Role of ribosome release in the basal level of expression of the *Escherichia coli* gene *pheA*

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In *Escherichia coli*, the expression of the phenylalanine biosynthetic operon *pheA* is regulated by an attenuation mechanism. In the presence of excess phenylalanine, gene expression was decreased to 10% of the fully deattenuated level. To understand the factors that determine the basal level of *pheA* expression, we examined the role of ribosome release from the leader peptide stop codon UGA. The transcriptional readthrough from the *pheA* attenuator decreased by over 2-fold in the presence of the defective release factor 2. However, a release factor 1 (UAG and UAA specific) mutation did not influence the basal level of *pheA* expression. These results support the proposal that the release of translating ribosomes from the leader peptide stop codon in stem 2 of the *pheA* attenuator plays a crucial role in determining the basal level of expression of this gene.

Introduction

The amino acid phenylalanine is synthesized in three steps from chorismate, the final intermediate in the common pathway of aromatic biosynthesis. In *Escherichia coli*, the first two of these steps are catalysed by the bifunctional enzyme chorismate mutase/prephenate dehydratase (EC 5.4.99.5/4.2.1.51), which is encoded by the gene *pheA* (Pittard, 1987). Nucleotide sequence analysis of the *pheA* leader region suggests that the *pheA* gene is regulated by an attenuation mechanism (Zurawski *et al.*, 1978). The leader region of *pheA* has a short open reading frame (*pheL*) capable of encoding a fifteen amino acid polypeptide, which is preceded by a potential ribosome binding sequence AGGA (Fig. 1a). The *pheA* leader transcript is 145 nucleotides long and has the potential to form several mutually exclusive secondary structures (Kolter & Yanofsky, 1982; Landick & Yanofsky, 1987; Yanofsky, 1988). These secondary structures include a stem and loop designated 3:4, which has the usual features of a transcription terminator (Fig. 1; Adhya & Gottesman, 1978; Yager & Von Hippel, 1987). It was demonstrated that the transcription terminates at the string of thymines immediately 3' to this stem and loop (Hudson & Davidson, 1984).

Previous studies have shown that the expression of the *pheA* gene is decreased by about 90% when the cells

are grown in the presence of phenylalanine (Im & Pittard, 1971; Gowrishankar & Pittard, 1982b; Gavini, 1988). This indicates that approximately 10% of the RNA polymerase molecules read through the attenuator terminator to ensure the basal level expression of *pheA*. Therefore, it is reasonable to assume that, under these physiological conditions, the formation of the antiterminator structure is favoured against the formation of the terminator about 10% of the time. By analogy with other well-characterized attenuator systems, it is the rate of movement of the leader-peptide-translating ribosome, in collaboration with the strength of the mutually exclusive secondary structures, that set up the basal level expression (Yanofsky, 1981; Yanofsky *et al.*, 1984; Yanofsky, 1988).

Studies on the mechanism of translational termination have shown that protein release factors play a crucial role in the release of ribosomes from mRNA at stop codons (Caskey, 1980; Hershey, 1987). In *E. coli*, the release factor 1 (RF1) recognizes the stop codons UAA and UAG, and release factor 2 (RF2) recognizes the stop codons UAA and UGA (Craigie *et al.*, 1985; Craigie & Caskey, 1986; Ryden *et al.*, 1986; Hershey, 1987; Grunberg-Manago, 1987). Ryden & Isaksson (1984) have isolated a temperature-sensitive mutation in release factor 1 (*prfA*[Ts]), and have shown that introduction of this mutation decreases the ribosome release from

mRNA at UAA and UAG stop codons. However, this mutation did not seem to affect the ribosome release from the stop codon UGA. On the other hand, the ribosome release from UGA and from UAA stop codons was affected by mutations in *prfB*, which encodes release factor 2 (Kawakami *et al.*, 1988).

Recently, Yanofsky and colleagues have shown that ribosome release from the stop codon located in stem 2 has a significant role in determining the basal level expression of the *trp* operon (Roesser & Yanofsky, 1988; Rosser *et al.*, 1989). Since the translation termination codon of the *pheA* leader peptide is located in stem 2 (Fig. 1), by analogy with the *trp* attenuator, ribosome release from this stop codon may play a significant role in determining the basal level expression of the *pheA* gene. In this report, by taking advantage of the existing *prfA* and *prfB* mutations, we have examined the role of ribosome release in determining the basal level expression of *pheA*.

Methods

Strains and growth conditions. The bacterial strains and phages used in this study are listed in Table 1. *E. coli* strains were grown in Luria broth, LB agar or minimal media supplemented with 0.2% glucose and appropriate growth factors (Maniatis *et al.* 1982). Lac⁺ phenotype was identified by growing cells on LB agar plates containing 0.05% 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) or MacConkey-lactose agar plates. Tetracycline was used at a concentration of 15 μ g ml⁻¹ when required. *E. coli* strains were routinely grown at 37 °C. The temperature sensitive *E. coli* strain UL213 and its derivatives were grown at 30 °C. When used to propagate bacteriophage P1, cells were grown in Z broth (LB supplemented with 5 mM-CaCl₂) and to propagate λ phage, cells were grown in Z broth supplemented with 0.2% maltose.

Construction of *E. coli* NG200. The λ ppheA-lac carries transcriptional fusion in which the *lacZ* expression is under the control of *pheA* promoter-attenuator (Gowrishankar & Pittard, 1982a). Hence all the constraints that apply to the expression of *pheA* can be easily monitored by measuring the β -galactosidase activity. This λ phage has been integrated into the chromosome of a variety of *E. coli* strains used in the study of the regulation of *pheA* expression (Gowrishankar & Pittard, 1982b; Gavini, 1988). To construct *E. coli* strain NG200, the *E. coli* NG3118 (*thi-l*, *lacU169*) was used to lysogenise with λ ppheA-lac. To isolate λ lysogens, the recipient strain was grown to exponential phase (OD₆₀₀ = 0.5) and 0.2 ml cells were mixed with 3 ml soft agar and layered on to Z-agar plates. These plates were incubated at 37 °C for 2 h. Samples of phage (10 μ l of 10⁵ p.f.u ml⁻¹) were spotted onto the lawn of recipient cells and the plates were incubated for 16 h at 30 °C. Colonies were isolated from the centres of the turbid plaques and were streaked onto plates which had been prespread with λ cIb2. Single colonies were purified and were tested for λ lysogeny. Monolysogens were identified as described previously (Simons *et al.*, 1987).

Construction of *E. coli* NG201. The temperature-sensitive release factor 1 mutation *prfA*(Ts) was transferred from *E. coli* strain UL213 to NG200 by P1 transduction to construct NG200:RF1. Since the *prfA*(Ts) mutation is linked to the tet^r transposon Tn10, the transfer of this mutation could be easily identified by selecting for tet^r transductants and testing them for temperature sensitivity at 42 °C. The P1 transduction was carried out as follows. The recipient strain was grown

in Z broth overnight and after concentrating 10-fold, 0.2 ml cell suspension was mixed with 10⁹ p.f.u of P1 lysate. This mixture was added to fresh Z broth to give a final volume of 5 ml. After incubation at 30 °C for 20 min for adsorption, the unadsorbed phage were removed by centrifugation and the cell pellets were washed three times with 0.1 M-sodium citrate (pH 5.5). Finally, these cell pellets were resuspended in 0.5 ml of 0.1 M-sodium citrate (pH 5.5) and appropriate dilutions were plated onto agar plates. The plates were incubated at 37 °C for 4 h and soft agar containing 15 μ g ml⁻¹ of tetracycline was layered onto the plates. The tet^r colonies carrying the *prfA*(Ts) mutations were selected by testing for their inability to grow at 42 °C.

Construction of *E. coli* NG203. The *E. coli* strain NG3118 (*thi-l*, *lacU169*) was used as a recipient in the P1 transduction experiment employed to transfer the release factor 2 mutation *prfB3* from the *E. coli* strain YN2980 (Kawakami *et al.*, 1988). The P1 transduction was carried out as described above. The *prfB3* mutation is 50% cotransducible with the transposon Tn10 carrying the tet^r marker. Therefore tet^r P1 transductants could be selected for the transfer of *prfB3* mutation. Since there is no marker (such as temperature sensitivity) associated with the *prfB3* allele, the transfer of this mutation was tested in a different manner from that of the RF1 mutation. It has previously been shown that the presence of release factor 2 mutation decreased the transcriptional readthrough from the *trp* attenuator 2-fold (Roesser & Yanofsky, 1988; Roesser *et al.*, 1989). We had constructed a transcriptional fusion of the promoter attenuator region of *trp* and truncated *lacZ* in the λ RS88 (Simons *et al.*, 1987). Ten tet^r P1 transductants were selected and were lysogenized with the λ phage carrying this *trp-lacZ* fusion. Lysogenization was carried out as described above and monolysogens were selected from each experiment. Comparison of the β -galactosidase produced from these 10 different monolysogens showed that 4 of them had a twofold decrease in the transcriptional readthrough from the *trp* attenuator. The original colonies from which these lysogens were derived were selected as containing the RF2 mutations. One of these colonies was selected for further studies and was designated NG202 (Table 1). The *E. coli* strain NG202 was then used to generate a monolysogen of λ ppheA-lac designated NG203 as described above.

Results

Effect of prfB3 allele on the basal level expression of pheA operon

The leader peptide of *pheA* is 15 residues long, and a stop codon UGA is positioned in stem 2 of the attenuator (Fig. 1). It is postulated that a translating ribosome occupies about 12 bases on the mRNA either side of the codon (Gold & Stormo, 1987). This would prevent stem 2 from base pairing with either stem 1 or stem 3 and would lead to a situation which favours the formation of the transcription terminator; i.e. the 3:4 stem-loop of the attenuator. However, if the ribosome is released from the stop codon, stem 2 will be free again and will base pair with either stem 1 or stem 3 depending upon their relative stabilities. Because of this reason, translation of the leader mRNA up to the stop codon, and release of the translating ribosome from the appropriately positioned UGA stop codon in stem 2, become important constraints in determining the basal level expression of this

the *prfB3* allele on the basal level expression of *pheA*. As described in Methods, the *E. coli* strain NG202 carrying this mutation was used to lysogenize with the λ *ppheA-lac* phage to generate the strain NG203. The amount of β -galactosidase produced from this λ *ppheA-lac* in the presence (NG203) and absence (NG200) of the *prfB3* allele is given in Table 2. It was observed that the presence of *prfB* mutation decreased the amount of

Table 1. *E. coli* strains and bacteriophages used in this study

Strains and phages	Characteristics	Source or reference
<i>E. coli</i>		
UL213	XA10C (Miller & Albertini, 1983)	(Ryden & Isaksson, 1984)
YN2980	<i>prfA</i> (Ts), Tn10	(Kawakami <i>et al.</i> , 1988)
NG3118	LS653 (Soll, 1974), <i>prfB3</i> , Tn10	N. Gavini (unpublished)
NG200	<i>thi1</i> , <i>lacU169</i>	This work
NG201	NG3118, λ <i>ppheA-lac</i>	This work
NG202	NG200, Tet ^r , <i>prfA</i> (Ts)	This work
NG203	NG3118, Tet ^r , <i>prfB3</i>	This work
NG250	NG202, λ <i>ppheA-lac</i>	This work
NG251	NG3118, λ <i>ptrp-lac</i>	This work
Phages		
λ <i>ppheA-lac</i>	Lac ⁺	(Gowrishankar & Pittard, 1982a)
λ <i>ptrp-lac</i>	Lac ⁺	N. Gavini and L. Pulakat (unpublished)
P1 virulent	Transducing phage	Laboratory stock

Table 2. β -galactosidase activity in strains carrying *ppheA-lac* with or without release factor mutations

Results are the average of six trials.

<i>E. coli</i> strain	Resident release factor mutation	Relevant resident <i>lac</i> fusion	β -Galactosidase activity*	
			30 °C	37 °C
NG200	None	<i>ppheA-lac</i>	123	125
NG201	RF1	<i>ppheA-lac</i>	125	119
NG203	RF2	<i>ppheA-lac</i>	NA	57
NG250	None	<i>ptrp-lac</i>	NA	250
NG251	RF2	<i>ptrp-lac</i>	NA	130

* Specific activity of β -galactosidase is expressed according to Miller (1972).NA Not applicable (since *prfB*(RF2) is not associated with a temperature sensitive phenotype).

β -galactosidase produced from the λ *ppheA-lac* by about 55% indicating that the transcriptional readthrough from the *pheA* attenuator decreased by over 2-fold when the defective release factor 2 was present in the cell. To verify the presence of the *prfB3* allele, the effect of this mutation on the expression of β -galactosidase produced from an *E. coli* strain lysogenized with λ *ptrp-lac* was also tested (as described in Methods) and the results are presented in Table 2. Measuring the transcriptional readthrough from the *trp* attenuator in the presence (NG251) and absence (NG250) of *prfB* mutation was selected as a method to test the presence of *prfB* mutation, since the synthesis of *trp* leader peptide also terminates at a UGA stop codon, and it was shown that

the *prfB* mutation decreased the basal level expression of the *trp* operon 2-fold (Roesser & Yanofsky, 1988; Roesser *et al.*, 1989).

A mutation in prfA does not affect the basal level expression of pheA

To test whether the twofold decrease found in the transcriptional readthrough from the *pheA* attenuator is specifically due to the presence of release factor 2 mutation, we introduced a *prfA* mutation which encodes defective release factor 1 into *E. coli* NG200 as described in Methods. The presence of the temperature sensitive *prfA* mutation could easily be verified since the *E. coli* strain NG201 carrying this mutation could not grow at 42 °C. At 37 °C, the ribosome release from the UAG codon is decreased when the *prfA* mutation is introduced in a strain (Ryden & Isaksson, 1984; Ryden *et al.*, 1986). The amount of β -galactosidase produced from the λ *ppheA-lac* in the presence (NG201) and absence (NG200) of the *prfA* mutation is given in Table 2. It was observed that the amount of β -galactosidase activity produced at 30 °C from NG201 was similar to the amount produced at 37 °C from the same strain and also similar to the amount produced from NG200 at both temperatures. These results indicate that the presence of defective release factor 1 does not affect the basal level transcriptional readthrough from the attenuator terminator of *pheA*. Therefore the 2-fold decrease in the basal level expression of *pheA* in the presence of the *prfB* mutation is specifically due to the decrease in the ribosome release from the *pheL* stop codon caused by the defective release factor 2.

Discussion

In this study we have examined the role of release of translating ribosomes from the leader peptide stop codon in determining the basal level expression of *pheA*. It has been shown previously that the *prfB3* mutation, which encodes a defective release factor 2, decreased the basal level of expression of the *trp* operon (Roesser & Yanofsky, 1988; Roesser *et al.*, 1989). The leader transcripts of both *trp* and *phe* operons have UGA translation termination codons. Therefore, using the *trp* attenuator as a control, we have tested the effect of the *prfB3* allele on the basal level expression of *pheA*. As shown in Table 2, the β -galactosidase activity of the λ *ppheA-lac* decreased by over 2-fold in the *prfB3* mutants. However, the *prfA* mutation, which encodes a defective release factor 1, did not affect the β -galactosidase activity (Table 2). Therefore, a deficiency in the function of release factor 2 specifically decreases the transcriptional readthrough from the attenuator terminator of *pheA*. Since the UGA translation termination codon of *pheL* is located in stem 2, the deficiency in the ribosome release would lead to stalling of the translating ribosome on the stop codon for longer than usual and occupying stem 2, preventing it from base-pairing with either stem 1 or stem 3 of the attenuator. Stem 3 will then become free to base-pair with stem 4 and this would result in more frequent transcription termination at the attenuator terminator. It is this situation that dictates the low basal level expression of *pheA* in a strain carrying the *prfB* mutation. Thus, identifying that the defective release factor 2 decreases the transcriptional readthrough from the *pheA* attenuator points out two significant factors that affect the attenuation regulation of *pheA*: firstly, that the translating ribosome should translate the leader peptide up to the stop codon and secondly, that the translating ribosome should release from the leader peptide stop codon efficiently to ensure normal basal level expression of the *pheA* gene.

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