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Functional mutants of phenylalanine transfer RNA from *Escherichia coli*

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The gene *pheV* from *Escherichia coli*, coding for $tRNA^{Phe}$ and carried on a plasmid, has been mutagenised with hydroxylamine. Mutants in the structural gene have been identified using two criteria: (i) de-attenuation of β-galactosidase expression, while under the control of the attenuator region of the *pheS,T* operon by means of an operon fusion; (ii) loss of ability to complement thermosensitivity of a mutant Phe-tRNA synthetase. Mutants showing de-attenuation were sequenced and two nucleotide changes identified: G44→A44 (found five times) and m^7G46→A46 (found once). Sequencing of mutants that lost complementation identified two further tRNA mutants, C2→U2 and G15→A15; the mutant m^7G46→A46 was also re-isolated by this criterion. Three of the mutants involve bases implicated in tertiary rather than secondary structure hydrogen bonding. One hypothesis for the mechanism of de-attenuation is that mutant $tRNA^{Phe}$ molecules compete with the wild-type $tRNA^{Phe}$ on the ribosome but are inefficient at some step in the elongation process.

Key words: E. coli $tRNA^{Phe}$/plasmid mutagenesis/attenuation/tertiary structure mutants

Introduction

In recent years important advances have been made, both conceptually and experimentally, in understanding the process in protein synthesis whereby the ribosome selects the aminoacyl-tRNA corresponding to the codon presented in the ribosomal A-site (for a review, see Kurland and Ehrenberg, 1984). In these studies, the availability of functional mutants in ribosomal proteins has played a key role (see Bohman *et al.*, 1984 and references therein). In contrast, mutational studies on tRNA molecules, with a view to understanding the functional importance of different regions of the molecule and the tertiary structural interactions which stabilise it, have in general been possible so far only in the case of certain tRNA suppressor species (Abelson *et al.*, 1970; McClain, 1977). As a result, such studies have rarely been pursued to the point of detailed kinetic analysis of tRNA function in protein synthesis. Thus, we lack corresponding mutants in tRNA species whose behaviour can be studied *in vitro* in systems which lend themselves to detailed kinetic analysis, in particular, poly (Phe) synthesis (Wagner *et al.*, 1982; Ruusala *et al.*, 1982). However, the isolation of mutants of *Escherichia coli* $tRNA^{Phe}$ has recently become easier. Two genes for this tRNA species have been cloned, (Caillet *et al.*, 1983; Schwartz *et al.*, 1983), facilitating their localized mutagenesis. Furthermore, it has been demonstrated that expression of the operon *pheS,T* coding for phenylalanyl-tRNA synthetase (PheRS), is controlled by attenuation (Springer *et al.*, 1983; Fayat *et al.*, 1983; Trudel *et al.*, 1984). According to this mechanism, the efficiency of transcription of a structural gene is controlled by the speed of translation of certain codons (in this case Phe codons) in a short upstream coding sequence. Efficient translation leads to transcription termination before the structural gene under attenuator control, whereas pauses in leader sequence translation favour terminator readthrough, allowing transcription to continue into the following gene (see, for example, Yanofsky, 1981).

Gene fusions constructed between the attenuator region of *pheS,T* and *lacZ* (Springer *et al.*, 1983, 1984) have been used for screening potential *pheV* functional mutants. Finally, the availability of PheRS thermosensitive mutants, complemented by plasmids carrying wild type *pheV* (Caillet *et al.*, 1983), has also allowed us to screen for *pheV* mutants by loss of complementation.

We report here the isolation and characterisation by gene sequence determination and measurement of de-attenuation *in vivo* of *pheS,T* expression, of nine $tRNA^{Phe}$ functional mutants, four of which proved to be distinct. One of these affects the amino acid acceptor stem. In the remaining three mutants, by analogy with yeast $tRNA^{Phe}$, the species of tRNA the best understood in structural terms (Robertus *et al.*, 1974; Kim *et al.*, 1974; Jack *et al.*, 1976), we conclude that the bases altered are involved in tertiary structural interactions in the molecule.

Results

Mutagenesis and isolation of mutants

To isolate functionally altered mutants of *E. coli* $tRNA^{Phe}$, we have mutagenised *in vitro* a plasmid (pPP15) carrying the gene *pheV*, cloned in a 350-bp DNA fragment in pBR322 (J. Caillet, unpublished data). Mutagenesis was performed with hydroxylamine, specific for transitions (G.C→A.T) *in vitro*, and under the conditions employed, subsequent transformation was between 25 and 100 times less efficient than with the untreated plasmid DNA, in approximate agreement with the observations of Volker and Showe (1980). Potential *pheV* mutants were detected in two different ways. In a strain (XA102λGS5) carrying a fusion between the operon *pheS,T* and *lacZ*, in which β-galactosidase synthesis is subject to attenuation by the control region of *pheS,T* (Springer *et al.*, 1983), β-galactosidase synthesis is repressed by the action of the attenuator and transformation by wild-type pPP15 does not lead to de-repression. In contrast, transformation with pPP15 after treatment with hydroxylamine leads to transformants showing higher levels of *lacZ* expression (deeper coloured colonies on X-gal indicator plates), at a frequency of ~1%. It should be noted that the fusion λGS5 leads to a low level of β-galactosidase synthesis, though control is normal (Springer *et al.*, 1983). This low level is useful in that it permits the use of X-gal indicator plates to detect increases of β-galactosidase of as little as 2-fold. Nine potential *pheV* mutants were obtained in this way and subjected to further analysis. In each case, the degree of de-repression of β-

Table I. De-repression by mutant plasmids of β-galactosidase synthesis from λGS5 in *trans*

Plasmid	*Units* β-gal/A_{650} unit
pPP15	3.6 ± 0.4
pPP15H1	6.6 ± 1.4
pPP15H2	5.7 ± 0.8
pPP15H3	6.2 ± 1.0
pPP15H5	8.6 ± 1.9
pPP15H6	6.9 ± 1.8
pPP15H7	6.5 ± 1.5

Transformants of strain XA102 (λGS5) by plasmid pPP15 and six mutants of this plasmid obtained by treatment with hydroxylamine (see text) were grown in MOPS/glucose medium (Neidhardt *et al.*, 1977) supplemented with methionine (50 μg/ml), proline (50 μg/ml) and tetracycline (10 μg/ml). β-galactosidase activity was determined according to Miller (1972b); the values shown are the averages of four measurements and are accompanied by standard deviations. Doubling times were between 80 and 90 min.

galactosidase synthesis was measured during growth in liquid medium. Six mutants showed significant de-repression, by a factor of approximately two (see Table I) consistent with their appearance on indicator plates. Subsequent sequence analysis (see below) showed that five of the clones isolated in this way all showed the same nucleotide replacement, different from that found in the remaining clone, pPP15H5.

Further potential mutants were obtained by screening tetracycline-resistant transformants of strain IBPC1671 for the ability to grow at 42°C. This strain has a temperature-sensitive PheRS as a result of mutation *pheS5*, which can be complemented by transformation with pPP15 and thus regain ability to grow at 42°C. Of 38 tetracycline-resistant transformants obtained with pPP15 mutagenized by exposure to hydroxylamine, four were found to be temperature-sensitive. Three of these were studied further. One explanation for failure to complement thermosensitive PheRS is that mutant pPP15 has acquired a thermosensitive defect in replication. In this case, in the presence of wild-type PheRS, clones should lose resistance to tetracycline at 42°C and thus fail to grow on selective media. In contrast, if the mutation affects the tRNA gene or its regulatory sequences, growth should occur at 42°C in the presence of tetracycline if wild-type PheRS is present owing to the introduction of the wild-type *pheS,T* operon. Thus, phage λB1 (Plumbridge *et al.*, 1980) carrying the wild-type gene *pheS* was introduced into the three selected clones, pPP15T6, pPP15T36 and pPP15T61. In fact, all three clones grew at 42°C, suggesting that failure to complement thermosensitivity in the absence of λB1 was due to mutations affecting *pheV* or its expression.

DNA sequencing of mutants in pheV

The *Pst*I-*Hpa*I fragment from pPP15 carrying *pheV* was excised from pPP15 and recloned in M13mp8 (Messing and Vieira, 1982). Using the dideoxynucleotide method (Sanger *et al.*, 1980), nine mutants were sequenced and nucleotide changes were found at four distinct positions in the tRNA structure. These are shown in Table II. Thus, mutant A44 was isolated independently five times by the criterion of de-attenuation, A46 once by this criterion and once by loss of complementation of PheRS thermosensitivity, and U2 and A15 were both isolated once by the latter criterion. It should be noted that mutant A44 also lost the ability to complement PheRS thermosensitivity, though it has not so far been isolated directly by this criterion.

Table II. Characterisation of $tRNA^{Phe}$ mutants

Plasmid	Nucleotide change	Phe acceptor capacity	β-galactosidase synthesised from	
		pmol/A_{260} unit	λML2	λMBA15-28
pBR322		19.5	164 ± 15 (3)	1322 ± 88 (6)
pPP15		40	154 ± 17 (6)	1009 ± 181 (6)
pPP15H1		50		
pPP15H2		43		
pPP15H3	G44→A44	40	381 ± 57 (10)	858 ± 50 (11)
pPP15H6		53		
pPP15H7		49		
pPP15H5	m^7 G46→A46	28	267 ± 10 (8)	774 ± 55 (6)
pPP15T36		41		
pPP15T6	C2→U2	27	183 ± 19 (4)	857 ± 79 (5)
pPP15T61	G15→A15	39	230 ± 7 (3)	1204 ± 143 (4)

Nucleotide replacements in *pheV* mutants were determined by DNA sequencing; nucleotide positions are numbered according to Sprinzl and Gauss (1982; see also Figure 1). Charge capacity of unfractionated tRNA extracted from strains transformed by mutant or control plasmids was measured by charging with [^{14}C]Phe and crude aminoacyl-tRNA synthetases. For determination of β-galactosidase activity in transformants carrying the wild-type attenuator: *lacZ* fusions (Y1R1 λML2), or fusions with an attenuator deletion (Y1R1 λMBA15-28), transformed cells were grown in MOPS/glucose medium (Neidhardt *et al.*, 1977) with 10 μg/ml tetracycline. Doubling time was between 120 and 150 min. Values of β-galactosidase activity, expressed in units/A_{650} unit of bacterial culture (Miller, 1972b), are followed by the standard deviation and, in parenthesis, the number of determinations.

Further characterisation of $tRNA^{Phe}$ mutants

The phenylalanine acceptance of unfractionated tRNA extracted from cells transformed by each mutant plasmid was measured *in vitro* using a crude aminoacyl-tRNA synthetase preparation. The values obtained (see Table II) showed that, with the possible exception of mutant U2, an increase of ~2-fold in acceptance capacity is seen compared to the control with pBR322 carrying no insertion. These values are similar to that seen with pPP15, carrying wild type *pheV* (Table II), though lower than those reported previously (Caillet *et al.*, 1983).

A further experiment was performed to test whether the de-repression of β-galactosidase synthesis observed (Table I) was indeed related to the function of the *pheS,T* attenuation region, as expected. The four mutant plasmids, together with pPP15 and pBR322 as controls, were each introduced into two strains, Y1R1(λML2) and Y1R1(λMBA15-28) (Springer *et al.*, 1983, 1984), both carrying operon fusions between the *pheS,T* control sequences and *lacZ*, but in the latter case deleted in the attenuator sequence. In transformants of Y1R1(λML2), significant de-repression was observed with mutants A44, A46 and A15 in agreement with the results in Table I, but not in the case of mutant U2. No de-repression was observed in the case of the fusion lacking the attenuator [Y1R1(λMBA15-28), see Table II], consistent with the notion that the attenuator was responsible for the de-repression observed previously; indeed, some decrease in β-galactosidase levels which might be significant was observed in some cases, though the reason for such a decrease is not clear. A preliminary attempt has been made to separate each mutant tRNA species from wild-type $tRNA^{Phe}$ by chromatographic methods. Total tRNA from strains transformed by each mutant plasmid pPP15 was extracted and charged with [^{3}H]Phe

then mixed with wild-type [^{14}C]Phe-tRNA^{Phe} and subjected to chromatography on RPC-5 as described in Materials and methods. The elution profiles of ^{3}H-labelled and ^{14}C-labelled material were identical with the exception of mutant U2, where a small percentage of the tritium label (1–2%) was eluted at a lower concentration of NaCl (0.6 M) than the main peak (0.8 M; results not shown).

Discussion

We describe here the isolation and initial characterisation of functional mutants of *E. coli* tRNA^{Phe}. Mutants have been successfully isolated by the application of two different criteria: de-repression of β-galactosidase synthesis under the control of the *pheS,T* attenuator region, and loss of ability to complement PheRS thermosensitivity. Those mutants isolated by the criterion of de-attenuation also showed loss of complementation.

All the positions where we have found nucleotide substitutions correspond to bases involved in hydrogen bonding interactions, one in a secondary structural interaction and three in tertiary structural interactions (see Figure 1). In the wild-type molecule all three tertiary structural interactions are of the type most often found in D_4V_5 tRNAs (i.e., those with four Watson-Crick interactions in the D-stem and five bases in the extra loop) if A.G and G.A are considered equivalent in position 26.44 (see Table III). The base pairs in the mutants, on the other hand, are of a type found only rarely in naturally occurring tRNA species. Thus it seems likely that the change from the most frequently found base pair to an uncommon pair will entail a perturbation of tertiary structure in the tRNA or a decrease in stability. These predictions are open to verification.

Further biochemical analysis will be necessary to explain the mechanism (or mechanisms) underlying the phenotype of these *pheV* mutants. In the case of mutants which show de-repression of β-galactosidase synthesis, one hypothesis is that the mutant tRNAs, charged and in the form of ternary complexes, compete with their wild-type counterpart in translation of phenylalanine codons on the ribosome, but are inefficient (i.e., slow) at some process in elongation, such as peptidyl transfer or translocation. In translation of the attenuator leader peptide, this would produce the same effect as starvation for Phe-tRNA^{Phe} and favour transcription of the following coding sequences. This process is amenable to study *in vitro* and we are undertaking experiments of this kind.

An alternative explanation for the phenotype of these mutants may be based on a hypothetical autoregulatory control of *pheV* expression. The introduction of many copies of a mutant *pheV* gene into the cell by means of a plasmid may reduce expression of the chromosomal tRNA^{Phe} genes without at the same time contributing compensatory amounts of Phe-tRNA in a form that the ribosome can accept. This might arise, for example, by a defect in charging or in the interaction between mutant Phe-tRNA and EF-Tu.GTP.

All mutants of tRNA^{Phe} that we have obtained fail to complement thermosensitive PheRS, whether or not they were isolated by this criterion. First of all, it should be recalled that we do not understand the mechanism by which pPP15, carrying wild-type *pheV*, can complement PheRS thermosensitivity. An increase in concentration of tRNA^{Phe} may compensate for an increased K_m of the mutant PheRS, or may stabilise a thermolabile enzyme. Thus, loss of complementation may

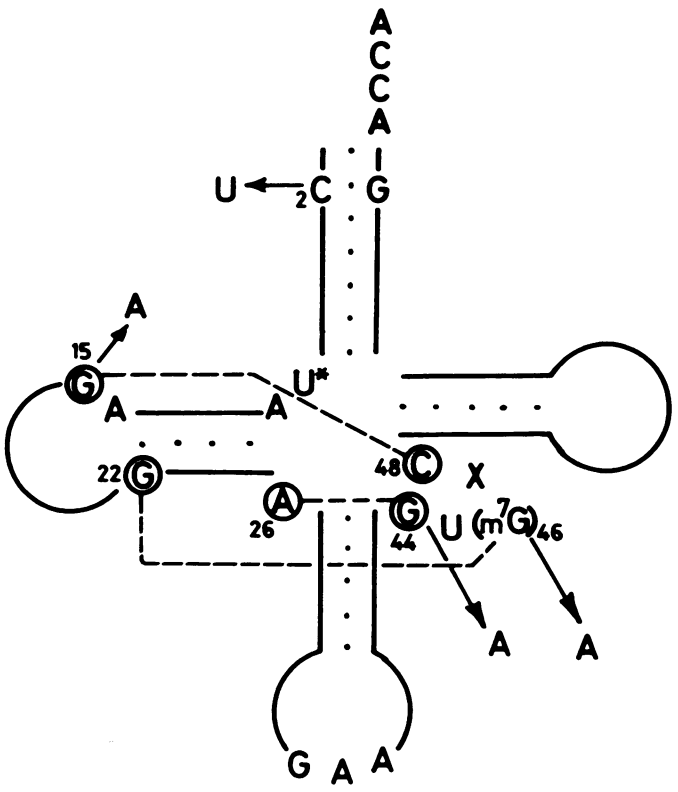

Fig. 1. *E. coli* tRNA^{Phe} mutants in a cloverleaf representation of *E. coli* tRNA^{Phe} showing positions of nucleotide substitutions in mutants and the hydrogen bonding interactions of the bases involved. Bases are numbered according to Sprinzl and Gauss (1982). Tertiary structural interactions are indicated by dotted lines.

Table III. Replaceable tertiary pairs in D_4 V_5 tRNAs

Tertiary interactions	Occurrence	Base pairs
15.48 (G.C)	81	G.C
	14	A.U
	3	N.C
	2	G.A
	1	A.C
	1	G.U
22.46 (G.G)	90	G.G
	4	A.G
	3	U.G
	3	G.A^{+}
	1	A.U
	1	G.U
	1	N.G
26.44 (A.G)	57	G.A
	27	A.G
	8	A.A*
	3	A.U
	2	G.G
	2	N.A
	1	C.G
	1	C.U
	1	G.C
	1	ψ.G

Tertiary structural interactions based on the sequences of D_4 V_5 tRNAs compiled by Sprinzl and Gauss (1984). The pairs in parenthesis are those found in wild-type *E. coli* tRNA^{Phe}.
$^{+}$:all are tRNAs from chloroplasts.
*: includes six sequences from mitochondria or chloroplasts.

Table IV. Strains used in this work

Bacteria strains	Relevant genotype	Origin and/or reference
IBPC1671	F⁻, *thi-1, argE3, his-4, proA2, lacY1, galK2, mtl-1, xyl-5, tsx-29, supE44, rpsL, recA1, pheS5*, λ⁻, λ^s	Plumbridge *et al.* (1980)
Xa102	F⁻, △(*lac pro*), *nalA, rpoB, metB, argEam, supE, ara*	Coulondre and Miller (1977)
Y1R1	F⁻, *trpR, lacZ U118, recA1*	Springer *et al.* (1984)
JM101	△(*lac pro*), *supE, thi, F', traD36, proAB, laci^{q}Z*△M15	Messing (1983)
λ Strains and plasmid	**Scorable markers**	**Origin and/or reference**
λB1	*imm21, ninR, thrS, infC, rplT, pheS, pheT*	Plumbridge *et al.* (1980)
λGS5	*cI857, nin5, rplT, pheST-lac* fusion	Springer *et al.* (1983)
λML2	*cI857, nin5, rplT, pheST-lac* fusion	Springer *et al.* (1983)
λMBA15-28	λML2 with a 192 bp deletion in the *pheST* attenuator	Springer *et al.* (1984)
pPP15	Tet^R derivative of pBR322 with a 350 bp *PstI-PvuI* fragment carrying a tRNA^{Phe} gene (*pheV*)	J. Caillet (unpublished data)

arise from mutations that reduce transcription or lead to incomplete maturation of the transcripts, or to tRNA^{Phe} molecules with a reduced affinity for PheRS. Mutant U2, which fails to complement thermosensitive PheRS but shows no de-attenuation, may fall into such categories. Experiments are in progress to test these hypotheses.

Materials and methods

Strains and plasmids

All the strains, plasmids and bacteriophages used in this work are shown in Table IV.

Mutagenesis

The plasmid pPP15, at a concentration of 0.75 mg/ml in 0.1 M phosphate buffer, pH 6; 10^{-3} M EDTA, was exposed to 0.8 M hydroxylamine for 3 h at 70°C (Volker and Showe, 1980) dialysed against 10^3 vol of 10 mM Tris-HCl, pH 8; 10^{-4} M EDTA for 5 h, and finally against 10^3 vol of 10 mM Tris-HCl, pH 8 for 15 h. These plasmid solutions were used directly for bacterial transformations.

Transformation and transfection

Prior to transformation, bacteria were treated with $CaCl_2$ (Cohen *et al.*, 1972); 0.2 ml aliquots (10^8 cells) were transformed with 1 μg DNA and plated on to selective medium. When screening for de-repression of β-galactosidase synthesis, plates contained 20 μg/ml of X-gal (5 bromo-4-chloro-3indolyl-β-D-galactoside, Sigma). The same method was used for transfection except for omission of the incubation for 1 h at 37°C.

DNA sequence determination

The *PstI-HpaI* restriction fragments carrying the wild-type *pheV* gene and its mutated derivatives were cloned into phage M13mp8 between its *PstI* and *HindII* sites (Messing and Vieira, 1982). Strain JM101 was transfected and plated on to minimal medium A (Miller, 1972a) with glucose (0.2%) and X-gal. Several white plaques were used for independent preparations of single-stranded DNA. DNA sequences were determined by the dideoxynucleotide triphosphate method of Sanger *et al.*, (1980) using [α^{32}P]dATP, and electrophoresis on 8% (w/v) polyacrylamide gels. Autoradiography was conducted overnight at −20°C on X-OMAT R film (Kodak).

tRNA

Total tRNA was extracted from transformed cells by the method of Stern and Littauer (1971). Aminoacylation was performed in 0.1 M Tris-HCl, pH 8; 5 mM $MgCl_2$; 7 mM ATP, 30 μM [^{14}C]Phe (450 Ci/mol) or [^{3}H]Phe (57 Ci/mmol), with 0.5 mg/ml 30 000 *g* supernatant protein fraction (Zubay *et al.*, 1970). After incubation for 15 min at 37°C, Phe-tRNA was precipitated with cold 10% trichloroacetic acid, collected on GF/C filters (Whatman), washed, dried and measured by liquid scintillation counting. For chromatography on RPC 5, tRNA was charged as described above and recovered by addition of 0.4 vol 2 M sodium acetate buffer pH 5 and 2 vol ethanol. Samples were redissolved in 50 mM sodium acetate buffer pH 5, 10 mM $MgCl_2$, 1.4 mM β-mercaptoethanol, 0.4 M NaCl and chromatographed at 20°C on a column of RPC-5 (90 cm x 1 cm) as described by Pearson *et al.*, (1971), eluting with a linear gradient (400 ml total volume) from 0.4 M NaCl to 0.9 M NaCl, in 50 mM sodium acetate buffer, pH5; 10 mM $MgCl_2$; 1.4 mM β-mercaptoethanol.

β-Galactosidase measurements

For these determinations, cells were cultured in minimal 3-(N-morpholino) propane sulphonic acid (MOPS) glucose medium (Neidhardt *et al.*, 1977) with 50 μg/ml methionine, 50 μg/ml proline and 10 μg/ml tetracycline. Enzyme activity was measured as described by Miller (1972b); one unit is defined as $(A_{420} - 1.75\ A_{550}) \times 10^3/t$, where t is the time of incubation in minutes.

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