated by the same regulators that govern the expression of virulence genes. To understand the transcriptional organization of the *apy* gene, the authors sequenced an 8023 bp *Pst*I fragment of the pINV of EIEC strain HN280, which encompasses *apy* as well as its adjacent genes. The *Pst*I fragment displays 99% identity with the corresponding fragment of pWR100, the pINV of *S. flexneri* strain M90T, and contains four genes. One of these genes, *ospB*, encodes a secreted protein of unknown activity and is located immediately upstream of *apy*. Analyses of sequence, Northern hybridization, RT-PCR and primer extension data and transcriptional fusions indicated that *ospB* and *apy* are co-transcribed as a 2 kb bicistronic, temperature-regulated mRNA from an upstream promoter that precedes *ospB*. The 2 kb mRNA is post-transcriptionally processed in the intercistronic *ospB–apy* region, leading to the considerable accumulation of a more stable 1 kb *apy*-specific mRNA (half-life of 2·2±0·3 min, versus 27±4 s for the 2 kb transcript). Upon temperature induction, peak expression of the *ospB–apy* operon occurs when bacteria enter into the late phases of bacterial growth, where the *apy*-specific transcript was found to be much more prevalent if compared to the *ospB–apy* transcript.

Keywords: ATP diphosphohydrolase, mRNA processing, polycistronic mRNA

INTRODUCTION

Shigella flexneri and enteroinvasive *Escherichia coli* (EIEC) are pathogenic micro-organisms that are known to cause disease in humans by very similar mechanisms of pathogenicity. Once ingested, bacteria reach the colonic mucosa and invade various cell types, including M cells, macrophages and epithelial cells. Invasion of the intestinal mucosa is followed by intracellular bacterial multiplication, spread of the infection to adjacent cells, induction of severe inflammation of the colon and destruction of the mucosa (Sansonetti, 2001). The expression of virulence genes is transcriptionally regulated by temperature: bacteria which are invasive when grown at 37 °C become non-invasive when grown at 30 °C (Dagberg & Uhlin, 1992; Dorman *et al.*, 1990; Hromockyi *et al.*, 1992; Jost & Adler, 1993). This thermoregulated expression is under the control of the global regulator *hns*, which prevents transcription,

This paper is dedicated to the memory of our dear friends and colleagues Giuseppe Carruba and Franco Tatò, who died prematurely.

Abbreviations: EIEC, enteroinvasive *Escherichia coli*; pINV, virulence plasmid; RT, reverse transcriptase.

The GenBank accession number for the sequence reported in this paper is AJ315184.

albeit at different levels, of the virulence-plasmid(pINV)-encoded regulatory genes *virB* and *virF* at 30 °C (Dagberg & Uhlin, 1992; Falconi *et al*., 1998; Jost & Adler, 1993; Porter & Dorman, 1994; Tobe *et al*., 1993). The activation of pINV-encoded virulence gene expression occurs in a co-ordinated fashion at 37 °C, where H-NS repression is relieved and VirF activates transcription of *virB* which, in turn, activates the transcription of unlinked operons encoding the *ipa*, *mxi* and *spa* invasion operons (Dagberg & Uhlin, 1992; Tobe *et al*., 1991, 1993).

In a previous study (Berlutti *et al*., 1998), we have shown that the pINV-carried *apy* gene, which encodes apyrase (ATP diphosphohydrolase), is regulated at the transcriptional level – like other virulence genes – by temperature, H-NS and the VirF/VirB regulatory cascade. Even though a specific role has not been assigned to *apy*, the finding that its transcription is controlled by the same regulatory network that governs the expression of virulence genes raises the possibility that *apy* might be involved in the pathogenicity of enteroinvasive bacteria. In this respect, it is worth mentioning that apyrase has been implicated in the decrease of dNTP levels occurring in host cells during intracellular multiplication as well as in the *Shigella*-induced process of actin polymerization (Mantis *et al*., 1996; Babu *et al*., 2002). Furthermore, it has also been suggested that apyrase could be considered either as a general cytotoxin (possibly involved in damaging cellular metabolism and eventually in cell death, and in the dephosphorylation of exogenous nucleotides to nucleosides necessary to cross the impermeable cytoplasmic membrane) or as an effector playing a role in some as yet unknown metabolic pathway not directly involved in pathogenicity (Berlutti *et al*., 1998; Fernandez-Prada *et al*., 1997; Zalkin & Nygaard, 1996; Zychlinsky *et al*., 1996).

In our previous study (Berlutti *et al*., 1998), Northern-hybridization analysis showed that *apy* is probably transcribed as part of a polycistronic, temperature-regulated mRNA. This indicated that unknown genes, located adjacent to *apy*, could be co-transcribed under the control of the same regulatory network. To address this issue and to better characterize the transcriptional organization of *apy*, we have cloned and sequenced an 8023 bp *Pst*I fragment of the pINV of the O135:K^-:H^- EIEC strain HN280, encompassing *apy* as well as its adjacent genes. Analyses of sequence, Northern hybridization, RT-PCR and primer extension data and transcriptional fusions indicated that *apy* is co-transcribed on a bicistronic, temperature-regulated mRNA together with a gene located upstream of *apy* and identical to *ospB*, a gene with unknown function that encodes a secreted protein (Buchrieser *et al*., 2000). These data also showed that transcription starts from a promoter element located upstream of *ospB*. The bicistronic mRNA is processed in the intercistronic *ospB–apy* mRNA region, leading to the considerable accumulation of a smaller, more stable, *apy*-specific mRNA.

The implications of the results presented in this work with regard to the origin and role of the *ospB–apy* operon within enteroinvasive micro-organisms are also discussed.

METHODS

Bacterial strains, plasmids and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Growth media used were Trypticase soy broth (BBL Microbiology Systems) and Luria–Bertani (LB) broth medium (Miller, 1972). The solid media contained 1·5 % agar. Congo red (Sigma) was added at 0·01 % to Trypticase soy agar, to determine Congo-red binding. Antibiotics (Sigma) were used at the following concentrations: 100 μg ampicillin ml^{-1}; 30 μg chloramphenicol ml^{-1}; 30 μg kanamycin ml^{-1}; 5 μg tetracycline ml^{-1}.

DNA manipulations. DNA extraction, isolation of plasmids, restriction digestion, electrophoresis, purification of DNA fragments, construction of recombinant plasmids, transformations and Southern hybridizations were performed by standard methods (Sambrook *et al*., 1989). pINV DNA preparations were performed as described previously (Zagaglia *et al*., 1991).

Overlapping DNA fragments, generated after digestion with different restriction enzymes of the ~8·3 kb *Pst*I *apy*-containing fragment of pHN290 (Table 1) cloned into pACYC177, were subcloned into pUC18. Double-stranded DNA inserts were sequenced by the dideoxy-chain-termination method using QIA-express forward and reverse sequencing primers (Diagen) and a commercial T7 sequencing kit (Pharmacia Biotech). Primers were 5′-end-labelled with carbocyanin (Pharmacia Biotech) and sequencing products were analysed with an ALF-express automated DNA sequencer (Pharmacia Biotech). The sequences of individual fragments were assembled to obtain the nucleotide sequence of an 8023 bp *Pst*I fragment that contained the entire *apy* locus. Sequence data were compared to known sequences by using the BLAST server (http://www.ncbi.nlm.nih.gov/BLAST/).

PCR analysis. The primers used throughout this study are listed in Table 2. Thermal cycling conditions were as described previously (Berlutti *et al*., 1998). Specific annealing temperatures for the individual primers are reported in Table 2.

RNA manipulations. Total bacterial RNA was extracted by a modification of the hot phenol method, as described previously (Colonna *et al*., 1995), and quantified spectrophotometrically (A_{260}). The quality of each RNA preparation was checked by visualization of rRNA bands in ethidium-bromide-stained agarose gels that were electophoresed under denaturing conditions.

For Northern (RNA) analysis, total RNA (10 μg samples) was denatured at 65 °C for 15 min in the presence of 2 M formaldehyde and 50 % formamide. It was then separated on a formaldehyde/MOPS/agarose gel and transferred to Hybond-C extra membranes (Amersham). DNA probes were prepared by PCR. PCR-generated DNA fragments were recovered from low-melting-agarose gels; they were then ^{32}P-labelled by random priming (Sambrook *et al*., 1989). After hybridization, dried membranes were analysed in an Instant Imager electronic autoradiographer (Canberra Packard), to quantify the amount of bound probe. Results were adjusted by subtracting the background level for the filters and were normalized by probing duplicate filters with the ^{32}P-labelled *rrnB* probe (7·5 kb *Bam*HI fragment) from pKK3535 (Brosius *et al*., 1981).

For RT-PCR experiments, total RNA was extracted from cultures of EIEC strain HN280 that had been grown overnight

Table 1. Bacterial strains and plasmids used in this study

Strain/plasmid	Relevant characteristics	Source/reference(s)
Strains		
HN280	Wild-type EIEC strain of serotype O135:K$^-$:H$^-$, contains pINV pHN280, invasive; *apy*$^+$	Berlutti *et al.* (1998); Colonna *et al.* (1995)
HN570	Δ*lac* derivative of strain HN280, invasive; *apy*$^+$	Colonna *et al.* (1995)
DH10b	*E. coli* K$^-$ 12 F$^-$ *mcrA* Δ(*mrr–hsdRMS–mcrBC*) *ϕ*80d*lacZ*ΔM15 Δ*lacX74 deoR recA1 ara*Δ*139* Δ(*ara leu*)*7697 galU galK λ*$^-$ *rpsL endA1 nupG*	Gibco-BRL
Plasmids		
pHN280	pINV of EIEC strain HN280; *apy*$^+$	Zagaglia *et al.* (1991)
pHN290	pTH10-mobilized, Tn*1*-labelled derivative of pINV pHN280; *apy*$^+$	Zagaglia *et al.* (1991)
pACYC177	Cloning plasmid vector; Ap^r Km^r	Rose (1988)
pUC18	Cloning plasmid vector; Ap^r	Yanisch-Perron *et al.* (1985)
pHN99	pACYC177-derived vector carrying the 8023 bp *Pst*I fragment of the pHN290; Ap^s Km^r	This study
pHNEH2.2	pUC18-derived vector carrying the 2·2 kb *Eco*RI–*Hin*cII fragment of the 8·3 kb fragment of pHN290; Ap^r	This study
pMP220	Promoter-probe plasmid vector; Tc^r	Spaink *et al.* (1987)
pHNOR1	pMP220 plasmid vector containing the PCR-generated *ospB–lacZ* operon fusion; Tc^r	This study
pHNAP1	pMP220 plasmid vector containing the PCR-generated *apy–lacZ* operon fusion; Tc^r	This study
pHNOR1-1	pMP220 vector containing the same DNA fragment as pHNOR1, but inserted in the opposite orientation; Tc^r	This study
pHNAP1-1	pMP220 vector containing the same DNA fragment as pHNAP1, but inserted in the opposite orientation; Tc^r	This study

Ap^r, ampicillin-resistant; Ap^s, ampicillin-sensitive; Km^r, kanamycin-resistant, Tc^r, tetracycline-resistant.

at 30 °C and then diluted 1:200 in fresh LB medium, incubated at 30 °C up to an OD_{600} of ∼0·2 and shifted rapidly to 37 °C until an OD_{600} of ∼1·1 was reached. RNA preparations were subjected to an additional treatment with RNase-free DNase. Reverse transcriptase (RT) assays were performed using SuperScript II RT (Gibco-BRL), according to the manufacturer's instructions, with the 3′-reverse oligonucleotide AGZ18 (Table 2) complementary to the 3′ extremity of the *apy* gene. PCR amplifications were done using cDNA as template in 30 cycles of reaction using the primer pair INTXR1 (5′-sense)/INTXL1 (3′-reverse), which amplified a specific *ospB* 501 bp fragment, or the primer pair AGZ17 (5′-sense)/AGZ18 (3′-reverse), which amplified a specific 672 bp *apy* fragment (Table 2). As controls, we performed RT assays without adding RT, without adding RNA or by adding pHN290 DNA instead of RNA. These controls were then subjected to PCR amplification as described above.

In the primer-extension experiments, oligonucleotide primers were 5′-end-labelled with [γ-^{32}P]-dATP by using T4 polynucleotide kinase. Each labelled oligonucleotide was hybridized with 10 μg of total RNA isolated from strain HN280 grown at 30 or 37 °C to an OD_{600} of ∼0·6. Reverse transcription experiments were carried out at 43 °C with avian myeloblastosis virus RT (US Biochemicals), according to the manufacturer's instructions. The 5′-labelled primers used for detecting the start sites were INTX2, complementary to nucleotides +104 to +85 of the *ospB–apy* bicistronic mRNA (the *ospB–apy* 5′ end marks position +1), and INTAPY1 and INTAPY2 (Table 2), complementary to nucleotides +187 to +169 and +116 to +97, respectively, of the *apy* mRNA (the *apy* 5′ end marks position +1). The resulting cDNAs were analysed on denaturing 7% polyacrylamide gels, along with a sequencing ladder that was generated by using the same oligonucleotides and pHNEH2.2 (Table 1) as template. Manual double-stranded DNA sequencing reactions were performed with the Sequenase DNA Sequencing Kit (version 2.0; US Biochemicals) and [^{35}S]-dATP.

The determination of mRNA half-lives was carried out using bacterial cells that had been grown in LB medium at 30 °C to an OD_{600} of ∼0·2 and then shifted rapidly to 37 °C. When cultures reached OD_{600} ∼1·1, rifampicin was added to a final concentration of 250 μg ml^{-1} and samples were taken at various time intervals. RNA was extracted as described above. Equal amounts (10 μg) of each RNA sample were separated on a formaldehyde/agarose gel and the resulting bands were transferred to a nitrocellulose membrane. RNAs were analysed by Northern-blot assay and hybridization with a ^{32}P-labelled *apy* probe. To normalize the quantity of RNA in each lane, duplicate filters were hybridized with the labelled oligonucleotide 5′-ACTACCATCGGCGCTACGGC-3′, which was used as a probe for 5S rRNA. The relative half-lives of the bicistronic and *apy* transcripts were obtained by determining (phosphorimager quantification) and plotting the normalized amounts of bound probe for each transcript at each time point. Half-lives were calculated from the slope of each plot, and the errors of these half-lives were estimated from the SD of the slopes.

Determination of promoter activity *in vivo*. Fragments encompassing the DNA regions containing putative transcriptional signals of the *apy* operon were generated by PCR using pHN290 DNA as template and primers carrying restriction sites at their ends. Plasmid pHNAP1 (carrying an *apy–lacZ* fusion) was constructed by cloning a 376 bp PCR-generated fragment [primers AP1 (5′-sense) and AP2 (3′-reverse); Table 2] into the reporter plasmid pMP220 (carrying a promoterless

Table 2. Primers used in this study

Oligonucleotide	DNA sequence*	Function and features	Annealing temperature (°C)†
INTXL1	5′-TTCTAAAAATGGCTGAATCATGG-3′	Amplification of *ospB* DNA	57
INTXR1	5′-AGGGAAAAAGCTCTTGACCATAG-3′	Amplification of *ospB* DNA	57
SPACL1	5′-AATTGTTTGCATTTAAGATGAAGTT-3′	Amplification of intergenic DNA region	55
SPACR1	5′-AATTTGAACAGAGTGATGGAAAAAC-3′	Amplification of intergenic DNA region	55
AGZ17	5′-CTGAAGGCAGAAGGTTTT-3′	Amplification of *apy* DNA	52
AGZ18	5′-TTATGGGGTCAGTTCATTGGT-3′	Amplification of *apy* DNA	52
INTX2	5′-AATATCTGATATGCTTTCAT-3′	Primer extension	NA
INTAPY1	5′-GACAAACTGTCTGGTGAAG-3′	Primer extension	NA
INTAPY2	5′-AATCATATTTGTAGCAATAC-3′	Primer extension	NA
OR1	5′-CGC**GTCGAC**AGAACATTACCGGAACAAGATAG-3′	5′ Primer for *ospB* promoter amplification; *Sal*I	57
OR2	5′-GGA**AGATCT**GATATGCTTTCATTCTTTTTATTGA-3′	3′ Primer for *ospB* promoter amplification; *Bgl*II	57
AP1	5′-CGC**GTCGAC**AATTGTTTGCATTTAAGATGAAGTT-3′	5′ Primer for *apy* promoter amplification; *Sal*I	57
AP2	5′-GGA**AGATCT**TTTGCTGAGGGGATAAAAATCATA-3′	3′ Primer for *apy* promoter amplification; *Bgl*II	57
OR1-1	5′-CGC**AGATCT**AGAACATTACCGGAACAAGATAG-3′	5′ Primer for *ospB* promoter amplification; *Bgl*II	57
OR2-1	5′-GGA**GTCGAC**GATATGCTTTCATTCTTTTTATTGA-3′	3′ Primer for *ospB* promoter amplification; *Sal*I	57
AP1-1	5′-CGC**AGATCT**AATTGTTTGCATTTAAGATGAAGTT-3′	5′ Primer for *apy* promoter amplification; *Bgl*II	57
AP2-1	5′-GGA**GTCGAC**TTTGCTGAGGGGATAAAAATCATA-3′	3′ Primer for *apy* promoter amplification; *Sal*I	57

* Restriction sites (*Sal*I and *Bgl*II) introduced at the 5′ end of the oligonucleotides are in bold type.

† NA, Not applicable (primer not used for PCR).

lacZ gene downstream of a multiple-cloning site; Spaink *et al.*, 1987), which was used to generate transcriptional fusions. Plasmid pHNOR1 (carrying an *ospB–lacZ* fusion) was constructed by cloning a 378 bp fragment [primers OR1 (5′-sense) and OR2 (3′-reverse); Table 2] into pMP220. To precisely assess promoter activity, the same DNA regions – but in the opposite orientation – were also cloned into pMP220 using primers AP1-1 (5′-sense) and AP2-1 (3′-reverse) for the *apy* gene and primers OR1-1 (5′-sense) and OR2-1 (3′-reverse) for the *ospB* gene (Table 2), thus generating recombinant plasmids pHNAP1-1 and pHNOR1-1, respectively. The recombinant plasmids were separately transformed into the Δ*lac* mutant invasive EIEC strain HN570 (Table 1). β-Galactosidase expression was assayed on SDS/chloroform-permeabilized cells, as described by Miller (1972).

RESULTS

Sequence analysis of the *Pst*I fragment encompassing *apy*

An 8023 bp *Pst*I fragment of pHN290 (Table 1) was cloned and sequenced (GenBank accession no. AJ315184), as described in Methods. The sequence of the *Pst*I fragment shows 99% identity with the corresponding *Pst*I fragment of plasmid pWR100, the pINV of *S. flexneri* strain M90T, and contains three ORFs on the same strand and a frameshift-inactivated gene (*ospC4*) located downstream of *apy* and in the opposite orientation with respect to the other three genes (Buchrieser *et al.*, 2000; Venkatasen *et al.*, 2001) (Fig. 1). Since these ORFs share a highly similar location and a very high sequence similarity (over 99% identity) with the pathogenesis-associated genes *icsP* (*sopA*), *ospB* and *apy* (*phoN2*) carried by the *Pst*I fragment of pWR100 (Buchrieser *et al.*, 2000; Venkatasen *et al.*, 2001), we have designated these ORFs as the *icsP*, *ospB* and *apy* genes.

Transcription of the *ospB–apy* operon

Previous results obtained in our laboratory suggested that *apy* could be part of a polycistronic transcriptional unit (Berlutti *et al.*, 1998). To better characterize the transcriptional organization of *apy*, a Northern-blot analysis was performed. Total RNA was extracted from exponentially growing cultures of EIEC strain HN280 grown in LB medium at 30 or 37 °C; this was hybridized

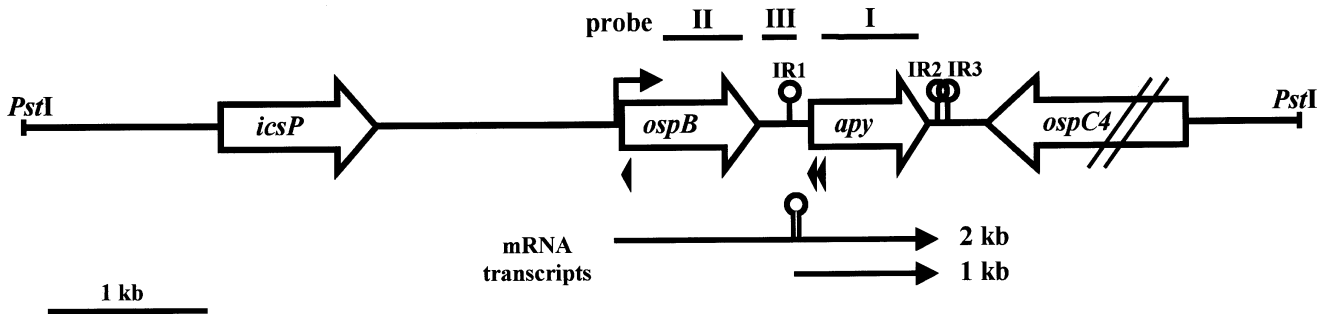

Fig. 1. Genetic and transcriptional organization of the *ospB–apy* operon. The 8023 bp *Pst*I fragment of the pINV of EIEC strain HN280 encompasses four genes (*ospC4* is a frameshift-inactivated gene; Buchrieser *et al.*, 2000). Open arrows represent the position and extent of the four genes. The bent arrow upstream of *ospB* indicates the transcriptional start site of the *ospB–apy* operon and the direction of transcription. The horizontal arrows below the map indicate the two mRNA transcripts (the 2 kb *ospB–apy* bicistronic transcript and the 1 kb *apy* transcript). The stem–loop structure in the 2 kb transcript marks the putative processing site of the bicistronic mRNA (shown in Fig. 5b). The inverted repeats (IRs) downstream of *ospB* (IR1) and *apy* (IR2 and IR3) are indicated on the map. The bars above the map (II, III and I) represent the PCR-amplified DNA fragments used as probes in Northern-blot experiments (see Results). The locations of the primers (Table 2) used in the primer-extension experiments are depicted by arrow heads below the *ospB* and *apy* genes.

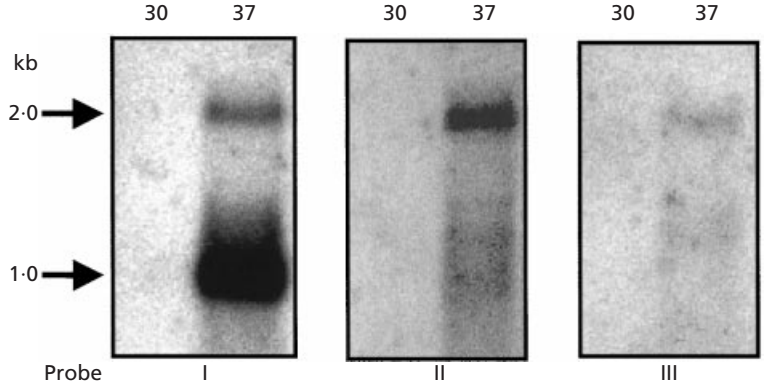

Fig. 2. Northern-blot analysis of the *ospB–apy* operon. Total RNA was extracted from cultures of HN280 grown at 30 or 37 °C to an OD_{600} of ~0·6. Each lane was loaded with 10 μg of total RNA and the filters were probed with ^{32}P-labelled DNA probes I, II or III (see text and Fig. 1).

with three different ^{32}P-labelled probes (Fig. 2). PCR-generated DNA fragments I, II and III were used as probes (Fig. 1). Probe I covers a 672 bp internal fragment of *apy* (primers AGZ17/AGZ18), probe II covers a 501 bp internal fragment of *ospB* (primers INTXL1/INTXR1) and probe III covers a 218 bp internal fragment of the intergenic *ospB–apy* region (primers SPACL1/SPACR1) (Table 2).

Two different transcripts were detected by probe I (the *apy*-specific probe) in RNA preparations from exponentially growing HN280 at 37 °C. The larger and less-abundant transcript (~2 kb) also hybridizes with probes II (the *ospB*-specific probe) and III (the intergenic *ospB–apy*-specific probe). This RNA species could correspond to a transcript starting from a putative promoter located upstream of *ospB* and ending downstream of *apy*. The smaller and more-abundant mRNA (~1 kb) was only detected with probe I, and it may represent a specific cleavage product of the large transcript or an independent mRNA expressed from an internal promoter located within the transcriptional unit. Phosphorimager quantification of the two transcripts showed that the 1 kb transcript was about eightfold more abundant than the 2 kb transcript. Prolonged exposure of the Northern blots also revealed the binding of probes II and III to smaller RNA species which were not resolved in discrete bands and which might represent degradation products generated from the 2 kb mRNA. No messages were detected with any probe in RNA preparations from HN280 grown at 30 °C, indicating that the transcription of *ospB* and *apy* is dramatically repressed at this non-permissive temperature (Fig. 2). These data indicated that EIEC strain HN280 produces two temperature-inducible *apy* mRNAs – a 1 kb transcript encoding *apy* alone and a 2 kb species encoding both *ospB* and *apy* – and that the two genes might be organized as a bicistronic operon.

RT-PCR analysis was used to assess whether *ospB* and *apy* are transcribed as a single message. Oligonucleotide AGZ18 (3′-reverse primer; Table 2), which is complementary to the *apy* mRNA, was annealed to total RNA isolated from HN280 grown at 30 or 37 °C, and RT was used to generate cDNA. PCR amplifications were carried out using RT products as templates and using two pairs of oligonucleotide primers (Table 2) designed to amplify fragments internal to the *ospB*

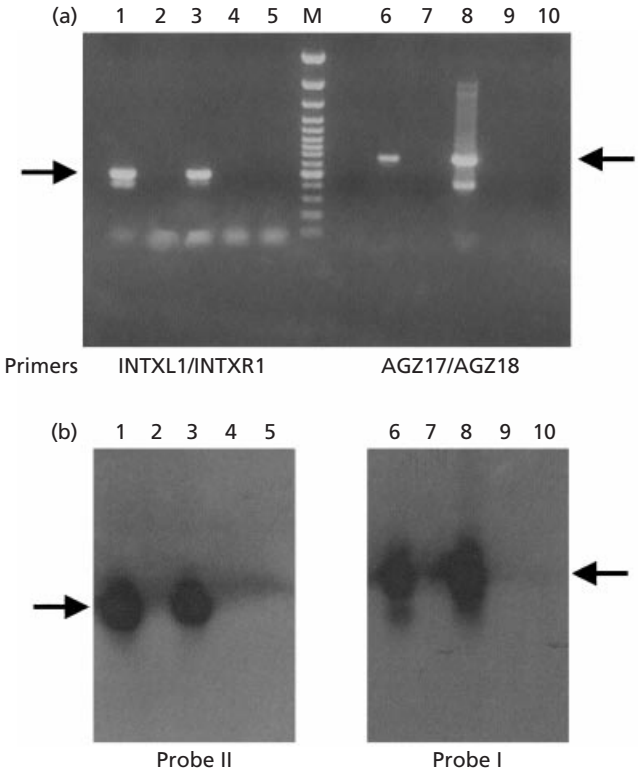

Fig. 3. RT-PCR analysis of the *ospB–apy* operon. (a) Ethidium-bromide-stained agarose gel of PCR-amplified products. RNA isolated from HN280 cultures grown at 30 or 37 °C was used as template to generate cDNAs (RT products). The cDNAs obtained from RNA preparations at 30 (lanes 4, 5, 9 and 10) and 37 °C (lanes 2, 3, 7 and 8) were then used as templates in PCR with the *ospB*-specific primers INTXL1/INTXR1 (lanes 2–5) and with the *apy*-specific primers AGZ17/AGZ18 (lanes 7–10) (Table 2). pHN290 DNA (Table 1) was used as a positive control in PCR with the same primers (INTXL1/INTXR1 in lane 1 and AGZ17/AGZ18 in lane 6). RT was omitted from control reactions (lanes 2, 4, 7 and 9). M, 100 bp DNA ladder (Gibco-BRL). (b) Southern blot of the ethidium-bromide-stained gel shown in (a), probed with the *ospB*-specific probe (lanes 1–5) and with the *apy*-specific probe (lanes 6–10). Arrows mark the expected PCR-products (a) and the corresponding hybridization bands (b).

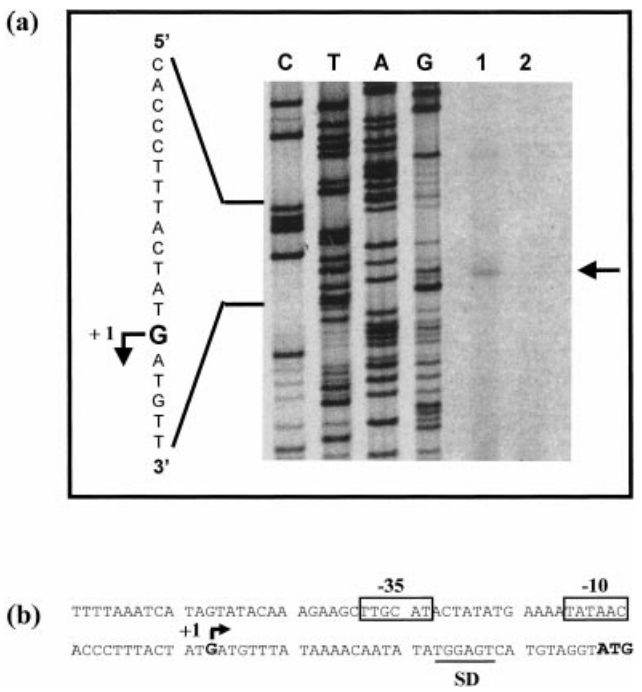

Fig. 4. Primer-extension analysis of the transcriptional start site of the 2 kb *ospB–apy* bicistronic transcript. (a) The auto-radiograph shows a primer-extension experiment performed on RNA extracted from strain HN280 grown at 37 (lane 1) and 30 °C (lane 2) with the anti-sense primer INTX2 (Table 2). Lanes C, T, A and G show the sequencing ladder generated with the same primer. The right arrow marks the end-point of the extended product. The bent arrow indicates the location of the transcriptional start (+1 site). The exposure of the ladder was reduced to prevent overexposure as compared to the primer-extension product. (b) Nucleotide sequence of the *ospB* promoter region. The −10 and −35 regions are indicated by open boxes and the bent arrow marks the transcription initiation site. The putative Shine–Dalgarno (SD) sequence is underlined and the ATG initiation codon of *ospB* is shown in bold.

coding region [INTXL1 (5′-sense) and INTXR1 (3′-reverse)] or to the *apy* coding region [AGZ17 (5′-sense) and AGZ18 (3′-reverse)]. Thus, if the mRNA was bicistronic, the cDNA would contain *ospB* and *apy* sequences, and 501 and 672 bp PCR products, respectively, would be amplified. Using this approach, we amplified the two expected DNA fragments from the RNA of HN280 grown at 37 °C and confirmed (by Southern hybridizations with the *ospB*- and *apy*-specific probes) that these fragments were, indeed, *ospB* and *apy* (Fig. 3a, b). As expected, no amplification products were detected from RNA preparations from HN280 grown at 30 °C. As a negative control, RNA annealed to primer AGZ18 and incubated without RT was used as the template for the PCR described above. Other positive and negative control mixtures contained pHN290 DNA or distilled water as templates. These results allowed us to unequivocally confirm that the *ospB* and *apy* genes are organized as a single bicistronic operon.

Determination of the transcriptional signals of the *ospB–apy* operon

Primer-extension analysis was carried out to determine the 5′ ends of the transcripts. Total RNA isolated from HN280 grown at 30 or 37 °C was annealed to primers complementary to regions proximal to the *ospB* and *apy* ATG start codons (3′-reverse primers; INTX2 for *ospB* and INTAPY1 or INTAPY2 for *apy*) (Table 2), as detailed in Methods. Using RNA isolated from HN280, a major *ospB* extension product was detected only upon growth at 37 °C (as expected, no product was detected when HN280 was grown at 30 °C) (Fig. 4a). This suggested the existence of a transcriptional start site located 35 nt upstream of the translational start codon. A sequence resembling a promoter element precedes the 5′ end: the predicted −35 (TTGCAT) and −10 (TATAAC) hexamers are separated by 12 nt and share

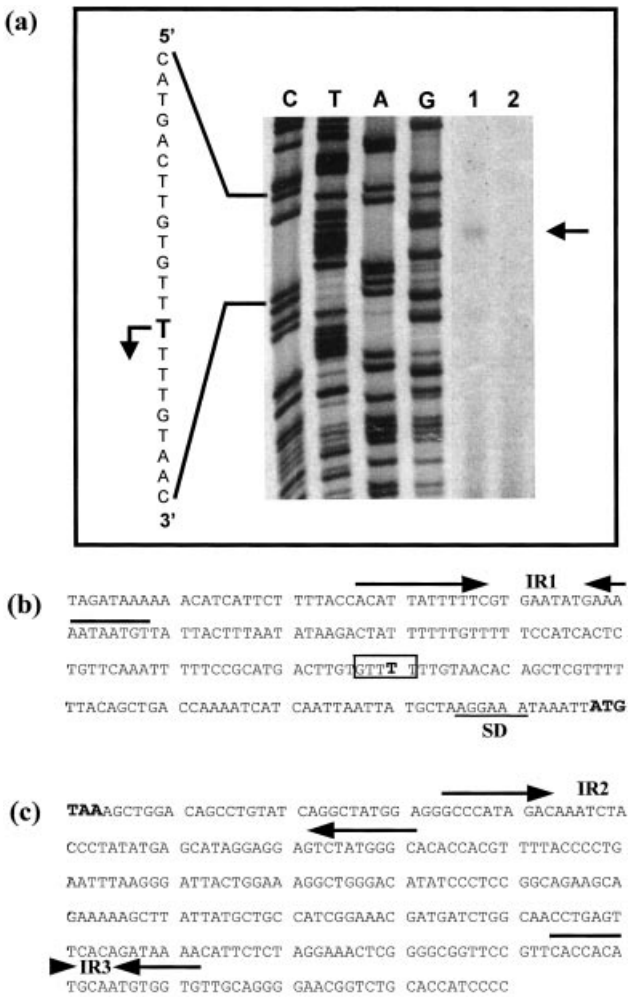

Fig. 5. Primer-extension analysis of the 5′ end of the 1 kb *apy* transcript. (a) The autoradiograph shows a primer-extension experiment performed on RNA extracted from strain HN280 grown at 37 (lane 1) and 30 °C (lane 2) with primer INTAPY2 (Table 2). Lanes C, T, A and G show the sequencing ladder generated with the same primer. The right arrow indicates the end-point of the extended product. The bent arrow indicates the location of the extended product. The exposure of the ladder was reduced to prevent overexposure as compared to the primer-extension product. (b) Nucleotide sequence of the DNA region upstream of the *apy* start codon. The 5′ end of the *apy* transcript is shown in bold within an open box indicating the putative RNase E consensus sequence. A putative Shine–Dalgarno (SD) sequence is underlined and the *apy* ATG initiation codon is shown in bold. The inverted repeat (IR1) is indicated by arrows. (c) Nucleotide sequence of the DNA region downstream of the *apy* stop codon. The TAA stop codon is shown in bold. Inverted repeat elements (IR2 and IR3) are indicated by arrows.

three and five residues, respectively, with the *E. coli* σ^{70} consensus sequence (TTGACA and TATAAT) (Fig. 4b). The −10 region maps 12 nt upstream of the start codon. Analysis of the DNA sequence in the intergenic *ospB–apy* region showed the presence of a 12 bp inverted repeat (IR1; $\Delta G°$, −6·7 kcal mol^{-1}) that could represent a site of inefficient termination of transcription originating from the *ospB* promoter (Fig. 5b). Moreover, a putative Shine–Dalgarno sequence, TGGAGT, precedes (9 nt) the *ospB* start codon (Fig. 4b).

A single *apy* product was detected in primer extensions performed using two separate primers (Fig. 5a, and data not shown). The 5′ end was found to map within the 328 bp intercistronic *ospB–apy* DNA region 68 nt upstream of the translational start codon, within a sequence (GUUUU) that closely resembles the proposed *E. coli* RNase E recognition consensus sequence (A/GAUUA/U) (Grunberg-Manago, 1999) (Fig. 5b). Inspection of the DNA sequence upstream of the 5′ end of *apy* showed no sequences resembling −10 and −35 consensus hexamers typical of promoters. This suggested that the small 1 kb *apy* transcript may not originate at the identified 5′ end but, rather, may result from post-transcriptional processing of the 2 kb bicistronic mRNA whose transcription initiates upstream of *ospB*, and that the *ospB–apy* operon might be subjected to post-transcriptional regulation which may also lead to differential gene expression (a putative RBS, AGGAAA, is located 6 nt upstream of the *apy* start codon; Fig. 5b).

Analysis of the sequence immediately downstream of *apy* reveals two inverted repeats, designated IR2 ($\Delta G°$, −8·7 kcal mol^{-1}) and IR3 ($\Delta G°$, −3·3 kcal mol^{-1}) (Fig. 5c). Although they must be considered imperfect terminators (because of the spacing or the span of the U nucleotides distal to the bases of the hairpin structures) they could nevertheless function as ρ-independent termination signals (Fig. 5c). The distance between the 5′ ends of the large *ospB–apy* bicistronic transcript and the small *apy* transcript, and the putative terminators located just downstream of *apy*, agrees very well with the sizes of the identified transcripts (2 and 1 kb) as determined by Northern-hybridization experiments (Fig. 2).

To confirm and further analyse the transcriptional organization of the *ospB* and *apy* genes and to determine promoter activity *in vivo*, two reporter gene constructs were prepared. *ospB* and *apy* putative promoter regions (a 378 bp DNA fragment encompassing 310 bp upstream of the *ospB* start codon and a 376 fragment encompassing 305 bp upstream of the *apy* start codon) were cloned upstream of the *lacZ* reporter gene of the promoter-probe plasmid pMP220, generating recombinant plasmids pHNOR1 and pHNAP1, respectively. Plasmids pHNOR1-1 and pHNAP1-1, which contained the same inserts as pHNOR1 and pHNAP1, respectively, but in an inverted orientation, were used as controls (see Methods for details). Since the expression of *apy* is regulated by the same regulatory network that governs the expression of virulence genes (Berlutti *et al.*, 1998), recombinant plasmids were separately transformed into the Δ*lac* EIEC strain HN570 (Table 1) and β-galactosidase activity was used as a measure of promoter expression (Miller, 1972). A comparison of the expression of the transcriptional fusions in HN570 grown at 37 °C revealed that pHNOR1 (*ospB–lacZ* fusion) produces about 32-fold more β-galactosidase activity than pHNAP1 (*apy–lacZ* fusion) (2689 versus 84

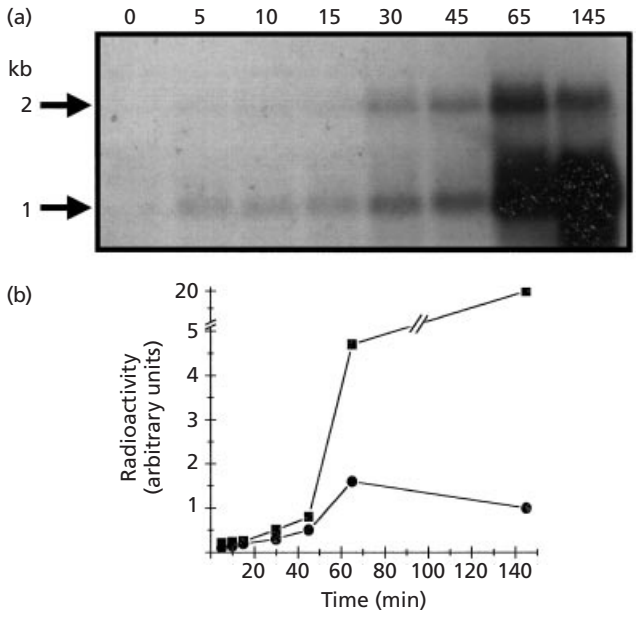

Fig. 6. (a) Time-course of *ospB–apy* operon transcriptional expression upon temperature induction. EIEC strain HN280 was grown in LB medium at 30 °C to an OD_{600} of ~0·2 (0 min) and transcription of the *ospB–apy* operon was induced by shifting the temperature to 37 °C (5–145 min). Total RNA was isolated at different time intervals and probed with probe I (the *apy*-specific probe). (b) Radioactivity was measured by phosphorimager quantification and normalized values were plotted as a function of time; 2 kb transcript, ●; 1 kb transcript, ■.

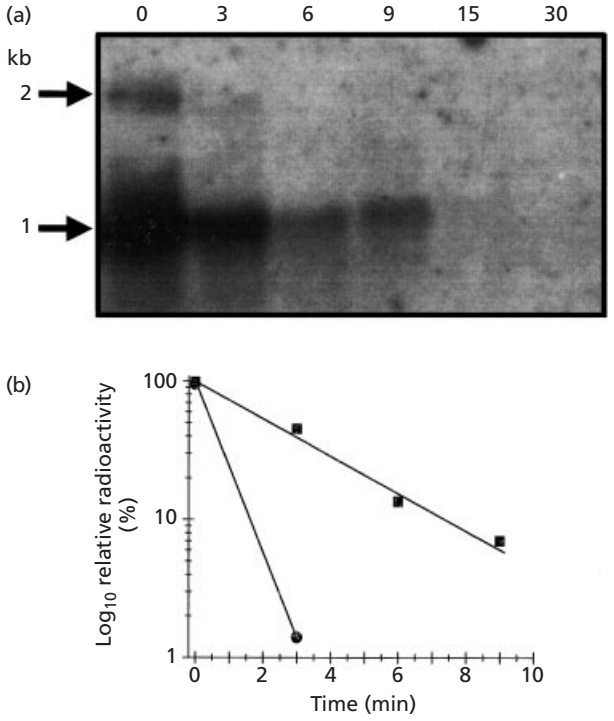

Fig. 7. Decay of *ospB–apy* operon transcripts. EIEC strain HN280 was grown in LB medium at 30 °C to an OD_{600} of ~0·2 and transcription of the *ospB–apy* operon was induced by rapid shifting of the temperature to 37 °C. When cultures reached OD_{600} ~1·1, rifampicin was added (250 μg ml^{-1} final concentration) and samples were taken at different time-point intervals (min) for Northern-blot analysis. The 2 and 1 kb transcripts were identified with probe I (the *apy*-specific probe). Half-lives of the mRNAs were determined by phosphorimager quantification of the radiolabelled bands and by plotting the values obtained from different blots [a typical Northern-blot experiment is shown in (a)] on a semi-logarithmic scale as a function of time (b), as described in Methods. The measured half-lives were 27±4 s for the 2 kb *ospB–apy* transcript (●) and 2·2±0·3 min for the 1 kb *apy* transcript (■) (mean±SD of four independent experiments).

units, respectively). The results are expressed as the means of four independent experiments; the SE was less than 10% of the mean for all results. Since a level of β-galactosidase activity similar to that obtained with pHNAP1 was detected with both pHNOR1-1 (92 units) and pHNAP1-1 (87 units), and since pMP220 alone scored 3 units, the low but detectable β-galactosidase activity of pHNAP1 may reflect unspecific basal transcriptional initiation, probably due to the high A+T content of the cloned DNA regions (data not shown). These results are consistent with the hypothesis that the expression of the *ospB–apy* operon is under the control of a promoter located upstream of *ospB*.

Time-course of *ospB–apy* operon expression upon induction by temperature

To better define the relative levels of the two transcripts as well as the extent to which *ospB–apy* transcription is influenced by temperature, we analysed the induction of transcription by Northern-blot hybridization. To this end, HN280 was grown at the non-permissive temperature of 30 °C, up to an OD_{600} of ~0·2, and then shifted rapidly to 37 °C. Total RNA was extracted at various time intervals and hybridized with the ^{32}P-labelled *apy*-specific probe I. As shown in Fig. 6a, the expression of the *ospB–apy* operon was strongly induced and peak expression was achieved when HN280 passed through the late-exponential to early-stationary phases of growth [i.e. 65 min (OD_{600} ~1·1) and 145 min (OD_{600} ~2·2), respectively, after the temperature shift]. At every time point the 1 kb transcript was more abundant than the 2 kb transcript (the ratios of the 2 and 1 kb transcripts at 65 and 145 min were 1:3 and 1:20, respectively) (Fig. 6b). These results indicated that a difference in stability might be responsible for the different levels of the two transcripts.

Stability of the 2 kb *ospB–apy* and 1 kb *apy* transcripts

To get a further insight into the stability of the bicistronic mRNA, the half-lives of the two transcripts were measured. Total RNA was extracted from HN280 grown at 37 °C at various time intervals after transcription had been blocked with rifampicin, and was analysed by Northern-blot hybridization with the *apy*-specific probe I (Fig. 7a). The half-lives of the two

transcripts were estimated by phosphorimager quantification (Fig. 7b), as described in Methods. We calculated the half-life of the 2 kb *ospB–apy* transcript to be 27 ± 4 s and the half-life of the 1 kb *apy* transcript to be $2{\cdot}2 \pm 0{\cdot}3$ min (means $\pm$ SD of four independent experiments).

The shorter half-life of the 2 kb transcript and the relatively high level of 1 kb *apy* transcript present (see above) indicate that in EIEC strain HN280, when entering the late phases of bacterial growth, the transcriptional level of *apy* is considerably higher than that of *ospB*.

DISCUSSION

apy is a potential pathogenesis-associated gene, located on the pINVs of virulent *Shigella* spp. and related EIEC strains, which encodes a periplasmic ATP-hydrolysing enzyme known as apyrase (Berlutti *et al*., 1998; Bhargava *et al*., 1995; Buchrieser *et al*., 2000; Venkatasen *et al*., 2001). To study the transcriptional organization of *apy*, we have sequenced an 8023 bp *Pst*I fragment of the pINV of the EIEC strain HN280 that carries *apy* as well as its adjacent genes. *ospB*, a gene which has been reported to encode a protein secreted by the Mxi/Spa type III secretion apparatus of *S. flexneri* (Buchrieser *et al*., 2000), is located just upstream of *apy* (Fig. 1).

In this work, we present evidence that *ospB* and *apy* are part of a bicistronic operon and that they are co-transcribed on a temperature-repressible mRNA. Two transcripts, a large one (approx. 2 kb) encoding *ospB* and *apy*, and a smaller one (about 1 kb) encoding *apy*, were clearly detected in HN280 grown at 37 °C (Figs 2 and 3). Based on the finding that *ospB* is co-transcribed with *apy* (this study), and that we have previously shown that the transcription of *apy* is under the control of the same regulatory network that governs the expression of the virulence genes *ipa*, *mxi* and *spa* (Berlutti *et al*., 1998), we hypothesize that the *ospB–apy* operon might play a role in the pathogenicity of enteroinvasive micro-organisms, even though a specific biological role for the two genes has not yet been assigned.

Primer-extension analysis identified the 5′ ends of the 2 and 1 kb transcripts (35 and 68 nt, respectively) to be upstream of the ATG translational start codons of *ospB* and *apy*. Inspection of the DNA region upstream of the 5′ end of *ospB* reveals sequences resembling *E. coli* σ^{70} -10 and -35 consensus promoter hexamers, although the span of the spacing between the -35 and the -10 (12 nt) consensus sequences and between the -10 and $+1$ site (12 nt) is not canonical (Fig. 4b).

Sequences resembling promoter elements were not found in the DNA region upstream of the 5′ end of *apy*, and the expression of the transcriptional fusions indicated the presence of promoter activity for *ospB–lacZ* fusions but not for *apy–lacZ* fusions. Moreover, the size of the 2 kb transcript is consistent with the transcription of the *ospB–apy* operon starting at the identified 5′ end ($+1$) of *ospB* and terminating at putative transcriptional terminators located downstream of *apy*. These data are consistent with the hypothesis of an *ospB–apy* bicistronic transcript starting from a promoter located upstream of *ospB*.

Thus, the 1 kb transcript encoding *apy* alone must result from post-transcriptional processing of the 2 kb transcript at the identified 5′ end, within the 328 bp *ospB–apy* intercistronic region. That this could be the case is further supported by the following observations: (i) probes II (the *ospB*-specific probe) and III (the intercistronic-specific probe), which both recognized the 2 kb *ospB–apy* transcript, also bound smaller RNA species that were not resolved into discrete bands (Fig. 2) and which are likely to represent degradation products of the processed 2·0 kb transcript at the newly formed 3′ end; (ii) the 5′ end of the 1 kb *apy* transcript maps within a sequence (GUUUU) (Fig. 5b) homologous to the consensus recognition sequence (A/GAUUA/U) proposed for RNase E (Grunberg-Manago, 1999); and (iii) computer analysis (not shown) revealed that the 5′ end of the 1 kb *apy* transcript lies within a single-stranded segment in a secondary structure, with a predicted ΔG° value of $-9{\cdot}4$ kcal mol^{-1}, as expected for RNase E cleavage sites (Grunberg-Manago, 1999). RNase E is a key enzyme that has been implicated in the cleavage and in the decay of several different RNA molecules in *E. coli*. mRNA decay is a process often initiated by specific RNase E cleavage followed by 3′ to 5′ exonucleolytic degradation at the newly formed 3′ ends (Grunberg-Manago, 1999). Further experiments are needed to precisely assess whether RNase E is involved in post-transcriptional processing of the 2 kb transcript.

Post-transcriptional processing of mRNA, rather than simply initiating mRNA decay, may also modulate gene expression, since the amount of a given gene product is also dependent on the stability of its own messenger. Segmental differences in mRNA stability have been demonstrated to be a mechanism of post-transcriptional regulation of gene expression in several operons of prokaryotic micro-organisms, including *E. coli* (Alifano *et al*., 1994; Baga *et al*., 1988; McCarthy *et al*., 1991; Naureckiene & Uhlin, 1996; Nilsson & Uhlin, 1991; Owolaby & Rosen, 1990; Ruiz-Echevarria *et al*., 1995), *Salmonella typhimurium* (Newbury *et al*., 1987) and *Rhodobacter capsulatus* (Alifano *et al*., 1994; Belasco *et al*., 1985). In this work, we have quantified the relative amounts and stability of the two transcripts from HN280 (i.e. the 1 and 2 kb transcripts) and have found that they are not present in equivalent amounts and that they have considerably different half-lives [the 1 kb *apy* mRNA shows an half-life approximately 4·9-fold higher than that of the 2 kb *ospB–apy* transcript ($2{\cdot}2 \pm 0{\cdot}3$ min versus 27 ± 4 s, respectively)]. Stem–loop structures have been reported to act as protective barriers against the degradation of upstream mRNA segments by 3′ exonucleases (Alifano *et al*., 1994; Grunberg-Manago, 1999). The predominance and higher stability of the 1 kb transcript over the 2 kb

transcript might reflect a stabilizing effect possibly mediated by the stem–loop sequences located downstream of the *apy* stop codon (IR2 and IR3; see Fig. 5c).

Although differences in the transcript levels may or may not be reflected in differences in the relative amounts of OspB and apyrase expressed, depending upon whether subsequent translation regulation occurs, the generation of a separate, more stable and more abundant *apy* transcript suggests that apyrase might stoichiometrically outnumber the amount of OspB present in virulent enteroinvasive micro-organisms. Moreover, peak expression of the *ospB–apy* operon was shown to occur when HN280 entered into the late phases of bacterial growth (Fig. 6), as has been reported for the virulence genes of *S. flexneri* (Bahrani *et al.*, 1997; Day & Maurelli, 2001). These results are in agreement with our previous findings which indicate that *S. flexneri* and EIEC strains display their highest levels of apyrase activity upon entry into the late phases of bacterial growth (Berlutti *et al.*, 1998).

Finally, the complete DNA sequence of pWR100 has been reported recently (Buchrieser *et al.*, 2000; Venkatesan *et al.*, 2001). Based on an unexpected proportion of known and putative insertion sequences (IS) and on the G+C content of the genes and of their flanking regions (used as a marker to trace the phylogenetic origin of the genes), it has been proposed that the pINV is composed of a mosaic of blocks of genes which have different origins, probably resulting from IS-mediated acquisition and assembly of DNA across bacterial species (Buchrieser *et al.*, 2000; Venkatesan *et al.*, 2001). In the case of *ospB* and *apy*, their G+C contents (33 mol% for *ospB*, similar to that of virulence genes, and 42 mol% for *apy*) suggest that these genes may have a different origin and that they have probably been acquired separately in the evolutionary assembly of the pINVs of enteroinvasive micro-organisms. Additional experiments are required to assess precisely whether these two genes play a role in virulence or a role in some unknown bacterial metabolic function not directly involved in pathogenesis. However, the findings that *ospB* and *apy* were probably acquired independently, are expressed from a single transcript and are located in a highly conserved DNA region in both *Shigella* and EIEC (Berlutti *et al.*, 1998; Bhargava *et al.*, 1995; Buchrieser *et al.*, 2000; Venkatesan *et al.*, 2001) indicate that such a gene organization must provide some selective advantage to enteroinvasive micro-organisms.

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