The nucleotide sequence of phenylalanine $tRNA_2$ of Drosophila melanogaster: four isoacceptors with one basic sequence

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Received 26 June 1979

ABSTRACT

The nucleotide sequence of Drosophila melanogaster phenylalanine $tRNA_2$ was determined to be: pG-C-C-G-A-A-A-U-A-m^2G-C-U-C-A-G-D-D-G-G-G-A-G-A-G-C-m^2_2G-Ψ-Ψ-A-G-A-C$^{(m)}$-U-G^m-A-A-m^1G-A-Ψ-C-U-A-A-A-G-m^7G-U(D)-C-C-C-C-G-G-T-Ψ-C-A-m^1A-U-C-C-C-G-G-G-U-U-U-C-G-G-C-A-C-C-A_{OH}. Upon RPC-5 chromatography at pH 3.8 $tRNA^{Phe}_2$ can be separated into four isoacceptors due to the partial modifications in positions 32 and 47. Thus the posttranscriptional modification of $tRNA^{Phe}_2$ transcribed from one gene (or many genes with identical sequences) results in four isoacceptors with the same basic sequence.

INTRODUCTION

The central role of tRNA in protein biosynthesis has been described in detail in pro- and eukaryotes[1,2]. Recently the interest has shifted towards the many regulatory roles of tRNA at different cellular levels[3,4]. Drosophila melanogaster seems to be the organism of choice among higher organisms for studies concerning tRNA and tRNA gene structure and function for several reasons. Transfer RNA genes can be localized on the polytene salivary gland chromosomes by "in situ" hybridization[5-8]. Many mutants have been described[9,10] and the sophisticated genetic techniques available facilitate the manipulation of the genome[11]. Furthermore, several selection procedures have been described allowing the screening of forward and backward mutations[12-14].

Molecular cloning techniques[15] have been applied for the isolation of plasmids containing Drosophila melanogaster tRNA genes[16] and transcription and processing of tRNA precursors have been studied "in vitro"[17]. However, these and other stu-

dies require the determination of the sequence of the mature tRNA with all its modifications. Transfer $RNA^{Phe}_{Drosophila}$ has therefore been isolated by anticodon-anticodon affinity chromatography[18] and its primary sequence determined by post labeling of non-radioactive tRNA[19,20].

MATERIALS AND METHODS

Purification of Drosophila $tRNA^{Phe}$: Transfer RNA was isolated from Drosophila melanogaster (Oregon strain) adults of mixed age and prefractionated on Sepharose 4B as described[6]. Fractions containing $tRNA^{Phe}$ were pooled and ethanol precipitated. About 40 A_{260} units were then loaded on an anticodon-anticodon affinity column[18]. Transfer $RNA^{Phe}_{Drosophila}$ forms a stable complex at 4°C with the column-bound $tRNA^{Glu}_{2}$ of E.coli and is eluted by raising the temperature to 35°C and adding EDTA.

Preparation of aminoacyl-tRNA synthetases and aminoacylation: For the isolation of the aminoacyl-tRNA synthetases we followed the procedure of Olexa[21]. Aminoacylation was done according to White and Tener[22].

Reverse phase chromatography: RPC-5 chromatography was performed on a column of 0.9x65 cm in two different buffer systems. Buffer 1[5]:10 mM sodium acetate, pH 4.5; 10 mM $MgCl_2$; 1 mM 2-mercaptoethanol. Buffer 2[23]: 10 mM sodium formate, pH 3.8; 1 mM EDTA; 1 mM 2-mercaptoethanol.

Two-dimensional polyacrylamide gel electrophoresis: The system of Fradin et al.[24] was used with the modifications introduced by Egg[25]. The gels were stained in ethidium bromide (10 µg/ml).

Sequence analysis: Most of the materials, enzymes and methods used were described previously[20]. Transfer RNA^{Phe} was digested completely with T_1 RNase or pancreatic RNase, labeled with γ-[^{32}P]-ATP and polynucleotide kinase, and subsequently fingerprinted. The 5'-end analysis of the 5'-[^{32}P]-oligonucleotides was done by complete digestion with nuclease P_1 and subsequent two-dimensional thin layer chromatography. The sequence of the

oligonucleotides was then established by partial digestion with snake venom phosphodiesterase or nuclease P_1 followed by analysis on DEAE-paper and/or two-dimensional homochromatography. In addition the method described by Stanley and Vassilenko[19] was used. Transfer RNA^{Phe} was digested with pure formamide (100°C for 10 min.), kinased and the fragments separated on a 20% polyacrylamide-8 M urea slab gel. The bands were cut out, eluted and their 5'-ends determined as described above.

Cleaveage of the phosphodiester bond adjacent to m^7G: The procedure was a modification of that described by Silberklang et al.[20]. Five µl of 50 mM NaOH were added to 4 µg of $tRNA_{2a}^{Phe}$ and incubated for 15 min. at room temperature, followed by addition of 1 µl 0.4 M acetic acid and 5 µl 0.3 N anilin-HCl, pH 4.5. After 4 hours at 37°C the sample was diluted with 40 µl of water and loaded on a small DEAE-cellulose column, equilibrated with 50 mM triethylammonium bicarbonate (TEAB). Elution of the fragments was done with 2 M TEAB. After repeated lyophilization the 5'-phosphate groups were removed with bacterial alkaline phosphatase as described by Silberklang et al.[20]. Half of the material was then kinased and analyzed by polyacrylamide gel electrophoresis. The appropriate bands were cut out, eluted and further analyzed.

Determination of the 5'-end sequence: To confirm the sequence at the 5'-end complete RNase T_1 and pancreatic RNase digests of uniformly labeled $tRNA_2^{Phe}$ were analyzed by degradation to mononucleotides with RNase T_2 and subsequent two-dimensional thin layer chromatography[26]. Only the 5'-oligonucleotides contain a nucleoside-5'-3'-diphosphate and can therefore easily be identified.

RESULTS

Demonstration of four $tRNA^{Phe}$ isoaccepting species: Chromatography on Sepharose 4B resolves Drosophila melanogaster tRNA into nine peaks[6]. As determined by aminoacylation $tRNA^{Phe}$ elutes mainly in fractions 2 and 3 (data not shown). Forty A_{260}

units were applied to a small anticodon-anticodon affinity column containing $tRNA_2^{Glu}$ of E.coli (anticodon U*UC) covalently bound to a supporting material. Transfer RNA^{Phe} which has a complementary anticodon is retained and can be eluted at 35°C in the presence of EDTA (Fig. 1a). This peak corresponds to the major $tRNA_2^{Phe}$ isoacceptor of White et al.[27] as determined by RPC-5 chromatography at pH 4.5 (not shown). The shaded area in Fig. 1a (about 2.5 A_{260} units) was pooled, ethanol precipitated and further fractionated on a two-dimensional polyacrylamide gel. Two distinct spots (not shown), designated $tRNA_{2a}^{Phe}$ and $tRNA_{2b}^{Phe}$, appear in about equal amounts. They were eluted, aminoacylated with [^{3}H]-Phe and [^{14}C]-Phe respectively and cochromatographed on RPC-5 at pH 3.8 using a 200 ml linear salt gradient from 0.55 - 0.80 M sodium chloride (Fig. 1b). Each of the two

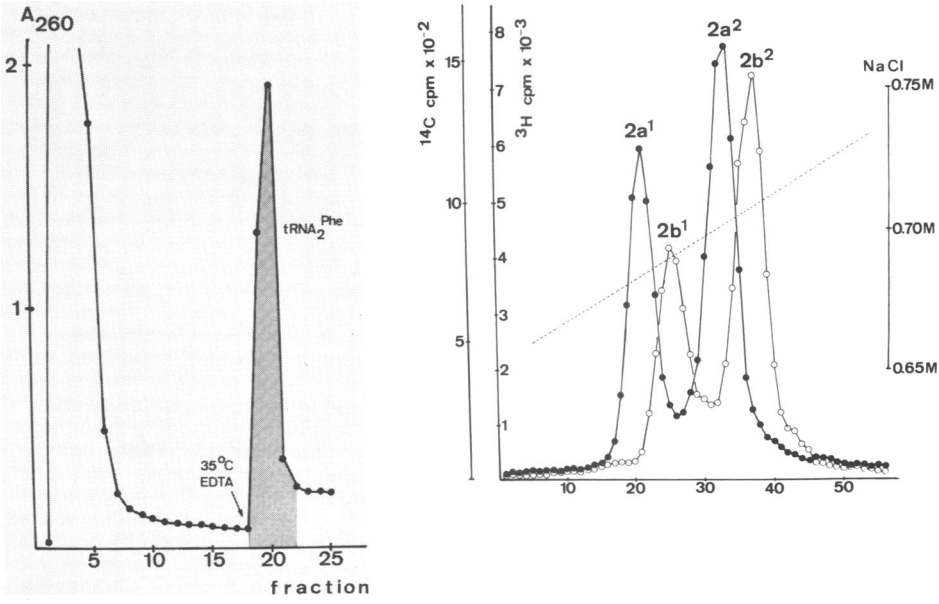

Figure 1

(a) Affinity chromatography of prefractionated Drosophila melanogaster tRNA on a E.coli $tRNA^{Glu}$ column.
(b) RPC-5 chromatography of [^{3}H]-Phe-$tRNA_{2b}^{Phe}$ (O—O) and [^{14}C]-Phe-$tRNA_{2a}^{Phe}$ (●—●).

isoacceptors is further resolved into two peaks, suggesting four $tRNA^{Phe}$ isoaccepting species possessing the same anticodon but slightly different sequences.

Sequence analysis: Most of the oligonucleotides present in complete T_1 RNase and pancreatic RNase digests have been sequenced by partial digestion with snake venom phosphodiesterase, two-dimensional homochromatography and/or electrophoresis on DEAE-paper. For a summary of sequenced fragments see Fig. 2. They are identical in $tRNA^{Phe}_{2a}$ and $tRNA^{Phe}_{2b}$.

Cleavage of RNase T_1 at the methylated G adjacent to the anticodon is not complete, whereas the 2-O-methylated G is fully resistant to nuclease action. This results in overlapping T_1 fragments in this region (Fig. 2).

Of special interest are two pancreatic RNase fragments, namely AGAC and $AGAC^mU$. The presence of the 2-O-methylated C can be demonstrated by electrophoresis of partial snake venom phosphodiesterase digests on DEAE-paper. Both fragments belong to the same position of $tRNA^{Phe}_2$ and their simultaneous appearence is due to a partial modification of the methylated C (see also below).

Further information was obtained by a direct sequencing method described by Stanley and Vassilenko[19]. Transfer RNA^{Phe}_{2a} and $tRNA^{Phe}_{2b}$ were digested with hot formamide. The fragments lacking a 5'-phosphate group can be labeled with γ-[^{32}P]ATP

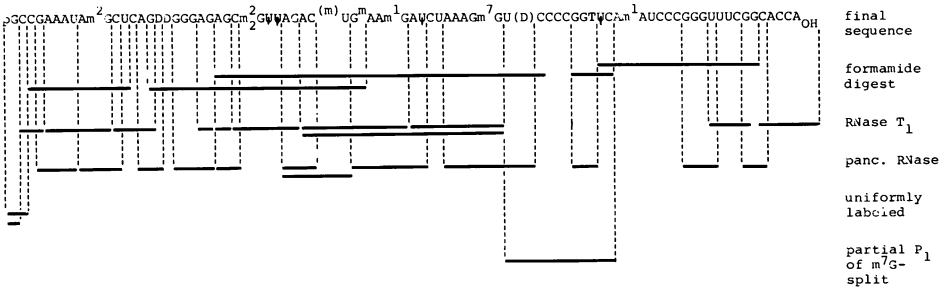

Figure 2

Compilation of sequences determined by different methods.

and polynucleotide kinase and are separated on a 20% polyacrylamide gel. All bands were cut out, eluted, fully digested with nuclease P_1 and analyzed by thin layer chromatography (Fig. 3a and b). The sequences obtained by these means are listed in Fig. 2. The partial modification of the C in the anticodon loop can also be demonstrated (see above). In addition to that there is a second sequence heterogeneity in the extra loop.At the 3'-side of m^7G there is either a D ($tRNA^{Phe}_{2a}$) or a U ($tRNA^{Phe}_{2b}$).

Probably due to the very strong base pairing in the TΨC-stem several bases are lacking on these gels. To overcome this problem $tRNA^{Phe}_{2a}$ was cleaved at the phosphodiester bond adjacent to m^7G, the 5'-phosphate group removed, kinased and run on a 20% polyacrylamide gel together with a formamide digest as marker. The main band migrates in the position expected for the shorter split fragment. Its 5'-end was determined to be pD (not shown). Partial digestion with nuclease P_1 and two-dimensional homochromatography reveals the sequence pDCCCCGG... adjacent to m^7G (Fig. 4).

Uniformly labeled $tRNA^{Phe}_2$ was used to determine the sequence at the 5'-end of the tRNA. RNase T_1 and pancreatic RNase oligonucleotides were fully digested with RNase T_2 and the base compositions analyzed by two-dimensional thin layer chromatography (not shown). The fragments derived from the 5'-end contain a nucleoside diphosphate and were determined to be pGp and pGpCp, respectively.

The complete sequence of $tRNA^{Phe}_2$ of Drosophila melanogaster with its partial modifications is shown in Fig. 5.

DISCUSSION

A study of the $tRNA^{Phe}$ isoacceptor pattern during the development of Drosophila melanogaster has been made by White et al.[27]. One major and two minor $tRNA^{Phe}$ peaks could be resolved upon RPC-5 chromatography at pH 4.5. Our results show, that the major peak can be separated into four isoacceptors by lowering the pH to 3.8 and adding EDTA. As the sequence data reveal this is due to partial modifications at two sites of the

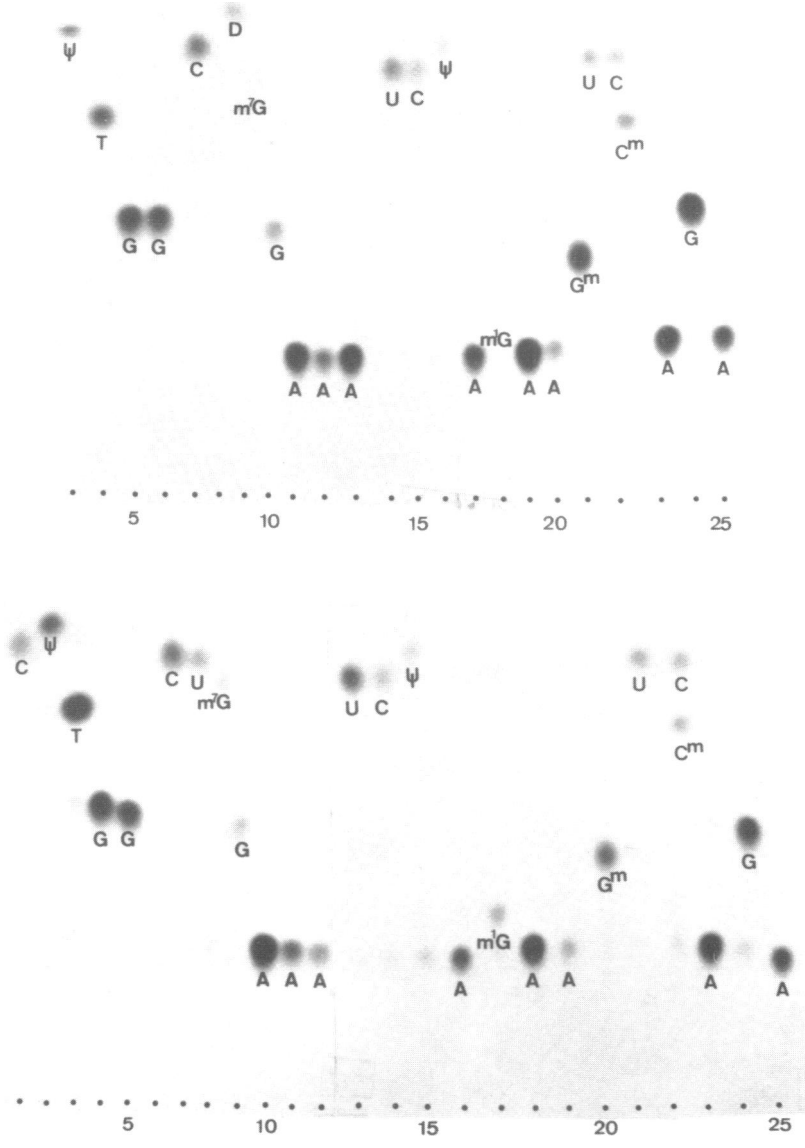

Figure 3

5'-end analysis of fragments recovered from a polyacrylamide gel electrophoresis of a 5'-[^{32}P]-labeled formamide digest of (a) $tRNA^{Phe}_{2a}$ and (b) $tRNA^{Phe}_{2b}$.

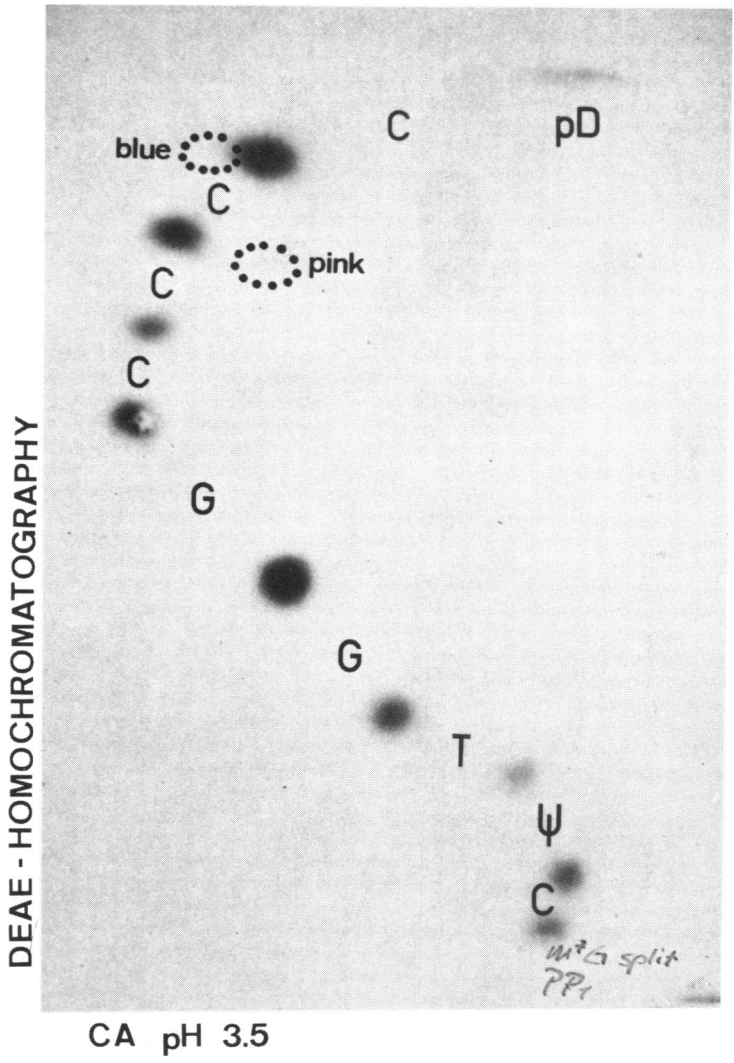

Figure 4

Autoradiogram of partial digest of m^7G-split material with nuclease P_1.

$tRNA^{Phe}$ molecule. Hence the four $tRNA^{Phe}$ isoacceptors are homogeneic, i.e. they differ only in the extent of modification. The flies used for the tRNA extraction were of mixed age. We

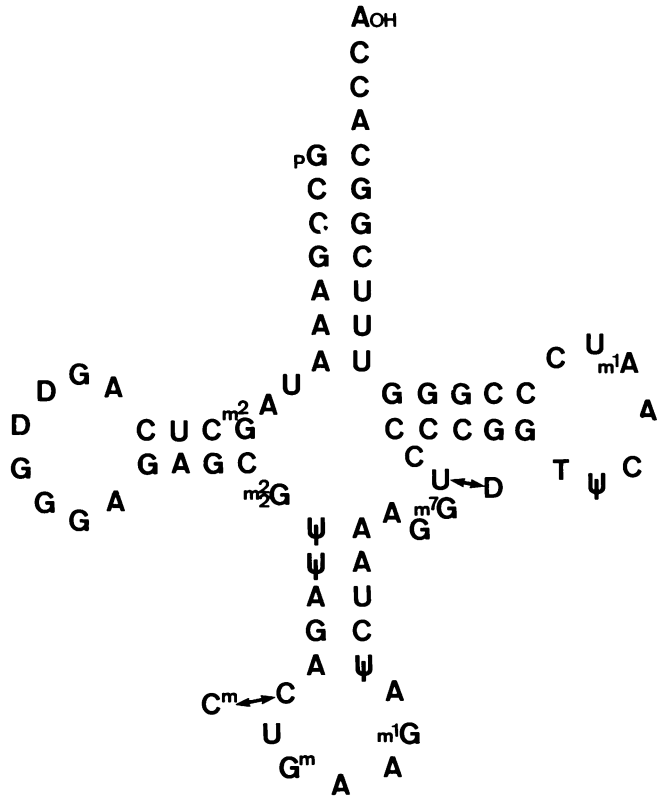

Figure 5

Cloverleaf model of Drosophila melanogaster $tRNA_2^{Phe}$ indicating the partial modifications in positions 32 and 47.

therefore do not know whether there are any major changes in the degree of modification during ontogeny, as found for example for the Q-base containing tRNAs[27,28].

A comparison of the nucleotide sequence of $tRNA^{Phe}_{Drosophila}$ with the sequences of $tRNA^{Phe}$ from other eukaryotes confirms the finding that tRNA genes from higher organisms are conservative[29]. Drosophila melanogaster $tRNA^{Phe}$ differs from mammalian[29] and Bombyx mori $tRNA^{Phe}$ (G.Keith, personal communication) in only two out of seventysix positions and in comparison with

the gene sequence of Xenopus laevis $tRNA^{Phe}$ in three positions[30]. The nucleotide sequence of yeast $tRNA^{Phe}$ differs in eighteen positions[29]. However, the functional similarity of $tRNA^{Phe}_{Drosophila}$ with $tRNA^{Phe}$ isolated from yeast is underlined by the finding that crosswise aminoacylations can be performed[31]. This is probably due to the fact that seventeen changes are found in the stem regions and only one in the TΨC-loop, demonstrating the low importance of the tRNA stem regions for the synthetase-tRNA interaction[32,33].

An interesting observation is that $tRNA^{Phe}_{Drosophila}$ does not contain the hypermodified base Y at the 3'-end of the anticodon as usually found in higher organisms[29,31]. Instead a 1-methyl G is found in position 37, as found in Mycoplasma[34].

The exact position of m^5C also found by White and Tener[31] could not be determined since the wandering spot method does not discriminate between C and m^5C. However, it is clear that the m^5C must be in the extra loop or in the TΨC-stem.

A partial modification in the positions 32 and 47 has not been found in any of the $tRNA^{Phe}$ sequences as of today of higher organisms[29] (G.Keith, personal communication). A high degree of modification is characteristic for tRNA molecules[35,36]. The importance of modifications is demonstrated impressively by its role e.g. in codon-anticodon stabilization[37], stabilization of tRNA structure[38], discrimination of codons[39], and regulation of the his- and other operons[40,41].

Transfer RNAs of higher organisms contain even a higher amount of modified nucleotides, pointing towards a wide variety of possible functions. Although many drastic changes in the modification of tRNA during the ontogeny of different organisms have been described[42,43] almost nothing is known about their biological role. It has been reported that the presence of ribothymidine in mammalian $tRNA^{Phe}$ increases the rate of translation[44] whereas the presence of the same modification reduces the translation capacity of $tRNA^{Gly}$ and other tRNAs in a "in vitro" protein synthesizing system[45]. Similar experiments and injections of tRNA into Xenopus laevis oocytes together with

a defined messenger RNA might give informations about the function of the modification in the four $tRNA^{Phe}$ isoacceptors found in Drosophila melanogaster.

ACKNOWLEDGEMENTS

This investigation was supported by grant No. 3.024.76 from the Swiss National Science Foundation, the Hescheler- and the Julius Klaus-Stiftung.

We wish to express our gratitude to Drs. S.Silverman and D.Söll for the introduction into the post-labeling sequencing technique and to M.Kutzer for excellent technical assistance.

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