Evidence that pheV, a gene for tRNA^{Phe} of E. coli is transcribed from tandem promoters

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ABSTRACT

A DNA fragment of 487 bp containing a gene for tRNA^{Phe} has been sequenced. Although the tRNA^{Phe} sequence is identical to that of pheU (which maps at 94.5 min) the surrounding sequences are quite different. This sequence is thus that of a second gene for tRNA^{Phe} (which we shall call pheV). In vitro transcription experiments and S1 mapping in vivo show the existence of two promoters separated by about 60 nucleotides. The second transcript starts only 3 nucleotides 5' from the tRNA^{Phe} structural sequence. A DNA sequence characteristic of a rho-independent terminator is located 30 nucleotides 3' of the end of the structural gene and is shown to function efficiently in vitro.

INTRODUCTION

Genes for tRNA have been found through the E. coli chromosome (1) and they are found in very different genetic environments. The majority of the tRNA genes identified are found in transcription units which carry several tRNA genes arranged head to tail (2, 3, 4, 5, 6). However, two possible examples of small transcription units containing a single tRNA gene have been reported (7, 8). Some tRNA genes are also found in ribosomal operons where they are between the genes for 16S and 23S and at the end of the operon (9). A few others are found in transcription units which also code for proteins (10, 11).

tRNA^{Phe} is one of the E. coli tRNA's for which a single major isoacceptor species has been purified and sequenced (12). A minor isoacceptor was also characterized but probably arises from a different post-transcriptional modification (12). Previously to the present investigation genomic Southern blotting data indicated that a single EcoRI fragment hybridized to tRNA^{Phe} (13). However, two groups (14, 7) have reported the identification of two different clones carrying a tRNA^{Phe} gene by phenotypic suppression of thermosensitive phenylalanyl-tRNA synthetase mutants. This implied that there are at least two loci for tRNA^{Phe}. The clone identified by Schwartz et al. (7) carries the gene called pheU which was found on a 9.5 kb EcoRI fragment probably corresponding to the EcoRI fragment originally seen on genomic

The gene pheU has been located at 94.5 min of the E. coli map. The second gene for tRNA^Phe which we now propose to call pheV was cloned on a 5.6 kb PstI fragment from two different E. coli genome libraries. We previously named this gene pheU since we were unaware of the existence of two genes.

This present paper shows a 487 bp sequence of DNA containing pheV and gives the characterization of several in vivo and in vitro transcripts covering pheV.

**MATERIAL AND METHODS**

**General techniques**

Plasmids carrying pheV used in this work are shown in Fig 1. They are isolated by phenotypic suppression of strains carrying the thermosensitive pheS5 allele. Standard recombinant DNA techniques were as described previously. Restriction endonucleases and other DNA modifying enzymes were from commercial suppliers and used as recommended unless otherwise stated. DNA fragments were purified from low melting point agarose gels. Strands were separated as described.

**In vitro transcription**

The same conditions were used for native supercoiled DNA and for purified fragments. The reaction mixture (50 μl) containing 20 mM Tris-HCl pH 8.1: 10 mM MgCl2: 0.1 mM Dithiothreitol: 1 mM EDTA: 150 mM KCl: 0.3-1 μg DNA and 1.5 μg E. coli RNA polymerase (holoenzyme) was preincubated for 5 minutes at 37°C. The reaction was initiated by the addition of all 4 nucleotides (0.2 mM ATP: CTP: GTP: 0.05 mM[α32P]UTP: 100 μCi/mM). After 15 min the reaction was stopped by addition of 200 μl of 10 mM EDTA. The samples were extracted with phenol, ethanol precipitated and analysed on 5% polyacrylamide gels containing 8.3 M urea. The gels were fixed with 20% ethanol: 10% Acetic Acid and dried before autoradiography at -80°C.

**Nuclease S1 Mapping**

The method used was basically that of Barry et al. The probes used were the separated strands of the 5' end labelled 780 bp Ddel fragment shown in Fig 5A. RNA was prepared by the hot phenol method from exponentially growing cells (strain 294 was used). Hybridization was performed in 50 μl of 80% formamide: 40 mM Pipes pH 6.4: 1 mM EDTA: 400 mM NaCl with 10^5 cpm of radioactive single strand probe and 0-50μg of RNA extracted from exponentially growing cells. The total quantity of RNA in each incubation was made up to 100 μg using rRNA. After denaturation at 75°C for 10 min hybridization was carried out at 53.5°C for 3 hours, under paraffin oil. The hybrids were treated with S1 by transferring to 0.4 ml of buffer containing 10^5 units/ml (Boehringer-Mannheim GFR) and digested at 37°C for 30 min. S1 resistant hybrids were recovered by ethanol precipitation and analysed on denaturing gels (5%
Fig 1: Structure of pCC2 and pPP15, two pheV carrying plasmids.

The plasmid pPP15 carries a 0.350 kb PstI-PvuI fragment of pPC1007 (14) cloned between the PstI and PvuI sites of pBR322. The plasmid pCC2 carries a 3.5 kb HindIII-ClaI fragment from pHD3 (14) between the HindIII and ClaI sites of pBR322. The plasmid pCC2 also carries a 200 bp ClaI fragment from pHD3 in the left ClaI site. As this ClaI fragment is irrelevant it is not shown on the Figure. The simple lines are E. coli DNA and the vector DNA is boxed. The location of trNA\(^{Phe}\) sequence is shown on each plasmid by a black box under the E. coli DNA line. The scales are shown on the Figure.

polyacrylamide containing 8.3 M urea). Gels were fixed, dried and autoradiographed with Cronex Hi-plus amplifying screens at -80°C for 2 days.

Sequencing

The nucleotide sequences of DNA fragments or strands were determined as described (20). DNA restriction fragments were 5’ end-labelled with polynucleotide kinase. The cleaved products were analysed on 16 %, 8 % and 6 % polyacrylamide gels containing 8.3 M urea (90x20x0.03 cm).

RESULTS

Isolation and sequence of a 487 bp DNA fragment containing a gene for trNA\(^{Phe}\) (pheV)

A gene for trNA\(^{Phe}\) (pheV) was originally isolated by phenotypic suppression of a thermosensitive phenylalanyl-tRNA synthetase mutant (14) as two cosmids coming from a collection of E. coli DNA partially digested with Sau3A and cloned in the BamHI site of pHC79. The same gene was also isolated from a clone of the Clarke-Carbon Collection (14). A 3.5 kb HindIII-ClaI fragment and a 0.35 kb PstI-PvuI fragment
Fig 2: Sequencing strategy for the PstI-HpaI and HpaI-Hpal restriction fragments of pPP15 and pCC2 plasmids. The sites of 5' end labelling are indicated by closed circles. The arrows indicate the direction and extent of sequence determined on each strand or fragment.

carrying pheV were subcloned in pBR322 to give respectively pCC2 and pPP15 (Fig 1). These two plasmids suppress the thermostable phenylalanine-tRNA synthetase mutation corresponding to the pheS5 allele. The 487 bp HpaI-Hpal fragment of pCC2 and the 246 bp PstI-Hpal fragment of pPP15 were sequenced using the strategy outlined in Fig 2. The actual sequence of the HpaI-Hpal fragment is shown in Fig 3 where the sequence corresponding to the tRNA^Phe is boxed. The structural part of pheV predicts a tRNA^Phe sequence identical to that of the isoacceptor species sequenced (12) and is identical to the structural part of pheLI (7). The surrounding sequences of pheV both 5' and 3' are different from that of pheLI. In addition for pheV, two promoter like sequences can be identified (21). One is immediately in front of the sequence corresponding to tRNA^Phe (P2 Fig 3) and shows some homology with the sequence in front of pheU where a promoter consensus sequence has also been observed. The second potential promoter is 60 bp before the gene (P1 Fig 3) and has no counterpart in pheU. The tRNA^Phe sequence is followed by a sequence characteristic of a rho-independent terminator (22). Computer analysis of the sequence using some programs (23) designed to find promoters shows P2 as a good promoter. P1 shows less homology with the consensus sequence.

In vitro transcription with native plasmids and DNA fragments

Original transcripts carrying tRNA genes undergo a series of nuclease attacks and are further modified to give the biological active form. This processing of transcripts is called maturation and can present a problem when it comes to detecting tRNA transcripts in vivo but not in vitro. Thus we first analysed transcription around pheV by studying RNA synthesis in vitro using the native supercoiled plasmids pPP15.
Fig 3: Nucleotide sequence of pheV and its flanking regions. The sequence is numbered from the Hpal3 site to the Hpal4 site as defined in Fig 2. The mature tRNA\textsuperscript{Phe} sequence is enclosed in a box. Sequences corresponding to the -35 and -10 regions of the consensus promoter sequence are underlined. Regions exhibiting dyad symmetry are indicated with overlined arrows. Relevant restriction enzymes sites are noted. The -35 and -10 regions of P2's secondary RNA polymerase binding site are overlined.

and pCC2 (Fig 1). As both plasmids derive from pBR322, the latter plasmid was used as control. Under the conditions used, the major transcript from pBR322 is a 104 nucleotides long RNA (Fig 4A, Lane 3) which is known to be implicated in the control of ColE1-type plasmid replication (24). The plasmid pPP15 directs the synthesis of pBR322's 104 RNA and a 115 nucleotides long RNA coming from the PstI-PvuI Insert (Fig 4A, Lane 2). The plasmid pCC2 which carries a 3.5 kb long ClaI insert, containing the PstI-PvuI fragment of pPP15, synthesizes the same RNA species as pPP15 plus a 170 nucleotides long RNA (Fig 4A, Lane 1). To further characterize these 115 and 170 nucleotides long transcripts, we examined RNA synthesis in vitro using the fragments Hpal3-Hpal4, Hpal2-Hpal4, Hpal1-Hpal4 from pCC2 (Fig 1). All fragments synthesize the same two products: a 105 and a 170 nucleotides long products (Fig 4B, Lanes 1, 2, 3). The apparent molecular weight discrepancy between 115 (found with the native plasmids) and 105 (found with the fragments) is explained by the fact that in the former case the gel was run hot and in the latter case not. As we suspect
Fig 4 : Analysis of in vitro transcription products :

A) using supercoiled plasmids : Lane 1. pCC2 ; Lane 2. pPP15 ; Lane 3. pBR322 ; Lane 4. molecular weight markers made from the large EcoRI-HindIII fragment of pBR322 digested by HpaII and subsequently 5' end labelled). In vitro transcription was performed as described under Material and Methods. Products were analysed on a 40x20x0.1 cm 5 % polyacrylamide gel in the presence of 8.3 M urea by electrophoresis at 63 W for 3 hours.

B) using purified DNA fragments : Lane 1. Hpal3-Hpal4 ; Lane 2. Hpal2-Hpal4 ; Lane 3. Hpal1-Hpal4 ; Lane 4 as Lane 4 in A. Products analysed as in A except electrophoresis was for 5 W for 16 hours.

(see later) this transcript to carry the tRNA\textsuperscript{Phe} which has a very stable secondary structure. this slight discrepancy is not surprising. From now on, we will refer to that transcript as 115. As this 115 nucleotides long transcript is made from Hpal3-Hpal4 and pPP15 (that is from the PstI-PvuII insert). It has to be synthesized from the common PstI-Hpal4 fragment (248 bp) whose sequence is shown in Fig 3. Transcription from the promoter like sequence P2 (Fig 3) to the rho-independent terminator - like structure after the tRNA\textsuperscript{Phe} would permit the synthesis of a RNA of almost exactly 115 nucleotides. Thus the 115 nucleotides long RNA most probably starts at P2, covers the tRNA\textsuperscript{Phe} gene and terminates just after the BglII site at the rho-independent terminator. The Hpal3-Hpal4 fragment also directs the synthesis of the
**Fig 5 : Nuclease S1 mapping experiments.**

A) Probes used in Nuclease S1 mapping experiments. The Ddel fragment was prepared from the purified Hpal2-Hpal4 fragment and 5' end labelled. D11 and D12 are defined as the strands which respectively do not and do hybridised with in vivo synthesized RNA.

B) Electrophoretic analysis of the protected against S1 digestion. Lanes 1-4, probe D11 and lanes 5-8, probe D12. Lanes 1 and 5, untreated probes. Lanes 2-4 and 6-8 samples were hybridised and S1 treated with 0 (Lanes 2 and 6) 25 µg (Lanes 3 and 7) and 50 µg (Lanes 4 and 8) RNA. Lanes 9 and 10: molecular weight markers. Lane 9: Hpal2-Hpal4 fragment digested by Ddel and 5' end labelled. Lane 10 as in lane 9 except recutting by PstI. Experiments were performed and the products analysed as described under Materials and Methods.

170 nucleotides long RNA synthesized from pCC2 but not from pPP15. This indicates that the 170 nucleotides long RNA initiates somewhere between Hpal3 and PstI. *In vivo* transcription data see (below) prove that this 170 nucleotides long transcript starts at
P1 (Fig 3) and also carries the structural part of pheV and probably terminates at the rho-independent terminator at the Ball site.

**In vivo S1 mapping of the pheV promoters**

The probes used for the Nuclease S1 mapping are shown in Fig 5A and consist of the separated strands of the Ddel1–Ddel2 fragment labelled with T4 polynucleotide kinase at their 5' ends. The site Ddel2 lies inside the structural part of pheV. Both strands of the Ddel1–Ddel2 fragment were used in the Nuclease S1 mapping experiments (Fig 5B). The strand D11 of Fig 5A is not protected by any RNA extracted from exponentially growing cells (Fig 5B, Lanes 3 and 4). Using the other strand D12 three fragments are protected from Nuclease S1 digestion by RNA from exponentially growing cells (Fig 5B, Lanes 7 and 8): a 780 bp long fragment which is the size of the probe, a 75 bp fragment and a 14 bp fragment. The two longest fragments are absent when only carrier ribosomal RNA (rRNA) is added before S1 digestion (Fig 5B, Lane 6) and their yield increase with increasing mRNA addition before Nuclease S1 digestion. This indicates that there are transcripts covering the whole Ddel fragment and that there is a transcript starting 75 bp in front of Ddel2. A 75 nucleotides long RNA corresponds well to the length expected for a transcript initiating at the P1 promoter-like sequence of Fig 3. The 14 bp long protected fragment is also observed when rRNA is added instead of RNA extracted from exponentially growing cells before S1 digestion (Fig 5B, Lane 6). The distance from the Ddel2 site to the beginning of tRNA\(^{\text{Phe}}\) is 14 bp. Thus the 14 bp long fragment most probably corresponds to hybridization with the mature tRNA. The fact that it is observed with rRNA only is presumably due to the fact that the rRNA is contaminated with tRNAs. This strong 14 bp long fragment also appears when no rRNA is added since there is also mature tRNA\(^{\text{Phe}}\) in the RNA extracted from exponentially growing cells. The existence of that strong 14 bp band corresponding to mature tRNA\(^{\text{Phe}}\) prevented us from observing any transcript from P2. Such a transcript would have protected about 17 bp of the probe from digestion and would not have been separated from the 14 bp long fragment we observe. In vivo transcripts starting at P2 were previously isolated using a completely different method (see in the discussion).

**DISCUSSION**

Schwartz et al. (7) have reported a 300 bp DNA sequence containing a structural gene for tRNA\(^{\text{Phe}}\), which they call pheU and have mapped to 94.5 min (15). Here we report a 487 bp sequence where a second tRNA\(^{\text{Phe}}\) gene is located. Although the structural part of tRNA\(^{\text{Phe}}\) is identical the sequences 5' and 3' are quite different. At the moment we cannot deny the possibility that there are, in addition, other genes for tRNA\(^{\text{Phe}}\). What perhaps is most unusual is that both pheU and pheV do not seem to be
Fig 6: Comparison of the promoter regions of the pheU and pheV genes and those of the rRNA operons in E. coli. The sequences are aligned by the last T of the -10 region. -10 and -35 regions are boxed. Sequences corresponding to the "stringent box" are underlined. P1 and P2 are respectively the upstream and downstream promoters. P2 is identical for rRNA A and B and for rRNA D. E and X (sequences are taken from (21)).

closely associated with other tRNA or rRNA genes. In fact the DNA sequence around pheU (7) suggests that the gene is transcribed from a promoter just in front of the tRNA encoding sequence and finishes immediately afterwards at a rho-independent terminator. For pheV the DNA sequence presented here suggests the existence of two promoters, one in the equivalent position to that of the pheU gene and another 80 bp in front of it. A sequence characteristic of a rho-independent terminator is also found immediately after pheV.

Our in vitro transcription experiments show that the 246 bp PstI-HpaI fragment containing the pheV promotes the synthesis of two transcripts of 115 and 170 nucleotides long. RNAs of these lengths are precisely what is expected for the two transcripts starting at P1 and P2 and finishing at the terminator after the tRNA gene.

Nuclease S1 mapping shows that the promoter P1 (which gives rise to the 170 nucleotides long transcript) functions in vivo and suggests the existence of a third promoter a long way upstream of P1. Since the whole Ddel band was specifically protected, this putative promoter must be at least 600 bp 5' of P1. The large amount of mature tRNA Phe in the RNA preparation from exponentially growing cells which hybridised to the labelled S1 probe prevented the identification of a transcript corresponding to P2. However, it is already known from the work of Vogeli et al. (25) that a precursor form of tRNA Phe exists with the 5' sequence of pppGUUGGC where the second G is the first nucleotide of mature tRNA Phe. This precursor could also be synthesized from pheU which has the same 3 nucleotides, GUU, 5' of the tRNA Phe sequence. It is however not unreasonable to assume that the same precursor is synthesized from the two genes. Strong analogies exist between the P2 promoter of
pheV and the putative promoter found immediately before pheU. The sequences immediately 5' to pheU and pheV (Fig 6) are very similar up to -15 (where +1 is the G located 3 nucleotides in front of the structural gene where transcription from P2 probably starts). The region around -35 is also similar but the two sequences differ considerably between -15 and -35 and 5' of the -35 sequence. Moreover, both promoters possess a "stringent box" or "discriminator sequence" apparently involved in the stringent response (26) between the -10 region and the transcription initiation site. For pheV (− GCGCCC−) the agreement with the consensus sequence −gGCCC−C− (26) is perfect. No such sequence is found for the P1 promoter of pheV (Fig 6).

The existence of tandem promoters for pheV is reminiscent of the situation for the ribosomal RNA operons (9). For rrnA, B, D, E, G and the hybrid rrnX genes, tandem promoters (P1 and P2) have been identified with -35 and -10 sequences which look like each other and are characteristic of strong promoters (Fig 6). A sequence characteristic of a "stringent box" or "discriminator sequence" is found associated with the first promoter P1 of each tandem pair and P1 for rrnA and rrnB has been shown to be stringently regulated (27-28). In this case pheV is both similar and dissimilar to rRNA operons, in the sense that a "stringent box" is detected but is associated with the second promoter P2 (Fig 6).

An other similarity between the upstream P1 promoters of rrn operons and the downstream P2 promoter of pheV is the possible existence of secondary RNA polymerase binding sites 5' to the -35 region. In the ribosomal operons secondary RNA polymerase binding sites are located about 15 nucleotides 5' to the -35 region of their upstream (P1) promoters (28). In the case of pheV the secondary RNA polymerase binding site located 5' to the -35 region of P2 would be made of a -10 like region (GTAAATT : bp 246-252 overlined in fig 3) and a -35 like region (ATGATA : bp 224-229 overlined in fig 3). As P2 is separated from P1 by only 20 nucleotides the secondary RNA polymerase binding site of P2 and the upstream promoter P1 overlap (Fig 3). This organisation of the P2 promoter of pheV is very similar to that of P1 of rrnB and rrnG and also to that of the leuV (26).

Finally we would like to mention another similarity. Around the Pribovnaw box of all the P2 promoters shown in Fig 8. It is possible to identify regions of hyphenated dyad symmetry which would put the Pribovnaw boxes within a loop, should it form in the DNA. Alternatively, the sequence falls within the transcript from the P1 promoter and so could form a stem and loop within the mRNA. At present we have no evidence that these structures are significant but we are struck by the consistency of their existence.

There is no reported evidence for an upstream promoter for pheU. The regulatory regions 3' of the structural part are similar in so far that in both cases the genes are followed by a rho-independent terminator, which in neither case was shown.
to be active in vivo. It seems however that in both cases the tRNA$^{\text{Phe}}$ genes are isolated on a small transcription unit.

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**REFERENCES**


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