AMINO ACID ACCEPTOR ACTIVITY OF tRNA-C-C-C-A[1]

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SUMMARY: Rabbit liver tRNA nucleotidyltransferase was used to synthesize modified tRNA molecules containing an additional CMP residue at the 3' terminus. In the first step tRNA-C-C was converted to tRNA-C-C-C by a minor activity of the purified enzyme. In the second step the lengthened molecules were converted to tRNA-C-C-C-A. AMP addition to tRNA-C-C-C occurred at about 50% the rate with tRNA-C-C. Aminoacylation studies indicated that tRNA-C-C-C-A was active for acceptance of at least 12 amino acids.

INTRODUCTION: All tRNA species so far examined contain the identical trinucleotide sequence, -C-C-A, at the 3' terminus which is required for the acceptor and transfer functions of these molecules (see 1 for references). Since these terminal nucleotides presumably interact with both the aminoacyl-tRNA synthetases and the peptidyl transferase site of ribosomes, modification of these residues should prove useful as a probe for studying protein-tRNA interactions during peptide synthesis. In addition, examination of the biological activity of tRNA molecules with modified 3' terminal regions will be of value for understanding the function of this common sequence.

A variety of modified nucleotides have been introduced into tRNA by use of the enzyme tRNA nucleotidyltransferase leading to alteration of either the base, sugar or phosphate residues of the -C-C-A sequence (1,2). An alternative approach to modification of this region would be to change its length. Previous work from this laboratory has shown that purified liver tRNA nucleotidyltransferase could be used to synthesize terminal sequences of varying length (3,4). In an earlier paper it was shown that one such tRNA molecule, tRNA-C-A, containing one less terminal nucleotide, was totally devoid of amino acid acceptor activity (5). In contrast, we report here that liver tRNA

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chains with one additional nucleotide residue, tRNA-C-C-C-A,can accept a variety of amino acids. Thang et al. have reported that yeast $tRNA^{Phe}$-C-C-C-A can accept phenylalanine and transfer it into protein (6).

MATERIALS AND METHODS: Rabbit liver tRNA nucleotidyltransferase purified through step 6 was prepared as described previously (8). Rabbit liver tRNA and tRNA-C-C were prepared as reported earlier (9). Rat liver aminoacyl-tRNA synthetases were prepared by the procedure of Yang and Novelli (10) except that the DEAE cellulose and Sephadex G-100 chromatography steps were reversed. α-[^{32}P] ATP and [^{14}C] CTP were purchased from Schwarz/Mann. Radioactive amino acids were from New England Nuclear. Ribonuclease T1 was obtained from Worthington Biochemical.

tRNA nucleotidyltransferase was assayed as described earlier (8). Details of individual experiments are described below and in the Legends. Conditions of the aminoacyl-tRNA synthetase assays are given in the Legend to Table I.

tRNA-C-C-C-A was synthesized from rabbit liver tRNA-C-C by the sequential addition of CMP and AMP using purified tRNA nucleotidyltransferase. The structure of the modified tRNA was determined at each stage of the preparation by alkaline hydrolysis and measurement of nucleosides and nucleotides on Dowex-1 as reported previously (8). For this purpose the first product, tRNA-C-C-C was synthesized with [^{14}C] CTP. The reaction mixture (9 ml) contained: 50 mM glycine-NaOH, pH 9.4; 5 mM $MgCl_2$; 15 mg tRNA-C-C; 0.5 mM [^{14}C] CTP (about 500 cpm per nmole) and 2.3 units of tRNA nucleotidyltransferase. The mixture was incubated for 3 hours at 37° and the reaction stopped by heating at 60° for 5 min. After cooling, the tRNA-C-C-[^{14}C]C product was purified on Sephadex G-25 containing silicic acid on top as described earlier (9).

tRNA-C-C-A and tRNA-C-C-[^{14}C]C-A were prepared by the addition of AMP to tRNA-C-C and tRNA-C-C-[^{14}C]C. Conditions for AMP addition were essentially identical to those for CMP addition. In some experiments the time of incubation was shortened by the use of additional enzyme.

RESULTS: Analysis of the tRNA-C-C-[^{14}C]C product by alkaline hydrolysis showed that 75% of the incorporated [^{14}C] CMP residues were terminal and 25% internal, or an average of 1.3 residues per chain. Quantitation of the number of termini indicated that CMP was incorporated into greater than 90% of the available tRNA chains. It has not been possible to synthesize a preparation that was solely tRNA-C-C-C, while still utilizing all the available tRNA chains, since the first product was a substrate for further CMP incorporation by tRNA nucleotidyltransferase.

In order to assess the ability of tRNA nucleotidyltransferase to catalyze the second step of the synthesis, the incorporation of AMP into tRNA-C-C-C, the rate and extent of this reaction was compared to that of AMP incorporation into the normal substrate, tRNA-C-C (Fig. 1). AMP was incorporated into tRNA-C-C-C at about 50% the rate into tRNA-C-C (Fig. 1A). However, the extent of AMP incorporation into both tRNAs reached an identical limit provided that sufficient

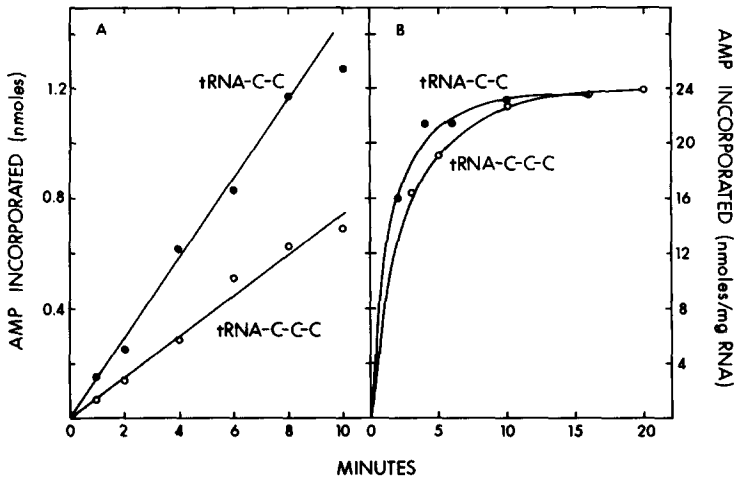

Fig. 1. Comparison of rate and extent of AMP incorporation into tRNA-C-C and tRNA-C-C-C. (A) Reaction mixtures contained in 200 µl: 50 mM glycine-NaOH, pH 9.4; 5 mM $MgCl_2$; 0.5 mM [^{32}P]ATP (about 1300 cpm/nmole); 1.6 µg of tRNA nucleotidyltransferase and either 80 µg tRNA-C-C or 100 µg tRNA-C-C-C. Samples were incubated for the indicated times at 37°. (B) Reaction mixtures were identical to those in (A) except that they contained 110 µg tRNA-C-C with 8 µg enzyme or 80 µg tRNA-C-C-C with 32 µg of enzyme. Samples were incubated for the indicated times at 37°.

enzyme was present with tRNA-C-C-C (Fig. 1B). Under these conditions only a single AMP residue was incorporated into each of the chains.

For the large scale preparation of tRNA-C-C-[^{14}C]C-A, unlabeled ATP was used as substrate, and the extent of AMP incorporation was determined by alkaline hydrolysis of the product and measurement of the number of terminal [^{14}C]CMP residues which were converted to penultimate residues. Such an analysis indicated that greater than 96% of the tRNA molecules were terminated with AMP. In some experiments we also determined how much more [^{32}P] AMP could be added to the purified product after the first cycle of AMP incorporation. These analyses also indicated that greater than 90% of the tRNA-C-C and tRNA-C-C-[^{14}C]C had already accepted AMP.

Further evidence for the synthesis of modified tRNA was obtained by high voltage electrophoresis of RNase T1 digests of tRNA-C-C-A and tRNA-C-C-C-A labeled with [^{32}P] in the terminal AMP. Although each of the digests gave a similar pattern upon electrophoresis, the labeled 3' oligonucleotides genera-

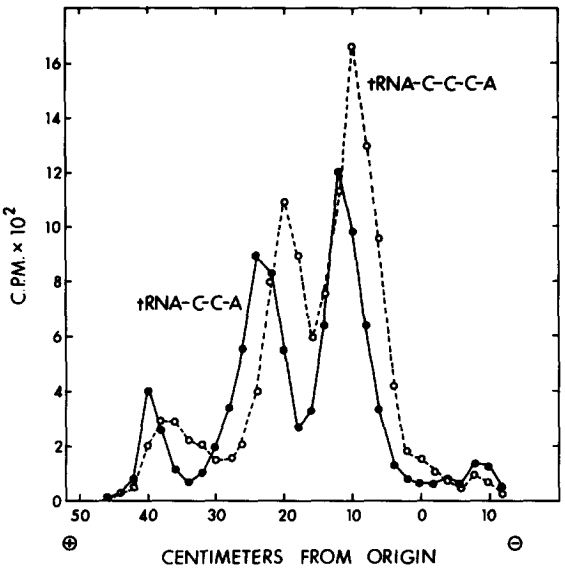

Fig. 2. Electrophoresis of ribonuclease T1 digests of tRNA-C-C-A and tRNA-C-C-C-A. tRNA-C-C-[^{32}P]A and tRNA-C-C-C-[^{32}P]A about 50 µg, were digested with 35 µg of T1 RNase at 37° for 30 min. Aliquots were spotted on Whatman 3 MM paper and electrophoresis run for 5.5 hours at 3000 volts in 0.02 M sodium citrate, pH 3.5. Strips of 2 cm were cut out and counted in toluene-based scintillation fluid.

ted from tRNA-C-C-C-A migrated more slowly. The electrophoretic data alone do not identify the product, but they do show that the 3' oligonucleotides from the modified tRNA have been altered, which is consistent with the analyses obtained by the other methods. Taken together, these data indicate that the major product of this synthetic scheme is tRNA-C-C-C-A. The preparation may be contaminated by as much as 30-40% with higher homologues containing additional CMP residues. However, our analysis indicates that, at most, only a 10-15% contamination with the normal amino acid acceptor, tRNA-C-C-A, could be present. This heterogeneity is due to the fact that liver tRNA nucleotidyltransferase is also a poly(C) polymerase which can add multiple CMP residues to tRNA-C-C (11). The presence of this activity necessitates stopping the reaction before all the chains have added more than one CMP residue, but as a consequence, some unmodified chains still remain. Nevertheless, this prepara-

tion is satisfactory for determining the amino acid acceptor activity of tRNAs with additional CMP residues since contamination by unmodified tRNA is relatively low.

The extent of amino acid acceptance by the lengthened tRNA was compared to that of tRNA-C-C-A using a mixture of rat liver aminoacyl-tRNA synthetases. The tRNA-C-C-A used in these experiments was prepared by the addition of AMP to tRNA-C-C in a manner identical to the preparation of tRNA-C-C-C-A. This served to correct for any damage to the tRNA during periodate oxidation which could affect acceptance of some amino acids (5). Since the amount of tRNA-C-C-C-A available was limited, the conditions of time and enzyme concentration required for maximal incorporation of each amino acid were first determined with unmodified tRNA, and then these conditions were also used for tRNA-C-C-C-A.

The relative incorporation of various amino acids into tRNA-C-C-C-A compared to tRNA-C-C-A is shown in Table I. Several of the amino acids, namely histidine, glutamic acid and aspartic acid were incorporated into tRNA-C-C-C-A at low levels which could be attributed to the contamination by normal tRNA. Another group of amino acids (alanine, leucine, valine, proline and isoleucine) were incorporated to levels of 40-60% of normal tRNA. This extent of incorporation is close to the maximum that might be expected if the tRNA chains containing more than three terminal CMP residues were not acceptors since they probably account for 30-40% of the total tRNA chains present. The remainder of the amino acids tested were incorporated to lower levels (20-40%), but still greater than would be expected from contamination of the preparation of tRNA-C-C-A. For this latter group it is not yet certain whether the partial incorporation is due to only a fraction of the isoaccepting tRNAs that are active as substrates or to inadequate conditions for maximal incorporation.

DISCUSSION: The data presented in this paper suggest that tRNA-C-C-C-A can act as an acceptor for a number of amino acids. These results indicate that a variety of liver aminoacyl-tRNA synthetases can tolerate the increased flexibility at the 3' end of the modified tRNA. This contrasts with the loss of

TABLE I

Extent of Aminoacylation of tRNA-C-C-C-A Compared to tRNA-C-C-A

Amino Acid	Relative incorporation
aspartic acid	0.12
glutamic acid	0.13
histidine	0.13
arginine	0.21
lysine	0.24
threonine	0.26
tyrosine	0.28
serine	0.30
methionine	0.32
glycine	0.37
proline	0.45
alanine	0.46
leucine	0.48
isoleucine	0.49
valine	0.54

Aminoacylation was measured in reaction mixtures of 100 µl containing: 0.25 M Tris-Cl, pH 7.0, 5 mM ATP; 5 mM $MgCl_2$; 10 µg bovine serum albumin; 0.2 mM EDTA; 0.25 mM [^{3}H] or [^{14}C] amino acid (5-40 cpm/pmole); 50-100 µg tRNA-C-C-A or tRNA-C-C-[^{14}C]C-A and 40 µl of mixed rat liver aminoacyl-tRNA synthetases. Reaction mixtures were incubated 10 to 20 min at 37° and stopped by the addition of 10% TCA containing 0.02 M sodium pyrophosphate. The precipitate was collected on GF/C filters, washed, dried and counted. Zero time blanks were subtracted from each assay. Incorporation is expressed relative to that into tRNA-C-C-A which is set at 1.00. Incorporation into tRNA-C-C-A varied from 0.2 to 2.5 nmoles/mg depending on the amino acid.

activity of tRNA-C-A chains (5). It would be of interest to repeat the studies reported here with purified tRNA species to determine whether the increased flexibility at the 3' end of tRNA might lead to mischarging. Our results suggest that for several amino acids not all of the isoaccepting species of

tRNA-C-C-C-A are active. This raises the possibility that the combined effect of both an extra CMP residue at the 3' terminus and a change in another part of the molecule could render a tRNA inactive, whereas either change alone does not lead to loss of activity. This may indicate interaction of residues in the 3' terminal sequence with other parts of the tRNA molecule.

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