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INVOLVEMENT OF RIBONUCLEASE III IN THE ENHANCEMENT OF EXPRESSION OF THE *speF-potE* OPERON ENCODING INDUCIBLE ORNITHINE DECARBOXYLASE AND POLYAMINE TRANSPORT PROTEIN

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SUMMARY: Since the *speF-potE* operon (pPT71 clone) encoding inducible ornithine decarboxylase (iODC) and polyamine transport potE protein is inducible at acidic pH, a gene encoding a protein involved in the enhancement of expression of the operon was searched for. Using the fused gene containing the upstream sequence of the *speF-potE* operon and the open reading frame of β-galactosidase as a reporter gene, a clone (pPTS23) which causes the increase of β-galactosidase activity at acidic pH was isolated. The clone also increased iODC activity at acidic pH and was identified as a gene encoding RNase III. This is the first example that RNase III increases the translational efficiency of mRNA derived from *Escherichia coli* gene by cutting the 5'-untranslated region of mRNA.

Polyamines, aliphatic cations present in almost all living organisms, are known to be necessary for normal cell growth (1). As the cellular concentration is regulated by both biosynthesis and transport, we tried to isolate clones of polyamine transport genes and obtained three clones in *Escherichia coli* (pPT104, pPT79 and pPT71) (2). Among the three clones, the systems encoded by pPT104 and pPT79 were spermidine-preferential and putrescine-specific uptake systems, respectively (3–5). In contrast, the system encoded by pPT71 catalyzed excretion of putrescine through the putrescine-ornithine antiport activity of potE protein (6). The operon encoded by pPT71 clone consisted of the genes for inducible ornithine decarboxylase, iODC (*speF*) and potE protein (*potE*) (7), and the expression was strongly induced in the presence of ornithine at acidic pH. The induction probably contributed to the neutralization of the medium via excretion of putrescine.

In this communication, we tried to identify inducible factor(s) on the expression of the operon at acidic pH, and found that RNase III is one such factor.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions — *E. coli* W3110, DH5α [F^- *φ80d lacZ ΔM15 Δ(lacZYA-argF)U169 recA1 hsd R17* (r_K^-, m_K^+) *supE44* λ^- *thi-1 gyrA relA1* (8),

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obtained from BRL], and MA261 [*speB speC thr leu ser thi* (9), a gift from Dr. W. K. Maas] were grown in LB medium aerobically or LB medium containing 50 mM MES-NaOH, pH 5.5, and 0.8% ornithine anaerobically. When necessary, 30 μg/ml chloramphenicol (CM), 100 μg/ml ampicillin (Amp) and 200 μg/ml erythromycin (EM) were added to the medium.

The plasmid pPT71.20 (7) containing *speF* and *potE* genes were fused with *lac* gene by small bacteriophage Mu transposable elements containing the *lac* operon structural gene according to the method of Castilho *et al.* (10), and fusion plasmid pPT71Φ*lac* was obtained. The plasmid pAC71Φ*lacZ* was then constructed by inserting the 3.5 kb *Bam*HI-*Csp*45I fragment treated with a Klenow fragment of the above fusion plasmid pPT71Φ*lac* into the 4.3 kb *Nru*I fragment of pPT71.20. The plasmid pMW71Φ*lacZ* was constructed by inserting the 4.0 kb *Bam*HI-*Dra*I fragment treated with a Klenow fragment of pAC71Φ*lacZ* into the *Pvu*II site of pMW119 (obtained from Nippon Gene, Japan), a low copy number of the plasmid (11). The plasmid contained 659 nucleotides of the upstream sequence of *speF* (gene for iODC), 153 nucleotides for the NH_2-terminal open reading frame of iODC, and 3054 nucleotides for the COOH-terminal open reading frame for β-galactosidase. The plasmid pMW71.20 was constructed by inserting the 4.3 kb *Bam*HI fragment of pPT71.20 into the *Bam*HI site of pMW119.

The 1.8 kb *Ava*I-*Hind*III fragment from pVA838 plasmid (12), which encodes EM^r determinant, was blunted by T4 DNA polymerase. The open reading frame of *lacZ* in the fusion plasmid pPT71Φ*lac* was cut by *Sac*I and blunted by T4 DNA polymerase, and the above 1.8 kb EM^r fragment was inserted into the *Sac*I site (plasmid *lacZ*::EM). *E. coli* W3110 *lacZ*::EM and MA261 *lacZ*::EM were isolated by linear transformation with the 8 kb *Bam*HI fragment from the plasmid *lacZ*::EM.

Enzyme Assays — Cells were cultured for 5 h. Assay of β-galactosidase was performed by the method of Miller (13). Assay for iODC was performed as described previously (14) except that the reaction mixture (0.35 ml) contained 100 mM Hepes-KOH (pH 7.1) and 0.1 mM GTP. Protein content was determined by the method of Lowry *et al.* (15).

Cloning and DNA Sequencing of the Gene for Activator Protein of speF-potE Operon — Total DNA from *E. coli* DH5α was partially digested by *Sau*3AI. The 3–6 kb DNA fragments separated by agarose gel electrophoresis were inserted into the *Bam*HI site of vector pACYC184 (16). Transformation of *E. coli* MA261 *lacZ*::EM/pMW71Φ*lacZ* with pACYC184 containing *E. coli* DNA fragments was carried out by the method of Maniatis *et al.* (17). The blue colonies of transformants grown on LB agar plates at pH 5.5 containing 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), 0.01% 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal), 30 μg/ml CM, 100 μg/ml Amp and 200 μg/ml EM were isolated. The clone producing the activator protein of the *speF-potE* operon was finally identified by measuring the β-galactosidase activity of the transformants. The DNA was sequenced by the dideoxy method of Sanger *et al.* (18), using the M13 phage system (19), and by authentic and synthesized primers.

Gene Mapping — *E. coli* gene mapping membrane (TaKaRa Biomedical, Japan) was hybridized with ^{32}P-labeled probe prepared from pPTS23 clone. The position of the gene for the activator protein was identified from hybridizing spots corresponding to Kohara's clones (20).

Determination of the 5'-end of iODC mRNA by Primer Extension — Primer extension was performed according to the method of McKnight and Kingsbury (21). Synthetic oligonucleotide, which hybridizes 20 to 43 nucleotides upstream from the initiation codon AUG of iODC mRNA (7), was end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. The ^{32}P-labeled oligonucleotide was then hybridized with total RNA (10 μg) isolated from *E. coli* (22) to prime the synthesis of cDNA with Moloney murine leukemia virus reverse transcriptase. The product of this reaction was analyzed by electrophoresis on 5% sequencing gel.

Others — Optimal computer folding of the 5'-untranslated region of iODC mRNA was performed according to the method of Zuker and Stiegler (23). *E. coli* RNase III-deficient mutant YN3330 (W3110 *rnc14*::miniTn10) was a gift from Dr. Y. Nakamura, Institute of Medical Science, University of Tokyo.

RESULTS

Cloning of the Gene for Activator Protein of the speF-potE Operon — Since the induction of the operon encoding genes for iODC and polyamine transport potE protein was observed at

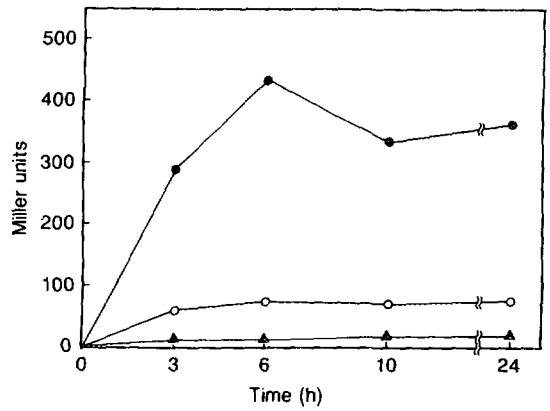

Fig. 1. β-Galactosidase activity of various *E. coli* strains carrying pMW71Φ*lacZ* plasmid. *E. coli* cells shown in the figure were cultured and β-galactosidase activity was measured as described in Materials and Methods at the designated times shown in the horizontal axis. ●, DH5α/pMW71Φ*lacZ* ; ○, W3110 *lacZ*::EM/pMW71Φ*lacZ* ; ▲, MA261 *lacZ*::EM/ pMW71Φ*lacZ* .

acidic pH in some *E. coli* cells, we looked for the gene encoding an activator protein using the fused gene containing the upstream sequence of the *speF-potE* operon (promoter and 5'-untranslated region) and the open reading frame of β-galactosidase as a reporter gene. First, the inducible activity of several *E. coli* strains was examined. As shown in Fig. 1, *E. coli* DH5α most strongly induced β-galactosidase activity, and the induction of β-galactosidase in *E. coli* MA261 was smallest. Therefore, *E. coli* MA261 *lacZ*::EM/pMW71Φ*lacZ* was used as a host strain, and pACYC184 containing DNA fragments from *E. coli* DH5α was transformed into the host strain. Clones were isolated from the colonies which became blue only at acidic pH. As shown in Fig. 2, the clone termed pPTS23 increased β-galactosidase activity by 3.7-fold.

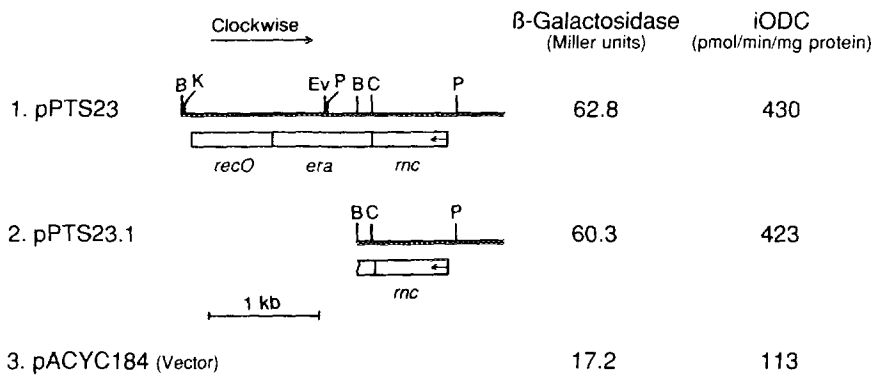

Fig. 2. Structure of clones and enhancement of β-galactosidase and inducible ornithine decarboxylase activities by the clones. The cloning of pPTS23 and subcloning of pPTS23.1 were performed as described in the text. Restriction enzymes shown in the figure are as follows: B, *Bam*HI; K, *Kpn*I; Ev, *Eco*RV; C, *Cla*I; P, *Pst*I. The position of *rnc* operon was determined by the nucleotide sequence. Small arrows indicate the direction of transcription. The activities of β-galactosidase and iODC were measured using *E. coli* MA261 *lacZ*::EM/ pMW71Φ*lacZ* and MA261/pMW71.20, respectively, transformed with the clones or vector shown in the figure.

Since *E. coli* MA261 is a deficient mutant of constitutive ornithine decarboxylase, only iODC existed in the cell lysate prepared from this strain. This was confirmed from the findings that ODC activity was only observed in cell lysate when cells were cultured at acidic pH and the optimal pH of ODC activity was 7.1 (7). The iODC activity in *E. coli* MA261 was also increased significantly by the clone pPTS23 (data not shown). To confirm these results, *E. coli* MA261 was then transformed by pMW71.20 encoding iODC. The iODC activity in the cell lysate was increased by the clone pPTS23 by 3.8-fold (Fig. 2). These results clearly show that the clone pPTS23 contains a gene encoding an activator protein of the *speF-potE* operon.

Identification of RNase III as an Activator Protein of the speF-potE Operon — The clone pPTS23 contained a 2.9 kb fragment, and the restriction enzyme map of the clone is shown in Fig. 2. Since the position of pPTS23 on *E. coli* chromosome was not determined from a comparison of our restriction enzyme map and the physical map constructed by Kohara *et al.* (24), it was determined by hybridization of the ^{32}P-labeled 3.2 kb *Kpn*I-*Sal*I fragment (*Sal*I site was from vector) with the *E. coli* gene mapping membrane. As shown in Fig. 3, the gene encoding an activator protein of the *speF-potE* operon was located at 55.5 min of *E. coli* chromosome, corresponding to λ phage clones 7G4 and 4A12.

The nucleotide sequence of pPTS23 was then determined. When the sequence was compared with that reported before (25–27), it was found that our clone contained the *rnc* operon as shown in Fig. 2. The *rnc* operon consisted of the genes encoding RNase III, era protein having a GTP-binding motif, and recO protein involved in recombination and repair pathway. As RNase III was the most likely as an activator protein among the three proteins, a subclone containing the gene for RNase III only, termed pPTS23.1, was constructed by removing the 1.6 kb *Bam*HI fragment from pPTS23, and enzyme activities of the transformant were measured. As shown in Fig. 2, both β-galactosidase and iODC activities were increased by pPTS23.1 to almost the same degree as pPTS23. These results clearly indicate that RNase III is one of the activator proteins of the *speF-potE* operon at acidic pH.

To clarify how RNase III is involved in the enhancement of expression of the *speF-potE* operon, primer extension analysis of iODC-potE mRNA was performed. The amount of iODC-potE mRNA did not change significantly by the clone pPTS23, judging from the intensity of the bands in lanes 2 and 3 of Fig. 4. When iODC-potE mRNA was extracted from RNase III-deficient *E. coli* cells, the 5'-end of the mRNA was the 138th nucleotide upstream from the initiation codon of the open reading frame (lane 1 in Fig. 4), which was close to the putative −10 and −35 regions of the promoter (7). When it was extracted from *E. coli* MA261/pMW71.20 + pACYC184, the mRNA starting from the 126th nucleotide upstream from the initiation codon also appeared (lane 2 in Fig. 4). When it was extracted from *E. coli* MA261/pMW71.20 + pPTS23, the mRNA starting from the 138th nucleotide upstream from the initiation codon almost disappeared. Instead, the mRNA starting from the 126th or 99th nucleotide upstream from the initiation codon increased (lane 3 in Fig. 4).

The possible secondary structure of the 5'-untranslated and initiation codon region of the mRNA was then constructed by optimal computer folding. As shown in Fig. 5, the mRNA starting from the 138th nucleotide upstream from the initiation codon has a complicated

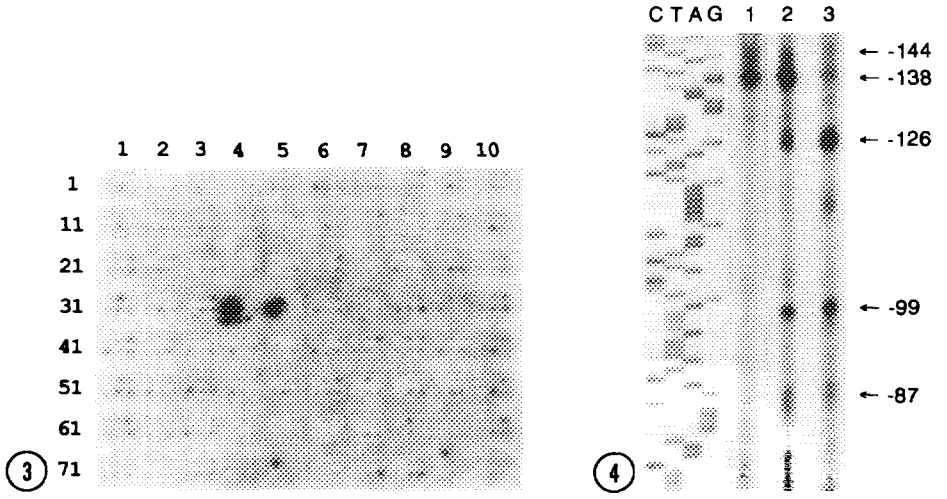

Fig. 3. Hybridization pattern of the *E. coli* gene mapping membrane probed with the clone pPTS23. Hybridizing spots corresponded to serial numbers 434 and 435, which correlate with clones 7G4 and 4A12 of Kohara et al. (24).

Fig. 4. Primer extension analysis of iODC-potE mRNA. The 24 nucleotides which hybridize with the upstream nucleotide sequence 20 – 43 from the initiation codon of iODC-potE mRNA were used as the primer. Lanes 1, 2 and 3 contain the primer-extension products of the RNA prepared from *E. coli* YN3330, RNase III-deficient mutant, MA261/pMW71.20 + pACYC184, and MA261/pMW71.20 + pPTS23. C, T, A, and G represent dideoxy nucleotide sequencing of the upstream region of the *speF-potE* operon. Numbering (–) of nucleotides (on the right) starts from the next nucleotide upstream from the initiation codon of the mRNA.

structure around the initiation codon and Shine-Dalgarno sequence. However, the initiation codon AUG and Shine-Dalgarno sequence were exposed on the surface of the mRNA starting from the 99th nucleotide upstream from the initiation codon. Thus, it is suggested that RNase III stimulates the translational efficiency of the iODC-potE mRNA by cutting the 5'-untranslated region of the mRNA.

DISCUSSION

Our data clearly show that RNase III enhances the expression of the *speF-potE* operon. This is probably due to the processing of the 5'-untranslated region of mRNA. Although this is the first example of mRNA derived from *E. coli* gene, stimulation of the synthesis of λ N protein and T7 0.3 gene protein by RNase III has been reported (28, 29). In addition, several studies have reported the inhibition of gene expression by RNase III processing within the 5'-untranslated region of mRNA. They were the genes for RNase III itself (30), polynucleotide phosphorylase (31), and β, β' subunits of RNA polymerase (32). Taken together, the findings suggest that RNase III plays important roles in various cellular processes, although *rnc* is expressed at low levels probably due to the poor codon usage characteristic of genes and is not indispensable for *E. coli* cells (27).

It is clear that ornithine is necessary for induction of the *speF-potE* operon. However, ornithine is not related to RNase III activity or synthesis. Thus, we still need to search for

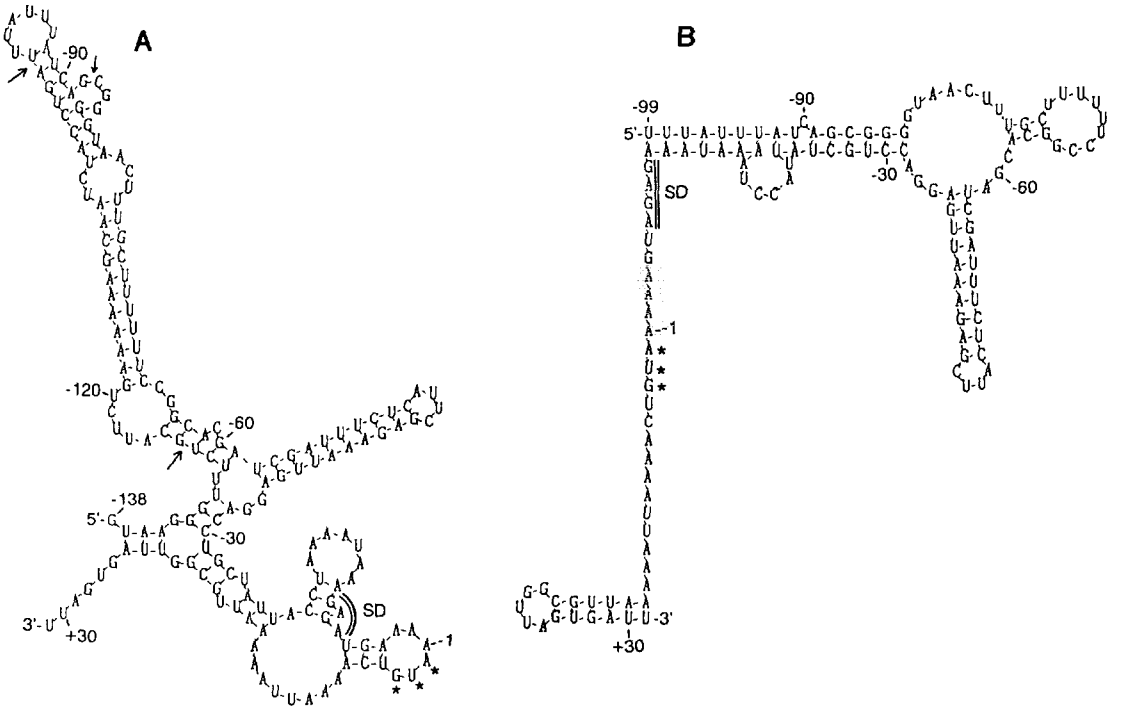

Fig. 5. The possible secondary structure of the 5'-untranslated region of iODC-potE mRNA. The secondary structure was constructed with a 138 (A) or 99 (B) nucleotide-long 5'-untranslated region plus the 31 nucleotide-long open reading frame region of iODC-potE mRNA. The initiation codon AUG and Shine-Dalgarno sequence were indicated by stars and double lines, respectively.

another activator protein of the *speF-potE* operon, whose activity or synthesis is regulated by ornithine.

The initiation site of iODC-potE mRNA transcription in the RNase III-deficient mutant was the 138th nucleotide upstream from the initiation codon for iODC. However, this is slightly far away from the putative –10 and –35 region of the promoter. There is a faint band at the 144th nucleotide upstream from the initiation codon (Fig. 4). This band became stronger when RNA was extracted from cells cultured at neutral pH (data not shown). Therefore, this may be explained by the following: the mRNA transcription starts at the 144th nucleotide upstream from the initiation codon, and the mRNA is processed at the 138th nucleotide by RNase(s) other than RNase III. The positions of the 99th and 126th nucleotides upstream from the initiation codon are not such plausible positions as recognition sites of RNase III (Fig. 5). The mRNA may also be processed further by other RNase(s) after hydrolysis by RNase III.

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