The Fractionation of Transfer Ribonucleic Acid from Roots of Pea Seedlings

LARRY N. VANDERHOEF2 AND JOE L. KEY3
Department of Botany and Plant Pathology, Purdue University, Lafayette, Indiana 47907

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ABSTRACT

Isoaccepting transfer RNA species for several amino acids were fractionated by reverse phase column chromatography. Transfer RNA from dividing cells of pea (Pisum sativum) root was compared to that from nondividing cells, and no relative quantitative or qualitative differences were noted for the transfer RNA species except those for serine and phenylalanine. However, certain artifactual differences in asparagine and threonine, methionine, serine, and phenylalanine. However, certain artifactual differences in height and weight were noted. Quantitative differences were observed in tyrosyl-transfer RNA's. Ribonuclease action on tRNA did not contribute to the tRNA species observed.

Transfer RNA is involved in the proper translation of the sequence of bases in mRNA into the prescribed order of amino acids in protein (24). Aminoacyl-tRNA is central in theories of translational regulation of enzyme synthesis (1, 25, 27); it is necessary for the end product repression of the enzymes of the amino acid biosynthetic pathways (12, 25, 26, 37) and for the stringent control of RNA synthesis (10, 11, 23, 38). Isoaccepting tRNAs (different tRNA's which accept the same amino acid) have been demonstrated in various organisms for several amino acids. This has been shown in some cases (e.g., Neurospora, rat liver, and Tetrahymena), to be partly due to mitochondria which have their own tRNA's (4, 5, 8, 30). In other cases, there are changes in tRNA populations which correlate with changes in metabolism or development (3, 13, 17, 20, 21, 28). Infection by phage (14, 16, 18, 33, 34, 36) and by Herpes virus (29) also leads to changes in the tRNA population in the host cells.

There have been three reports of isoaccepting species of tRNA in higher plants. Vold and Sypherd (32) studied tRNA changes in germinating wheat seedlings. Their data showed slight changes in the tRNA populations as assayed at 0 and 48 hr of germination. Anderson and Cherry (2) found six leucyl-tRNA species in soybean seedlings, but found that only four of these could be charged by synthetases extracted from the hypocotyl, whereas all six were charged by synthetases extracted from cotyledons. Furthermore, the two hypocotyl tRNA's which were not charged by hypocotyl synthetases were present in relatively smaller amounts in the hypocotyl than in the cotyledon. Williams (37) studied in detail the formation of leucyl-tRNA, and demonstrated at least four leucine isoacceptor tRNA's in bean.

This is a study of isoaccepting tRNA species for eight amino acids from pea root. Transfer RNA populations of dividing cells are compared to those of nondividing cells.

MATERIALS AND METHODS

Plant Material. Alaska peas (Pisum sativum, var. Alaska) were germinated in moist absorbent paper for 4 days at about 25 C. The roots were either excised for immediate synthetase or tRNA extraction, or were excised onto Dry Ice and stored at −20 C for further extraction.

Extraction and Purification of tRNA. The roots were divided into three sections (7) for the extraction of tRNA: the apical 2 mm (meristematic tissue), the next 5 mm (elongation region), and the remaining approximately 2 cm of root (maturing region). The elongating tissue was usually discarded, and total RNA was extracted (19) from the rapidly dividing cells of the meristem and from the nondividing, fully elongated cells of the maturing tissue. Purification of tRNA has been previously described (31). The total RNA pellet was thoroughly suspended in 3.0 mM sodium acetate, pH 6.0, and the RNA that dissolved was collected by centrifugation after ethanol precipitation. The salt-soluble RNA was further purified by DEAE-cellulose chromatography. A 1 mg/ml solution of the final RNA product (90-95% 4S tRNA and 5-10% 5S tRNA) gave an A_{260} value of 16.8 in 1 mM MgCl_2. Approximately 85 to 90% of the tRNA molecules had an intact −CpCpA 3' terminus (31). All steps in this procedure were performed at 0 to 4 C.

Extraction of Synthetases. The synthetases were extracted from whole roots of 4-day pea seedlings (31). Tissue was minced with a razor blade in 1 tissue volume of 0.4 M sucrose containing 10 mM MgCl_2, 0.5 mM diethiothreitol, and 10 mM HEPES, pH 7.5. After homogenization in a Polytron homogenizer, the supernatant was collected by centrifugation (30 min at 30,000g) and was passed through Miracloth. The protein, which precipitated from the supernatant between 0.4 and 0.7 (NH_4)_2SO_4 saturation, was dissolved in pH 7.5 10 mM HEPES, containing 0.5 mM diethiothreitol. One volume of phosphate gel (20 mg/ml) was added dropwise to this solution and was stirred for 15 min. The gel was collected by centrifugation, and the protein was eluted from it with 15% (NH_4)_2SO_4 (w/v) in pH 7.5 KH_2PO_4. The gel was then pelleted by centrifugation and discarded. The protein was precipitated from the supernatant by slowly increasing (NH_4)_2SO_4 concentration to 0.8 saturation with crystalline (NH_4)_2SO_4. The precipitate...
was dissolved in pH 7.5 10 mM HEPES buffer, containing 0.5 mM dithiothreitol. All steps were performed at 0 to 4 C.

**Aminocyclation of Transfer RNA.** Aminocyclation (charging) of tRNA, unless specifically stated otherwise, was carried out as follows. Incubation was at 37 C in 1 ml containing 100 μmoles of HEPES, pH 7.5, 1 μmole of ATP, 5 μmoles of MgCl₂, 0.1 to 10 μmoles of tRNA, 0.01 μmole of ¹⁴C-amino acid (50–500 mc/m mole), and 100 to 300 μg of protein (31). When aminocyclation was done for the fractionation of isoaccepting species of tRNA, the reaction was terminated by transferring the assay tubes to an ice bath with the addition of 2 ml of ice-cold buffer I (10 mM sodium acetate, pH 4.5, containing 0.3 M NaCl, 10 mM MgCl₂, and 0.1 mM dithiothreitol). The tRNA was added to a 1 x 3 cm DEAE-cellulose column previously equilibrated with buffer I. A 6-ml wash with buffer I was followed by elution of the charged tRNA with 7 ml of buffer II (buffer I, with the 0.3 M NaCl replaced with 1.0 M NaCl). The aminocyl-tRNA was precipitated from the eluate with 2.5 volumes of absolute ethanol, with 0.02% sodium azide and varying NaCl concentrations, to prevent the growth of microorganisms. The total radioactive amino acid charged to tRNA was determined by precipitating a 0.10-ml aliquot of this 3 ml solution and then by counting as described below. The rest of the 3-ml sample was chromatographed. Two tRNA preparations were compared by charging one with ¹³C-amino acid and the other with ¹⁴H-amino acid, followed by cofractionation.

The reversal of labeled amino acid in a parallel experiment was necessary to avoid differences resulting in traces of contaminating radioactivity in the ¹⁴H-amino acids (e.g., serine in Fig. 2).

**Freon Chromatography.** Reverse phase chromatography was performed according to Weiss and Kelmers (35) on a 1 x 220 cm column with these modifications: (a) The washed Freon-Aliquot solution was dried for 3 hr in a desiccator over silica gel crystals; (b) the coated Chromosorb W was suspended in buffer I, containing 0.02% sodium azide, and was placed under a 30-pound vacuum to infuse the Chromosorb W; (c) the column was packed under 20 pounds of pressure. The charged tRNA was eluted with a 2-liter linear gradient (the gradient solutions contained buffer I with 0.02% sodium azide and varying NaCl concentrations, depending upon the particular aminocyl-tRNA to be fractionated). The column was run under gravity flow with a pressure differential of 160 cm, giving a flow rate of approximately 90 ml per hr; 10-ml fractions were collected and chilled to 4 C. RNA was precipitated by adding 1.5 ml of 50% trichloroacetic acid (w/v). The precipitates were collected on glass fiber discs and were washed with three 2-ml washes of cold 0.5% trichloroacetic acid and then by two 2-ml washes of Hokin's reagent (935 ml of ethanol, 60 ml of glacial acetic acid, 1.6 ml of 10 N NaOH). The filter discs were then dried under heat lamps, were placed in vials containing 5 ml of scintillation solution (0.3 g of dimethyl p-bis [2-(5-phenyloxazolyl)]benzene, and 5 g of 2,5-diphenyloxazole per liter toluene), and were counted in a scintillation spectrometer for determination of the distribution of ¹⁴C-aminocayl tRNA and ¹⁴H-aminocacyl tRNA on the column. Experiments of all column fractionations were repeated at least four times.

**RESULTS AND DISCUSSION**

The percentage of total tRNA charged by a single amino acid was determined for several amino acids for tRNA from dividing and nondividing cells (Table I). If one extrapolates from the percentages for these six amino acids, it can be said that a larger percentage of tRNA from dividing cells (70%) can be charged than for tRNA from nondividing cells (54%).

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Percentage of Total tRNA Charged</th>
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<tbody>
<tr>
<td></td>
<td>tRNA from dividing cells</td>
</tr>
<tr>
<td>Leucine</td>
<td>4.1</td>
</tr>
<tr>
<td>Serine</td>
<td>4.1</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>1.7</td>
</tr>
<tr>
<td>Proline</td>
<td>5.0</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>4.2</td>
</tr>
<tr>
<td>Lysine</td>
<td>4.8</td>
</tr>
<tr>
<td>Total</td>
<td>20.9</td>
</tr>
<tr>
<td>Average per amino acid</td>
<td>3.5</td>
</tr>
<tr>
<td>Projected total for 20 amino acids</td>
<td>70</td>
</tr>
</tbody>
</table>

Leucine, lysine, proline, threonine, methionine, serine, phenylalanine, and tyrosine were reacted separately with tRNA, and their isoaccepting species of tRNA were fractionated by reverse phase column chromatography (35). Cocromatography of two populations, representing the different tissues under study, of aminomycin-tRNA was performed, one being charged with ¹³H-amino acid and the other with ¹⁴C-amino acid. In all cases the experiments were repeated with reversal of the ¹³H- and ¹⁴C-labeled amino acids charged to the respective tRNA populations. Five of the amino acids, leucine, lysine, proline, threonine, and methionine, showed profiles that were always identical for tRNA preparations from dividing and maturing tissues (Fig. 1). These tRNA's underwent no relative change when the cells developed from the dividing to the nondividing state.

Serine and phenylalanine also gave identical profiles (Fig. 2) for tRNA from both dividing and nondividing cells, with the possible exception of the fraction of phenylalanine-tRNA which eluted at the highest salt concentration (denoted by the arrows). These amino acids, however, both gave spurious tRNA peaks. In the case of serine, the peaks were associated with the ¹³H-amino acid (compare Fig. 2A and B). That is, no matter what the source of tRNA, when ¹³H-serine was used to charge the tRNA, two peaks appeared which were not present when ¹⁴C-amino acid was used (note arrows of Fig. 2). Other results suggest that this was most likely the result of a minor contaminating amino acid in the ¹³H-serine. In the case of phenylalanine, the variable peak (arrow in Fig. 2C and D) was not specifically associated with either ¹³C- or ¹⁴H-phenylalanine. Also, it was not specifically associated with tRNA from either dividing or nondividing cells (Fig. 2C). Rather, it may have been an unstable tRNA or, possibly, a conformer which arose during some part of the procedure. Sueoka has found that Escherichia coli phenylalanine-tRNA showed an identical artifact. He believes that this is conformer formation because of "slipping" of the hydrogen bonds (N. Sueoka, personal communication).

Of the eight amino acids studied, only tyrosine showed consistent quantitative differences in the relative amounts of the tRNA species present in dividing and nondividing cells (Fig. 3). Reversing the amino acids gave an identical result. When the profiles were normalized about peak I, there was approximately...
Fig. 1. Reverse phase column chromatography of tRNA from pea root. Transfer RNA was charged with radioactive amino acid, purified, adsorbed to a Freon column, and eluted with a linear NaCl gradient as described in the text. The five aminoacyl-tRNA's illustrated showed no relative quantitative or qualitative differences between dividing and nondividing cells. Dashed line: tRNA from dividing cells charged with H-amino acids; solid line: tRNA from nondividing cells charged with C-amino acids. A reversal of the isomers gave the same results for these amino acids. Leu: Leucine; Lys: lysine; Pro: proline; Thr: threonine; Met: methionine.

1.5 times as much tyrosyl-tRNA II and 2.2 times as much tyrosyl-tRNA III in dividing cells as in nondividing cells (Table II and Fig. 3). This is in agreement with the observation that tRNA from nondividing cells charges less tyrosine than that from dividing cells (Table I), thus, a decrease in the relative amount of tyrosine tRNA II and III relative to tRNA I seems to be the most likely explanation of the changing ratios during development. When tRNA from the elongating tissue (the 5-mm segment between the dividing cells of the meristem and the fully elongated cells of the maturing region) was charged and cofractionated with charged tRNA from the meristem, differences in peak ratios for the three tyrosine-tRNA's were not as great as when tRNA's from meristematic and maturing tissue were compared (Table III, Fig. 4). The amount of peak II, relative to peak I, had not changed at all, and Peak III was still present in greater amounts than in maturing tissue, giving a ratio of 1.6 (Fig. 4, Table III). The major apparent decreases in tyrosyl-tRNA's II and III occurred in the elongating to maturing transition, with some relative decrease in tyrosyl-tRNA III occurring in the dividing to elongating transition. This was confirmed with the appropriate cross experiments (Fig. 4A to C). The profiles of the three tyrosyl-tRNA's from all three tissues were normalized about peak I (Fig. 4D) for clarity.

It was previously shown (31) that the tRNA used in these experiments was quite free from RNase damage, e.g., melting did not release unchargable fragments, the Cpcpa 3' terminus was intact in 90% of the tRNA molecules. These experiments were necessary inasmuch as it is feasible that tRNA could be damaged such that it would still charge but would separate from the population of identical undamaged molecules during reverse phase chromatography, thus giving an artifactual species.

To substantiate the above results, tyrosyl-tRNA was melted, then quick-cooled, and compared to untreated tRNA by reverse phase column chromatography. There were no differences, and, hence, endonuclease damage and conformer formation were eliminated as possible sources of artifact. In addition, the amino-
The profiles of the three tRNA peaks were normalized about peak I (Fig. 4). The ratios for the other two peaks were then calculated.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Peak Ratios (1:II:III)</th>
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<tr>
<td>Dividing</td>
<td>1.0:2.8:3.0</td>
</tr>
<tr>
<td>Elongating</td>
<td>1.0:2.8:1.7</td>
</tr>
<tr>
<td>Maturing</td>
<td>1.0:1.9:1.4</td>
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The acylation incubation time was varied (10 min versus 30 min) to determine whether RNase damage or other changes occurred during this time. Again, there were no differences in the subsequent chromatographic profiles.

**CONCLUSIONS**

The correlation of changes in amounts of certain species of tRNA with changes in metabolism, as has just been described for pea root tyrosyl-tRNA's, may be the result, rather than the cause, of some cellular event; however, it is becoming more apparent that some degree of translational regulation may occur in higher organisms, and that these changes in tyrosyl-tRNA's might well have a causal function. It is not difficult to hypothesize a role of tRNA concentration in cell regulatory mechanisms. For instance, if a particular tRNA species was a modulator tRNA or an initiator tRNA for the synthesis of a particular enzyme, then the concentration of that molecule, when present in limiting amounts, would control the rate of enzyme synthesis. A role of this sort for tRNA has frequently been discussed, and much work, especially with bacteria, has been done in the area. Hopefully, work with higher organisms, organisms in which the operon theory of control and regulation of gene function (34) does not appear to explain all of the available data (6, 9), will eventually give answers to the complex questions of transcriptional and translational regulation.

**LITERATURE CITED**
