*MULTIPLE ISOACCEPTING TRANSFER RNA'S IN A MOUSE PLASMA CELL TUMOR**

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That every amino acid has its specific transfer RNA (tRNA) for activation during protein synthesis[1] has become well known. The existence of physically heterogeneous tRNA's for a single amino acid, which will be named "isoaccepting tRNA" in this paper, was first described by Doctor *et al.*[2] in leucine and threonine tRNA's of yeast separated by countercurrent distribution. As the technique of separation became improved, more amino acids with isoaccepting tRNA's and more complicated multiplicity of certain amino acid-specific tRNA's were demonstrated.[3–9] For example, *Escherichia coli* has been shown to have various isoaccepting tRNA's—five for leucine,[7, 8, 10] four for serine,[8] three for proline,[4] and two for most of the other amino acids. The separation and identification of these isoaccepting tRNA's have become important because it has become evident that multiple tRNA's with different coding properties specific for a single amino acid may be physically separable,[11–14] and that modifications in these systems may play an important role in cellular regulatory mechanisms (see review by Novelli[15]). Most studies on isoaccepting tRNA's, however, have been performed with microorganisms, especially *E. coli* and yeast. No complete study on the heterogeneity of mammalian tRNA is yet available, although it has been shown that tRNA's from mammalian liver may be more heterogeneous than those from organisms of lower phyla.[16, 17] In the present communication we report the results of such a study in a mouse plasma cell tumor and demonstrate the applicability of a newly developed method, reversed phase Freon column chromatography,[18] which employs a new solvent system[19] and has a resolving power for analyzing multiple isoaccepting tRNA's in this tissue.

Materials and Methods.—Tissue and animal: MOPC-31C tumor (originally obtained from Dr. M. Potter), a plasma cell tumor that produces immunoglobulin-G-like myeloma protein, was maintained by serial 2-week transplantations in BALB/c Cum mice.[20] The solid tumor that formed 2 weeks after transplantation was usually clean and free from necrosis, and was used for preparing aminoacyl-tRNA synthetases and tRNA.

Preparation of aminoacyl-tRNA synthetases and tRNA: Aminoacyl-tRNA synthetases were prepared by modifying the method of Muench and Berg.[21] The minced tissue was homogenized in buffered medium (0.01 *M* Tris-HCl, pH 7.5; 0.01 *M* Mg acetate; 0.01 *M* β-mercaptoethanol; 0.05 *M* KCl; 0.25 *M* sucrose; 10% glycerol) and after removal of particulate components by several centrifugations, including a final one at 198,000 × *g* for 2 hr, the soluble portion was passed through a G-100 Sephadex column (3 × 70 cm) in a solution containing 0.01 *M* K-PO_4 at pH 7.5, 0.02 *M* β-mercaptoethanol, 0.005 *M* KCl, and 10% glycerol, and it was resolved mainly into three absorbance peaks (280 mμ): a straw-colored peak containing excluded materials of high molecular weight in which most of the synthetase activities were found, a reddish peak including hemoglobin, and a third peak containing tRNA. The first protein peak and the front half of the second protein peak were pooled and poured onto a DEAE-cellulose column which had been

equilibrated with the same buffer. About 50–60% of the protein, which consisted of the bulk of myeloma proteins, was not retained, whereas the synthetases were absorbed. After thorough washing of the column, the active fraction containing all 20 synthetases was eluted with a solution of lower pH (0.01 *M* K-PO_4, pH 6.5) and higher salt concentration (0.25 *M* KCl). The solution was made up to 2.0 mg protein/ml in 50% glycerol and stored in 2-ml vials at −20°C.

To prepare mixed tRNA, the following procedure was used: The minced tissue was homogenized in 2.5 vol (w/v) of medium containing 0.01 *M* Tris-HCl (pH 8.0), 0.01 *M* Mg acetate, 0.05 *M* KCl, 0.6 *M* sucrose, and 1 mg/ml washed bentonite (Macaloid). The postmitochondrial supernatant fluid, obtained by centrifugation (3020 × *g* for 10 min; 10800 × *g* for 15 min; 34800 × *g* for 15 min), was diluted with an equal volume of 0.15 *M* NaCl containing 0.001 *M* EDTA-disodium and 2.0 mg bentonite/ml, and the mixture was subjected to phenol extraction at 4°C. Nucleic acids were precipitated from the aqueous phase by adding ethanol to a final concentration of 75% and allowed to stand at −20°C overnight. Ribosomal RNA was then removed by 1 *M* NaCl precipitation in the presence of 1 mg/ml bentonite. The soluble RNA, shown by sucrose density gradient centrifugation to consist exclusively of 4–5*S* material, was further fractionated by DEAE-cellulose column chromatography by using a stepwise NaCl gradient. The 0.25–0.65 *M* NaCl fraction was collected, and the mixed tRNA was obtained by ethanol precipitation. To discharge the endogenous amino acids from tRNA, the aqueous solution of RNA was always brought to 3% potassium acetate before ethanol precipitation and the final product of mixed tRNA was incubated in 0.3 *M* Tris-HCl (pH 7.5) at 35°C for 30 min. Throughout the entire procedure, 0.01 *M* Mg acetate and 0.001 *M* EDTA-disodium were present in the solution of tRNA. With one OD_{260} unit equal to 2 mμmoles of tRNA, one mole of this tRNA preparation was found to accept a total of 0.50 to 0.55 mole of amino acids. The yield of tRNA by this isolation procedure was 60–65 mg/100 gm of fresh tumor tissue.

Chromatography: Reversed phase Freon column chromatography was performed essentially as described by Weiss and Kelmers,[18] except that the column was packed under gravity and a linear gradient of 0.25–0.65 *M* or 0.35–0.65 *M* NaCl containing 0.01 *M* Na acetate buffer at pH 4.5, 0.01 *M* Mg acetate, 0.001 *M* EDTA was employed. After the tRNA was applied, the column was eluted with a gradient volume of 2 liters at a flow rate of 1 ml/min at room temperature. Optical density tracings at 260 mμ were obtained, and 10-ml fractions were collected. The tRNA of each fraction was precipitated by mixing the fraction with 2.5 vol of ethanol and allowing the mixture to stand overnight at −20°C. The precipitates were collected by filtration on Millipore (type HA, pore size 0.45 μ) filters, and washed with 70% ethanol. The tRNA on the Millipore filter could be eluted by shaking the filter with 1 ml of 0.05 *M* NaCl, 0.01 *M* Mg acetate, and 0.001 *M* EDTA in a glass vial at 4°C for 30 min. The elute was then assayed for its amino acid accepting activity (postchromatography charging).

The reaction mixture of 0.1 to 0.2 ml for assaying amino acid accepting activity contained per ml: Tris-HCl, pH 7.5, 100 μmoles; KCl, 10 μmoles; β-mercaptoethanol, 1 μmole; ATP, 4 μmoles; Mg acetate, 4–20 μmoles; 1–2 μc of C^{14}-amino acid; 0.1–0.2 ml (0.5–1.4 OD_{260} units) of the 10× concentrated tRNA fraction; and 0.2 mg of mixed synthetase protein. The optimal Mg/ATP ratio for activation of each amino acid had been determined (1 for leucine, 2 for glutamic acid, 3 to 5 for all the other amino acids) and was used accordingly. The incubation was performed at 22°C for 45 min. Preliminary experiments showed that under these conditions all aminoacylations reached a maximum after 10 min of incubation and maintained a plateau up to 60 min, and that quantitative results could be obtained. The incorporated radioactivity was determined by the filter paper disc method.[22]

C^{14}-aminoacyl tRNA was prepared by chilling the reaction mixture (usually 5 ml) at the end of a 30-min incubation at 30°C, adding an equal volume of 1 *M* Na acetate, pH 4.5, and extracting with phenol. The aqueous layer was twice precipitated in 75% ethanol containing 0.01 *M* acetate buffer, pH 4.5, at −20°C overnight and then dis-

solved in the initial buffer for chromatography (prechromatography charging). Usually 5 to 10 times as much of uncharged tRNA was added as carrier. The chromatographic fractions were divided into two portions, 4 ml and 6 ml, and separately precipitated by ethanol. The Millipore filter which contained the precipitate of the 4-ml portion was washed, dried, and counted directly by the liquid scintillation method; this detected the location of precharged aminoacyl tRNA's. The precipitate of the 6-ml portion was used for assaying amino acid accepting activity, as described above, to detect the uncharged tRNA's as well as to precharged ones. By this method the charged and uncharged tRNA's specific for an amino acid can be localized and compared in the same chromatographic run.

C^{14}-amino acids: Seventeen uniformly labeled C^{14}-L-amino acids were purchased from the New England Nuclear Corporation, Inc. The specific activities, expressed in mc/mmole, were: alanine, 117; arginine, 240; asparagine, 46; aspartate, 164; glutamate, 205; glycine, 80; histidine, 222; isoleucine, 250; leucine, 275; lysine, 200; methionine, 198; phenylalanine, 367; proline, 200; serine, 115; threonine, 160; tyrosine, 393; valine, 208. Three C^{14}-DL-cysteine HCl (5.05 mc/mmole) and uniformly labeled C^{14}-L-glutamine HCl (6.58 mc/mmole) were products of Schwarz BioResearch, Inc. Uniformly labeled C^{14}-DL-tryptophan (20 mc/mmole) was purchased from the Nuclear Chicago Corporation, Inc.

Results and Discussion.—Shown in Figure 1 is the complete profile of various isoaccepting tRNA's of mouse plasma cell tumor, MOPC-31C, from the reversed phase Freon column chromatography as detected by postchromatography aminoacylation. It can be seen that this column was extremely efficient for separating mammalian tRNA's, as well as *E. coli* tRNA's.[18] In some cases the peak was so sharp that it was included in only a few fractions. Except for tryptophan, multiple peaks were observed for all other 19 amino acids, each having its own characteristic pattern of eluted peaks. Compared with the profile of *E. coli* tRNA obtained under similar chromatographic conditions in other studies,[18] this mammalian tumor tRNA was evidently more heterogeneous with regard to most of the amino acid accepting activities. This feature of phylogenetically evolved complexity in isoaccepting tRNA's has also been noted in lysine tRNA's of yeast and rat liver by using countercurrent distribution,[16] in lysine and threonine tRNA's of yeast, carp, frog, and rat liver by using MAK column chromatography,[17] and in six amino acid tRNA's of *E. coli* and guinea pig liver by using reversed phase isoamylacetate column chromatography.[23] Another feature of particular interest is that the multiplicity of isoaccepting tRNA's observed for an amino acid in the present study seems to be related to the number of its published synonym codons.[24] For tryptophan, with one codon, only a single peak of acceptor was detected; and for arginine, leucine, and serine, which have six codons, more peaks were observed. There were, however, exceptions in lysine, aspartic acid, tyrosine, and methionine; for these, the acceptor tRNA appeared in more than two peaks.

To interpret the occurrence of multiple isoaccepting tRNA's in our chromatographic studies of this tumor tRNA preparation, two questions should be considered: (1) Are they experimental artifacts? (2) Are there actually multiple forms of these tRNA's *in vivo*? Experimental artifacts which have been reported to cause the formation of multiple fractions for a single tRNA species during separation were examined and summarized as follows:

(a) *Aggregation:* Schleich and Goldstein[25] have shown that by gel filtration

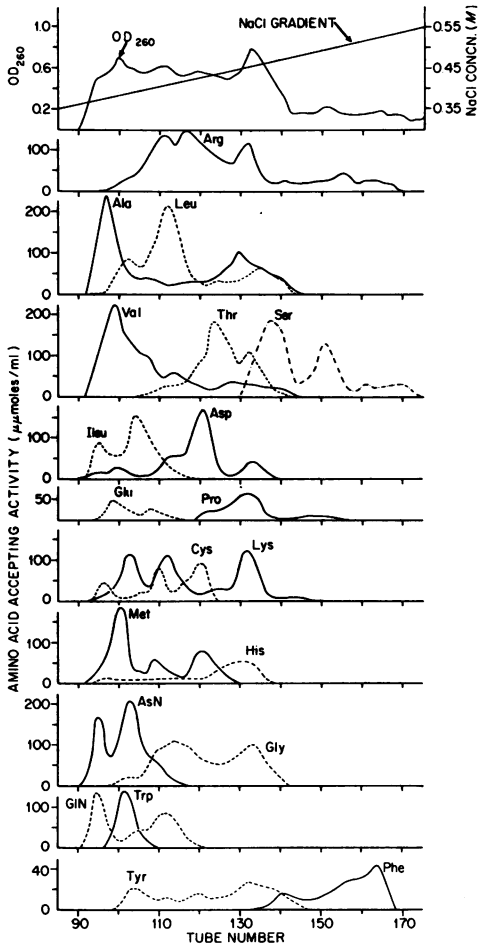

FIG. 1.—The complete elution profiles of all 20 amino acid tRNA's from mouse plasma cell tumor, MOPC-31C, on the reversed phase Freon column chromatography. 207 OD_{260} units were applied on a column of 1 × 240 cm and eluted with 2 liters of 0.25 to 0.65 *M* NaCl gradient. Fractions of 10 ml were collected and concentrated 10× for amino acid accepting assay. For details see *Materials and Methods*.

E. coli tRNA could be separated into several components with different molecular weights possibly reflecting aggregation, and the aggregates could be dissociated by urea treatment. Since tRNA in aggregated form rarely accepts amino acids[25] and since we detected multiple peaks by aminoacylation, it seems unlikely that any of the peaks are tRNA aggregates. However, it might be possible that the aggregates could be dissociated during chromatography and thereby eluted at a different salt concentration from the original monomer peak. This was virtually ruled out by an experiment to be described below.

(b) *Partial degradation:* Nishimura and Novelli[26] showed that *E. coli* tRNA, after being digested by *Bacillus subtilis* RNase, still retained many of its amino acid accepting activities but behaved differently on MAK column chromatography and lost some of its amino acid transfer functions. To check if our tRNA preparation had been contaminated with RNase, the preparation was allowed to stand at room temperature in the initial buffer of the column for 24 hours, re-precipitated with ethanol, and tested for its activities to accept

leucine, alanine, threonine, methionine, aspartic acid, proline, and valine. No decrease in these activities was found. Furthermore, the concentrated chromatographic fractions were divided into two portions, one being heated at 85°C for six minutes, which should lead to irreversible breakdown of the functional conformation of RNase-nicked tRNA, and the other serving as the control. The peak tubes of alanine, valine, aspartic acid, and lysine tRNA's were then aminoacylated with their respective C^{14}-amino acids. There was neither a significant decrease in accepting activities nor an obliteration of any peak after the heat treatment. Thus the possibility that the multiple isoaccepting peaks of tRNA are induced by RNase contamination seems unlikely in the present case.

(c) *Differential removal of pCpCpA terminus:* Cantoni and his group[27] showed that serine tRNA, lacking the terminal adenosine, was separated by countercurrent distribution from intact serine tRNA. During assay for activity the species lacking the terminal adenosine showed full activity. Because crude activating enzymes may contain the pCpCpA pyrophosphorylase, and ATP is used in the assay, the terminal adenosine can be added, restoring activity to the molecule, and giving the erroneous assumption that the separate fractions contain different species of serine tRNA. To test whether this phenomenon might be involved in our observation of multiple isoaccepting tRNA's, our tumor tRNA preparation was reacted with a partially purified pCpCpA pyrophosphorylase of *E. coli* in the presence of radioactive ATP; about 5 per cent of tumor tRNA's were found lacking the pA terminus. In addition, we used an assay system containing *E. coli* tRNA from which the terminal adenosine had been removed to test for possible presence of the pCpCpA pyrophosphorylase in the tumor synthetase preparation; we detected no pyrophosphorylase activity in our synthetase preparation.[28]

The possibility that multiple isoaccepting tRNA's are not artifacts was further substantiated by comparing the profiles of charged and uncharged tRNA's in the same chromatographic run. Figures 2 and 3 show such studies for lysine- and leucine-specific tRNA's. The same number of peaks was usually obtained whether tRNA was charged with amino acid or uncharged before chromatography. Possibly because of the effect of aminoacylation on molecular conformation,[29] the charged aminoacyl tRNA tended to chromatograph in sharper peaks than did the uncharged tRNA molecules. The second peak of leucine tRNA could be resolved into two peaks if the tRNA was charged prior to chromatography. The charged and uncharged tRNA's appeared in different positions in the chromatogram, and this difference varied according to the kind of amino acid. Lysyl tRNA's, for example, were eluted at a lower salt concentration than uncharged lysine tRNA's, while the reverse seemed to be true for leucine. This kind of phenomenon has also been observed in MAK column chromatography of *E. coli* tRNA's.[30] Our preliminary results also indicated that this effect of aminoacylation on the chromatographic location might differ among various isoaccepting tRNA's.

Since the possibility that artifacts could account for the observation of multiple isoaccepting tRNA's had been virtually excluded, the significance of their existence in the cell was then considered. Whether these isoaccepting tRNA's,

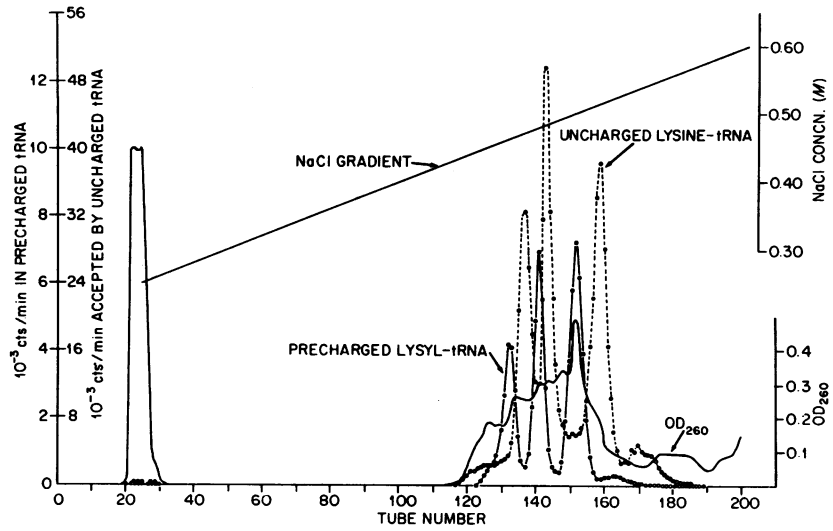

FIG. 2.—Chromatography of lysyl and lysine tRNA's on reversed phase Freon column. Five OD_{260} units were charged with C^{14} lysine and chromatographed with 40 OD_{260} units of uncharged tRNA of the same preparation. Cpm accepted by the uncharged lysine tRNA was determined by reducing the total activity with the radioactivity due to the precharged lysyl tRNA's.

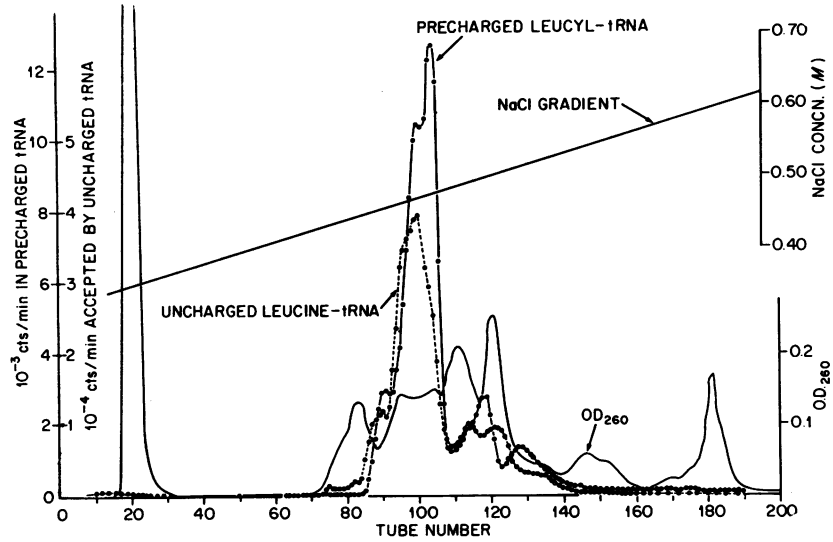

FIG. 3.—Chromatography of leucyl and leucine tRNA's on reversed phase Freon column. Chromatographic conditions were same as in Figs. 1 and 2 except that 0.35 to 0.65 *M* NaCl gradient was employed. Six OD_{260} units of C^{14}-leucine-charged tRNA and 25 OD_{260} units of uncharged tRNA were used.

represent different synonym codons remains to be elucidated. If the published table of codons assigned for each amino acid[24] is applicable to this tumor tissue, one codon may have more than one entity of isoaccepting tRNA for lysine,

aspartic acid, and tyrosine. Recently Barnett and Brown[31] have shown that heterogeneity of tRNA in *Neurospora* can be attributed to differential compartmentation in the cytoplasm and mitochondria. Since the tRNA for the present study was prepared from the post-mitochondrial fraction of the tissue, it is unlikely that some isoaccepting tRNA's may be from mitochondria. Studies are now in progress on the biological role that these isoaccepting tRNA's may play in the synthesis of heterogeneous immunoglobulins in the mammalian system.

Summary.—Multiple isoaccepting tRNA's for 20 amino acids from mouse plasma cell tumor, MOPC-31C, have been resolved on a reversed phase Freon chromatography column. Multiplicity of isoaccepting tRNA's may be related to the number of synonym codons. Heterogeneity was more extensive in this tissue than that reported for *E. coli*. The possibilities that the isoaccepting peaks might be due to aggregation, partial degradation, or differential removal of pCpCpA terminal have been virtually excluded. It was found that charged aminoacyl tRNA's were eluted differently than the uncharged correspondents.

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