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THE NUCLEOTIDE SEQUENCE OF RAT LIVER tRNA^{Asn}

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SUMMARY. The major species of asparagine specific tRNA was isolated from rat liver, degraded to oligonucleotides, and shown to have the nucleotide sequence pG-U-C-U-C-U-G-U-m^{1}G-m^{2}G-C-G-C-A-A-D-C-G-G-D-X-A-G-C-G-C-m$^2_2$G-ψ-ψ-C-G-G-C-U-Q-U-U-t^{6}A-A-C-C-G-A-A-A-G-m^{7}G-D-U-G-G-U-G-G-Z-ψ-C-G-m^{1}A-G-C-C-C-A-C-C-C-A-G-G-G-A-C-G-C-C-A_{OH}. Although this tRNA contains several modified nucleotides in their expected positions, it is unique in having X, 3-(3-Amino-3-carboxy-n-propyl)uridine in loop I rather than in loop III; Q, 7-(4,5-cis-dihydroxyl-1-cyclopenten-3-yl-aminomethyl)-7-deazaguanosine in the wobble position of loop II; and Z, an unknown, and presently uncharacterized nucleoside, at position 23 from the 3' terminus usually occupied by ribothymidine.

We recently reported that rat liver and Walker 256 tumor tRNA differed in their chromatographic elution from acylated DBAE-cellulose and Aminex A-28 (1). These differences, namely the presence of additional isoaccepting species of tRNA^{Asn}, tRNA^{Asp}, tRNA^{His} and tRNA^{Tyr}, were ascribed to the lack of Q (or Q*) nucleoside in the tumor tRNA. Since our laboratory has been interested in studying the role of modified nucleotides and alterations in tumor tRNA, we have determined the complete nucleotide sequence of the normal rat liver tRNA^{Asn} so that its sequence can be directly compared with that of the altered tumor tRNA^{Asn}.

With the recent advances in rapid, sensitive post-labeling techniques (2-6), the unique nucleotide sequence analysis for a desired tRNA can now be determined when only a small amount of pure tRNA is available. The development and implementation of these techniques now make it possible to compare the primary structure of a normal tRNA with its altered tumor counterpart. In addition, as these procedures require only minute quantities of tRNA for the complete nucleotide sequence analysis, the remaining tRNA can then be employed for subsequent studies on the functional significance of these altered tRNAs.

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MATERIALS AND METHODS. The isolation of rat liver $tRNA^{Asn}$ to a purity of 1.2 nmoles per A_{260} unit has previously been reported (1). Conditions for digestion by ribonucleases and procedures for the resolution of the complete digestion products were identical to those reported earlier for the human placenta $tRNA^{Phe}$ and $tRNA^{Val}$ (2,3). The procedures employed for the analysis of the 5' terminal nucleoside and sequential degradation of each oligonucleotide fragment were as described (4,5). The sequentially degraded and tritium post-labeled oligonucleotides were then resolved using the two-dimensional PEI-cellulose TLC procedure described in detail earlier (6). In addition, the tRNA was labeled with ^{32}P according to the published procedures (7,8), after prior removal of the 5' terminal phosphate by treatment with ribonuclease free bacterial alkaline phosphatase (7). It was essential that the commercial BAP be treated as described (9), since the contaminating ribonucleases created several nicks, thereby making interpretation of the subsequent gels impossible. The ^{32}P-labeled $tRNA^{Asn}$ was further purified on 15% cross-linked polyacrylamide slab gels as reported (8). After partial alkaline and ribonuclease digestion (8), the fragments were resolved on a 20% polyacrylamide gel (8) containing 7 M urea, Tris-borate pH 8.3, with a 3 cm 5% spacer gel (10) containing Tris-HCl pH 3.6, and the bands located by exposure to Kodak XR-1 medical X-ray film at -70° (11).

RESULTS AND DISCUSSION. The tracings of the two-dimensional PEI-cellulose TLC maps of the complete RNase T_1 and RNase A digestion products of rat liver $tRNA^{Asn}$ are shown in Figures 1 and 2, respectively. A summary of the nucleotide sequences and molar ratios for each fragment is shown in Tables I and II. These sequences were determined with the following protocol: (a) After complete digestion to nucleotides, each fragment's base composition was determined by tritium trialcohol mapping (4). (b) The 5' terminal nucleoside was determined by incorporation of ^{32}P as described (7,12). By use of these first two analysis procedures, the nucleotide sequence of all di- and trinucleotides could be established. (c) With fragments which were tetranucleotides or longer, we employed the recently reported two dimensional PEI-cellulose TLC procedure for resolution of partial digestion products which were labeled at their various 3' termini with tritium (6). By subsequent analysis of each tritium labeled terminus the unique nucleotide sequence of all fragments but one, T-18, were deduced.

In the case of fragment T-18, digestion with snake venom phosphodiesterase did not proceed through the modified nucleoside t^6A, and therefore other methods were required to deduce its final sequence as shown in Table I. In addition, since the trialcohol derivative of Q nucleoside remains at the origin in the

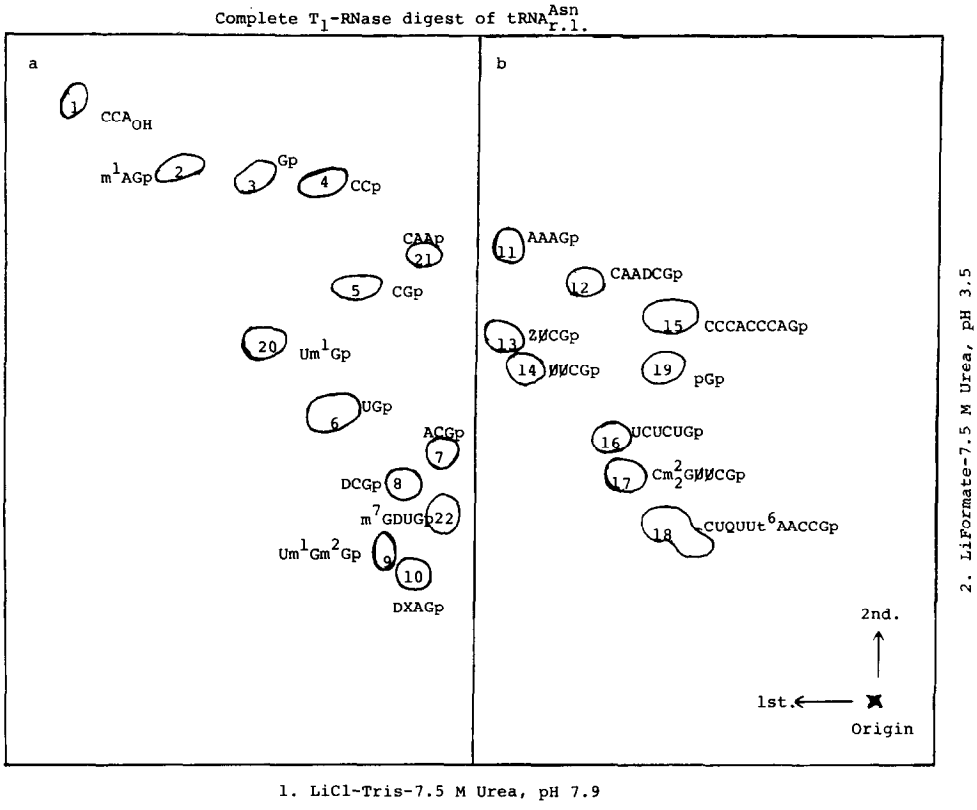

Figure 1. PEI-cellulose TLC map of RNase T_1 digest of rat liver $tRNA^{Asn}$. The first dimension was developed with increasing concentrations (0.0~0.4M) of LiCl in 0.3M Tris-HCl, 7.5M urea (pH 7.9). The plate was then cut into two strips prior to development in the second dimension with stepwise Li-formate gradient (a. 0.0~0.7M; b. 0.0~1.5M) containing 7.5M urea (pH 3.5).

cellulose TLC employed for the analysis of tritium labeled nucleosides, additional techniques were required for the direct analysis of Q. By employing both the ^3H and ^{32}P base analysis procedures (7) (data not shown), we demonstrated that Q nucleoside was present at the 5' terminus of the dinucleotide Q-Up in fragment A-1. In addition, the complete nucleoside analysis of fragment T-18 gave C_3, U_3, A, t^6A, Q, and G, thereby demonstrating the presence of Q in both the RNase T_1 and RNase A digests. Further analysis of the two-dimensional PEI-cellulose TLC partial snake venom digestion of T-18 indicated that the 3' terminal sequence was t^6A-A-C-C-Gp, while the 5' terminal

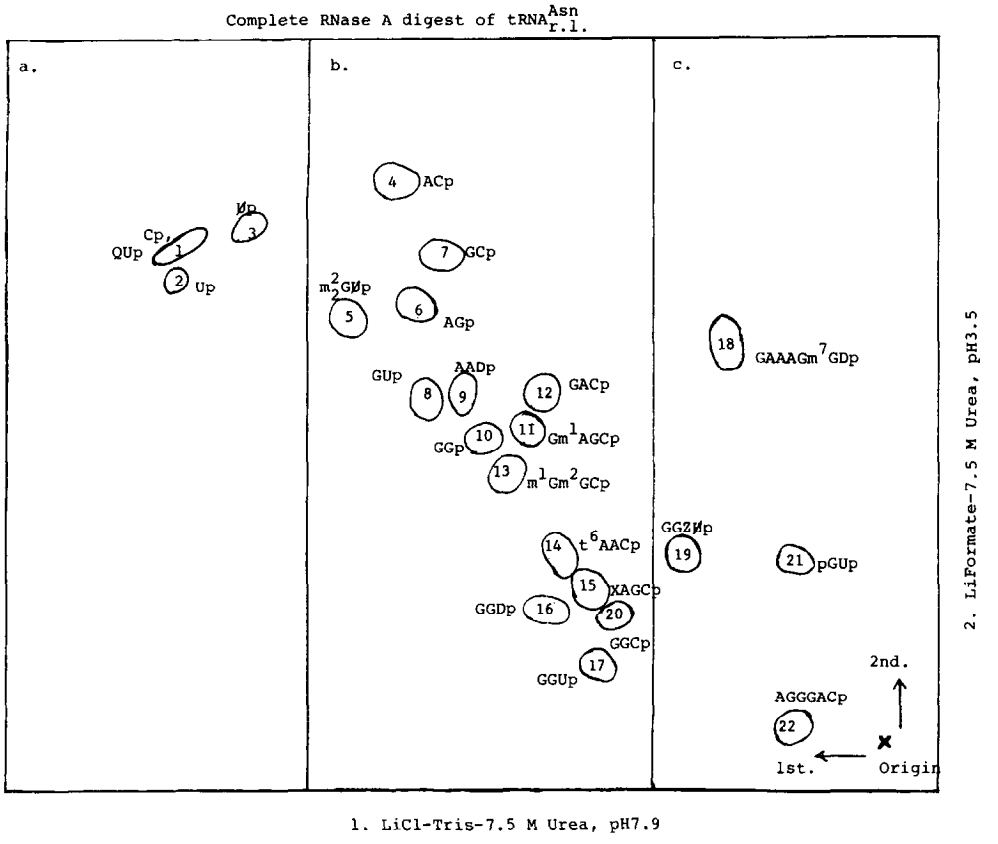

Figure 2. PEI-cellulose TLC map of RNase A digest of rat liver tRNA^{Asn}. The solvents used in both dimensions were identical to those employed in Figure 1, except that the plate was cut into three strips prior to development in the second dimension with stepwise gradient of Li-formate (a. 0.0~0.2M; b. 0.0~0.4M; c. 0~1.5M) containing 7.5M urea (pH 3.5).

contained cytosine. As Q-Up was found in molar yields in the RNase A digest and Q nucleoside was present in T-18, this oligonucleotide must contain Py-Q-Up as part of its sequence. By employing the recently reported separation of 5' ^{32}P-labeled fragments on 15% polyacrylamide gels (8), after partial digestion with alkali or various ribonucleases, we could directly deduce the complete nucleotide sequence of this unique oligonucleotide. As shown in Figure 3, the alkali digest (lanes 2 and 3) yielded 10 fragments, each one nucleotide shorter than the preceding one. By partial digestion with RNase U_2 (lane 4), the adenosine was

Table I

Sequence and Molar Ratio of Oligonucleotides Obtained From the Complete Digestion of Rat Liver $tRNA^{Asn}$ with Ribonuclease T_1

[a]Spot no.	Compound	Molar ratio [b]Observed	[c]Calculated
1	[d]C-C-A_{OH}	0.5	1
2	m^1A-Gp	1.1	1
3	Gp	6.0	6
4	[d]C-Cp	0.5	0
5	C-Gp	2.2	2
6	U-Gp	1.0	1
7	A-C-Gp	1.1	1
8	[e]D-C-Gp	0.5	0
9	[f]U-m^1G-m^2Gp	0.3	1
10	D-X-A-Gp	0.9	1
11	A-A-A-Gp	0.9	1
12	[e]C-A-A-D-C-Gp	0.5	1
13	Z-ψ-C-Gp	1.1	1
14	[g]ψ-ψ-C-Gp	0.4	0
15	C-C-C-A-C-C-C-A-Gp	0.9	1
16	U-C-U-C-U-Gp	1.0	1
17	[g]C-m_2^2G-ψ-ψ-C-Gp	0.7	1
18	C-U-Q-U-U-t^6A-A-C-C-Gp	0.9	1
19	pGp	0.9	1
20	[f]U-m^1Gp	0.6	0
21	[e]C-A-Ap	0.5	0
22	m^7G-D-U-Gp	0.7	1

[a]See Figure 1 for the position of each spot on the PEI-cellulose thin layer.

[b]The molar ratio of Gp was derived from the fragments of the complete RNase A digest (see Table II). All other molar ratios were determined directly from the RNase T_1 digest.

[c]Molar ratios as predicted from the final derived sequence shown in Fig. 5.

[d]Depending on the digestion conditions, C-C-A_{OH} is split further to C-Cp.

[e]The fragment C-A-A-D-C-Gp is split further to give C-A-Ap and D-C-Gp.

[f]The fragment U-m^1G-m^2Gp is split further to give U-m^1Gp.

[g]The fragment C-m_2^2G-ψ-ψ-C-Gp is split further to give ψ-ψ-C-Gp, the fragment C-m_2^2Gp was not recovered from the RNase T_1 digest.

Table II

Sequence and Molar Ratio of Oligonucleotides Obtained From the Complete Digestion of Rat Liver $tRNA^{Asn}$ with Ribonuclease A

[a]Spot no.	Compound	Molar ratio [b]Observed	[c]Calculated
none	[b]Adenosine	0	1
1	[b]Cp, Q-Up	11, 1.2	11, 1
2	[b]Up	5.0	5
3	Up	1.0	1
4	A-Cp	1.5	1
5	m_2^2G-Up	1.0	1
6	[d]A-Gp	0.2	0
7	G-Cp	3.2	3
8	G-Up	0.9	1
9	A-A-Dp	0.8	1
10	[d]G-Gp	0.5	0
11	G-m^1A-G-Cp	1.0	1
12	[d]G-A-Cp	0.5	0
13	m^1G-m^2G-Cp	1.2	1
14	t^6A-A-Cp	1.0	1
15	X-A-G-Cp	0.9	1
16	G-G-Dp	0.9	1
17	G-G-Up	1.1	1
18	G-A-A-A-G-m^7G-Dp	0.9	1
19	G-G-Z-ψp	1.0	1
20	G-G-Cp	0.9	1
21	pG-Up	1.1	1
22	[d]A-G-G-G-A-Cp	0.6	1

[a]See Figure 2 for the position of each spot on the PEI-cellulose thin layer, spot #1 contains two fragments Cp and Q-Up. Adenosine can not be detected on the anion-exchange thin layer.

[b]The molar ratios of adenosine and nucleoside monophosphates were derived from the fragments of the complete RNase T_1 digest of rat liver $tRNA^{Asn}$ (see Table I). All other molar ratios were determined directly from the RNase A digest.

[c]See footnote c of Table I.

[d]Depending on the digestion conditions, the fragment A-G-G-G-A-Cp is split further to give A-Gp, G-Gp and G-A-Cp.

placed at position 7, as expected from the two-dimensional PEI-cellulose TLC analysis. In contrast, partial digestion of this

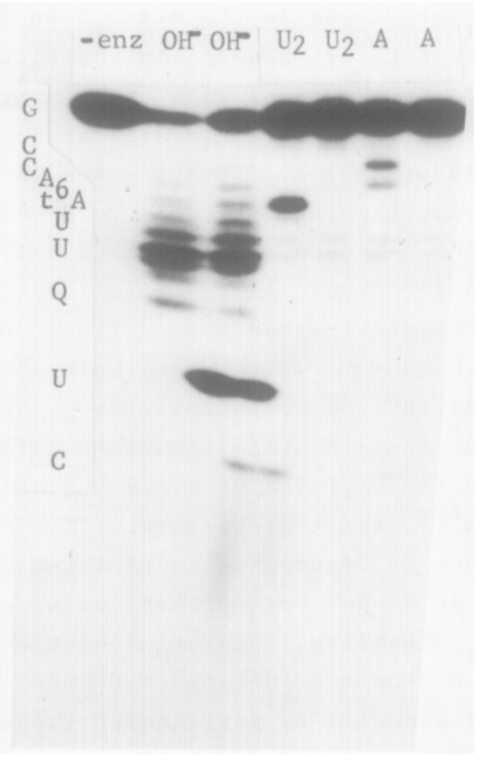

Figure 3. Autoradiograph of partial enzymic digests of 5'-[^{32}P] labeled T-18 fragment from Figure 1. Incubations of tRNA were at two different levels of CO_3^- buffer (cleavage at every base), RNase U_2 (cleavage at adenosine), and RNase A (cleavage at each pyrimidine). Separation was by electrophoresis on a 20% polyacrylamide slab gel.

fragment with RNase A (lanes 6 and 7) yielded the series of oligonucleotides which placed the pyrimidines at positions 1,2,4,5,8, and 9 from the 5' terminus, thereby unequivocally establishing the sequence of T-18 as C-U-Q-U-U-t^6A-A-C-C-Gp.

From the data presented in Tables I and II, it is not possible to write the complete nucleotide sequence for rat liver $tRNA^{Asn}$, assuming the modified nucleotides occur in their usual positions (13), the normal rules of base pairing apply, and the structure can be written in the classical cloverleaf form. This difficulty is partly due to the presence of fragments T-22 and P-18 which overlap to give the sequence G-A-A-A-G-m^7G-D-U-Gp and the X nucleoside containing fragments (T-10 and P-15) which give the sequence G-D-X-A-G-Cp. Since, to date, the modified nucleosides

X and m^7G have only been found in the minor loop (loop III), then either these two fragments are present in the minor loop, which would place this tRNA in the class with other large minor loop tRNAs, or one of these fragments is located at some other position in the tRNA. Additional difficulty comes in determining the location of the overlapping fragments T-13 and P-19, which yield the sequence G-G-Z-ψ-C-Gp, and fragment T-17, which yields the sequence G-C-m_2^2G-ψ-ψ-C-Gp. In order to place these and the other RNase T_1 and RNase A fragments in a final, unique sequence, we employed a partial specific digestion technique with separation of the ^{32}P-5' labeled tRNA on polyacrylamide gel electrophoresis (8,14). As shown in Figure 4, this technique allowed us to deduce unequivocally the position of every guanosine and adenosine residue in $tRNA^{Asn}$, and thereby obtain the unique nucleotide sequence as shown in Figure 5. In addition, the presence of the X nucleoside in loop I was further confirmed by obtaining the partial RNase T_1 digestion fragment A-D-C-G-G-D-X-A-G-C-G-C-m_2^2G-ψ-ψ-C-G-G-C-U-Q-U-Up and pG-U-C-U-C-U-G-U-m^1G-m^2G-C-G-C-A-A-D-C-Gp (data not shown). By positioning these large overlapping fragments at the 5' terminus, and by inspecting the sequencing gel (Figure 4), the complete unique nucleotide sequence of rat liver $tRNA^{Asn}$ can be written as shown in Figure 5.

From inspection of the complete nucleotide sequence of rat liver $tRNA^{Asn}$, as shown in Figure 5, several conclusions can be drawn. (a) This tRNA contains all the "universal" nucleosides ascribed to elongator tRNAs in their proper positions (15), including the uridine at position 8, adenosine at both the beginning and end of the unpaired loop I, GpG dinucleotide in loop I, uridine adjacent to the 5' side of the anticodon triplet, a C-G base pair just prior to loop IV, the sequence ψ-C-Pu-A in loop IV, and the sequence $C\text{-}C\text{-}A_{OH}$ as the 3' terminus. (b) As predicted (15), the nucleotides A_{15} -U_{49} could possibly form a base pair in the tertiary structure. (c) All stem and loop regions fit the present model of tRNA secondary structure, with the only unpaired nucleotides being the U_4 and G_{70} in the acceptor stem, a phenomenon also observed with several other tRNAs (16).

A comparison of the primary structure of the rat liver $tRNA^{Asn}$ with the E. coli $tRNA^{Asn}$ (17) reveals several identical regions. These include portions of the acceptor stem, almost the entire loop I region, the entire anticodon loop, and portions

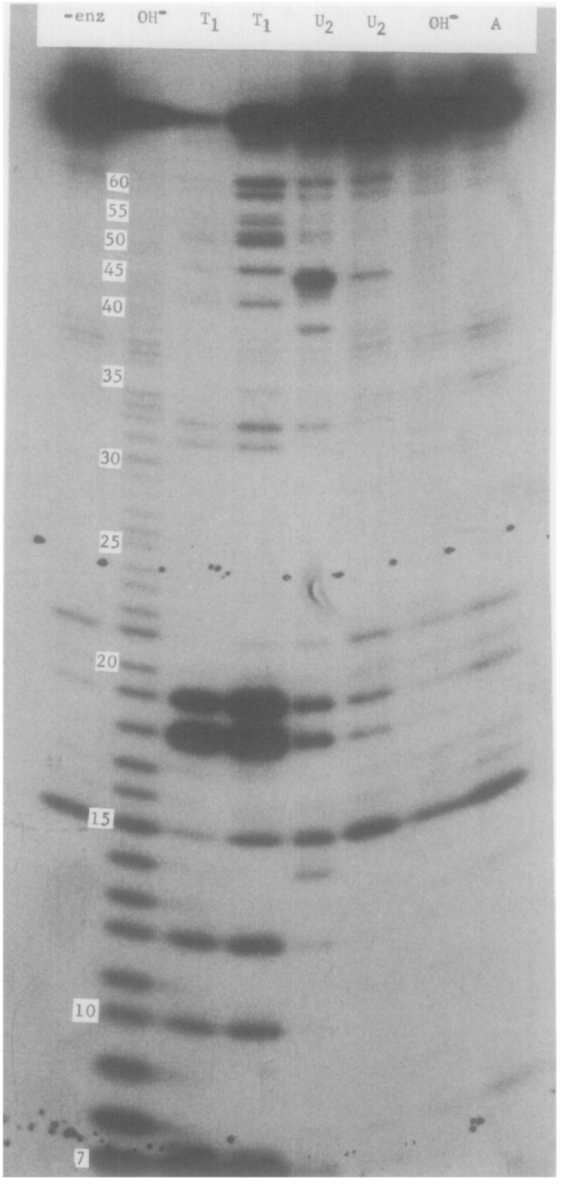

Figure 4. Autoradiograph of 5'-[^{32}P] labeled rat liver tRNA^{Asn} partially cleaved at every guanine (T_1), adenine (U_2), and each base (OH^-) with two different levels of enzymes. Separation by size was on 20% polyacrylamide slab gel with a 5% spacer layer.

of loop IV and its adjacent stem. These results indicate approximately 60% sequence homology between the tRNA^{Asn} of procaryote and eucaryote origin.

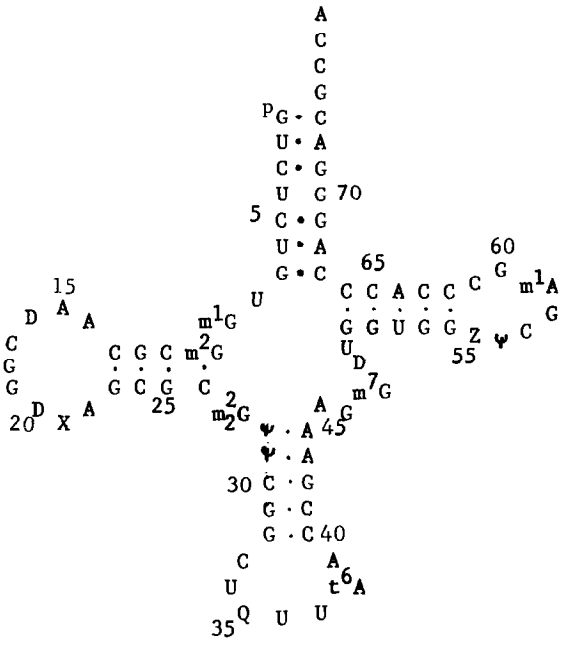

Figure 5. The nucleotide sequence of rat liver $tRNA^{Asn}$ arranged in cloverleaf form.

The major unique features of the rat liver $tRNA^{Asn}$ are the location of X nucleoside in loop I, rather than in loop III as reported for E. coli X containing tRNAs (18-22), and the replacement of the usual rT, 2'-O-methyl rT, ψ or A at position 23 from the 3' terminus by a previously unreported nucleoside, termed Z. In regard to the presence of X nucleoside in mammalian tRNA, Friedman has reported the phenoxyacetylation of X nucleoside in several rat liver tRNAs (23,24), Randerath has described the presence of X nucleoside in mammalian tRNAs from several sources (25), Ohashi, et al have briefly described a mammalian enzyme preparation which synthesizes X nucleoside (26), and Katz (27) has described the effect of cyanogen bromide treatment on the mobility of X containing tRNAs on anion exchange columns.

In all of the aforementioned reports, no experimental evidence has been presented which describes the location of X nucleoside in mammalian tRNA. It is therefore interesting to note that this modified uridine is present in the loop I sequence

D-X-A in the rat liver $tRNA^{Asn}$ while it is present in the loop III sequence m^7G-X-C in E. coli tRNAs. In regard to the unknown Z nucleoside, at present our data (to be published elsewhere) indicate that it is not identical to any reported E. coli or mammalian nucleosides, as judged by mobility in several solvents on cellulose, PEI-cellulose, or silica gel TLC.

We have previously reported the similarities in nucleotide compositions of the rat liver and human placenta $tRNA^{Asn}$ (1). This data, as well as the observation (B. Roe, C. Y. Chen and E. Y. Chen, unpublished results) that the RNase T_1 digest of the human placenta $tRNA^{Asn}$ contains fragments similar to those shown in Figure 1, indicate that the primary structure of both tRNAs might be identical, as is the case with mammalian $tRNA_i^{Met}$ (28), $tRNA^{Phe}$ (3), and $tRNA^{Val}$ (2). These observations support the hypothesis (2) that the primary sequence of the structural genes for several tRNAs are conserved in tissues isolated from many evolutionary divergent species. In order to expand these observations to other mammalian (including human) species, the complete nucleotide sequence of both the human placenta and beef liver $tRNA^{Asn}$ is presently under investigation.

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