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PRIMARY STRUCTURE OF *BOMBYX MORI* POSTERIOR SILKGLAND $tRNA^{Phe}$

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SUMMARY

$tRNA^{Phe}$ was isolated from posterior silkgland from Bombyx mori and hydrolysed to mixtures of oligonucleotides. $[^{32}P]5'$ end labelling of the oligonucleotides and sequence study indicates that the major component of Bombyx mori $tRNA^{Phe}$ is similar to mammalian $tRNA^{Phe}$, the minor component differing from the major one by one nucleotide only.

INTRODUCTION

Phenylalanine tRNAs have been the subject of numerous studies. The nucleotide sequences of 13 of them are known : 6 from eukaryotes, 4 from prokaryotes, 1 from mitochondria and 2 from chloroplasts (1, 2). Eukaryotic $tRNAs^{Phe}$ show a high degree of similarity : identical sequences were found in the known mammalian or plant $tRNAs^{Phe}$. A similar situation has also been found in eukaryotes for the initiator tRNAs and for $tRNAs^{Trp}$ (1).

Preliminary results reported that the characteristic Wye-type base found in eukaryotic $tRNAs^{Phe}$ is absent in silkworm (3) as well as in drosophila $tRNAs^{Phe}$ (4), but no other comparisons between insect and other eukaryotic $tRNAs^{Phe}$ were made. Therefore it was of interest to determine the primary structure of an insect $tRNA^{Phe}$.

Presently we report the purification and the nucleotide sequence determination of the *Bombyx mori* posterior silkgland $tRNA^{Phe}$. Comparison with the other known $tRNAs^{Phe}$ reveal a very high degree of homology with mammalian $tRNAs^{Phe}$.

MATERIALS AND METHODS

Purification of $tRNA^{Phe}$. $tRNA^{Phe}$ is a "minor" tRNA species in the posterior silkgland of *Bombyx mori* during the functional adaptation of its tRNA population to fibroin biosynthesis. The posterior silkglands from hybrids of European strair 200 and 300 were dissected at the end of the larval instar. Transfer RNA was extracted and $tRNA^{Phe}$ was purified by counter-current distribution (1500 transfers) (3) to 25% purity (Fig. 1) and further subjected to a Sepharose 4B column filtration using a reverse gradient of $(NH_4)_2SO_4$ at 20° (5) followed by 20% polyacrylamide slab gel electrophoresis at pH 8.3 (not shown). The tRNA was extracted from the gel as already published (2).

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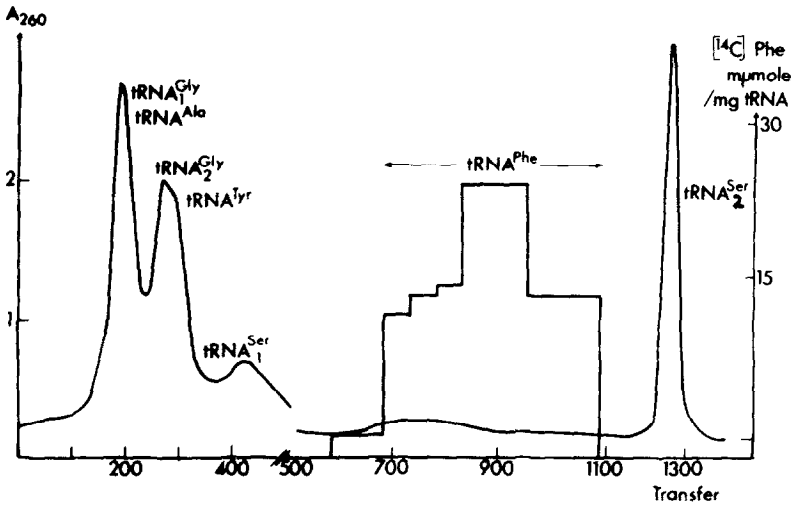

Fig. 1. Purification of *Bombyx mori* $tRNA^{Phe}$ by counter-current distribution performed as described in ref. 3.

Sequencing methods. 5 A_{260} units of the $tRNA^{Phe}$ were digested either with pancreatic RNAase or with T_1 RNAase (6), and the fragments separated by two dimensional electrophoresis using cellogel (Chemetron, Milano) in the first dimension and DEAE-cellulose paper in the second dimension (Fig. 2) (7). After elution (7) each U.V. absorbing spot was further studied by determining its nucleoside composition (8).

The sequences of the oligonucleotides longer than trinucleotides were determined by 5'$|^{32}P|$ or 3'$|^{32}P|$postlabellings followed by the "wandering spot" technique. 5'$|^{32}P|$ labelling was performed as described by Silberklang *et al.* (9) with T_4 polynucleotide kinase prepared according to Richardson (10) and Panet *et al.* (11). $\gamma|^{32}P|$ ATP (400-2000 Ci/mmole) was prepared by a method derived from Glynn and Chappel (12) and Maxam and Gilbert (13) and purified on a DEAE-cellulose column (0.1 ml) eluted by a triethylammonium (pH 8) gradient from 0 to 0.5 M (100 ml total volume). 3'$|^{32}P|$ labelling was performed as described by Bruce and Uhlenbeck (14) with T_4 RNA ligase (Enzo Biochem). $|^{32}P|$pCp was prepared by reacting $\gamma|^{32}P|$ ATP, Cp and T_4 polynucleotide kinase as above. After kination, the reaction mixture was heated for 2 min at 100°C and used in the T_4 RNA ligase assay without further treatment. The separation of the 5' and 3' labelled fragments was done by two-dimensional electrophoresis as mentioned above, or by electrophoresis on cellogel followed by homochromatography on DEAE-cellulose thin layer. The methods used for sequencing each oligonucleotide were identical to those reported for chloroplastic $tRNA^{Phe}$ primary structure determination (16).

Partial digests of $tRNA^{Phe}$ were obtained by incubating 2-10 μg tRNA in 10 μl dimethylsulfoxyde (DMSO) at 100° for 10 min. The digestion products were postlabelled and separated by electrophoresis on cellogel and homochromatography as mentioned above, or by two-dimensional electrophoresis on polyacrylamide gel (13, 16) followed by total hydrolysis with P_1 nuclease (P.L. Biochemicals) and analysis of the 5'$|^{32}P|$ nucleotides on thin layer chromatography (6, 17). The latter procedure was used especially for confirmation of the minor nucleotides and for obtention of overlapping sequences in the D-, anticodon-, and T-ψ regions.

The *Bombyx mori* $tRNA^{Phe}$ could not be dephosphorylated and 5'$|^{32}P|$ postlabelled as described for many other tRNAs. However smaller fragments, quarters and halves, from the 5' end, could be labelled. They were prepared by digesting the tRNA with S_1 nuclease (Miles) (18) under the following conditions : 50 μl

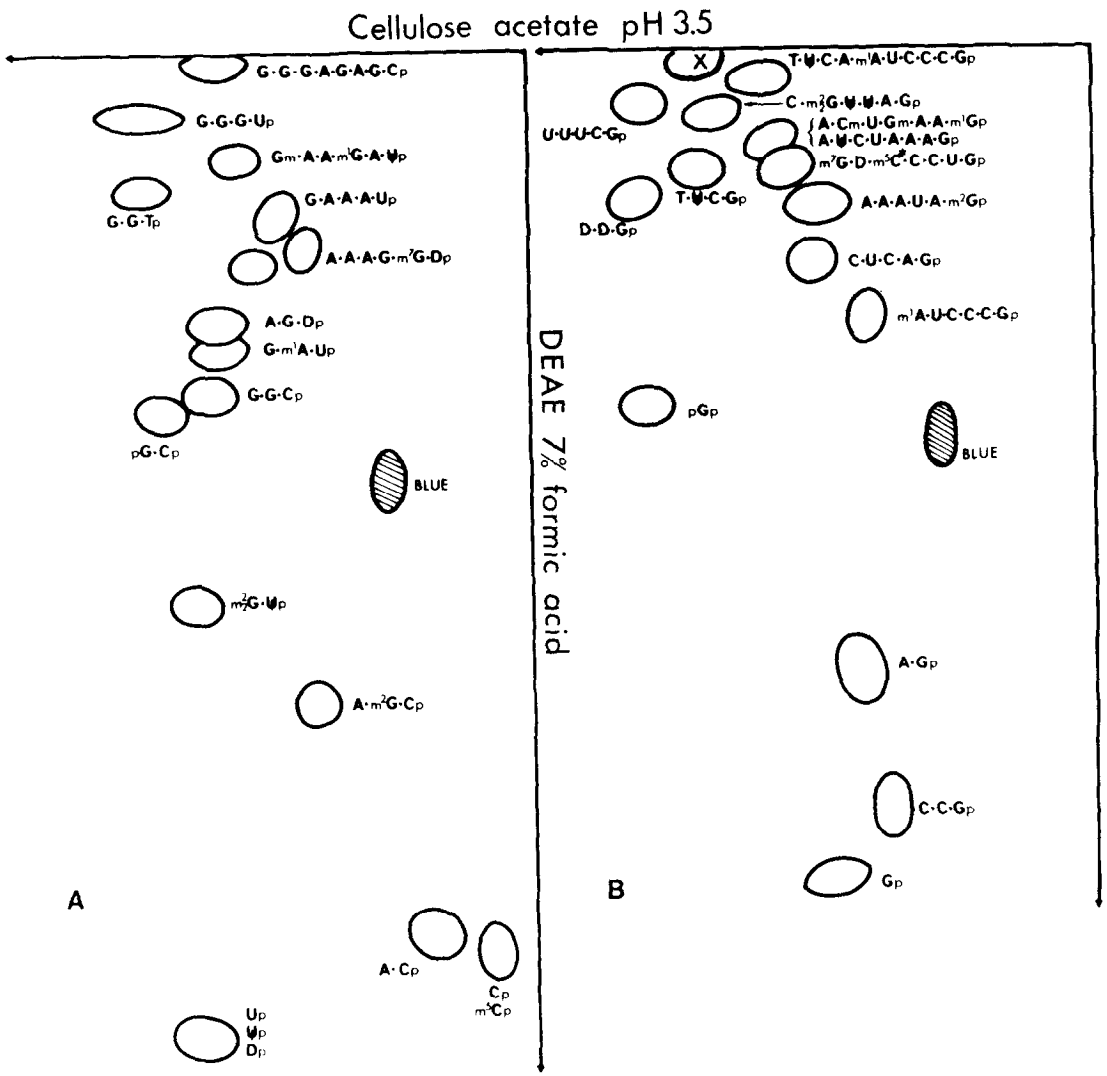

Fig. 2. Two dimensional fractionation patterns of complete RNAase A digest (A) and RNAase T_1 digest (B). For m^5C^* see Discussion and Fig. 4. A-m^1A-Up could not be detected, because only 10% of the molecules have this sequence. Oligonucleotide X corresponds to the partially T_1 RNAase resistant oligonucleotide : A-Cm-U-Gm-A-A-m^1G-A-ψ-C~U-A-A~A-Gp.

Na acetate 0.05 M pH 4.5, NaCl 0.3 M, $ZnCl_2$ 1 mM and glycerol 5% containing 125 units S_1 nuclease and 25 µg tRNA were incubated overnight at 18°C. After phenol extraction and several washings with ethanol, the fragments were dephosphorylated, 5'|^{32}p| postlabelled and separated on 20% polyacrylamide sequencing gels (13). The radioactive spots were eluted (13) and further analysed and sequenced by the "wandering spot" technique (9) or on two-dimensional electrophoresis on polyacrylamide gel (13, 16), after partial hydrolysis in 10 µl DMSO at 100° for 60 min in presence of 5-10 µg carrier tRNA.

RESULTS

In order to determine unambigously the rare nucleotide containing oligonucleotides and the molar ratio of the mono-, di- and trinucleotides, exhaustive hydrolyses of $tRNA^{Phe}$ with pancreatic RNAase and T_1 RNAase were done, followed by fingerprinting of the digests. The nucleotide composition of the non-radioactive spots was studied. For the determination of the sequence of the oligonucleotides we used the $|^{32}P|$ postlabelling techniques as mentioned in "Materials and Methods". The fingerprints and the sequence of the oligonucleotides are shown in Fig. 2. Both non-radioactive and $5'|^{32}P|$ labelled digests give identical fingerprints.

Most of the oligonucleotides found in silkworm $tRNA^{Phe}$ exist also in mammalian $tRNAs^{Phe}$ (18). The only exceptions concern the heterogeneity in the T-ψ stem, where part of the population of silkworm $tRNA^{Phe}$ has T-ψ-C-A (20%) instead of T-ψ-C-G (80%).

The primary structure was deduced from (i) the overlapping sequences of the oligonucleotides obtained by exhaustive nuclease digestions (ii), the sequences of long fragments obtained by S_1 nuclease splitting (iii), partial digestion of tRNA in DMSO followed by $5'|\gamma^{32}P|$-ATP labelling and separation by two-dimensional electrophoresis on cellogel/DEAE-cellulose paper or two-dimensional gel electrophoresis as mentioned in "Materials and Methods". Analyses of each radioactive spot after total P_1 nuclease hydrolysis gave part of the sequence from G-G-T-ψ to the C-C-A end, from C_3 to D_{16} and from $D\text{-}D_{17}$ to m^7G-D. The sequence from m^7G-D to G-G-T was established by the "wandering spot" technique applied to fragments ending with D_{47} or C_{48} obtained using the

Fig. 3. Autoradiograms of partial digests of post-labelled fragments of *Bombyx mori* $tRNA^{Phe}$.

A. Two dimensional gel electrophoresis of a partial digest of $5'|^{32}P|$ labelled 5' half molecule obtained by S_1 nuclease cleavage of *Bombyx mori* $tRNA^{Phe}$. The sequence shown is from A_5 to A_{31}.

B. Two dimensional homochromatography of the same fragment as in A, but showing the 5' end of the tRNA.

C. Two dimensional homochromatography of a partial digest of $3'|^{32}P|$ labelled anticodon fragment (labelling was with $|^{32}P|$pCp and T4 RNA ligase as described in Materials and Methods). First spot near the Blue marker corresponds to $G|^{32}P|pCp$.

D. Two dimensional homochromatography of a partial digest of $5'|^{32}P|$ labelled 3' OH half molecule of *Bombyx mori* $tRNA^{Phe}$ obtained by S_1 nuclease cleavage. The spots track shown is from A_{38} to ψ_{55}. The high intensities and large surfaces of spots a and b account for the unusual "reverse mobility shift" of m^7G : indeed spot a contains the fragments $pA_{38}\text{-}A_{44}$ and $pA_{38}\text{-}m^7G_{46}$, and spot b contains the fragments $pA_{38}\text{-}G_{45}$ and $pA_{38}\text{-}C_{48}$.

E. Two dimensional homochromatography of a $5'|^{32}P|$ labelled partial digest of $tRNA^{Phe}$. Each spot from the homochromatography was eluted, digested with P_1 nuclease and further analysed on TLC. The nucleotides shown in the pannel E correspond to the results of these analyses. The sequence shown is from G_{63} to C_{75}.

XC or B is Xylene Cyanol Blue, BB is Bromophenol Blue. All homochromatographies were performed as described in Material and Methods using 25 mM KOH-strength "homomix".

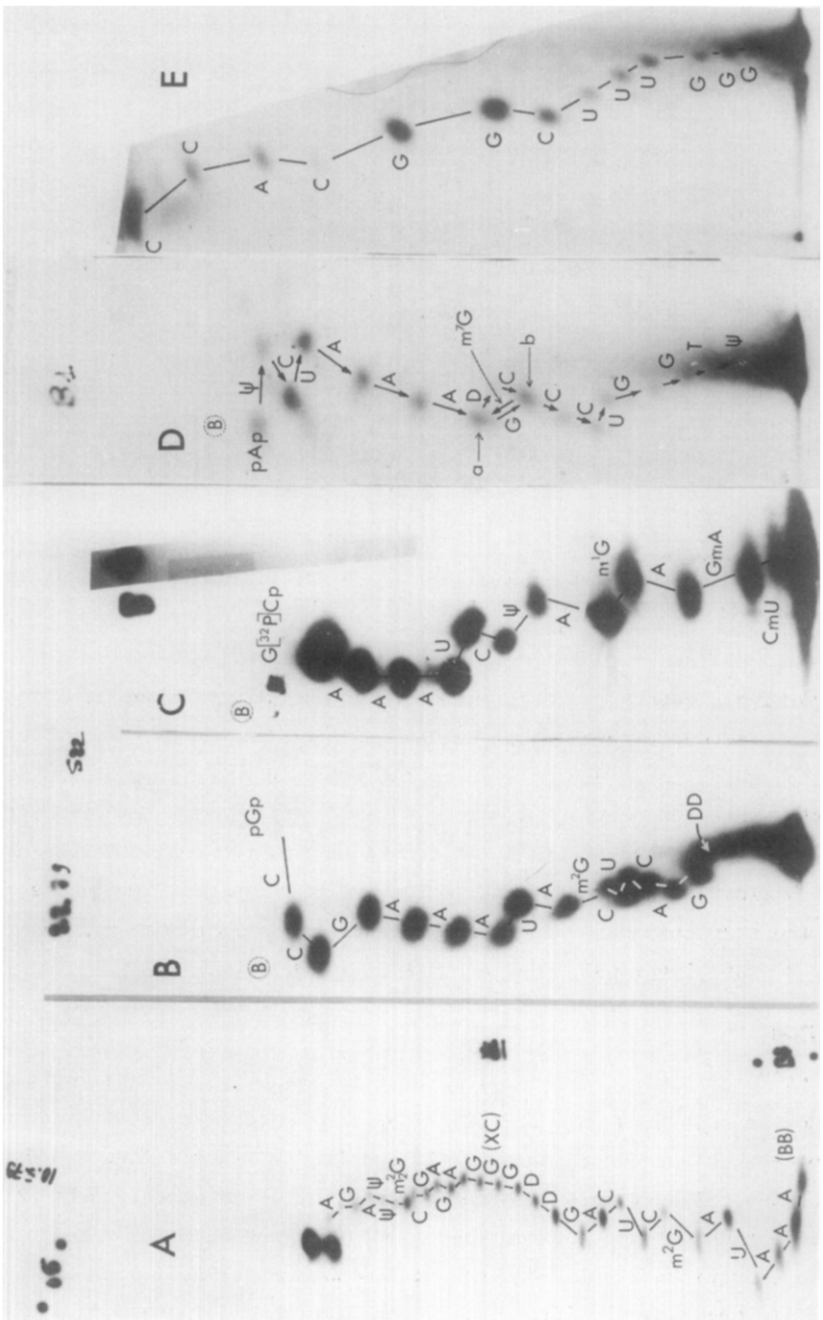

Figure 3

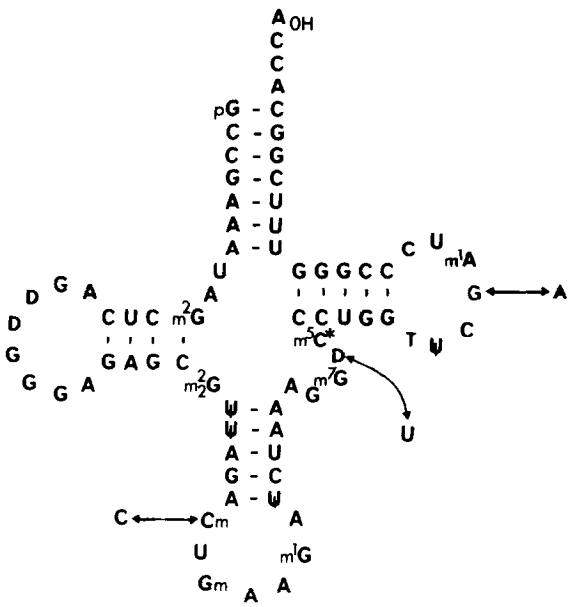

Fig. 4. Cloverleaf model of *Bombyx mori* $tRNA^{Phe}$ indicating the post-transcriptional undermodifications in positions 32 and 47, and the heterogeneity in position 57. m^5C^* as mentionned in the Discussion, could be either in position 48 or, as found in Mammals, in position 49.

DMSO hydrolysis technique. This technique confirmed the heterogeneity in the T-ψ-C-G(A) sequence. Furthermore, undermodifications (5-10%) were found in other positions, which could not be seen in the study of the oligonucleotides of the exhaustive RNAase digests: Cm_{32} or C_{32}, D_{47} or U_{47}. The 5' end sequence was also established by the "wandering spot" technique on quarter and halves from the 5' end obtained by S_1 nuclease splitting and 5'$|^{32}P|$ labelling as described above. The structure determination of fragments by different methods is shown in Fig. 3. The cloverleaf model of $tRNA^{Phe}$ is shown in Fig. 4.

DISCUSSION

$tRNA^{Phe}$ of *Bombyx mori* despite the heterogeneity and undermodifications could not be separated into different peaks by counter-current distribution or Sepharose-4B column chromatography. It resolved sometimes into two different bands on 20% polyacrylamide sequencing gels but no detectable difference in the sequence could be found between these two different bands.

The comparison of the sequence of *Bombyx mori* $tRNA^{Phe}$ with other known $tRNAs^{Phe}$ reveals its similarity with mammalian $tRNA^{Phe}$, the modified nucleotides being not taken into account. The only differences being the heterogeneity G_{57} or A_{57} instead of only G_{57} in mammalian $tRNA^{Phe}$. Other differences between this $tRNA^{Phe}$ and higher organisms $tRNA^{Phe}$ concern the absence of the hypermodified

Wyosine nucleotide adjacent to the anticodon as previously mentioned. Instead we found m^1G, as it is in Mycoplasma (20) and yeast mitochondria (2).

Other differences are the absence of modified A_{18}. Concerning the position for m^5C it may be either in position 48 or in position 49 as found in mammals, but the exact position could not be confirmed.

Our results confirm nevertheless, as previously suggested for $tRNAs^{Phe}$ (19), $tRNAs^{Trp}$ (21) and initiator tRNAs (1), that the primary structures of these specific tRNAs from higher organisms are highly conserved within the considered kingdoms. During our study, Altwegg (personal communication) studied *Drosophila melanogaster* $tRNA^{Phe}$, this tRNA differs from *Bombyx mori* $tRNA^{Phe}$ in only two positions : C_{51} instead of U_{51} and A_{57} instead of the heterogeneity G_{57} and A_{57}.

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