The Nucleotide Sequence of tRNA\textsuperscript{Val} of Drosophila melanogaster

CHLOROACETALDEHYDE MODIFICATION AS AN AID TO RNA SEQUENCING*

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The nucleotide sequence of tRNA\textsuperscript{Val} from Drosophila melanogaster was determined by labeling the 5' end of the tRNA with \[\text{\textsuperscript{32P}}\text{pCp}\] and then end-labeled RNA was purified by gel electrophoresis. The sequence was determined using several recently developed techniques. The previously published nucleotide analysis of the major Drosophila valine-accepting tRNAs (2) facilitated interpretation of the results obtained. The nucleotide sequence determined for tRNA\textsuperscript{Val} corresponds to the genes found in plasmid pDts5.

MATERIALS AND METHODS

General

Ribonuclease T\textsubscript{1}, T\textsubscript{2}, and U\textsubscript{1} and P\textsubscript{1} nucleases were purchased from Worthington. Ribonuclease Phy I from Enzo Biochem., phage T4 polynucleotide kinase from New England Biolabs, Beverly, MA, and RNA ligase from P-L Biochemicals. \[\text{\textsuperscript{32P}}\text{pCp}\] was prepared as described previously (3). [\(\text{\textsuperscript{32P}}\text{pCp}\)] was synthesized by the procedure of Masamune and Gilbert (4) or purchased from Amersham Corp. [\(\text{\textsuperscript{32P}}\text{pCp}\)] was prepared by incubating a 10-\(\mu\)l reaction mixture containing 1.2 mM C\textsubscript{p} (2' and 3'), 20 mM Tris HCl, pH 8.3, 10 mM MgCl\textsubscript{2}, 10 mM dithiothreitol, 35 \(\mu\)M [\(\text{\textsuperscript{32P}}\text{pCp}\)] ATP (>300 Ci/mmol), and 2 units of T4 polynucleotide kinase for 60 min at 37 \(^\circ\)C. The reaction was stopped by heating the mixture to 100 \(^\circ\)C for 1 min. The [\(\text{\textsuperscript{32P}}\text{pCp}\)] was used without further purification.

Formamide (98%, Mallinckrodt) was deionized by stirring it consecutively with Dowex-1 (OH\textsuperscript{-}) and Dowex-50 (H\textsuperscript{+}) resin and was stored at 4 \(^\circ\)C.

End-labeling of tRNA—An [\(\text{\textsuperscript{32P}}\text{pCp}\)] AMP residue was incorporated into the 3' end of tRNA\textsuperscript{Val} by incubating 10 \(\mu\)g of snake venom phosphodiesterase-treated tRNA (3) in 10 \(\mu\)l of 100 mM Tris-HCl buffer, pH 9.0, containing 100 mM KCl, 10 mM magnesium acetate, 2 mM dithiothreitol, 1 mM EDTA, 0.15 mM CTP, 60 \(\mu\)M [\(\text{\textsuperscript{32P}}\text{pCp}\)] ATP

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†- The abbreviations used are: MOPS, 4-morpholinopropanesulfonic acid; PEI-cellulose, polyethyleneimine-impregnated cellulose; TLC, thin layer chromatography.
presumably because of strong secondary structure in this region. Modification of the RNA with chloroacetaldehyde allowed the sequence to be resolved. A 50% solution (v/v) of chloroacetaldehyde was prepared by heating 25 μl of 97% chloroacetaldehyde dimethyl acetal (Aldrich) with an equal volume of 0.2 M HCl in a sealed glass tube at 100 °C for 30 min. End-labeled tRNA plus carrier tRNA (2-4 μg) was dissolved in 15 μl of 1 M sodium acetate buffer, pH 4.0, 1 mM EDTA containing 2 μl of the chloroacetaldehyde solution. The solution was sealed in a glass capillary tube and heated to 100 °C for 2 min, then chilled on ice. The contents of the tube were expelled into 100 μl of 0.1 M sodium acetate buffer, pH 4.0, containing 0.1 mM EDTA and the solution incubated at 80 °C for 20 min. The RNA was precipitated with ethanol and its sequence determined by the gel read-off method.

RESULTS AND DISCUSSION

The sequence of tRNAVal, determined in this study, is shown in cloverleaf form in Fig. 1. It contains 76 nucleotide residues and has all the invariant and other strongly conserved nucleotides expected in a cytoplasmic tRNA (12). The residue at position 4 was observed to be resistant to the formamide-induced hydrolysis used in sequencing the 5' end of the molecule by two-dimensional homochromatography. Ribose-methylated nucleotides are present at position 4 of all sequenced eukaryotic glycine and proline tRNAs (13) and tRNAArg of D. melanogaster. Of the two ribose-methylated nucleosides detected in the nucleoside analysis (2) the 2'-O-methylcytidine had been found at position 32 by the Stanley and Vassilenko method. Therefore, the 2'-O-methylcytidine residue was assigned to position 4.

Determination of the nucleotide sequence by the Stanley and Vassilenko method indicated that a modified nucleotide was present at position 9. The mobility of this nucleotide on PEI-cellulose or cellulose TLC plates in a number of solvent systems did not match published mobility values for various modified nucleotides in the same systems but did suggest that the unknown nucleotide contained a modified guanosine residue. On the basis of the nucleoside analysis (2), 1-methylguanosine (m'G) was assigned to position 9. This assignment is consistent with the observation that m'G is found only at position 9 in previously sequenced eukaryotic tRNAs (13).

About 50% of the uridine residues at position 20 are modified to 3-(3-amino-3-carboxypropyl)uridine (acpU) (Fig. 3D). Authentic acpU-5' phosphate, isolated from a nuclease P1 digest of crude Escherichia coli tRNA and characterized by its UV absorption spectrum, positive ninhydrin reaction, and the chromatographic properties of the nucleoside (14), was used as a standard for identification of the modified nucleotide. The presence of acpU in tRNAVal is consistent with the work of White (15) who has shown that Drosophila tRNAVal reacts with cyanogen bromide and the N-hydroxyuccimicin ester of naphthoxyacetic acid, reagents thought to react with the amino group of acpU in tRNAs. In prokaryotes and plant chloroplasts, acpU is found exclusively 3' to the 7-methylguanosine in the variable arm of several tRNAs (13) while in eukaryotes acpU has been found only at position 20 of rat liver tRNAArg (15).

The series of five cytidine residues at positions 47-51 could not be determined by either the gel read-off or Stanley and Vassilenko methods. Electrophoresis of partial enzymatic or formamide digests of tRNAVal on denaturing polyacrylamide gels exhibited both strong band compression and incomplete enzymatic cleavage in the variable loop and T stem regions. In yeast tRNAArg these regions are involved in strong secondary and tertiary interactions with other parts of the molecule (17). Similar interactions in tRNAVal may be particularly strong because of the high G-C content of these regions. The observed band compression indicates that the products of such RNase cleavage do occur fold back on themselves and migrate anomalously during polyacrylamide gel electrophoresis.

At pH 4.0, chloroacetaldehyde reacts with nonbase-paired cytidine and adenosine residues in nucleic acids to form etheno derivatives (18-21) that cannot form Watson-Crick base pairs. Thus, if tRNA were denatured and then modified with chloroacetaldehyde, it should lose much of its secondary structure. The series of five cytidine residues at positions 47-51 could not be read because of band compression. B, tRNAVal was labeled at the 3' end as above. The end-labeled tRNA was modified with chloroacetaldehyde (“Materials and Methods”) and sequenced by the gel read-off method. 10-μl reaction volumes contained T1, 0.1 unit of RNase T1; A-I, 0.01 unit of RNase A; A-2, 0.005 unit of RNase A; L, 66% formamide at 100 °C for 30 min. The sequence from positions 45-52 could not be read because of band compression. Therefore, the 2'-O-methylcytidine residue was assigned to position 4.

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This is surprising considering the strict requirement of RNase A for an unsubstituted nitrogen at position 3 of pyrimidines in its substrate (22). Thus, the ethenocytidine residues produced by modification with chloroacetaldehyde would not be expected to be sites for RNase A cleavage. Tolman et al. (23) modified dinucleoside monophosphates containing cytidine with chloroacetaldehyde and examined the sensitivity of the etheno derivatives to hydrolysis by RNase A. They found ethenocytidylyl uridine (eCpU) and eCpeC to be completely resistant to hydrolysis while eCpG and eCpeG showed slight hydrolysis after prolonged RNase A treatment. It is possible, therefore, that RNase A recognizes the modified cytidine residues but it is more likely that modification was not complete and that unmodified cytidine residues were the sites of RNase A cleavage.

The nucleoside analysis (2) suggests that tRNA\(^{\text{Val}}\) contains two 5-methylcytidine (m\(^5\)C) residues per molecule. One of these was located at position 38; the other is assigned to a probable position at residue 48 or 49 since m\(^5\)C has been found in this region of other eukaryotic valine tRNAs (13). The bands corresponding to C-49 in Fig. 2B move anomalously fast compared to the surrounding cytidine bands. This suggests modification of C-49.

The T loops of mammalian tRNA\(^{\text{Val}}\)\(^{(\text{IAC})}\) and tRNA\(^{\text{Val}}\)\(^{(\text{IAC})}\) of human placenta are unique among sequenced cytoplasmic tRNAs in having a uridine residue at position 54 and an adenosine at position 60 (24-27). These two nucleosides could base pair to produce a tRNA with a six-base pair T stem and a five-nucleotide T loop. Jank et al. (28) have provided some evidence favoring such a structure. Mammalian tRNA\(^{\text{Val}}\)\(^{(\text{IAC})}\) is also unusual in its coding properties. According to the wobble hypothesis (29) an IAC anticodon should decode GUA, GUC, and GUU codons but not GUG. Ribosome-binding experiments show that mammalian tRNA\(^{\text{IAC}}\) binds to ribosomes in the presence of all four valine triplets but most strongly with GUG (27). Unlike the mammalian tRNA, Drosophila tRNA\(^{\text{Val}}\) has a typical T loop with rT at position 54 and C at position 60. tRNA\(^{\text{Val}}\) binds strongly to ribosomes in response to GUC, GUA, and GUU as predicted by the wobble hypothesis and only weakly to GUG (2). Comparison of the two tRNA structures suggests that the anomalous coding properties of the mammalian tRNA\(^{\text{Val}}\)\(^{(\text{IAC})}\) may be related to its unusual T loop structure.

The sequence of tRNA\(^{\text{Val}}\) matches that of the two valine tRNA genes of the recombinant plasmid pDt55. The tRNA\(^{\text{Val}}\)\(^{(\text{IAC})}\) and tRNA\(^{\text{Val}}\)\(^{(\text{IAC})}\) of human placenta are unique among sequenced cytoplasmic tRNAs in having a uridine residue at position 54 and an adenosine at position 60 (24-27). Two nucleosides could base pair to produce a tRNA with a six-base pair T stem and a five-nucleotide T loop. Jank et al. (28) have provided some evidence favoring such a structure. Mammalian tRNA\(^{\text{Val}}\)\(^{(\text{IAC})}\) is also unusual in its coding properties. According to the wobble hypothesis (29) an IAC anticodon should decode GUA, GUC, and GUU codons but not GUG. Ribosome-binding experiments show that mammalian tRNA\(^{\text{Val}}\)\(^{(\text{IAC})}\) binds to ribosomes in the presence of all four valine triplets but most strongly with GUG (27). Unlike the mammalian tRNA, Drosophila tRNA\(^{\text{Val}}\) has a typical T loop with rT at position 54 and C at position 60. tRNA\(^{\text{Val}}\) binds strongly to ribosomes in response to GUC, GUA, and GUU as predicted by the wobble hypothesis and only weakly to GUG (2). Comparison of the two tRNA structures suggests that the anomalous coding properties of the mammalian tRNA\(^{\text{Val}}\)\(^{(\text{IAC})}\) may be related to its unusual T loop structure.

The sequence of tRNA\(^{\text{Val}}\) matches that of the two valine tRNAs from mammals and insects determined to date (13) shows a great deal of homology between equivalent tRNAs of different animal phyla. If differences due to post-transcriptional modifications are excluded, these homologies range from 100% for tRNA\(^{\text{Val}}\)\(^{(\text{IAC})}\) of Drosophila and rabbit liver to 92% for tRNA\(^{\text{Val}}\)\(^{(\text{IAC})}\) of mammals and Drosophila. Drosophila tRNA\(^{\text{Val}}\) differs from mammalian tRNA\(^{\text{Val}}\)\(^{(\text{IAC})}\) at 10 sites (87% homology), making it less like its mammalian counterpart than any insect tRNA sequenced to date. Interestingly, the tRNA\(^{\text{Val}}\)\(^{(\text{IAC})}\)-like genes described in the accompanying paper (1) differ from mammalian tRNA\(^{\text{Val}}\)\(^{(\text{IAC})}\) at only seven sites (91% homology). tRNA\(^{\text{Val}}\) is 81% homologous to yeast tRNA\(^{\text{Val}}\)\(^{(\text{IAC})}\). This strong homology is similar to that between the initiator methionine tRNAs of the two species (82%) and contrasts with the lower degree of homology for the other tRNAs for which sequence data are available (tRNA\(^{\text{Leu}}\)\(^{(\text{UUA})}\), 68%; tRNA\(^{\text{Val}}\)\(^{(\text{UCG})}\), 74%; tRNA\(^{\text{Pro}}\)\(^{(\text{CUC})}\), 76%) (13).
The nucleotide sequence of tRNA$_{Val}^{\text{Drosophila}}$ seems to have been strongly conserved during the evolution of the eukaryotes. Studies of other tRNAs$_{Val}^{\text{Drosophila}}$ are in progress.

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