THE ABSENCE OF RIBOTHYMIDINE IN SPECIFIC EUKARYOTIC TRANSFER RNAS: I. GLYCINE AND THREONINE tRNAS OF WHEAT EMBRYO.*

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Summary

Ribothymidine, generally considered a universal nucleotide in tRNA, is completely absent in five specific wheat embryo tRNAs. These consist of two species of glycine tRNA and three species of threonine tRNA. These tRNAs, all extensively purified, are acceptable substrates for E. coli - ribothymidine forming-uracil methylase, which produces one mole of ribothymidine per mole of tRNA. These five tRNAs account for about 90% of the wheat embryo tRNAs which are substrates for this methylase. Nucleotide sequence analysis of one of these tRNAs, $tRNA_I^{Gly}$, confirmed both the complete absence of ribothymidine at position 23 from the 3'end, and the presence of uridine at that site instead. In addition, it is shown that methylation with E. coli uracil methylase quantitatively converts uridine at position 23 to ribothymidine, while no other uridine in the molecule is affected.

Using E. coli uracil methylase as an assay we have detected this class of ribothymidine lacking tRNA, in each case consisting of a few specific species, in other higher organisms, such as wheat seedling, fetal calf liver and beef liver, in addition to wheat embryo. We could not detect this class of tRNA in E. coli or yeast tRNA.

Except for the eukaryotic tRNAs which initiate protein synthesis (1,2) and specific Staphylococcus epidermidus tRNAs which are not involved in protein synthesis (3) all of the approximately 50 tRNAs sequenced to date contain ribothymidine as the 23rd nucleotide from the 3'end (4). Due to this very wide occurence, ribothymidine is considered a universal nucleotide in tRNA. Ribothymidine is generally thought to play a role in protein synthesis. Indeed there is an excellent correlation in the tRNAs sequenced to date of the presence of ribothymidine in a tRNA and the functioning of that tRNA in the non-initiation steps of protein synthesis. However, the precise role of ribothymidine in tRNA remains unclear and, at least in E. coli, this modified base appears not to be essential for protein synthesis (5-8).

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Although the great majority of wheat embryo tRNAs contain ribothymidine (9) including the only wheat tRNA sequenced to date, wheat $tRNA^{Phe}$ (10), we have now found that this particular base modification is completely absent in several wheat embryo tRNAs (11). In addition, we have found that this class of ribothymidine-lacking tRNAs, which in each case consists of a few specific species, is also present in other higher eukaryotes such as wheat seedling, fetal calf liver and beef liver. These tRNAs were shown, in part, to lack ribothymidine by their ability to be methylated with crude E. coli-ribothymidine forming-uracil methylase and (methyl ^{14}C) S-adenosyl-L-methionine (SAM) as the methyl donor. In this paper we discuss the ribothymidine-lacking tRNAs of wheat embryo.

MATERIALS AND METHODS: Wheat embryo tRNA was isolated using phenol extraction, high salt precipitation and DEAE cellulose chromatography as previously described (10). Crude methylase from E. coli MRE-600 was prepared using the same procedure employed to prepare aminoacyl tRNA synthetases and is described in detail elsewhere (12). This procedure consists essentially of DEAE cellulose chromatography, ammonium sulfate precipitation and Sephadex G-25 chromatography.

The methylation reaction was performed in a 1.0 ml incubation mixture consisting of: 50 mM phosphate pH 8.2; 1 mM EDTA; 1 mM $MgCl_2$; 50 uM (^{14}C) SAM (10 Ci/mol); 0.1 to 5.0 A_{260} units of tRNA and a saturating amount of crude E. coli uracil methylase. Incubation was at 30°C for 3-6 hours and the reaction was terminated by the addition of 2 ml of cold 10% trichloroacetic acid (TCA). The precipitate was collected on a Whatman GF/A filter (previously moistened with cold 2% TCA), washed 5 times with 5 ml portions of cold 2% TCA, dried at 150°C for 30 minutes and counted in Omnifluor-Toluene. Aminoacylation reactions were performed at pH 7.6 as previously described (13).

Pancreatic ribonuclease (Worthington) and ribonuclease T_1 (Calbiochem) digestions, and the separation of these digestion products on DEAE cellulose have been described in detail elsewhere (14). Ribonuclease T_2 (Calbiochem) digestions were run as described (15). The two dimensional thin layer chromatographic system consists of cellulose plates (Eastman Chromogram Sheets #13255) 20 x 20 cm, with the solvent systems: first dimension, isobutyric acid -1M ammonium hydroxide, 50:30, v/v; second dimension, isopropanol- concentrated hydrochloric acid- water, 68:17.1:14.4, v/v/v as previously described (14).

RESULTS AND DISCUSSION: Five specific wheat embryo tRNAs, consisting of two species of glycine tRNA and three species of threonine tRNA, completely lack ribothymidine. This was shown, in part, by their ability to be methylated with crude E. coli - ribothymidine forming-uracil methylase (see Table I). Each of these five tRNAs could be quantitatively methylated by the crude E. coli uracil methylase, i.e., to the extent of one mole of methyl group attached per mole of tRNA. In each case the sole product of the methylation reaction was shown to be ribothymidine by ribonuclease T_2 digestion of the methylated tRNA and two-dimensional thin layer chromatographic analysis of

the nucleotides produced. Thus, although crude E. coli methylase was used, ribothymidine was the only methylated product produced.

These five tRNAs were isolated using their ability to be methylated with the crude E. coli uracil methylase as an assay, and account for approximately 90% of the methyl accepting tRNAs of wheat embryo. The two glycine tRNAs were each obtained in pure form and the three threonine tRNAs were each obtained about 60% pure. These tRNAs were isolated using BD-cellulose (16) and high pressure liquid chromatography on RPC #5 (17) both at neutral and acidic pH. Throughout the isolation of these tRNAs there was a complete coincidence of the methyl acceptance and the glycine or threonine acceptor activity.

To further confirm the absence of ribothymidine and to more completely characterize this class of tRNAs, we examined pure wheat embryo $tRNA^{Gly}_{I}$ in further detail. In separate experiments, this tRNA was digested with either RNase T_1 or pancreatic RNase and the products separated on DEAE cellulose columns. All of these digestion products were completely analyzed and none of them contained ribothymidine, thus confirming its absence in this molecule. The same results were obtained with pure wheat embryo $tRNA^{Gly}_{2}$. One of the RNase T_1 digestion products of $tRNA^{Gly}_{I}$ was found to be a tetranucleotide with the sequence U-Ψ-C-Gp, instead of the usual tetranucleotide T-Ψ-C-Gp found in other tRNAs. We have confirmed that this RNase T_1 fragment (U-Ψ-C-Gp) is located in the "ribothymidine loop" by the isolation of a partial RNase T_1 product consisting of one third of the molecule containing the 3'end (see Fig. 1). In addition to having ribothymidine replaced by uracil, the adjacent nucleotide Ψ (at position 22 from the 3' end) which is also essentially a universal nucleotide, is partially replaced by guanine. Thus there are two sequences in this region of the molecule, approximately 2/3 of the molecules contain U-Ψ-C-Gp, and 1/3 of the molecules have this sequence replaced by U-G-C-Gp. Interestingly, this sequence U-Gp, in place of the usually found T-Ψp, is the exact sequence found in the Staphylococcus epidermidus glycine tRNAs sequenced by Roberts (3), which do not function in protein synthesis.

Next, it remained to be shown that E. coli uracil methylase specifically converts uridine at position 23 to ribothymidine and does not methylate uridine located at other sites in the molecule. Therefore wheat embryo $tRNA^{Gly}_{I}$ was methylated by crude E. coli uracil methylase with ^{14}C-SAM. Since there are two sequences in the "ribothymidine loop" of the molecule, following RNase T_1 digestion we should obtain two labeled oligonucleotides, one with the sequence T-Ψ-C-Gp and the other should be a dinucleotide with the sequence T-Gp and they should be in the ratio 2:1. As shown in Fig. 2, this

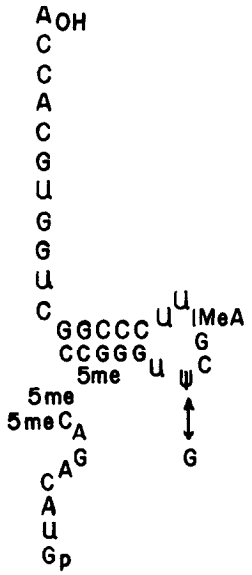

Figure 1

The 3' Terminus of Wheat Germ $tRNA^{Gly}_{I}$

This fragment was obtained from a partial ribonuclease T_1 digest and was purified as described (12).

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is exactly what was observed. In addition to confirming the degeneracy in this region of the molecule, this experiment also shows that the E. coli uracil methylase only methylates position 23 from the 3' end. No other uridine in any other location was found to be converted to ribothymidine. This was also confirmed by complete pancreatic RNase digestion of this methylated tRNA as shown in Fig. 3, where the sole methylated product obtained was the ribothymidine in the sequence G-G-G-Tp.

Wheat embryo $tRNA^{Gly}_{I}$ is apparently not sequestered from the tRNA modifying enzymes since it does contain the modified nucleotides 2'OmeC, Ψ, m^1A, m^1G, m^5C and diHU. The complete sequence of this tRNA has been determined and will be reported elsewhere (18). The mechanism whereby the five specific tRNAs of Table I do not contain ribothymidine, whereas the great majority of wheat embryo tRNAs do contain this specific base modification is unknown. This may simply be due to the specificity of the wheat embryo-ribothymidine forming-uracil methylase, or possibly because these tRNAs are specifically sequestered from this specific methylase, or perhaps a less straightforward mechanism is involved. This question is currently being pursued by the isolation of the wheat embryo uracil methylase.

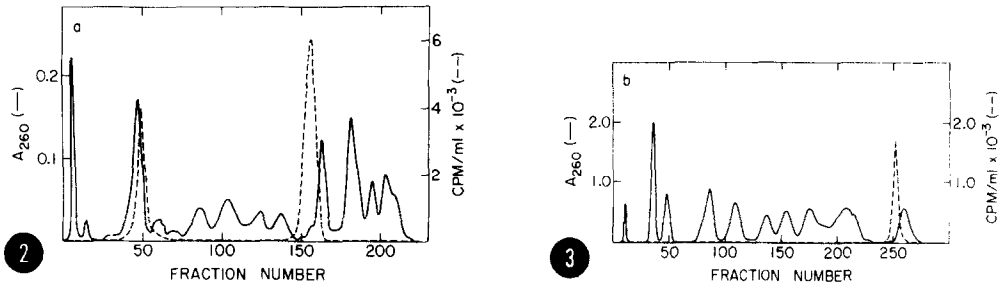

Figure 2

Ribonuclease T_1 Digestion Pattern of ^{14}C-Methylated Wheat Germ $tRNA^{Gly}_I$

0.7 mg of pure wheat germ $tRNA^{Gly}_I$ was methylated essentially as described in Materials and Methods. The reaction was allowed to proceed for 4h at which time 10% of the tRNA molecules were methylated, the remainder serving as carrier. The reaction was terminated by the addition of two volumes of water saturated phenol and the tRNA precipitated from the extracted aqueous phase with two volumes of 100% ethanol and several drops of 2M sodium acetate. The tRNA was then centrifuged and the pellet washed three times with 100% ethanol. After lyophilization, the dried tRNA was dissolved in 0.05M Tris (pH 7.6) and digested with 175 units of ribonuclease T_1 for 6h at 37°C. The digested sample was loaded onto a DEAE Cellulose (carbonate) column (0.4 cm x 100 cm) equilibrated in 0.025M $(NH_4)_2CO_3$ (pH 8.7). Two linear gradients 0.025M to 0.25M $(NH_4)_2CO_3$ (pH 8.8) (100 ml each) and 0.25M to 0.60M $(NH_4)_2CO_3$ (pH 8.8) (50 ml each) were generated in succession. The second gradient began at fraction 155. The column, run at 250 lb/inch^2, had a flow rate of 1.2 ml/min and a total running time of 4h. The first radioactive peak (fractions 40 to 55) was found to be T-Gp by ribonuclease T_2 digestion followed by thin layer chromatography (TLC) as described. Similarly, the second radioactive peak (fractions 145 to 165) was shown to be T-Ψ-C-Gp.

Figure 3

Pancreatic Ribonuclease Digestion Pattern of ^{14}C-Methylated Wheat Germ $tRNA^{Gly}_I$

0.03 mg (0.6 A_{260} units) of pure wheat germ $tRNA^{Gly}_I$ was methylated to completion essentially as described in Materials and Methods. The reaction was terminated by the addition of two volumes of water saturated phenol and 2 mg of wheat germ $tRNA^{Gly}_I$ was added to the extracted aqueous phase as carrier. The tRNA was precipitated with two volumes of 100% ethanol and several drops of 2M sodium acetate. After lyophilization, the dried tRNA was dissolved in 0.05M Tris (pH 7.6) and digested with 0.1 mg of pancreatic ribonuclease for 12h at 37°C. The digested sample was loaded onto a DEAE Cellulose (carbonate) column (0.4 cm x 40 cm) equilibrated with 0.015M $(NH_4)_2CO_3$ (pH 8.7). Two linear gradients 0.015M to 0.25M $(NH_4)_2CO_3$ (pH 8.8) (100 ml each) and 0.25M to 0.80M $(NH_4)_2CO_3$ (pH 8.8)(70 ml each) were generated in succession. The second gradient began at fraction 207. The column, run at 250 lb/inch^2, had a flow rate of .8 ml/min and a total running time of 5⅓h. The only radioactive peak (fractions 245 to 257) was found to be G-G-G-Tp by digestion with 0.3M KOH at 37°C for 16 h followed by TLC as described.

Table 1

Wheat Embryo tRNAs methylated by E. coli Uracil Methylase

tRNA	Purity nmoles amino acid accepted/A_{260} unit	Ribothymidine Formed nmoles/nmole tRNA
Wheat Gly 1	1.6	.98
Wheat Gly 2	1.5	.90
Wheat Thr 1	.8	.90
Wheat Thr 2	.8	1.10
Wheat Thr 3	.8	.92

In wheat embryo we have found four species of glycine tRNA, separable on RPC #5 (Plaskon) at pH 7.0, only two of which, $tRNA^{Gly}_1$ and $tRNA^{Gly}_2$, are substrates for E. coli uracil methylase. The other two glycine tRNAs are not substrates for E. coli uracil methylase, probably because they already contain ribothymidine in the normal position. In the case of threonine, we have found three threonine tRNAs in wheat embryo, separable on BD-cellulose at pH 4.5, which completely lack ribothymidine. The possible presence of additional threonine tRNAs which are not substrates for the E. coli uracil methylase cannot be excluded.

As mentioned above, we wished to determine if other organisms contain a class of tRNAs which specifically lack ribothymidine. The crude tRNAs of wheat seedling (10-20 day old), fetal calf liver and beef liver have therefore been examined and here too it was found that a few specific tRNAs lack ribothymidine. In addition, we have examined the crude tRNA from E. coli and yeast and could not find any tRNAs which were substrates for E. coli uracil methylase. This is in agreement with the many E. coli and yeast tRNA sequences which are known, none of which contains uridine at position 23 from the 3' end. Thus it appears that this class of tRNAs, lacking ribothymidine and having uridine in its place, may be limited to higher organisms.

The approach of using E. coli uracil methylase as an assay to detect tRNAs which lack ribothymidine has distinct limitations. For example, only tRNAs which have uridine at position 23 can be detected by this approach, and species such as the eukaryotic initiator tRNAs, which have adenosine at that site (1,2) are missed using this detection procedure. In addition, it is certainly possible that some tRNAs which have uridine at position 23 are not acceptable substrates for the E. coli uracil methylase. Thus this procedure allows one to determine the minimum number of tRNAs which lack ribothymidine in a specific organism, while the total number of such tRNAs may be somewhat larger.

The functional significance of the complete absence of ribothymidine in specific eukaryotic tRNAs is unknown. It is tempting to propose, however, that these specific tRNAs are involved in translational control processes and that the absence of ribothymidine, perhaps at specific developmental stages, retards the functioning of that specific tRNA. Indeed the nucleotide sequence T-Ψ-C-G was found to compete with tRNA for ribosome binding (19). Perhaps efficient interactions of tRNAs with the acceptor sites of eukaryotic ribosomes require the presence of ribothymidine at position 23. Attempts to find support for this model are in progress.

References

1. Simsek, M., Ziegenmeyer, J., Heckman, J. and RajBhandary, U.L. (1973) Proc. Natl. Acad. Sci. U.S. 70, 1041-1045.
2. Petrissant, G. (1973) Proc. Natl. Acad. Sci. U.S. 70, 1046-1049.
3. Roberts, R.J. (1972) Nature New Biol. 237, 44-45.
4. Dirheimer, G., Ebel, J.P., Bonnet, J., Gangloff, J., Keith, G., Krebs, B., Kuntzel, B., Roy, A., Weissenbach, J. and Werner, C. (1972) Biochimie 54, 27-144.
5. Bjork, G.R. and Isaksson, L.A. (1970) J. Mol. Biol. 51, 83-100.
6. Bjork, G.R. and Neidhardt, F.C. (1971) Cancer Res. 31, 706-709.
7. Johnson, L., Hayashi, H. and Soll, D. (1970) Biochemistry 9, 2823-2831.
8. Yang, S., Reinitz, E.R. and Gefter, M.L. (1973) Arch. Biochem. Biophys. 157, 55-62.
9. Hall, R. (1971) The Modified Nucleosides in Nucleic Acids p. 166. Columbia University Press, New York.
10. Dudock, B.S., Katz, G., Taylor, E.K. and Holley, R.W. (1969) Proc. Natl. Acad. Sci. U.S. 62,941-945.
11. Roe, B., Mignery, R., Marcu, K., Sirover, M. and Dudock, B. (1973) Fed. Proc. 32, 585.
12. Roe, B., Marcu, K. and Dudock, B. (1973) Biochim. Biophys. Acta 319, 25-36.
13. Dudock, B., DiPeri, C., Scileppi, K. and Reszelbach, R. (1971) Proc. Natl. Acad. Sci. U.S. 68, 681-684.
14. Katz, G. and Dudock, B.S. (1969) J. Biol. Chem. 244, 3062-3068.
15. Harada, F., Kimura, F. and Nishimura, S. (1971) Biochemistry 10, 3269-3277.
16. Gillam, I., Millward, S., Blew, D., von Tigerstrom, M., Wimmer, E. and Tener, G.M. (1967) Biochemistry 6, 3043-3056.
17. Pearson, R.L., Weiss, J.F. and Kelmers, A.D. (1971) Biochim. Biophys. Acta 228, 770-774.
18. Mignery, R., Marcu, K. and Dudock, B. manuscript in preparation.
19. Ofengand, J. and Henes, C. (1969) J. Biol. Chem. 244, 6241-6253.