Characterization of the 5' flanking region of the *Escherichia coli* *ppa* gene encoding inorganic pyrophosphatase: mutations in the ribosome-binding site decrease the level of *ppa* mRNA

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We have previously cloned and sequenced the *ppa* gene, encoding inorganic pyrophosphatase (PPase), of *Escherichia coli* K12 [Lahti, R., Pitkäranta, T., Valve, E., Ilta, I., Kukko-Kalske, E. & Heinonen, J. (1988) *Journal of Bacteriology* 170, 5901–5907]. In this work mutations were constructed in the 5' flanking region of *E. coli ppa* and the effect on expression was determined. The minimum length of the fully active *ppa* 5' flanking region was shown to be 117 bp. Further deletion decreased the activity, and upon deletion to nucleotide –37 the promoter activity was totally lost. A clear point of inflection was observed in the inactivation upon deletion over the nucleotide –50. This is consistent with the fact that by binding to promoters RNA polymerase holoenzyme generally covers the –50 to +20 region in *E. coli* genes. When the –35 sequence of *ppa*, AAGACA, was mutated to AAAACA, *ppa* expression, as measured by PPase production, decreased to 20% of the wild-type, whereas by the change of the –10 sequence, TATAAT, to TTTAAT or TATAAA, the *ppa* gene was totally inactivated. Furthermore, when the ribosome-binding site (RBS) sequence, AGGAAA, was altered to AAGAAA, PPase production decreased to 19% of the wild-type. Surprisingly, when the RBS sequence was mutated to the consensus RBS sequence, AGGAGG, the intracellular levels of both *ppa* mRNA and PPase decreased drastically. The implications of these results are discussed.

Introduction

Inorganic pyrophosphatase (EC 3.6.1.1; PPase) is ubiquitous in nature and plays an important role in energy metabolism, providing a thermodynamic pull for biosynthetic reactions such as protein, RNA and DNA synthesis (Kornberg, 1962). According to Peller (1976), nucleic acid synthesis would be energetically impossible *in vivo* if it were not coupled to the hydrolysis of pyrophosphate (PP_i), catalysed by PPase. In addition, various findings suggest that PPase might have important roles not only in the regulation of macromolecular synthesis and growth (Airas & Cramer, 1986; Dignam & Deutscher, 1979; Heinonen & Kukko, 1977; Kent & Guterman, 1982) but also in evolutionary events by affecting the accuracy with which DNA molecules are copied during chromosome duplication (Bourguignon-

van Horen *et al.*, 1982; Herbomel & Ninio, 1980; Lecomte *et al.*, 1986).

While the occurrence, reaction mechanism, structural and kinetic properties of this important enzyme have been studied extensively (Cooperman, 1982; Welsh *et al.*, 1983; Terzyan *et al.*, 1984; Lahti & Jokinen, 1985; Gonzales & Cooperman, 1986; Samejima *et al.*, 1988; Lahti *et al.*, 1990a, b, 1991) the regulation of PPase has received little attention (Klemme, 1976; Lahti, 1983; Kukko-Kalske *et al.*, 1989). PPase is synthesized constitutively in *Escherichia coli*. Thus the composition of the culture medium generally exerts no specific effects on the level of the enzyme (Josse & Wong, 1971). However, the production of *E. coli* PPase can be stimulated 1.5- to 3-fold by partial inhibition of DNA synthesis (Heinonen & Kukko, 1977). Possibly inhibition of DNA synthesis affects PPase synthesis indirectly, by disturbing nucleotide metabolism, for example (Kukko-Kalske & Heinonen, 1985).

The *E. coli ppa* gene encoding PPase was recently

Abbreviations: PPase, inorganic pyrophosphatase; RBS, ribosome-binding site (= Shine-Dalgarno sequence, SD).

cloned and sequenced, and the 5' end of the *ppa* mRNA was identified by primer extension mapping (Lahti *et al.*, 1988). To identify the upstream regions important for *ppa* expression, we made a series of deletion and substitution mutations in the 5' flanking region. The effects of these changes on PPase production are described in this paper.

Methods

Chemicals. Restriction endonucleases, T4 DNA ligase, T4 DNA polymerase, exonuclease III (from *E. coli* B) and alkaline phosphatase from calf intestine were from Boehringer Mannheim. Mung bean nuclease was from Promega Biotec, and [³⁵S]dATP (500 Ci mmol⁻¹; 18.5 TBq mmol⁻¹) was from NEN Du Pont. T4 polynucleotide kinase was from Pharmacia, sequenase from USB, and antibiotics, egg white lysozyme and low-gelling-temperature agarose (type VII) were from Sigma.

Bacterial strains, plasmids and culture conditions. The following strains of *E. coli* were used. CJ236 (Kunkel *et al.*, 1987) was used as the host to prepare uracil-containing template DNA for the site-specific mutagenesis (Kunkel *et al.*, 1987). JM103 (Messing, 1983) was used as a host in the mutagenesis. HB101 (Maniatis *et al.*, 1982) was used as the host for plasmid preparations and deletion series. SY634 (Syvänen *et al.*, 1982) was used as the host for making the cointegrates.

The plasmid pTP1, containing the intact *E. coli ppa* gene (Lahti *et al.*, 1988), was used as the donor of the *ppa* fragments manipulated in this work. M13mp18 (Messing, 1983) was used as a vector in the oligonucleotide-directed mutagenesis, whereas pUC18 (Norrander *et al.*, 1983) was the vector in other manipulations.

The strains were grown in 2 × YT medium (Rodríguez & Tait, 1983) or on LA plates (Maniatis *et al.*, 1982). Ampicillin (100 µg ml⁻¹) was added when required. Phage transductions were plated on H-plates (Maniatis *et al.*, 1982).

DNA manipulation methods. Restriction endonuclease digestions of the plasmids were performed as suggested by the suppliers. Other DNA manipulations, including purification of plasmids, isolation of restriction fragments from low-gelling-temperature agarose gel, dephosphorylation of the linearized vectors with calf intestinal phosphatase, ligation, and transformation by the CaCl₂ method were carried out as described by Maniatis *et al.* (1982).

Construction of the deletion series. A series of unidirectional deletions of the *ppa* 5' flanking region was produced by the exonuclease III procedure (Henikoff, 1984). The extent of deletions was checked by sequencing with the dideoxy chain-termination method (Sanger *et al.*, 1977).

Production of the internal base substitutions. Base substitutions were produced by the site-specific mutagenesis method of Kunkel *et al.* (1987). Oligonucleotides for mutagenesis (and also for use as *ppa*-specific sequencing primers) were synthesized using the phosphoramidite method in an automated DNA synthesizer (Cyclone, BioResearch Inc.). Oligonucleotides were used unpurified as sequencing primers and for mutagenesis they were purified by HPLC in a reverse-phase column (Spherisorb ODS-2, 5 µm, 4 × 100 mm, reverse-phase cartridge). Mutagenic oligonucleotides were phosphorylated at their 5' end by T4 polynucleotide kinase. After mutagenesis, the whole *ppa* gene was resequenced to confirm the mutations and to ensure that no other mutations had arisen. In this sequencing a universal primer and four *ppa*-specific primers (Lahti *et al.*, 1990b) were used.

Integration. The series of deletions and the *in vitro*-mutated *ppa* genes were integrated by homologous recombination into the chromosome of *E. coli* SY634 (Syvänen *et al.*, 1982) by the method of Gutterson & Koshland (1983).

RNA methods. The plasmid-encoded production of *ppa* mRNA was monitored by slot-blot analysis (Maniatis *et al.*, 1982). RNA was isolated as described by Igo & Losick (1986). A 5' ³²P-labelled 19-mer oligonucleotide (5'-TACCCGACGGACGTTGAG-3'), complementary to nucleotides 44 to 62 of *ppa* mRNA (Lahti *et al.*, 1988) was used as a probe in the hybridization.

Enzyme assay. PPase activity of cell samples was determined by the method of Heinonen & Lahti (1981).

Results

Deletions by restriction endonuclease digestions

We have previously cloned the *E. coli ppa* gene as a 1.3 kb *Sau3AI* fragment into the *Bam*HI site of a runaway vector, pOU61, yielding a plasmid designated as pTP1 (Lahti *et al.*, 1988). A partial restriction map of the *ppa* gene is shown in Fig. 1 (for a more detailed map see Lahti *et al.*, 1988). For promoter analysis, we first compared the activities of *Bam*HI-*Acc*I (1267 bp), *Acc*I-*Acc*I (989 bp) and *Dra*I-*Acc*I (884 bp) fragments (Fig. 1). These fragments were ligated into the *Sma*I site of pUC18 in the opposite orientation to *lacp* and the resulting plasmids were designated as pMP1, pMP2 and pMP3, respectively. The orientation of the fragments was checked by nucleotide sequencing. The plasmids were transformed into *E. coli* HB101, and the promoter activities of the deletants were monitored by measuring the plasmid-encoded PPase activities of the transformants.

The cells containing pMP1 and pMP2 had equal PPase activities (about 70 times higher than that of HB101/pUC18), whereas the cells with pMP3 had the same level of PPase activity as HB101/pUC18. This indicates that the 5' *Bam*HI-*Acc*I fragment (280 bp) (Fig. 1) is not required for expression of the *ppa* gene. Deletion of the upstream *Bam*HI-*Dra*I fragment from the *ppa* gene totally abolished the expression of *ppa*. Hence, the *Acc*I-*Dra*I fragment of 105 bp contains sequences that are necessary for *ppa* promoter function.

Fig. 1. Partial restriction map of *E. coli ppa*. The open reading frame encoding *E. coli* PPase is shown by an open bar. The map is numbered by designating the transcription initiation site as +1. For a more complete restriction map of the *E. coli ppa* gene see Lahti *et al.* (1988).

Fig. 2. Effects of deletions in the 5' flanking region on *ppa* expression. The plasmid pMP2 was constructed by inserting the *AccI*-*AccI* (989 bp) fragment of the *E. coli ppa* gene into the *SmaI* site of pUC18 in the opposite orientation to *lacp*. The deletion series was produced by unidirectional exonuclease III digestion of pMP2 double-digested with *SphI* and *SalI*. All of the deletions shown by arrows have the same left boundary (the *AccI* site at nucleotide -85; Fig. 1). The numbers represent the right boundaries of the deletion. (a) The deletant-encoded PPase activities. The deletants were transformed into *E. coli* HB101. The transformants contained both the chromosomal and plasmid-encoded PPase. The plasmid-encoded PPase was about 70-fold amplified over the chromosomal PPase. The chromosome-encoded PPase activity (HB101/pUC18) was taken into account as a blank value in the measurements. The relative value of 100% corresponds to a specific activity of 700 mkat kg⁻¹; the subtracted background value was 10 mkat kg⁻¹. The abscissa shows deletion end-points along *ppa*. (b) Scheme of the 5' flanking region of *ppa* gene. Abbreviations: '-35' and '-10', the regions typical for *E. coli* consensus promoters; TIS, transcription initiation site; SD, Shine-Dalgarno sequence (= RBS); ATG, initiation codon.

Unidirectional deletions with exonuclease III

To further identify the regions important for *ppa* expression a series of deletions from the 5' end of the *ppa* gene (*AccI*-*AccI* fragment, Fig. 1) was produced by unidirectional digestion with exonuclease III as described by Henikoff (1984). Prior to the exonuclease

III digestion, the plasmid pMP2 was digested with *SalI* and *SphI*. The deletants were self-ligated and transformed into HB101. The extent of the deletions and their effects on *ppa* expression were determined by sequencing and by monitoring the PPase activities of the transformants, respectively (Fig. 2).

Four base pairs could be removed by exonuclease III from the upstream *AccI* site (Fig. 1) without any loss of activity. Further deletion resulted in a severe drop in the promoter activity. Upon deletion to nucleotide -37 the activity was totally lost (Fig. 2). Hence, the upstream *AccI* site (Fig. 1) seems to be located right at the 5' border of the *ppa* promoter, and the *AccI*-*DraI* fragment represents the minimum length of the fully active *ppa* promoter.

Site-directed mutagenesis study

The roles of the '-35', '-10' hexamers (Reznikoff & McClure, 1986) and the Shine-Dalgarno sequence (Shine & Dalgarno, 1974; Stormo, 1986) in the expression of *ppa* were studied by internal substitutions. For this purpose, a 0.8 kb *AccI*-*BglI* fragment (Fig. 1) containing the whole functional *ppa* gene was transferred from pTP1 (Lahti *et al.*, 1988) into the *SmaI* site of the replicative form of M13mp18 DNA in the opposite orientation to *lacp*. Base substitutions in the *ppa* 5' flanking region were made in this construction by oligonucleotide-directed mutagenesis as described by Kunkel *et al.* (1987). The oligonucleotides used for the mutagenesis are shown in Fig. 3. After mutagenesis we resequenced the whole *AccI*-*BglI* fragment to confirm the mutations and to ensure that no other mutations had arisen. The mutated *ppa* genes were transferred from M13mp18 as *EcoRI*-*HindIII* fragments into the pUC18 vector, and then transformed into *E. coli* HB101.

ppa expression of the mutants is shown as PPase production in Fig. 3. As expected, all the four mutations that reduced the homology of the *ppa* 5' end with the consensus *E. coli* 5' flanking region decreased *ppa* expression. When the -35 sequence of *ppa*, AAGACA, was mutated to AAAACA, *ppa* expression decreased to 20% of the wild-type, whereas by the change of the -10 sequence, TATAAT, to TTTAAT or TATAAA, the *ppa* gene was totally inactivated. Furthermore, when the RBS sequence AGGAAA, was changed to AAGAAA, PPase production decreased to 19% of the wild-type (Fig. 3). These results clearly emphasize the role of the -35, -10 and RBS sequences in the expression of the *E. coli ppa* gene. Surprisingly, the mutation of the RBS to the consensus sequence (AGGAGG) almost totally inactivated the *ppa* gene (Fig. 3). This inactivation was also evident in the plasmid-encoded *ppa* mRNA levels (Fig. 4).

Fig. 3. Oligonucleotides used for the site-directed mutagenesis of the 5' flanking region of *E. coli ppa*. The partial nucleotide sequence of *E. coli ppa* is from Lahti *et al.* (1988). For comparison, the sequence landmarks of the *E. coli* consensus gene with their spacing in parentheses are shown. These include the -35 and -10 sequences typical of *E. coli* promoters, and the Shine-Dalgarno (SD, = RBS) sequence. TIS, translation initiation site. Conserved sequences are underlined, and the nucleotides mutated are shown by asterisks. Percentage PPase activities encoded by the native *ppa* gene and its mutants are shown in parentheses. PPase activity of *E. coli* HB101/pUC18 was used as a blank value in the assays. The relative value of 100% corresponds to a specific activity of 700 mkat kg⁻¹; the subtracted background value was 10 mkat kg⁻¹. The upstream inverted repeat sequences are shown by solid arrows; the sequences responsible for the formation of the putative secondary structure in the RBS consensus mutant are shown by dashed arrows.

Fig. 4. Plasmid-encoded transcription of the wild-type *ppa* gene and its mutants. The wild-type *ppa* gene and its mutants produced *in vitro* were inserted into the polylinker region of pUC18 in the opposite orientation to *lacp*. The plasmids were transformed into *E. coli* HB101, and total RNA was isolated from the transformants. Two and four micrograms of total RNA were spotted onto nitrocellulose and hybridized with a 5' ³²P-labelled oligonucleotide (5'-TACCCGACGGGACGTTGAG-3') complementary to nucleotides 44 to 62 of *ppa* mRNA (Lahti *et al.*, 1988). Background, total RNA isolated from HB101 transformed with pUC18; WT *ppa*, total RNA isolated from the transformant containing the intact, wild-type *ppa* inserted into pUC18. The -35 and -10 sequences typical of *E. coli* promoters and the Shine-Dalgarno (SD, = RBS) sequence were mutated as indicated in Fig. 3. The mutant sequences are shown in parentheses. The corresponding wild-type sequences are shown in Fig. 3.

Discussion

Chen *et al.* (1990) recently proved that PPase is essential for the growth of *E. coli*. They showed that the minimal amount of PPase required for normal growth is 4–5% of the wild-type PPase activity. Accordingly, the native chromosomal *ppa* gene could not be replaced by the *ppa* variants studied here, since the activities of several variants were less than 4% of the wild-type activity (Figs 2 and 3). Instead, cointegrates were produced as described by Guttererson & Koshland (1983). They contained both the wild-type *ppa* gene and its promoter

variants on the chromosome side by side, separated from each other only by the DNA segment of the vector carrying the *bla* gene encoding β -lactamase. Hence, in these cointegrates there was only a twofold difference between 100% and 0% *ppa* expression, and so the activity measurements using the cointegrates were somewhat less accurate than those with the transformants producing PPase from a multicopy plasmid. However, the relative specific activities in the two cases were virtually identical, indicating that at least in this case gene expression can be reliably studied with multicopy number plasmid constructions.

The deletions from the 5' end of *ppa* with restriction endonucleases showed that the *AccI*–*DraI* fragment (Fig. 1) contains elements that are necessary for the expression of the *ppa* gene. Furthermore, the upstream *AccI* site (from nucleotides -86 to -81) was shown to be right at the 5' end of the fully active promoter. *ppa* expression started to fall by deletion of the upstream region when position -80 was reached, and expression was zero at residue -37 (Fig. 2). Thus the sequences upstream of the -35 sequence are important for the function of the *ppa* promoter. Similar conclusions have been reached with several other *E. coli* promoters (Gaal *et al.*, 1989, and references therein).

There is no significant homology between the *ppa* upstream sequence (Fig. 3) and the upstream activating sequences of genes regulated by positively acting transcription factors such as CRP (Magasanik & Neidhardt, 1987), FNR (Knappe & Sawers, 1990) and PHOB (Makino *et al.*, 1986). Instead, there is an inverted repeat sequence, CGTCTTNA, centred upon nucleotide -64 (shown by arrows in Fig. 3). Upon deletion from nucleotide -81 to -74 the left arm of the inverted repeat was removed (Fig. 3) and the promoter activity was decreased from 100% to 78% (Fig. 2). This suggests that the inverted repeat sequence is a weak upstream activating sequence, the role of which on *ppa* expression will be further tested by site-directed mutagenesis.

There was a clear point of inflection in the inactivation of *ppa* expression upon deletion over the nucleotide -50 (Fig. 2). This is consistent with the fact that by binding to promoters RNA polymerase holoenzyme generally covers approximately the region from -50 to +20 in *E. coli* genes (von Hippel *et al.*, 1984; Brunner & Bujard, 1987). Interestingly, by the deletion of a single nucleotide, G₃₈, the activity of the *ppa* promoter decreased from 22% to 0% of normal. This important G is 2 bp upstream of the -35 region (Fig. 3) that is supposed to be involved in the recognition of the promoter by RNA polymerase (Stormo, 1986).

The typical *E. coli* sigma 70 promoter elements (Reznikoff & McClure, 1986; Harley & Reynolds, 1987) identified immediately upstream of the *ppa* mRNA 5'

end were modified by mutagenesis and as expected all the single base substitutions that reduced the homology of the *ppa* 5' end with the consensus *E. coli* 5' flanking region drastically decreased *ppa* expression, thus verifying the identification of the -35, -10 and RBS sequences.

Surprisingly, when the RBS sequence (Stormo, 1986), AGGAAA, was mutated to the consensus sequence AGGAGG, *ppa* expression was not increased, as expected, but totally lost. It was verified by sequencing that no secondary mutations were produced during the mutagenesis. This unexpected result was further analysed by measuring the plasmid-encoded *ppa* mRNA levels of the mutants by the slot-blot technique (Fig. 4). In both RBS mutants, as in the other mutants, the *ppa* mRNA level decreased corresponding to the PPase production.

The RBS sequence is a signal for translation initiation, and thus it is not expected to have such a profound effect on transcription. RBS mutations might lead to decreased mRNA levels by weakening the binding of mRNA to ribosomes, because ribosomes are known to stabilize mRNA (Belasco & Higgins, 1988). In general, the mutation to the consensus RBS sequence is expected to strengthen the binding between ribosomes and mRNA and thus to increase mRNA stability. However, if one looks at the sequences shown in Fig. 3 by dashed arrows it is clear that, as a result of this RBS consensus mutation, a new and strong secondary structure can be formed ($\Delta G = -7.4 \text{ kcal mol}^{-1}$; $-30.96 \text{ kJ mol}^{-1}$) in which 11 of the 14 bases at the RBS (nucleotides 35–22) can pair with the upstream leader (nucleotides 4–17) RNA. The free energy of formation of this kind of secondary structure in the native *ppa* mRNA is so low ($\Delta G = -1.1 \text{ kcal mol}^{-1}$; 4.60 kJ mol^{-1}) that the structure does not exist in the wild-type strain. This secondary structure could in principle preclude translation by preventing the binding of ribosomes to *ppa* mRNA and thus leading to a decrease in mRNA stability. This hypothesis will be tested by making double mutants in which the secondary structure produced by the consensus RBS mutation is eliminated and by testing the stability of the various *ppa* mRNA variants.

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