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Nucleotide sequence of starfish initiator tRNA

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ABSTRACT

The nucleotide sequence of starfish ovary initiator tRNA was determined to be pA-G-C-A-G-A-G-U-m^1G-m^2G-C-G-C-A-G-U-G-G-A-A-G-C-G-U-G-C-U-G-G-G-C-C-C-A-U-t^6A-A-C-C-C-A-G-A-G-m^7G-D-m^5C-C-G-A-G-G-A-Ψ-C-G-m^1A-A-A-C-C-U-C-G-C-U-C-U-G-C-U-A-C-C-A_{OH}. The sequence was determined by a combination of the two different post-labeling techniques. Two-dimensional cellulose thin-layer chromatography was adopted for analysis of 5'-terminal nucleotides of tRNA fragments produced by formamide treatment.

The nucleotide sequence of starfish initiator tRNA is very similar to that of mammalian cytoplasmic initiator tRNAs, but has seven different nucleotide residues and two modifications:residue 55 is Ψ instead of U, and residue 26 is unmodified G instead of m^2G.

INTRODUCTION

Sequencing of tRNA has been greatly simplified by introduction of post-labeling techniques (1,2,3,4,5). One of these methods, called the rapid read-off gel technique, is similar in principle to the method for DNA sequencing of Maxam and Gilbert (6) except that several base-specific RNases are used instead of chemical methods for degradation (1,2,4). Since the RNases do not attack the tRNA molecule evenly, presumably owing to differences in conformational rigidity along the polynucleotide chain, it has often been found difficult to assign the sequence in a certain region of tRNA by applying this method. In addition, assignment of modified nucleoside is impossible with this technique. Stanley and Vassilenko (5) solved this problem by using partial alkaline hydrolysis with formamide, which reduced the effect of the secondary structure of tRNA. The method consisted of partial alkaline hydrolysis, followed by kination of the 5'-end of oligonucleotides, fractionation of the oligonucleotides labeled at the 5'-end by polyacrylamide gel electrophoresis, and analysis of the nucleoside 3',5'-diphosphate as the 5'-terminal in each oligonucleotide by paper electrophoresis after alkaline hydrolysis. To obtain clearer results for assignment of modified nucleosides,

we adopted two-dimensional cellulose thin-layer chromatography (7) instead of one-dimensional paper electrophoresis, and also used nuclease P_1 digestion instead of alkaline digestion to obtain the nucleoside 5'-monophosphate as the 5'-terminal. Two-dimensional chromatography was necessary, because the efficiencies of kination of some modified nucleotides are very low, and so the background of contaminants derived from non-specific hydrolysis of major nucleotides often masks the modified nucleotide. To save cost and time in two-dimensional chromatography, we used thin-layer plates of small size (5 x 5 cm). In this way, more than 40 of these plates could be put in each casette for radioautography, and only 30 minutes was required for development of chromatograms in each dimension.

In this communication we report the application of this modification of Stanley and Vassilenko's procedure for the total sequence analysis of starfish initiator tRNA. The rapid read-off gel technique was also adopted for clarification of some umbiguous results. It is of interest to know the nucleotide sequence of initiator tRNAs from different species to understand the significance of conservation of the sequence of initiator tRNA during evolution. We found that the sequence A-U-C-G that is found in the T-Ψ-C-loop of mammalian cytoplasmic initiator tRNAs is completely replaced by A-Ψ-C-G in starfish initiator tRNA. Moreover residue 26(8) is under-modified, being G in starfish initiator tRNA, whereas it is m^2G in mammalian cytoplasmic initiator tRNA (9). Otherwise the nucleotide sequence of starfish initiator tRNA was found to be similar to that of mammalian cytoplasmic initiator tRNA, except for seven differences in bases.

MATERIALS AND METHODS

Isolation of starfish ovary initiator tRNA. Unfractionated tRNA was prepared from whole ovaries of starfish (*Asterina amurensis*) by homogenization with phenol and fractionation with isopropyl alcohol (10,11). Approximately 10,000 A_{260} units of unfractionated tRNA was obtained from 1 kg of cells. It was first fractionated by DEAE-Sephadex A-50 column chromatography at pH 7.5 using a column of 1.5 x 180 cm and a total of 3 ℓ of elution buffer. A single peak of methionine acceptor activity, assayed with *E. coli* aminoacyl-tRNA synthetase, was recovered in later fractions, while methionine acceptor activity, measured with homologous enzyme, was detected in all fractions. Since the initiator tRNA is only charged by *E. coli* enzyme (12), the fractions enriched with methionine acceptor activity assayed with *E. coli*

enzyme were collected. The fraction rich in initiator tRNA thus obtained (1100 A_{260} units) was fractionated by chromatography on a column (1.5 x 80 cm) of BD cellulose as described previously. Initiator tRNA was eluted at the front of the A_{260} profile as a sharp single peak. This fraction (50 A_{260} units) was further fractionated by RPC-5 column chromatography (0.8 x 90 cm) as described by Peason *et al.* (13). Twenty A_{260} units of initiator tRNA fraction was obtained. Final purification of initiator tRNA was achieved by RPC-5 column chromatography at pH 4.3 using a column of 0.8 x 90 cm. Linear gradient elution was achieved with 250 ml of 0.02 M sodium acetate buffer (pH 4.3)-0.01 M $MgCl_2$-2 mM β-mercaptoethanol-0.5 M NaCl in the mixing chamber and 250 ml of 0.02 M sodium acetate (pH 4.3)-0.01 M $MgCl_2$-2 mM β-mercaptoethanol-1 M NaCl in the reservoir. The final yield of purified initiator tRNA was 5 A_{260} units.

Aminoacyl-tRNA synthetases and aminoacylation and formylation reactions. Crude aminoacyl-tRNA synthetase from starfish ovary cells was prepared as described by Nishimura and Weinstein (14). Crude *E. coli* aminoacyl-tRNA synthetase was prepared as described previously (15). The extents of aminoacylation and formylation of starfish initiator tRNA were measured as reported previously (12).

Preparation of 5'-terminal labeled starfish initiator tRNA and rapid read-off sequencing of the tRNA. 5'-Labeling of tRNA with ^{32}P was carried out essentially as described by Silberklang *et al.* (3). The exact conditions were as follows: To remove the 5'-terminal phosphate of tRNA, 0.01 A_{260} unit of initiator tRNA was incubated with 0.0025 unit of *E. coli* alkaline phosphomonoesterase in 3 µl of 50 mM Tris-HCl buffer (pH 7.5) at 55°C for 30 min. Then 0.5 µl of 35 mM nitrilotriacetic acid (pH 7.5, adjusted by NaOH) was added and the mixture was incubated at room temperature for 20 min and heated at 100°C for 90 sec to inactivate *E. coli* alkaline phosphomonoesterase. To the reaction mixture (3.5 µl) were added 0.15 µmol of $MgCl_2$, 0.15 µmol of β-mercaptoethanol, 2.5 units of T_4 polynucleotide kinase and 2.4 pmol of [γ-^{32}P]ATP to give a final volume of 10 µl. The mixture was incubated at 37°C for 30 min and then intact tRNA labeled at the 5'-terminal was separated by polyacrylamide gel electrophoresis on 15 % gel. Labeled tRNA was extracted from the gel with 1.5 ml of a solution of 0.5 M ammonium acetate-0.01 M magnesium acetate-0.1 % sodium dodecyl sulfate-0.1 mM EDTA. Then 0.5 A_{260} unit of carrier *E. coli* tRNA was added to the extract, and the labeled tRNA was precipitated with ethanol and dissolved in 30 µl of H_2O.

Partial digestion of the 5'-terminal labeled tRNA with RNases and alkali was performed as described by Brownlee *et al.* (2) with some modifications. RNase T_1 or RNase A digestion was carried in 10 µl of reaction mixture consisting of 0.1 M Tris-HCl (pH 7.5), 10 mM EDTA (pH 7.0), 0.017 A_{260} unit of tRNA and 0.01 unit of RNase T_1 or 0.5 ng of RNase A. The reaction mixture was incubated at 4°C for 30 min. Digestion with RNase U_2 or RNase T_1 was performed in 10 µl of reaction mixture consisting of 50 mM sodium acetate buffer (pH 4.5), 2 mM EDTA (pH 5.0), 0.017 A_{260} unit of tRNA and 0.01 unit of RNase U_2 or 0.005 unit of RNase T_2. The incubation was carried out at 37°C for 30 min. For limited alkaline hydrolysis of tRNA, 0.25 A_{260} unit of tRNA was incubated at 90°C for 15 min in 10 µl of 50 mM $NaHCO_3$-Na_2CO_3 (pH 9.0) and 1 mM EDTA.

Sequencing of tRNA by limited alkaline hydrolysis of tRNA with formamide. The procedure described by Stanley and Vassilenko (5) was followed, except that two-dimensional thin-layer cellulose chromatography was adopted instead of one-dimensional paper electrophoresis for identification of the 5'-end of the nucleotide residue in each oligonucleotide. Nuclease P_1 digestion was used instead of alkaline hydrolysis for characterization of the 5'-terminal nucleotides as nucleoside 5'-monophosphates rather than nucleoside 3',5'-diphosphates. Starfish initiator $tRNA^{Met}$ (0.01 A_{260} unit) was heated at 100°C for 15 min in a sealed capillary tube containing 10 µl of formamide. The partially hydrolyzed tRNA was collected by ethanol precipitation and phosphorylated in 10 µl of reaction mixture consisting of 50 mM Tris-HCl (pH 7.5), 15 mM $MgCl_2$, 15 mM β-mercaptoethanol, 2.5 units of T_4 polynucleotide kinase and 0.24 µM [γ-^{32}P]ATP. The incubation was carried out at 37°C for 30 min. The 5'-terminal labeled oligonucleotides were separated by polyacrylamide gel electrophoresis, the bands of each oligonucleotide were cut out of the gel and homogenized with a glass rod in a 1.5 ml Eppendorf centrifuge tube with 0.5 ml of the buffer consisting of 0.5 M ammonium acetate, 0.01 M magnesium acetate, 0.1 % sodium dodecylsulfate and 0.1 mM EDTA. The pieces of gel were removed and labeled oligonucleotides were precipitated with ethanol after addition of 0.25 A_{260} unit of carrier tRNA. The resulting material was then incubated with 1 µg of nuclease P_1 in 10 µl of 20 mM ammonium acetate buffer (pH 5.3) at 37°C for 3 hr to obtain the ^{32}P-labeled terminal 5'-nucleotide. A portion of the material was spotted on a thin-layer Avicel SF cellulose plate (5 x 5 cm) and subjected to chromatography (7) with solvent A (isobutyric acid-0.5 M ammonia, 5:3, v/v) in the first dimension and solvent B (isopropyl alcohol-conc. HCl-H_2O,

70:15:15, v/v/v) in the second dimension.

Analysis of modified nucleosides by tritium labeling. The composition of modified nucleosides in starfish initiator tRNA was determined by a tritium derivative method as described by Randerath *et al.*(16).

Materials. Uniformly labeled [^{14}C]methionine, ^{3}H-labeled potassium brohydrate (specific activity, 15 Ci/mmol) and [γ-^{32}P]ATP (specific activity, 5500 Ci/mmol) were obtained from New England Nuclear Corp. RNase T_1, RNase T_2 and RNase U_2 were products of Sankyo Co. *E. coli* alkaline phosphomonoesterase, RNase A, nuclease P_1 and T_4 polynucleotide kinase were purchased from Worthington Biochemical Corp., Sigma Chemical Co., Yamasa Shoyu Co. Ltd., and Boehringer Mannheim Corp., respectively. Avicel SF cellulose thin-layer plates (5 x 5 cm) were obtained from Funakoshi Pharmaceutical Co., Tokyo. Plastic sheets precoated with cellulose, used for analysis of tritiated modified components, were purchased from Brinkman Instruments Inc., Westbury, N.Y. Acrylamide and N,N'-methylene-bisacrylamide, used for preparation of polyacrylamide gel, were obtained from Eastman Kodak Co.

RESULTS AND DISCUSSION

Properties of starfish initiator tRNA. Starfish initiator tRNA was isolated from unfractionated starfish ovary tRNA by successive column chromatographies as described in the Materials and Methods. The reasons for concluding that the methionine tRNA isolated is initiator tRNA are as follows: [1] this tRNA, like yeast initiator $tRNA^{Met}$ (12), was aminoacylated by *E. coli* aminoacyl-tRNA synthetase; [2] it was formylated by *E. coli* formyltransferase (data not shown); [3] its binding to *E. coli* ribosomes was stimulated by GUG as well as AUG (data not shown).

Modified nucleosides present in starfish initiator tRNA. The composition of the modified nucleosides of starfish initiator tRNA was analysed by the tritium derivative method developed by Randerath *et al.*(16). As shown in Fig. 1, the tritiated alcohol derivatives of dihydrouridine (D) pseudouridine (Ψ), 1-methyladenosine(m^1A), 5-methylcytosine(m^5C), 1-methylguanine (m^1G), $\underline{N}^2$-monomethylguanosine(m^2G), 7-methylguanosine(m^7G) and $\underline{N}$-[$\underline{N}$-(9-β-$\underline{D}$-ribofuranosylpurin-6-yl)carbamoyl]threonine(t^6A) were clearly seen in the radioautogram. On the other hand, ribothymidine(T) was not detected as in the case of other eukaryotic initiator tRNAs. $\underline{N}^2$,$\underline{N}^2$-Dimethylguanosine(m^2_2G),

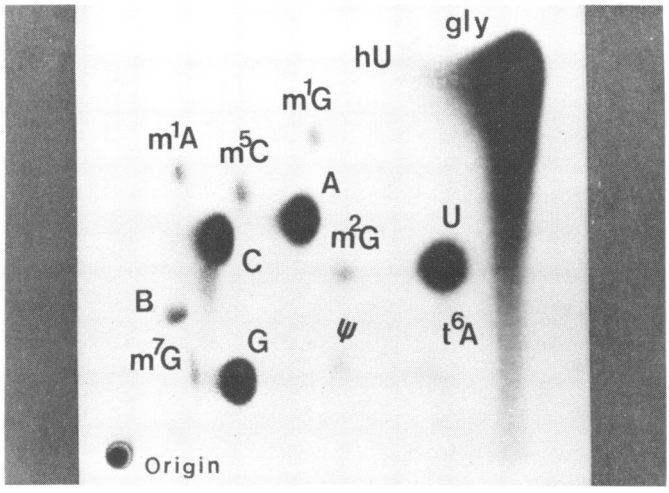

Fig. 1. Fluorogram of tritium-labeled digest of starfish initiator tRNA chromatographed on a cellulose thin-layer plate.

which is present in several eukaryotic initiator tRNAs (9), was also not present in starfish initiator tRNA. These findings were helpful in determining the positions of the modified nucleosides in the sequence of the tRNA in later experiments.

Sequence analysis of starfish initiator tRNA. For obtaining the primary structure of starfish initiator tRNA, we mainly used data obtained by a modification of the limited formamide hydrolysis method described by Stanley and Vassilenko (5) as shown in Fig. 2. The modifications we adopted were the use of nuclease P_1 instead of alkaline hydrolysis to obtain nucleoside 5'-monophosphate from the 5'-end of the fragments, and two-dimensional thin-layer cellulose chromatography instead of one-dimensional paper electrophoresis for better resolution of the modified nucleotides. Typical results with respect to the sequence containing modified nucleosides are shown in Fig. 3. The intensities of modified nucleotides expected as 5'-terminal residues of fragments were low, and sometimes much less than these of contaminating minor nucleotides formed by non-specific hydrolysis. This seems to be due partly to the low efficiency of phosphorylation of modified nucleoside 3'-phosphates, and partly to alkaline degradation of some modified nucleosides, such as m^1A and t^6A. Nevertheless all the 5'-terminal nucleotides, including modified nucleosides, can be clearly read out as in the

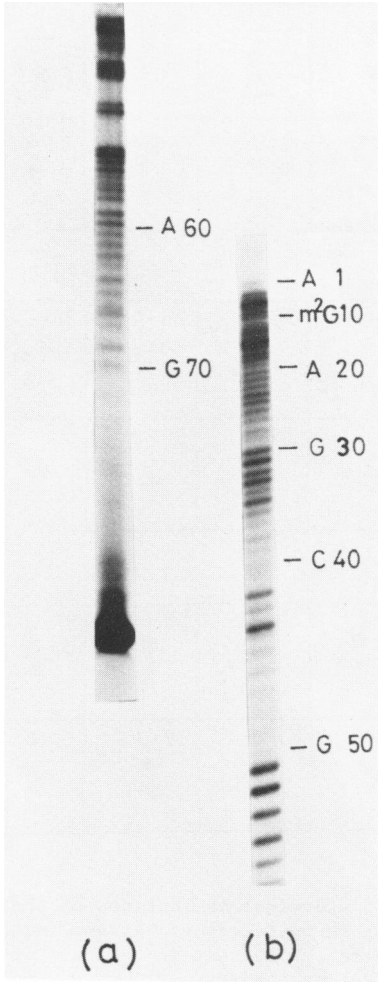

Fig. 2. Autoradiogram of 5'-^{32}P-labeled oligonucleotides fractionated on a 20 % polyacrylamide-7 M urea gel. Electrophoresis was carried out for 7 hr (a) and 20 hr (b) at 600 V (gel dimensions: 40 x 20 x 0.2 cm).

case of A-G-U-m^{1}G-m^{2}G-C-G-C-A-G-U (residue 6 to 16), G-G-G-C-C-C-A-U-t^{6}A-A (residue 29 to 38) and A-G-G-A-Ψ-C-G-m^{1}A-A-A-C (residue 51 to 61), respectively as seen in Fig. 3. The total primary structure of starfish initiator tRNA could be deduced, except for some ambiguity in the region of residue 49 to 53, and in the 5'- and 3'-terminal regions. The ambiguities about residues 1 to 6, and the nucleotide at the fourth position from the 3'-end are

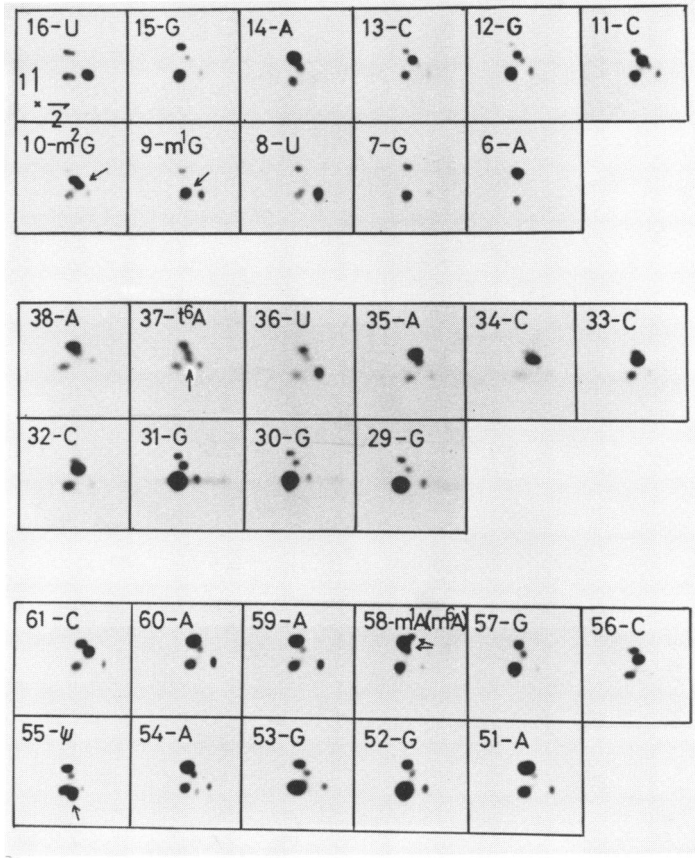

Fig. 3. Analysis of the 5'-terminal nucleotides of the fragments of tRNA by two-dimensional thin-layer chromatography. Arrows indicate modified nucleotides as shown in the square. Note that in the case of m^1A, it is partly converted to m^6A under alkaline condition.

due, respectively, to the limit of resolution of large RNA fragments and the difficulty of precipitating small RNA fragments with ethanol. To confirm these sequences, we used the rapid read-off sequencing method (1,2,4). The 5'-labeled tRNA was partially hydrolyzed by RNase T_1, RNase A, RNase U_2 or RNase T_2, and the digests were fractionated on 20 % polyacrylamide-7 M urea gel. As shown in Fig. 4, the sequences of residues 1 to 6 and 49 to 53 were read out as A-G-Py-A-G-A and Py-G-A-G-G, respectively. The results obtained by the rapid read-off gel technique were also useful for comfirmation of other parts of the sequence. In addition, fractionation of the 5'-end

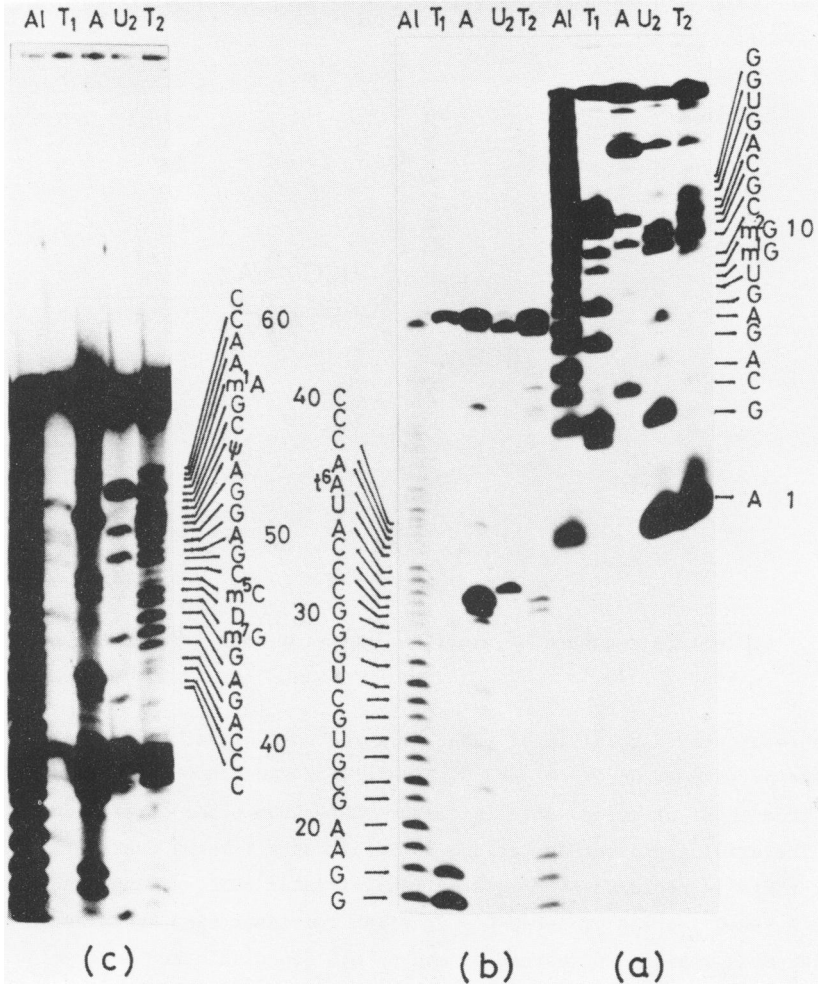

Fig. 4. Autoradiograms of partial enzymatic and alkaline digests of 5'-^{32}P-labeled starfish ovary initiator tRNA. Al shows the ladder obtained by heating the tRNA in alkali. T_1, A, U_2 and T_2 shows the ladder obtained by incubation with these ribonucleases. Details are described in the Methods. Gel electrophoresis was carried out on a 20 % polyacrylamide-7 M urea gel (gel dimensions: 40 x 20 x 0.2 cm) for 7 hr (a), 20 hr (b) and 27 hr (c) at 600 V.

labeled RNase A digest by Sanger's finger-printing procedure (17) revealed the presence of oligonucleotides, A-G-C and A-C (data not shown). Thus, the sequences at the 5'- and 3'- ends were unambigously determined as pA-G-C-A-G-A-G and C-U-G-C-U-A-C-C-A_{OH}, respectively. Fig. 5 shows the total primary

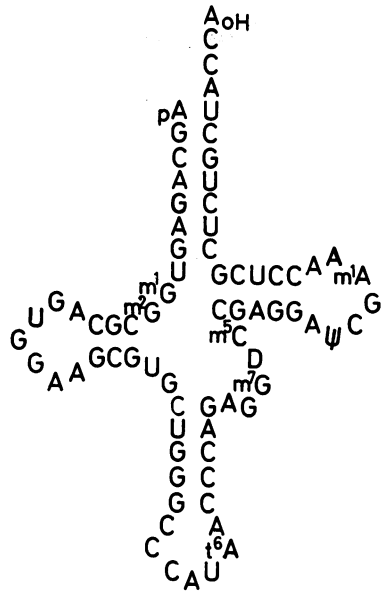

Fig. 5. Nucleotide sequence of starfish initiator tRNA arranged in a clover-leaf form.

structure of starfish initiator tRNA, arranged as a clover leaf form.

The primary structure of starfish initiator tRNA shows several differences from those of cytoplasmic initiator tRNAs from other eukaryotic cells (9). The uridine present as residue 55 in almost all known eukaryotic initiator tRNAs is replaced by Ψ in starfish initiator tRNA, giving the sequence A-Ψ-C-G instead of A-U-C-G. Starfish initiator tRNA contains unmodified G as residue 26 instead of m^2G or m^2_2G found in other eukaryotic initiator tRNAs. In addition to these differences with respect to post-transcriptional modification, starfish initiator tRNA has seven different bases from those in mammalian initiator tRNAs; namely residues 16, 49, 50, 51, 63, 64 and 65 in starfish initiator tRNA are U, C, G, A, U, C and G instead of C, G, A, U, A, U and C. Gillum *et al.* (18) showed that initiator tRNA from the liver or testis of salmon, which is a primitive verterbrate, has an identical sequence and the same modifications as mammalian initiator tRNAs. The starfish belongs to the Echinodermata, which were separated from the Chordata at an early stage of evolution. Wheat germ initiator tRNA, which was sequenced very recently [U.L. RajBhandary, personal communication (9)], also contains A-Ψ-C-G instead of A-U-C-G. This tRNA, however, shows

greater differences than starfish initiator tRNA from mammalian initiator tRNAs with respect to its nucleotide sequence and modifications. Thus, from the evolutionary view point, it is interesting that divergence of the sequence and modification of initiator tRNAs are consistent with the process of evolution.

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