A Comparison of 1-Methyladenine-Containing Sequences in Transfer Ribonucleic Acid from Yeast and from Rat Liver*

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ABSTRACT: The nucleotide sequences surrounding 1-methyladenylic acid in transfer ribonucleic acid from rat liver and from yeast were investigated by fractionation of pancreatic ribonuclease digests of transfer ribonucleic acid samples. Six 1-methyladenine-containing oligonucleotides were isolated from rat liver transfer ribonucleic acid digests, and the sequences were identified as guanylyl-(3′,5′)-1-methyladenylyl-(3′,5′)-uridylic 3′-acid, adenylyl-(3′,5′)-1-methyladenylyl-(3′, 5′)-uridylic 3′-acid, guanylyl-(3′,5′)-1-methyladenylyl-(3′,-5′)-cytidylic 3′-acid, guanylyl-(3′,5′)-1-methyladenylyl-(3′,5′)-adenylyl-(3′,5′)-uridylic 3′-acid, adenylyl-(3′,-5′)-1-methyladenylyl-(3′,5′)-adenylyl-(3′,5′)-uridylic 3′-acid, and guanylyl-(3′,5′)-1-methyladenylyl-(3′,5)-adenylyl-(3′,5′)-cytidylic 3′-acid. The same six oligonucleotides were found in yeast transfer ribonucleic acid. About 30% of yeast transfer ribonucleic acid molecules contain no 1-methyladenine, and the majority of these could be methylated *in vitro* using a purified transfer ribonucleic acid methylase preparation derived from rat liver.

When [^{14}C]*S*-adenosylmethionine was used as methyl donor for methylation, the radioactive 1-methyladenine so produced was found in the same six oligonucleotides as were found in yeast and liver transfer ribonucleic acid. The basis by which a transfer ribonucleic acid methylase recognized its substrate has been discussed in terms of these results.

In 1962, Fleissner and Borek discovered a group of enzymes which they called tRNA methylases. These enzymes were able to transfer methyl groups from *S*-adenosylmethionine to tRNA, and were found in a wide variety of tissues and cell types (Srinivasan and Borek, 1963). In general, tRNA methylases extracted from one organism were unable to further methylate tRNA from the same organism, since this tRNA was already fully methylated *in vivo*. However, tRNA extracted from one organism could usually be further methylated by tRNA methylases from another (Srinivasan and Borek, 1963). These observations raised the question of the nature of the acceptor site on the tRNA molecule. What was the difference between the acceptor site which was methylated *in vivo* by an homologous tRNA methylase, and one which was methylated only *in vitro* by an heterologous methylase?

Yeast tRNA contains less 1-methyladenine than rat liver tRNA (Staehelin, 1966); its content of 0.9% indicates that only 70% of the tRNA chains are methylated. On the other hand, rat liver tRNA contains 1.3% methyladenine (Baguley and Staehelin, 1968b). Furthermore, yeast tRNA can be methylated *in vitro* using 1-adenine methylase from rat liver (Baguley and Staehelin, 1968b). We have compared the sequence of nucleotides surrounding the 1-methyladenine in rat liver tRNA with that occurring in yeast tRNA and in *in vitro* methylated yeast tRNA. For this purpose a pancreatic RNase digest of tRNA was fractionated and the 1-methyladenine-containing sequences were isolated and identified.[1]

Methods

tRNA. tRNA was prepared from rat liver according to the method of Delihas and Staehelin (1966) and from brewer's yeast according to the method of Monier *et al.* (1960).

Chromatography. Pancreatic RNase digests were prepared (Staehelin, 1961) and chromatographed on columns (1 × 50 cm) of DEAE-cellulose (Whatman DE 52, Whatman Co., England), using a gradient of sodium acetate (pH 7.0, 0.01–0.4 M) in 7 M urea (Bell *et al.*, 1964). Fractions corresponding to each desired oligonucleotide fraction were combined and diluted 5–10-fold with water. The oligonucleotide fraction was adsorbed on a column (1 × 1 cm) of DEAE-cellulose, washed with 0.02 M triethylammonium bicarbonate (pH 7.5) (60 ml), and eluted with 1 M triethylammonium bicarbonate (10 ml). The buffer was removed by rotary evaporation.

Sequence Analysis Methods. Conditions for digestion with pancreatic RNase (Boehringer, Mannheim) and T_1 RNase (Sankyo Co., Tokyo) were described by Armstrong *et al.* (1964). Micrococcal nuclease (Worthington Biochemical Corp., Freehold, N. J., 13,400 units/mg)

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[1] Nonstandard abbreviations are: mononucleotides: Ap, Gp, Cp, Up: adenylic, guanylic, cytidylic, uridylic 3′-acids. 1MeAp, 6MeAp, 1MeGp, 2MeGp, DiMeGp, ψp: 1-methyladenylic, N^6-methyladenylic, 1-methylguanylic, N^2-methylguanylic, N^2-dimethylguanylic, pseudouridylic 3′-acids, respectively; oligonucleotides: *e.g.*, ApGpCp: adenylyl-(3′,5′)-guanylyl-(3′,5′)-cytidylic 3′-acid

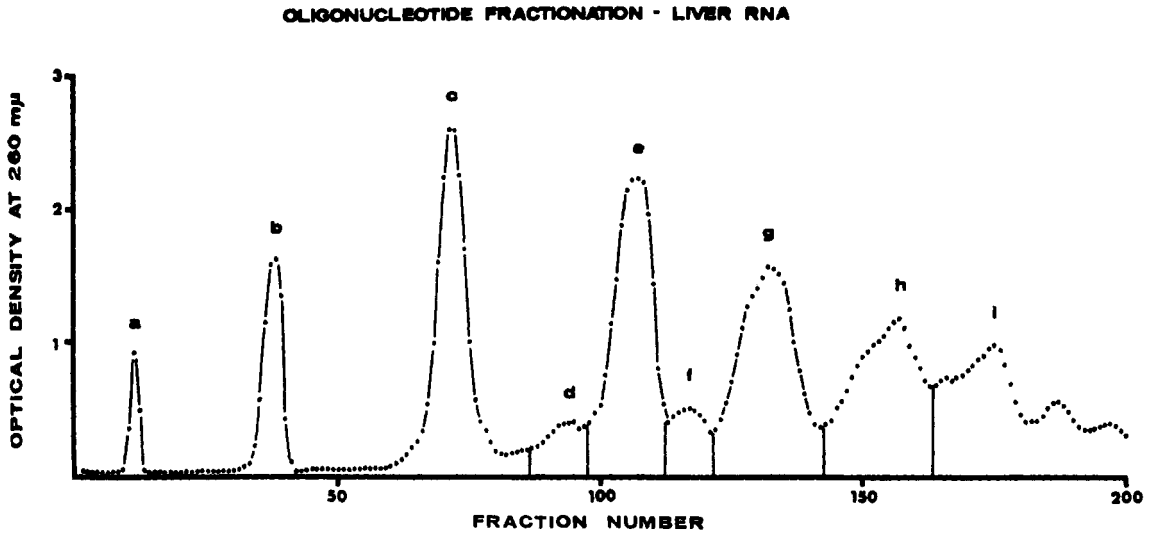

FIGURE 1: DEAE-cellulose chromatography in 7 M urea of a pancreatic RNAase digest of rat liver tRNA (500 A_{260} units). Fraction size 4.5 ml, flow rate 16 ml/hr. Optical density at 260 mμ (·····). For identification of fractions, see text.

TABLE I: Nucleotide Analysis of Oligonucleotide Peaks from DEAE-urea Columns.

Peak		Amt (A_{260})	% Nucleotide Composition				
			Gp	Ap	Cp	Up	MeAp
d	Liver	10	23	4	8	29	23
	Yeast	13	20	7	9	24	15
e, dinucleotides	Liver	70	30	17	26	20	
	Yeast	92	34	18	25	19	0.3
f	Liver	14	21	31	4	23	23
	Yeast	20	30	20	15	12	7
g, trinucleotides	Liver	88	40	22	13	19	1
	Yeast	98	38	28	18	13	0.6
h, tetranucleotides	Liver	72	43	27	9	10	
	Yeast	76	43	28	11	9	

was stored frozen at an enzyme concentration of 1 mg/ml in 0.1% bovine serum albumin. Oligonucleotide (1 A_{260} unit) was incubated at 37° for 1 hr in a solution (0.2 ml) containing enzyme (2 μl), 0.05 M Tris-Cl (pH 8.0), and 0.015 M $CaCl_2$ (Feldmann, 1967). Conditions for digestion with *Escherichia coli* alkaline phosphatase (Worthington Biochemical Corp., Freehold, N. J.) and venom phosphodiesterase (Worthington Biochemical Corp., Freehold, N. J.) and the methods of alkaline hydrolysis, two-dimensional thin-layer chromatography, and thin-layer electrophoresis have been previously described (Baguley and Staehelin, 1968a).

Methylation Conditions. 1-Adenine methylase was purified from liver homogenates of normal or leukemic Fisher rats by centrifugation, DEAE-cellulose chromatography, Sephadex G-200 chromatography, and $(NH_4)_2SO_4$ precipitation as previously described (Baguley and Staehelin, 1968b). For large-scale methylation of yeast tRNA, the reaction mixture contained 0.4 M ammonium acetate (pH 9.25), 0.1 mM dithiothreitol, 50 μM [^{14}C-methyl]*S*-adenosylmethionine (27,000 cpm/mμmole, Radiochemical Centre, Amersham), brewer's yeast tRNA (1 mg/ml), and 1-adenine methylase (2 units/ml), where 1 enzyme unit is defined as previously (Baguley and Staehelin, 1968b). Incubation was carried out at 37° for 1 hr, and tRNA was recovered by phenol extraction of the reaction mixture and precipitation of RNA from the supernatant with two volumes of ethanol.

Radioactivity Measurement. Radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer, using 0.6% 2-(4′-*t*-butylphenyl)-5-(4″-biphenyl)-1,3,4-oxdiazole (CIBA Ltd., Basle) in toluene as scintillation fluid. Samples of 0.2 ml from urea columns were mixed with ethanol (5 ml) and scintillation fluid

TABLE II: Identification of Sequence.[a]

Sequence	Method of Analysis	Products
Gp1MeApUp [0.75]	Alkaline hydrolysis	Gp, 6MeAp, Up
	(i) Phosphatase	Gp1MeApU [0.4]
	(ii) Phosphodiesterase	G, pMeA, pU
Ap1MeApUp [0.56]	Alkaline hydrolysis	Ap, 6MeAp, Up
	(i) Phosphatase	Ap1MeApU [0.05]
	(ii) Phosphodiesterase	A, pMeA, pU
Gp1MeApCp [0.41]	Alkaline hydrolysis	Gp, 6MeAp, Cp
	(i) Phosphatase	Gp1MeApC [−0.11]
	(ii) Phosphodiesterase	G, pMeA, pC
Gp1MeApApUp [0.72]	Alkaline hydrolysis	Gp, 6MeAp, Ap, Up
	(i) Phosphatase	Gp1MeApApU [0.35]
	(ii) Phosphodiesterase	G, p1MeA, pA, pU
	or (ii) T_1RNAse	Gp, 1MeApApU [0.05]
	(iii) Phosphodiesterase	1MeA, pA, pU
Ap1MeApApUp [0.57]	Phosphatase + diesterase	2A, MeA, U
	(i) Phosphatase	Ap1MeApApU [0.10]
	(ii) Phosphodiesterase	A + pMeA + pA + pU
	or (ii) Micrococcal nuclease	Ap1MeAp, Ap, 1MeAp, ApU
Gp1MeApApCp [0.37]	Phosphatase + diesterase	G, MeA, A, C
	(i) Phosphatase	Gp1MeApApC
	(ii) T_1RNAse	Gp + 1MeApApC
	(iii) Phosphodiesterase	1MeA, pA, pC

[a] (1) The figure in brackets following each oligonucleotide expresses the electrophoretic mobility at pH 2.5 with respect to Up. (2) Since pH 9 incubation conditions for phosphomonoesterase and phosphodiesterase caused some conversion of 1MeA into 6MeA, the term "MeA" is used to express a mixture of the two types. (3) Micrococcal RNase digestion products were chromatographed in propyl alcohol–NH_3. (4) Lack of material allowed only tentative identification of the hydrolysis products of the sequence Gp1MeApApCp, which were identified mainly by their chromatographic positions.

(10 ml). Autoradiographs of thin-layer plates were made with Gevaert X-ray film, using exposures of 1–5 days.

Results

1-Methyladenine-Containing Sequences in Rat Liver tRNA. When rat liver tRNA was alkali hydrolyzed, 1.3% of the liberated nucleotides was found to be N^6-methyladenylic acid (Baguley and Staehelin, 1968b). This nucleotide is actually present in the tRNA as 1-methyladenylic acid (Dunn, 1961) and is converted into N^6-methyladenylic acid under the conditions of alkaline hydrolysis (Brookes and Lawley, 1960). Rat liver tRNA therefore contains approximately one 1-methyladenine per tRNA chain. In order to investigate the environment of the 1-methyladenine in the primary structure of the tRNA molecule, a pancreatic RNase digest of rat liver tRNA was fractionated by DEAE-cellulose column chromatography. A gradient of sodium acetate in 7 M urea was utilized in order to fractionate oligonucleotides according to their negative charge. A similar type of experiment carried out by Bell *et al.* (1964) with yeast tRNA indicated that methyladenine was found predominately in a dinucleotide fraction.

Figure 1 shows a typical oligonucleotide fractionation under our conditions. Oligonucleotide fractions were recovered by adsorption to and elution from small DEAE-cellulose columns. Nucleotide analysis (alkaline hydrolysis followed by two-dimensional chromatography) of aliquots of each of these fractions (Table I) indicated that fractions e, g, and h contained mainly di-, tri-, and tetranucleotides, respectively, because of the purine:pyrimidine ratios. Fractions a, b, and c were assumed to consist of nucleosides, cyclic mononucleotides, and mononucleotides, respectively (Staehelin, 1963).

The distribution of the minor nucleotides was, as previously found (Bell *et al.*, 1964; Staehelin, 1964), nonrandom. N^2-Dimethylguanylic acid was found in fractions d and e, 1-methylguanylic and N^2-methylguanylic acids were found in fractions f and g, and ribothymidylic acid was found mainly in fraction h. The existence of the oligonucleotides DiMeGpCp, DiMeGpψp, and 1MeGp2MeGpCp in yeast tRNA has been reported by

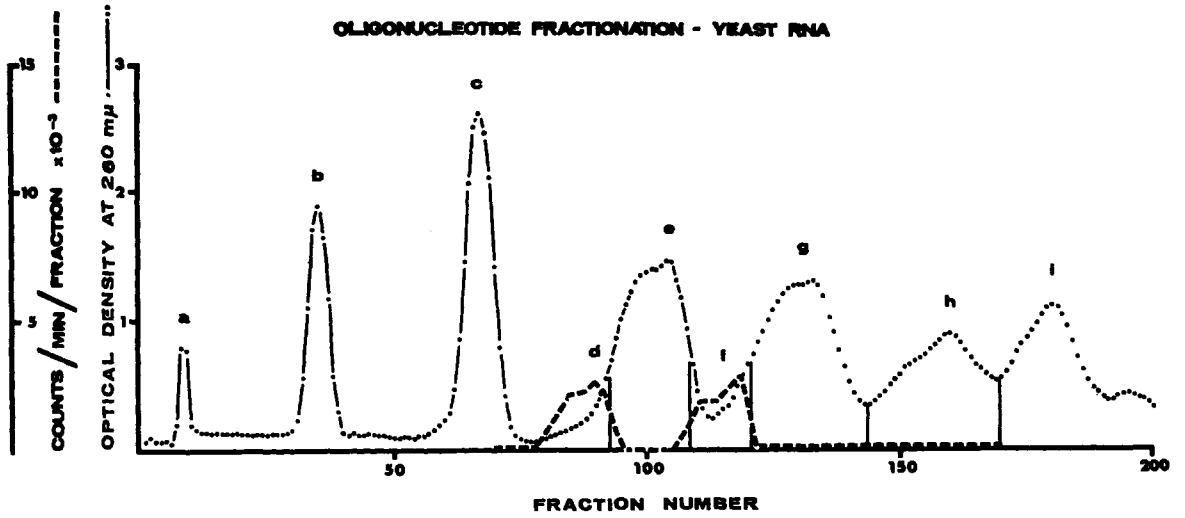

FIGURE 2: DEAE-cellulose chromatography in 7 M urea of a pancreatic RNase digest of yeast tRNA (550 A_{260} units). A portion of the RNA had previously been methylated *in vitro* using a 1-adenine methylase preparation from rat liver. Fraction size 4.5 ml, flow rate 22 ml/hr. Optical density at 260 mμ (·····); [^{14}C]methyl radioactivity (——). For identification of fractions, see text.

Staehelin (1964). The 1-methyladenylic acid containing fractions d and f were eluted just prior to the major dinucleotide and trinucleotide, respectively. The base composition of fraction d, however, suggested that this fraction contained tri- rather than dinucleotides. The possession of a positive charge on 1-methyladenylic acid at this pH (Brookes and Lawley, 1960) explained why these trinucleotides eluted at a position one full charge ahead of other trinucleotides. An aliquot from peak f was dephosphorylated, and then heated at pH 9 and 100° for 1 hr, a procedure which converted 1-methyladenylic acid into 6-methyladenylic acid (which has no positive charge at pH 7) without hydrolyzing phosphodiester bonds (Baguley and Staehelin, 1968a). The N^6-methyladenylic acid containing material then eluted from a DEAE-urea column in the position of a dephosphorylated tetranucleotide.

Fractions d and f were then each fractionated by electrophoresis into a number of bands, and the nucleotide content of each band was determined. Methyladenylic acid containing bands were further purified by dephosphorylation with alkaline phosphatase followed by electrophoresis. The methods used for the sequence identification of these components are summarized in Table II. Material in fraction d yielded the sequences GpMeApUp, ApMeApUp, and GpMeApCp, while material in peak f yielded GpMeApApUp, ApMeApApUp, and possibly GpMeApApCp. The approximate content of each sequence in rat liver tRNA is shown in Table III. Material in peak g, which contained a small amount of 1-methyladenylic acid, was not investigated. It is therefore possible that other sequences occur but were not detected. However, at least 90% of the 1-methyladenylic acid occuring in rat liver tRNA was accounted for in the six identified sequences.

TABLE III: Approximate Contents of Methyladenine-Containing Sequences in Liver and Yeast tRNA.

Sequence	Sequences/Molecule: Rat Liver tRNA	Sequences/Molecule: Yeast tRNA	% of Radioactivity *in Vitro* Methylation
Total trinucleotide sequences	0.35	0.30	47
GpMeApUp	0.20	0.20	35
ApMeApUp	0.05	0.05	4
GpMeApCp	0.10	0.05	8
Total tetranucleotide sequences	0.55	0.25	53
GpMeApApUp	0.30	0.10	23
ApMeApApUp	0.20	0.10	20
GpMeApApCp	0.05	0.05	10

1-Methyladenine-Containing Sequences in Yeast tRNA. Yeast tRNA was investigated in the same manner as was rat tRNA. However, a slight modification of the procedure enabled simultaneous investigation of the 1-methyladenine-containing sequences produced *in vitro* in yeast tRNA by the action of 1-adenine methylase derived from rat liver. Livers from leukemic rats were used as a convenient source of enzyme since their methylase content is higher than normal (Baguley and Staehelin, 1968b). Yeast tRNA (3 mg) was methylated to an extent of 1 mμmole of methyl group incorporation/mg of tRNA. Methylation was carried out with a limiting amount of enzyme to avoid methylation by enzymes other than 1-adenine methylase. The methylated RNA was isolated from the reaction mixture, mixed with further yeast tRNA (17 mg), and hydrolyzed with pancreatic RNase. The digest was chromatographed on DEAE-cellulose in the presence of urea. Because the 1-methyladenine produced *in vitro* amounted to only 0.6% of the total 1-

methyladenine in the digest, optical density quantities of 1-methyladenine-containing oligonucleotides reflected their occurrence in yeast tRNA, whereas radioactivity represented sequences methylated *in vitro* (Figure 2).

The optical density profile of eluted oligonucleotides was similar to that obtained for the rat liver tRNA digest. The distribution of methylated guanylic acids was similar to that of liver RNA, but ribothymidylic acid was found in peaks e and g as well as in h. 1-Methyladenylic acid was found to a smaller extent in fraction f than in fraction d, in contrast to the results obtained for liver tRNA (Table I). Further resolution of material from fractions d and f, by the same electrophoretic techniques as used before, yielded six 1-methyladenine-containing oligonucleotides of nucleoside composition identical with those found in liver tRNA. Furthermore, the electrophoretic position of each oligonucleotide corresponded to a radioactive component, of which the radioactivity was shown to be associated with methyladenine. The content in yeast tRNA of each 1-methyladenine-containing sequence and also the relative contents of each of the six radioactive bands detected by autoradiography are shown in Table III.

Since Tsutsui *et al.* (1966) have suggested that tRNA methylases from tumur tissues have altered substrate specificity, it was of interest to know if 1-adenine methylase from livers of normal and leukemic rats recognized the same sequences in yeast tRNA. The experiment was therefore repeated using 1-adenine methylase prepared from normal rat liver. Again, radioactivity was found associated with all six 1-methyladenine-containing oligonucleotides. The question of substrate specificity of 1-adenine methylase from leukemic tissue has been discussed in a separate publication (Baguley and Staehelin, 1968b).

Discussion

Brewer's yeast tRNA contains about 0.7 1-methyladenine group/molecule (Staehelin, 1966), whereas rat liver tRNA contains approximately one per molecule (Baguley and Staehelin, 1968b). We have therefore asked the question why 30% of yeast tRNA molecules contain no 1-methyladenine.

Since yeast tRNA can be methylated *in vitro* by 1-adenine methylase from rat tissue to a level of at least 0.2 methyl group/molecule (Baguley and Staehelin, 1968b), there appears to be a number of sites for adenine methylation in yeast tRNA which are not recognized by yeast adenine methylases. Phillips and Kjellin-Stråby (1967) have shown that the 1-methyladenine content of yeast tRNA is always less than one residue per molecule under a number of different growth conditions. Furthermore, they have shown that when yeast tRNA is incubated with *S*-adenosylmethionine and yeast methylases, little further methylation of adenine occurs. It therefore seems possible that the yeast adenine methylase is incapable of recognizing certain tRNA species. This supposition is supported by the published structures of individual yeast tRNA species, some of which contain 1-methyladenine and others of which do not (see later discussion).

In the present work, we have investigated the possibility that the sequences of nucleotides surrounding sites of *in vitro* adenine methylation differ from those surrounding sites of *in vivo* methylation. This would provide an explanation as to why yeast adenine methylase does not recognize all tRNA species. In a previous publication (Baguley and Staehelin, 1968a), we have shown that the sequence of nucleotides surrounding sites of adenine methylation in *E. coli* tRNA can be conveniently studied by analysis of oligonucleotides released by pancreatic RNase digestion. We have therefore looked for quantitative changes between sequences methylated *in vivo* in yeast or rat liver tRNA, and those methylated *in vitro* in yeast tRNA using adenine methylase from rat liver. Our results show that in all three cases the same six sequences were present. These were identified as Gp1MeApUp, Ap1MeApUp, Gp1MeApCp, Gp1MeApApUp, Ap1MeApApUp, and Gp1MeApApCp.

Since the sequences which are not methylated *in vivo* in yeast tRNA are similar to those which are methylated, it appears that the sequence surrounding the adenine methylation site does not determine which yeast tRNA molecules are methylated *in vivo*. (Nevertheless, surrounding sequence could still be important for enzyme recognition.) The tertiary structure of tRNA is therefore implicated, a factor which has already been shown to be important in recognition of tRNA by the aminoacyl synthetases (Lindahl *et al.*, 1966; Gartland and Sueoka, 1966).

It is interesting to compare the present results with those obtained for *E. coli* tRNA, which contains no 1-methyladenine (Dunn, 1961). *E. coli* tRNA may be methylated *in vitro*, using rat tissue enzyme, to a level approaching one 1-methyladenine per molecule (Baguley and Staehelin, 1968b). The majority of the 1-methyladenine is found in the sequence Gp1MeApApUp (Baguley and Staehelin, 1968a). Studies using *E. coli* B tRNA methylated *in vitro* to this maximum extent have shown that approximately 81% of the 1-methyladenine so formed is found in this sequence, while 12% is found in Ap1MeApApUp and 7% is found in Gp1MeApUp (B.C. Baguley, unpublished results). Thus all three types of tRNA have similar nucleotide sequences in this region.

The elucidation, in recent years, of the primary structure of a number of different tRNA species greatly facilitates the discussion about sites of methylation. The results so far show that 1-methyladenine is located specifically in the ribothymidine-containing loop, 19 nucleotides from the amino acid acceptor end. 1-Methyladenine occurs in the sequence Gp1MeApUp in phenylalanine tRNA (Raj Bhandary *et al.*, 1967) and valine tRNA from yeast (Baev *et al.*, 1966; Takemura *et al.*, 1968) and in the sequence Gp1MeApCp in tyrosine tRNA from yeast (Madison *et al.*, 1966). Of those molecules not containing 1-methyladenine, alanine tRNA from yeast contains in the corresponding position the sequence GpApUp (Holley *et al.*, 1965), serine tRNA I from yeast (Zachau *et al.*, 1966) and *N*-formylmethionine tRNA from *E. coli* (Dube *et al.*, 1968) contain ApApApUp, and tyrosine tRNA from *E. coli* contains

GpApApUp (Goodman *et al.*, 1968). It is therefore likely that *in vitro* 1-adenine methylation occurs in this loop, an assumption which has now been verified in the case of serine tRNA I from yeast (B. C. Baguley, W. Wehrli, and M. Staehelin, manuscript in preparation).

The "cloverleaf" model for alanyl RNA, as proposed by Holley and coworkers (1965), contains the following loop of seven nucleotides.

$$\begin{array}{l}\ldots(\mathrm{Gp})\mathrm{Tp}\psi\mathrm{pCpGpApUpUp}(\mathrm{Cp}) \\ \quad 1\ 2\ 3\ 4\ 5\ 6\ 7\end{array}$$

Although it is not yet known if the "cloverleaf" model is general for all tRNA molecules, there is now reasonable evidence that a sequence related to that above is found in all tRNA molecules. In fact, on the basis of our present results and of published tRNA sequence data, one can enumerate the restrictions in the generalized structure of the above loop. Zamir *et al.* (1965) have shown that the sequence (Gp)TpψpCpGp occurs commonly in *E. coli*, yeast, and rat liver tRNA. The first three positions in the loop are therefore taken up by Tp, ψp, and Cp, respectively. The possibility that positions 1 and/or 2 may sometimes be occupied by unmodified uridine nucleotides has been suggested (Zamir *et al.*, 1965; Sarkar and Comb, 1966). This is very probable in rat liver tRNA, which contains less than one thymidine residue per molecule (Baguley and Staehelin, 1968b). In the remainder of the loop, position 4 is taken up by Gp or Ap, position 5 is always Ap or 1MeAp, position 6 is unrestricted (but is usually Ap or Up), and position 7 is probably a pyrimidine nucleotide.

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