Stoichiometry of Yeast Nucleotidyl Transferase and Effect on the Addition Reaction of Stepwise Removal of Nucleotides from the Acceptor End of the Transfer Ribonucleic Acid*

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ABSTRACT: A highly purified preparation of yeast nucleotidyl transferase was used to study the forward and reverse reactions of this enzyme with yeast tRNA. Freshly isolated yeast tRNA consists of the following population of molecules: 25% of the chains have the complete terminal triplet pCpCpA, 50% are lacking the terminal AMP residue, and 25% are lacking both the terminal AMP residue and the adjacent CMP residue. Essentially none of the chains are lacking the second CMP residue. Further, pyrophosphorolysis, the reverse of the addition reaction, will not remove this second CMP residue. If, however, the entire triplet is removed by periodate–amine degradation, the transferase is competent to replace it. The periodate–amine stepwise degradation technique of Khym and Uziel (*Biochemistry 7*, 422 (1968)) was used to examine the effect on the transferase reaction of removal of nucleotides one at a time from the acceptor end of unfractionated tRNA from yeast, rabbit liver, and *Escherichia coli* and of purified yeast tRNA specific for phenylalanine, alanine, and arginine. It was found that the enzyme adds only those nucleotides necessary to complete the terminal pCpCpA triplet and that if four nucleotides are removed, the enzyme is inactive with that tRNA species. It was further shown, with the purified tRNA fractions, that the enzyme restores amino acid acceptor capacity to those tRNA species that have lost it by virtue of removal of their terminal triplets.

Studies of the stoichiometry of nucleotidyl transferase, the enzyme which catalyzes the reaction

$$\text{tRNA} + \text{CTP} + \text{ATP} \xrightarrow{Mg^{2+}} \text{tRNApCpCpA}^{1} + \text{PP}_i$$

have been handicapped by the inhomogeneity of the tRNA used as substrate. tRNA is composed of many molecular species with mixed end-group composition. In some of the earlier studies, pyrophosphorolysis, the reverse of the above reaction in the presence of excess pyrophosphate, was used to prepare tRNA lacking the pCpCpA triplet. However, we have found this technique to be unsatisfactory.

In the present work, we have used tRNA fractions highly enriched for accepting single amino acids and the periodate–amine degradation technique of Khym and Uziel (1968), which permits stepwise removal of nucleotides from the acceptor end of tRNA. We have determined the end-group composition of freshly isolated yeast tRNA, and we have observed the inadequacies of the pyrophosphorolysis technique for removal of terminal nucleotides.

A second area of interest is the effect, on the above reaction, of the sequential removal of nucleotides from the pCpCpA end of tRNA. The removal was accomplished by use of the periodate–amine technique mentioned above, and its effect was studied in unfractionated tRNA from yeast, rabbit liver, and *Escherichia coli* and in the purified tRNA species from yeast. It was shown that the nucleotidyl transferase restored amino acid accepting capacity to those purified tRNAs from which three nucleotides had been removed, but that in all cases, removal of the fourth nucleotide, counting the terminal adenylic acid as one, rendered the tRNA unable to accept nucleotides from the nucleotidyl transferase.

Materials and Methods

Enzymes. The terminal adding enzyme was purified from yeast according to a method reported in the preceding paper. Bacterial alkaline phosphatase was purchased from Worthington Enzymes (Grade BAP/C).

tRNA. Rabbit liver tRNA was prepared by phenol extraction. Rabbit liver (500 g) was frozen in liquid nitrogen and powdered with a pestle in a mortar containing liquid nitrogen. To this powder were added 500 ml of buffer, 0.1 M Tris (pH 7.5), 0.01 M $MgCl_2$, 0.05 M KCl and 0.005 M EDTA, and 500 ml of phenol. The mixture was stirred for 30 min and then centrifuged to separate the aqueous phase from the phenol phase. Then the phenol phase was reextracted with another aliquot of the buffer. The two aqueous phases were pooled and the RNA precipitated by the addition of 2.5 volumes of absolute ethanol (−20° overnight). The precipitate was collected, dried, and dissolved in water. Insoluble material was removed by centrifugation and the RNA was reprecipitated with ethanol. This precipitate was dissolved in water (approximately 300 ml, A_{260} 140) and loaded on a 500 ml (5 × 25 cm) benzoylated DEAE-cellulose column

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[1] Abbreviations used are: tRNApXpCpCpA is tRNA with the complete pCpCpA triplet and X is the fourth nucleotide.

TABLE I: Periodate–Amine Stepwise Degradation of Bulk tRNA from Yeast.[a]

Step	cpm of ^3H	cpm of ^{14}C	% of Total ^3H	% of Total ^{14}C
0	15.9×10^5	4.6×10^5	100	100
1	1.0×10^5	4.1×10^5	8	90
2	13.5×10^5	0.5×10^5	85	10
3	0.8×10^5		6	0

[a] Successive degradations of freshly isolated, unfractionated yeast tRNA which had been incubated with [^3H]CTP and [^{14}C]ATP in the presence of the terminal adding enzyme. The 0 row is the amount of label in the tRNA and the succeeding rows are the amounts of label removed by the first, second, and third rounds of periodate–amine cleavage.

TABLE II: Effect of Pyrophosphorolysis on the Extent of Addition by Nucleotidyl Transferase.[a]

Pyrophosphorylation	nmoles Incorpd[b] C	nmoles Incorpd[b] A	pX	Probable Distribution (%) of End Groups pXpC	pXpCpC	pXpCpCpA
0	1.1	1.8	0	25	50	25
1	1.2	2.8	0	25	75	0
2	1.9	2.2	0	50	50	0
3	2.0	1.9	0	50	50	0

[a] Yeast tRNA (6 mg) was pyrophosphorolyzed by incubation with 1 mg of nucleotidyl transferase at 37° for 1 hr in the following medium: 4 mM potassium pyrophosphate, 8 mM $MgSO_4$, and 50 mM potassium phosphate buffer (pH 7.5). The tRNA was recovered by alcohol precipitation. One-third of the tRNA was set aside and the remainder was pyrophosphorolyzed again under the same conditions, keeping the enzyme to tRNA ratio constant. The sample was again divided and half of it pyrophosphorolyzed a third time. The original tRNA and the three pyrophosphorolyzed samples were then assayed for their capacity to accept AMP and CMP. The assays were as described in Materials and Methods, except that only one nucleoside triphosphate was added to each incubation. [b] Each incubation contained 4 nmoles of tRNA. The specific activities of the precursors were: ATP, 2 μCi/μmole; CTP, 3.33 μCi/μmole.

which had been equilibrated with 0.01 M potassium acetate buffer (pH 5.4). Elution with 0.2 M NaCl removed about 50% of the A_{260} as low molecular weight material. The tRNA was eluted from the column in 1 M NaCl–10% EtOH, and precipitated with ethanol. The precipitated tRNA was collected, dried, dissolved in water (15 ml, A_{260} 530), and stored at −20°.

E. coli tRNA was purchased from General Biochemicals, Inc., and yeast tRNA from Boehringer Mannheim Co. Prior to use, these tRNAs were chromatographed on benzoylated DEAE-cellulose columns and stored as described above. This step removes the ultraviolet-absorbing material which is present in most tRNA preparations but which will not accept amino acids or nucleotides.

The purified yeast tRNAs for phenylalanine, alanine, and arginine were prepared according to the methods of Litt (1968) and Gillam *et al.* (1968) and assayed according to a modification of the method of Madison *et al.* (1968). These had the following $A_{260}:A_{280}$ ratios and fractions of chains accepting the given amino acid: phenylalanine, 2.09, 0.45; alanine, 2.05, 0.60; arginine, 1.92, 0.30.

Periodate–Amine Degradation. The sequential removal of single nucleotides from the amino acid accepting end of tRNA was effected by the periodate–amine technique of Khym and Uziel (1968). The tRNA is carried through the reaction as the cetyltrimethylammonium salt which is soluble in concentrated (≥1 M) salt solutions but insoluble upon dilution (≤0.3 M). This four-step process involves (1) oxidation of the terminal ribose by periodate in 1.0 M lysine buffer (pH 8), (2) recovery of 3′-phosphoryl-tRNA by dilution of the reaction mixture, (3) enzymatic cleavage of the monoesterified phosphate with bacterial alkaline phosphatase, and (4) recovery of the tRNA. The tRNA thus prepared is one nucleotide shorter than the starting material, and is in a form amenable to further removals. The cetyltrimethylammonium-tRNA salt is converted to the sodium salt for use in the addition reaction by extraction with 70% ethanol 0.1 M sodium acetate according to the method of Ralph and Bellamy (1964).

Addition Reaction. The addition reaction is done in the following reaction mixture (total volume 0.125 ml): 150 μM CTP, 2 mM ATP, 10 mM Mg^{2+}, 25 mM glycine buffer (pH 9.5), 30 μM tRNA (assuming a millimolar ϵ_{260} value of 600 (Morris and Herbert, 1970); and 0.02 OD_{280} of enzyme protein. The labeled nucleoside triphosphates were purchased from Schwarz BioResearch, Inc. The tRNA and enzyme levels were chosen so that the reaction would be completed in a 40-min incubation. After the 40-min incubation at 37°, the tRNA was precipitated by the addition of 0.02 ml of 3 M perchloric acid and was plated on glass fiber filter discs (Whatman GF/C). The disks were washed with 25 ml of 0.3 M perchloric acid, dried, and counted, either in a low-background planchet counter (Beckman Instruments, Inc. Lowbeta II) or in a liquid scintillation spectrometer (Nuclear-Chicago Model 720).

Results

The end-group composition of freshly isolated yeast tRNA was determined in three ways. Table I shows the results of the stepwise periodate–amine degradation of such tRNA which has been exhaustively labeled with [^3H]CMP and [^{14}C]AMP. The tRNA accepted 0.7 nmole of AMP and 0.3 nmole of CMP per nmole of tRNA. One can clearly see that the first degradation removed almost all of the AMP and very little of the CMP, and the second degradation removed essentially all of the CMP and the remainder of the AMP. The base removed by the third round of periodate–amine degradation contained less than 5% of the original tritium radioactivity. These data suggest that the end-group composi-

TABLE III: Extent of the Addition Reaction after Stepwise Periodate–Amine Degradations.[a]

	Yeast		Rabbit		*E. coli*	
Step	C	A	C	A	C	A
1	0.05	0.76 (1)	0.02	0.36 (1)	0.07	0.68 (1)
2	0.55 (0.72)	0.64 (0.84)	0.34 (0.95)	0.25 (0.70)	0.81 (1.19)	0.69 (1)
3	1.24 (1.64)	0.59 (0.78)	0.60 (1.67)	0.22 (0.61)	0.89 (1.31)	0.24 (0.35)
4	0.15	0.09	0.05	0.07	0.11	0.06

[a] The conditions were those described in Materials and Methods except that the incubation time was 40 min. The reactions of yeast and rabbit tRNA were complete by that time, but the reaction of *E. coli* tRNA probably was not. The incubations each contained approximately 4 nmoles of tRNA. In this table, the values have been normalized to 1 nmole of tRNA. The numbers in parentheses are a further normalization to the amount of AMP incorporated after one cleavage. The specific activities of the precursors were: ATP, 2600 cpm/nmole; CTP, 5400 cpm/nmole.

tion of freshly isolated tRNA is approximately as follows: one-fourth of the chains have the end-group XpCpCpA; one-half, the end-group XpCpC; one-fourth, the end-group XpC; and essentially none of the chains are lacking the entire terminal triplet.

Corroboration for this view is provided by the experiment reported in Figure 1. Bulk yeast tRNA was incubated with labeled CTP in the presence of nucleotidyl transferase but with no ATP in the incubation. Radioactive tRNA was isolated and hydrolyzed in alkali and chromatographed on a Dowex 1 formate column in the presence of authentic cytidine, adenosine, and 2′,3′-CMP. More than 95% of the radioactivity was recovered in a position corresponding to cytidine, indicating that less than 5% of the tRNA chains were able to accept two CMP residues, because if two CMP residues were accepted, one of them would appear in the hydrolysate as 2′,3′-CMP. The small peak of radioactivity between the cytidine and 2′,3′-CMP positions is 5′-CMP, resulting from the hydrolysis of CTP incompletely separated from the tRNA after the incubation.

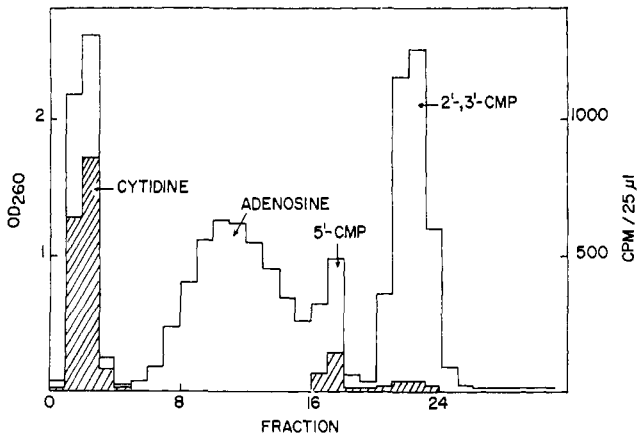

FIGURE 1: Dowex 1 chromatography of an alkaline hydrolysate of terminally labeled tRNA. 4×10^5 cpm of [^3H]tRNApCpC plus 8 A_{260} of cytidine, 7 A_{260} of adenosine, and 12 A_{260} of 2′,3′-CMP were hydrolyzed with 0.2 N NaOH for 45 min at 80°. After hydrolysis, the sample (0.55 ml) was loaded onto a 4 × 150 mm Dowex 1 formate column. The column was eluted with seven 2-ml aliquots of water and a 320-ml linear gradient from 0 to 0.3 N formic acid. More than 98% of the radioactivity loaded was recovered.

Further support is provided by studies on the nucleotide acceptor capacity of bulk yeast tRNA after pyrophosphorolysis. These data are shown in Table II and, taken with those above, clearly indicate that bulk yeast tRNA, as isolated, is of mixed end-group composition, and that pyrophosphorolysis is unsuited for the complete removal of the pCpCpA end-group of yeast tRNA.

Replacement of End Groups Removed by Periodate–Amine Cleavage. Table III shows the incorporation of AMP and CMP into unfractionated tRNA from yeast, rabbit liver, and *E. coli* after one, two, three, or four cycles of periodate–amine degradation. All of the tRNA were exhaustively charged with nonradioactive AMP and CMP before the first oxidation. The numbers for yeast and rabbit liver tRNAs represent true acceptor capacities of the given tRNA samples. The reaction with *E. coli* tRNA is not complete at 40 min, the incubation time used, so those numbers show only the extent of addition to periodate–amine-degraded *E. coli* tRNA after 40 min.

The most striking observation is that of the reaction of the enzyme with tRNA which had had four nucleotides removed. In all cases, essentially no CMP or AMP was incorporated.

It should also be noted that the tRNA is apparently being nonspecifically degraded by the periodate–amine treatment. That is, the amount of AMP accepted by once-degraded tRNA is slightly higher than the amount accepted by twice-degraded material. The cause of the nonspecific degradation is not clear. Possibilities include nuclease contamination of the bacterial alkaline phosphatase, and alkaline hydrolysis during the periodate oxidation which is run at elevated pH and temperature. Attempts to increase the level of incorporation by careful handling or slight modification of the procedure were unsuccessful.

Amino Acid Acceptor Activity of Reassembled tRNA. To see if there were differences among the tRNAs from yeast with regard to terminal addition, $tRNA_{Phe}$, $tRNA_{Ala}$, and $tRNA_{Arg}$ were subjected to periodate–amine degradation and reacted with the enzyme. After reaction with the terminal adding enzyme, the amino acid acceptor capacity of the tRNA was measured. The results of this experiment are shown in Table IV. Once again, the tRNAs from which

TABLE IV: Stepwise Degradation, Reassembly, and Amino Acid Acceptor Activity of $tRNA_{Phe}$, $tRNA_{Arg}$, and $tRNA_{Ala}$.

RNA	tRNA Purity[a]	Step	Bases Removed[b]	Bases Accepted (nmoles) C	Bases Accepted (nmoles) A	Reaction Extent[c]	Amino Acid Incorp[d]
$tRNA_{Phe}$	45	3	ACC	1.96	1.06	64	25 (28)[e]
$tRNA_{Phe}$		4	ACCA	0.39	0.17	8	
$tRNA_{Ala}$	60	3	ACC	1.54	1.14	60	10 (36)
$tRNA_{Ala}$		4	ACC, A/G = 4	0.53	0.15	7	
$tRNA_{Arg}$	30	3	ACC	1.36	0.85	45	15 (14)
$tRNA_{Arg}$		4	ACC, A/G = 1	0.23	0.07	3	

The degradation and assay conditions are those described in Materials and Methods. The incubation times are 40 min and the precursor specific activities are [^{14}C]ATP = 3×10^3 cpm/nmole and [^3H]CTP = 6×10^4 cpm/nmole. The amino acid acceptor activities were measured using enzymes purified from yeast. [a] Per cent of chains accepting given amino acid. [b] Determined spectrophotometrically. [c] Per cent of theoretical AMP incorporation assuming $A_{260}^{1mg/ml}$ 24 and molecular weight of tRNA = 2.5×10^4. [d] Per cent of chains labeled. [e] Numbers in parentheses are the products of columns 2 and 7.

four nucleotides had been removed were incapable of accepting AMP or CMP, and the three-times degraded material accepted AMP and CMP in a ratio of 1:2 and accepted amino acids to the extent that one would predict from the levels of AMP incorporation. The fact that the incorporation did not reach theoretical levels is probably due to the nonspecific degradation mentioned above.

Discussion

From the data in Tables III and IV, it is clear that the stoichiometry of the reaction is such that only those nucleotides required to complete the terminal triplet are incorporated, and that this is probably true of all the tRNA chains from yeast. That is, if one nucleotide has been removed, the enzyme will add only one AMP. If two or three residues have been removed, one CMP and one AMP or two CMP residues and one AMP, respectively, are incorporated. In no case does removal of a single nucleotide stimulate the incorporation of more than one nucleotide to replace the one removed, and removal of four nucleotides prevents the incorporation of any. This is so despite the fact that many tRNAs repeat all or part of the terminal triplet in the adjacent positions. For example, $tRNA_{Ala}$ has the terminal sequence pCpCpApCpCpA. Yet, after four periodate–amine degradations, there is no incorporation even though the remaining terminus is pCpC, which in native tRNA readily accepts AMP.

Thus, it is obvious that the enzyme can sense some measure of the length of the tRNA or of some rather long sequence unless removal of nucleotide number four disrupts the entire secondary structure. Since tRNAs differ in length and sequence, one must search for a guide site that is common to all tRNA with which the enzyme interacts. A candidate for one of the guide sites of the enzyme is the pentanucleotide pGpTpψpCpG, which is the same distance from the terminal AMP in all yeast tRNAs that have been sequenced; *i.e.*, if the terminal AMP is numbered 1, then the pseudouridine is always numbered 22 (Zachau, 1969).

It is interesting to note the species differences among the three tRNA sources. The data indicate that the yeast enzyme can probably add nucleotides to all the tRNA chains in yeast, but only half of the periodate–amine-degraded tRNA chains from rabbit liver. One possible source of the difference would be the sensitivity of a residue to the periodate treatment. If this residue were present in approximately 20% of the yeast tRNA chains and 60% of the rabbit tRNA chains, one would expect the results shown in Table III. In Table II are shown data which gave rise to this speculation. One observes that after one pyrophosphorolysis, all of the chains accept either CMP or AMP. This is in contrast to the data in Table III, in which, after one round of periodate–amine degradation, only 80% of the chains accept CMP or AMP. In the case of *E. coli*, preliminary kinetic data suggest that the level of incorporation with *E. coli* tRNA is as great as that with yeast tRNA, but that the rate is one-half as fast. *E. coli* tRNA is known to have thiol groups which might be susceptible to periodate.

As mentioned in Results, essentially none of the tRNA as isolated from yeast is missing the entire terminal triplet, and pyrophosphorolysis by yeast enzyme can remove only the first two nucleotides (see Table II). The former observation does not depend on the method of isolation (E. Herbert, 1960, unpublished data). However, when the triplet is removed chemically by periodate–amine degradation, the enzyme can replace it. The nature of the difference between the two CMP residues which allows the transferase to add both, but remove only the first, is a very interesting area for future work.

A closely related question is whether or not the second CMP residue turns over in yeast tRNA. Holt (1962) reports that both CMP residues exchange with the CTP pool in rabbit reticulocytes. The first turns over much more rapidly than the second, which recalls the question raised above.

Now that highly enriched enzyme and homogeneous substrate are available, this speculation can perhaps be resolved.

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References

Gillam, I., Blew, D., Warrington, R. C., von Tigerstrom, M., and Tener, G. M. (1968), *Biochemistry 7*, 3459.

Holt, C. E., III (1962), Ph.D. Thesis, Massachusetts Institute of Technology.

Khym, J. X., and Uziel, M. (1968), *Biochemistry 7*, 422.

Litt, M. (1968), *Biochem. Biophys. Res. Commun. 32*, 507.

Madison, J. T., Everett, G. A., and Kung, H. (1968), *J. Biol. Chem. 242*, 1318.

Morris, R. W., and Herbert, E. (1970), *Biochemistry 9*, 4819.

Ralph, R. K., and Bellamy, A. T. (1964), *Biochim. Biophys. Acta 87*, 9.

Zachau, H. G. (1969), *Angew. Chem. Int. Ed. Engl. 8*, 711.