Cuatrecasas, P., Taniuchi, H., and Anfinsen, C. B. (1968), *Brookhaven Symp. Biol. 21*, 172.
Cunningham, L., Catlin, B. W., and Privat De Garilhe, M. (1956), *J. Amer. Chem. Soc. 78*, 4642.
Dirksen, M. L., and Dekker, C. A. (1960), *Biochem. Biophys. Res. Commun. 2*, 147.
Rammler, D. H., Yengoyan, L., Paul, A. V., and Bax, P. C. (1967), *Biochemistry 6*, 1828.
Reddi, K. K. (1958), *Nature (London) 182*, 1308.
Reddi, K. K. (1967), *Methods Enzymol. 12*, 257.
Roberts, W. K., Dekker, C. A., Rushizky, G. W., and Knight, C. A. (1962), *Biochim. Biophys. Acta 55*, 674.
Taniuchi, H., and Anfinsen, C. B. (1966), *J. Biol. Chem. 241*, 4366.
von Hippel, P. H., and Felsenfeld, G. (1964), *Biochemistry 3*, 27.

Specificity of Rat Liver Lysine Transfer Ribonucleic Acid for Codon Recognition*

L. P. Liu and B. J. Ortwerth†

ABSTRACT: Rat liver lysine tRNA was separated into two well-defined isoaccepting species by reversed-phase Freon chromatography, and the coding properties of each peak were determined in an *Escherichia coli* ribosomal-binding assay. Lys-tRNA$_{I}$ binding was specific for ApApG, whereas Lys-tRNA$_{II}$ was specific for ApApA. In a tRNA-dependent, cell-free, protein-synthesizing system from rat liver, the addition of tRNA$_{II}^{Lys}$ gave a 2- to 4-fold stimulation of lysine incorporation with a poly(A) messenger, whereas tRNA$_{I}^{Lys}$ was only slightly active. When precharged samples of Lys-tRNA$_{I}$ and Lys-tRNA$_{II}$ were added to the rat liver protein-synthesizing system, Lys-tRNA$_{II}$ readily incorporated lysine with poly(A), whereas Lys-tRNA$_{I}$ was inactive. Both Lys-tRNAs were active with a poly(AG) (3:2) message and the ratio of incorporation of Lys-tRNA$_{II}$:Lys-tRNA$_{I}$ approximated the ratio of AAA:AAG in the poly(AG) random copolymer. Both peaks were also active in a rabbit reticulocyte system with Lys-tRNA$_{I}$ giving a 2-fold greater incorporation of lysine into hemoglobin. Therefore, tRNA$_{I}^{Lys}$ and tRNA$_{II}^{Lys}$ in the rat liver appear to be specific for translating the codons AAG and AAA, respectively. The unusual specificity of tRNA$_{II}^{Lys}$ for AAA may be due to a thiolated base in the anticodon region since I_2 oxidation of Lys-tRNA$_{II}$ resulted in a loss of ribosomal-binding activity.

Fractionation of tRNA from a tissue frequently results in multiple peaks of acceptance activity for the same amino acid (Hatfield and Portugal, 1970; Nishimura and Weinstein, 1969; Söll and RajBhandary, 1967; Caskey *et al.*, 1968). Lysine tRNA may be fractionated into two isoaccepting species from *Escherichia coli* (Söll and RajBhandary, 1967), guinea pig liver (Caskey *et al.*, 1968), and from rat liver (Nishimura and Weinstein, 1969). The coding specificity of these two lysine tRNA species for the condons AAA and AAG vary in the literature. With *E. coli*, Söll and RajBhandary (1967) reported that both peaks synthesized protein in response to poly(A) and poly(A-A-G) messages. With guinea pig liver, Caskey *et al.* (1968) concluded that one Lys-tRNA peak binds with ApApG and the other binds with both ApApA and ApApG in an *E. coli* ribosomal-binding assay. Nishimura and Weinstein (1969) however, reported that both peaks of rat liver Lys-tRNA[1] responded equally well to poly(A) and poly(AG) (3:1) in a ribosomal-binding assay. Carbon *et al.* (1965) showed that Lys-tRNA$_{I}$ binds with ApApG and poly(AG), whereas a mixture of Lys-tRNA$_{I}$ and Lys-tRNA$_{II}$ binds with poly(A) as well. We wish to report in this communication that rat liver tRNA$_{I}^{Lys}$ and tRNA$_{II}^{Lys}$ recognize ApApG and ApApA, respectively, in an *E. coli* ribosomal-binding assay. Furthermore, that the specificity observed in the ribosomal-binding assay is also reflected in a poly(A)- and poly(AG)-dependent protein-synthesizing system. A preliminary account of this work has been presented (Liu and Ortwerth, 1971).

Material and Methods

Preparation of Rat Liver tRNA. tRNA was prepared from livers of Wistar rats by phenol extraction and ethanol precipitation. Livers were homogenized in 2 volumes of buffer containing 0.15 M KCl, 0.01 M Tris-HCl (pH 7.5), 0.01 M $MgCl_2$, 0.001 M Na_2EDTA, and 0.02% polyvinyl sulfate. The homogenate was centrifuged for 20 min at 30,000*g*. The supernatant fraction was extracted with an equal volume of water-saturated phenol at 4° for 60 min. The aqueous layer, after centrifugation, was precipitated with 2.5 volumes of 95% ethanol and stored overnight at −18°. The precipitated nucleic acids were dissolved in a minimum amount of buffer C (0.05 M NaCl, 0.01 M Tris-HCl (pH 7.5), 0.01 M $MgCl_2$, and 0.001 M Na_2EDTA). The tRNA was further purified by applying it to a 2.0 × 30 cm DEAE-cellulose column which

* From the Departments of Ophthalmology and Biochemistry, University of Missouri, Columbia, Missouri 65201. *Received August 30, 1971.* Supported in part by a General Research Support grant (FR 5387-07 182) and in part by the Lions Eye Tissue Bank of the University of Missouri.

[1] Abbreviations used are: tRNA$_{I}^{Lys}$, the first major peak of lysine tRNA to elute from a reversed-phase Freon column; tRNA$_{II}^{Lys}$, the second major peak of lysine tRNA to elute from a reversed-phase Freon column; [^{14}C]Lys-tRNA, tRNA aminoacylated with [^{14}C]lysine; poly(AG) (3:2), a random copolymer containing adenine and guanine residues in a ratio of 3:2.

has been previously equilibrated with buffer C. The column was then washed with approximated 250 ml of buffer C and 250 ml of buffer C containing 0.3 M NaCl. The tRNA was eluted with buffer C containing 0.7 M NaCl. Fractions containing the 260-nm-absorbing material were pooled and the tRNA was precipitated with 2.5 volumes of 95% ethanol. The precipitate was collected, dissolved in buffer C, and stored at −15°.

Preparation of Rat Liver tRNA Synthetases. Rat livers were homogenized in 2.0 volumes of a buffer containing 0.25 M sucrose, 0.05 M KCl, 0.01 M Tris-HCl (pH 7.5), 0.01 M $MgCl_2$, 0.01 M β-mercaptoethanol, and 10% glycerol. The homogenate was centrifuged for 20 min at 30,000*g*, and the resulting supernatant fraction was centrifuged for 75 min at 160,000*g*. A crude synthetase preparation was isolated from the 160,000*g* supernatant by reverse-flow Sephadex G-75 gel filtration. A sample of 10–40 ml was placed on a 2.5 × 80 cm Sephadex G-75 column which had been previously equilibrated with a buffer containing 0.01 M KCl, 0.05 M Tris-HCl (pH 7.5), 0.01 M $MgCl_2$, 0.01 M β-mercaptoethanol, and 10% glycerol. The fractions which eluted in the void volume were pooled and used as a source of tRNA synthetases. This preparation was stored in 20% glycerol at −15°.

Fractionation of $tRNA_I^{Lys}$ and $tRNA_{II}^{Lys}$. Rat liver tRNA was applied to a 1 × 240 cm reversed-phase Freon column and fractionated by the method of Weiss and Kelmers (1967). A linear gradient of 0.25–0.60 M NaCl containing 0.01 M sodium acetate (pH 4.5), 0.01 M $MgCl_2$, 0.001 M Na_2EDTA, and 0.03 M β-mercaptoethanol was used when uncharged tRNA was fractionated. With precharged samples a 0.30–0.60 M NaCl gradient was used in the fractionation. The two fractions containing lysine-acceptance activity were pooled and the tRNA was precipitated with 2.5 volumes of 95% ethanol. The precipitate was filtered on a Millipore filter and eluted with 1.0 ml of deionized water and stored at −15°.

Preparation of [^3H]- or [^{14}C]Lys-tRNA. Rat liver tRNA was charged with lysine in a reaction mixture containing 0.16 M Tris-HCl (pH 7.5), 0.032 M ATP, 0.08 M KCl, 0.032 M $MgCl_2$, 0.016 M β-mercaptoethanol, 0.25 ml of rat liver synthetases, 5 μCi of [^{14}C]lysine (specific activity 269 mCi/mmole), or 20 μCi of [^{3}H]lysine (3.2 Ci/mmole), and 10 A_{260} units of fractionated rat liver $tRNA_I^{Lys}$ or $tRNA_{II}^{Lys}$ in a final volume of 3.0 ml. The reaction mixture was incubated for 45 min at room temperature. The mixture was then applied to a 1.3 × 6.5 cm DEAE-cellulose column which had been previously equilibrated with 0.25 M NaCl, 0.01 M sodium acetate, 0.01 M $MgCl_2$, and 0.001 M Na_2EDTA. Lysyl-tRNA was eluted by the same buffer containing 0.7 M NaCl. The radioactive peak was pooled and concentrated as described in the fractionation of lysyl-tRNA.

Ribosomal-Binding Assay. The procedure of Nirenberg and Leder (1964) was used for the ribosomal-binding assays. The ApApA and ApApG triplets were obtained from Miles Laboratories, Inc., Kankakee, Ill. Poly(A) and Poly(AG) (3:2) were obtained from Sigma Chemical Co., St. Louis, Mo. The reaction mixture (0.05 ml) contained 0.1 M Tris-acetate (pH 7.2), 0.02 M magnesium acetate, 0.05 M KCl, 2.5 A_{260} units of *E. coli* ribosomes, and 0.2 A_{260} unit of triplet or 40 μg of poly(A) or poly(AG) (3:2). [^{14}C]- or [^{3}H]Lys-tRNA was added as specified in the figure legends. Incubations were carried out at 25° for 15 min.

Preparation of Rat Liver Soluble Enzymes and Ribosomes. Liver tissue was homogenized in one volume of media A containing 0.35 M sucrose, 0.025 M KCl, 0.01 M $MgCl_2$, 0.035 M Tris-HCl (pH 7.5), and 0.01 M β-mercaptoethanol. The homogenate was centrifuged for 25 min at 32,000*g* and at 160,000*g* for 75 min. The resulting supernatant fraction was adjusted to contain 0.35 M KCl and mixed with DEAE-cellulose which had been previously equilibrated with media A containing no sucrose. This slurry was filtered by suction and the filtrate applied to a 1.5 × 50 cm Sephadex G-25 column. The column was eluted with a buffer containing 0.01 M KCl, 0.05 M Tris-HCl (pH 7.5), 0.01 M $MgCl_2$, 0.01 M β-mercaptoethanol, and 10% glycerol. The fractions emerging in the void volume were pooled and used in place of the conventional pH 5.0 fraction. The pellets containing the microsomes from the 160,000*g* centrifugation were resuspended in media A and sufficient sodium deoxycholate was added to give a 0.5% solution. This solution was then layered over medium B (0.9 M sucrose, 0.025 M KCl, 0.001 M $MgCl_2$, and 0.01 M β-mercaptoethanol and centrifuged for 75 min at 160,000*g*. The pellets were resuspended in media A to a concentration of 4–5 mg of ribosomal protein/ml. Ribosomes prepared in this manner contained no polysomes, but endogenous mRNA was present. Attempts to remove this RNA were unsuccessful.

Amino Acid Incorporation. The incorporation of lysine into protein was determined by the filter paper disk method of Mans and Novelli (1961). The reaction mixture contained 0.075 M Tris-HCl (pH 7.5), 0.01 M $MgCl_2$, 0.001 M ATP, 0.0002 M GTP, 0.04 M KCl, 0.08 M $(NH_4)Cl$, 0.01 M β-mercaptoethanol, 0.01 M phosphoenopyruvate, 40 μg of pyruvate kinase, 50 μg of poly(A) or poly(AG) (3:2), 0.5 μCi of [^{14}C]-lysine, 1.0–2.0 mg of rat liver soluble enzymes, 200 μg of ribosomal protein, and tRNA as specified in a final volume of 0.5 ml. Incubations were carried out at 37° and 0.1-ml aliquots were removed at different time intervals. Aliquots were spotted on Whatman 3MM filter paper disk, dried, and washed twice with 5% trichloroacetic acid at room temperature and once with 5% trichloroacetic acid at 90°. All trichloroacetic acid solutions contained 0.25% sodium tungstate and were adjusted to pH 2.0 (Gardner *et al.*, 1962). The disks were then washed in ethanol–ether (2:1, v/v) and ether, dried, and counted in a Packard Tri-Carb liquid scintillation counter.

Reticulocyte Lysate Preparation. Reticulocytes were obtained from the blood of phenylhydrazine-treated rabbits. The cells were collected by centrifugation in a refrigerated clinical centrifuge and washed twice in a 0.9% NaCl solution (Adamson *et al.*, 1968). The packed cells were hemolyzed at 4° by the addition of an equal volume of water and stirred for 5 min. This cell lysate was centrifuged at 30,000*g* for 10 min and the supernatant fraction was stored in liquid nitrogen. Each reaction mixture contained 0.04 M Tris-HCl (pH 7.5), 0.004 M $MgCl_2$, 0.001 M ATP, 0.0002 M GTP, 0.005 M phosphoenolpyruvate, 0.005 M β-mercaptoethanol, 20 μg of pyruvate kinase, 0.002 M lysine, 0.0001 M 19 other amino acids, [^{3}H]Lys-$tRNA_I$ or [^{3}H]Lys-$tRNA_{II}$ (40,000 cpm), and 0.2 ml of reticulocyte lysate in a final volume of 0.5 ml. Aliquots were removed at different intervals and spotted on on Whatman No. 3MM filter paper disks. The disks were first washed with 0.1 N HCl–acetone and then as described above for the lysine incorporation experiments.

Results

Fractionation of Lys-tRNA. When rat liver [^{3}H]Lys-tRNA was chromatographed on a reversed-phase Freon column, the results in Figure 1 were obtained. Two well-defined [^{3}H]lysyl-tRNA peaks were eluted with the 0.3–0.6 M NaCl

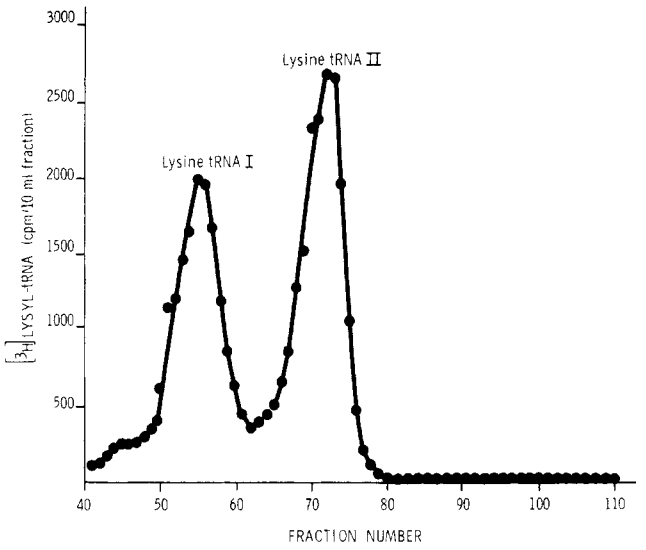

FIGURE 1: Elution profile of rat liver [^{3}H]Lys-tRNA from a 1.0 × 240 cm reversed-phase Freon column. [^{3}H]Lys-tRNA (40,000 cpm) was applied and the column was eluted with a 0.3–0.7 M NaCl linear gradient. The tRNA in each fraction (10 ml) was precipitated with 2.0 ml of 50% trichloroacetic acid, filtered on Millipore filters (0.45 μ pore size), dried, and counted. Recoveries of 90% were routinely obtained.

gradient. Similar separations were routinely obtained with $tRNA_I^{Lys}$ and $tRNA_{II}^{Lys}$ accounting for 43 and 57% of the total radioactivity, respectively.

E. coli Ribosomal Binding of Lys-tRNA$_I$ and Lys-tRNA$_{II}$. When pooled samples of [^{14}C]Lys-$tRNA_I$ and Lys-$tRNA_{II}$ were used in an *E. coli* ribosomal-binding assay, the results shown in Table I were found. This table shows the picomoles of [^{14}C]Lys-tRNA bound to *E. coli* ribosomes in the presence of ApApA, ApApG, poly(A), and poly(AG) (3:2). Lys-tRNA was found to bind with ApApG and poly(AG) (3:2), whereas Lys-$tRNA_{II}$ was found to bind with ApApA, poly(A), and poly(AG) (3:2). These data show that $tRNA_I^{Lys}$ is specific for the codon AAG and $tRNA_{II}^{Lys}$ is specific for the condon AAA. The small stimulation of Lys-$tRNA_I$ binding with ApApA and Lys-$tRNA_{II}$ binding with ApApG was shown to be due to the incomplete separation of the two Lys-tRNAs by reversed-phase Freon chromatography. Carbon *et al.* (1965), reported that mild iodine oxidation of $tRNA^{Lys}$ resulted in the specific inactivation of $tRNA_{II}^{Lys}$. A similar iodine oxidation was carried out in our laboratory with rat liver Lys-tRNA. When this preparation was used in a ribosomal-binding assay (Table II), our results showed that only ApApG stimulated the binding of [^{3}H]Lys-tRNA to ribosomes. These ribosomal-binding results agree with those of Carbon and Hung (1966) and were identical with those obtained in Table I with Lys-$tRNA_I$ except that the small binding seen with ApApA was lost. This suggests that the ApApA binding with Lys-$tRNA_I$ was due to the presence of a $tRNA_{II}^{Lys}$ impurity. This was confirmed by chromatography of the iodine-oxidized Lys-tRNA on a reversed-phase Freon column. We found that only 90% of the iodine-resistant tRNA chromatographed as Lys-$tRNA_I$ and that 10% chromatographed in the position of Lys-$tRNA_{II}$. Also treatment of our $tRNA_I^{lys}$ preparation with iodine caused a 10–15% loss of acceptor activity indicating the presence of a $tRNA_{II}^{Lys}$ contaminant.

TABLE I: Coding Response of Fractionated Rat Liver [^{14}C]Lys-tRNA.

	Δpmoles of [^{14}C]Lys-tRNA Bound to Ribosomes[a]	
Template	I	II
ApApA	0.10	0.54
ApApG	0.56	0.16
Poly(A)	0.07	0.53
Poly(AG) (3:2)	0.41	0.48
None[b]	0.44	0.88

[a] Δpmoles of [^{14}C]Lys-tRNA bound to ribosomes in the presence of template minus binding in the absence of template. [b] pmoles of [^{14}C]Lys-tRNA bound to ribosomes in the absence of template. Each 50-μl reaction mixture contained 0.1 M Tris-acetate (pH 7.2), 0.02 M magnesium acetate, 0.05 M KCl, 8.1 pmoles (2400 cpm) of [^{14}C]Lys-tRNAI or [^{14}C]Lys-tRNAII, 0.2 A_{260} unit of trinucleotide or 40 μg of poly(A) or poly(AG) (3:2), and 2.5 A_{260} units of *E. coli* ribosomes. Incubation was at 25° for 10 min.

TABLE II: Effect of Iodine Oxidation on Ribosomal Binding.[a]

Template	Δpmoles of [^{3}H]Lys-tRNA Bound to Ribosomes: tRNA Charged[b] after Ox	tRNA Charged[c] before Ox
ApApA	0.01	0
ApApG	0.37	0.14

[a] The reaction mixture was the same as shown in Table I except that [^{3}H]Lys-tRNA (specific activity 3.2 Ci/mmole) was used. Iodine oxidation was carried out according to Carbon *et al.* (1965). [b] 4.3 pmoles (3000 cpm) of [^{3}H]Lys-tRNA added. [c] 2.9 pmoles (2000 cpm) of [^{3}H]Lys-tRNA added.

In the above experiments the acceptance activity $tRNA_{II}^{Lys}$ was specifically inactivated by iodine oxidation. Whether this oxidation also inactivated the ribosomal-binding ability of this tRNA was tested by treating precharged [^{3}H]Lys-tRNA with iodine in the usual manner. Our results (also shown in Table II) indicate that the iodine oxidation also prevented the binding of [^{3}H]Lys-$tRNA_{II}$ since no ApApA-dependent binding was seen.

Poly(A)- and Poly(AG)- (3:2) Dependent Incorporation of [^{14}C]Lysine with $tRNA_I^{Lys}$ and $tRNA_{II}^{Lys}$. Since the ribosomal-binding assay appears to give different results in different laboratories, experiments were carried out to confirm the coding properties of $tRNA_I^{Lys}$ and $tRNA_{II}^{Lys}$ in a rat liver protein-synthesizing system. Rat liver $tRNA^{Lys}$ was fractionated into two peaks by reversed-phase Freon chromatography. Each peak of lysine acceptance was precipitated with ethanol, filtered, and eluted in deionized water. Equal amounts of $tRNA_I^{Lys}$ or $tRNA_{II}^{Lys}$, based on lysine-acceptance activity were added to a tRNA-dependent, protein-synthesizing system from rat liver. Figure 2 shows the poly(A)-dependent incorporation of [^{14}C]lysine in the presence of added $tRNA_I^{Lys}$ or $tRNA_{II}^{Lys}$. The addition of $tRNA_{II}^{Lys}$ to a poly(A)-dependent reaction markedly stimulated the incorporation

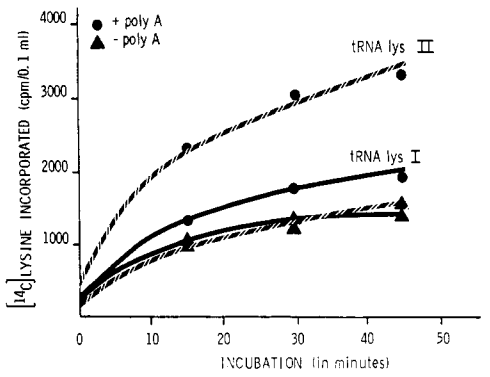

FIGURE 2: Poly(A)-dependent incorporation of [^{14}C]lysine stimulated by the addition of $tRNA_I^{Lys}$ (solid lines) or $tRNA_{II}^{Lys}$ (dashed lines). Conditions were those described in Materials and Methods. Each reaction mixture contained 50 pmoles of $tRNA^{Lys}$ as determined by acceptance activity. At the indicated times, 0.1-ml aliquots were withdrawn and the lysine incorporation into trichloroacetic acid precipitable material was determined.

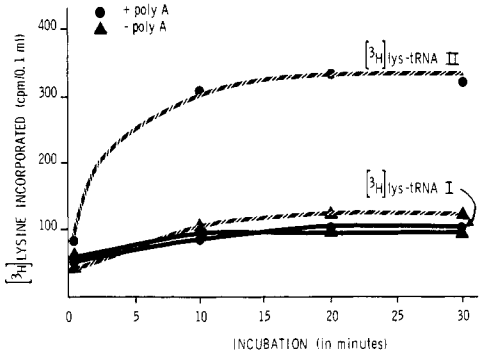

FIGURE 4: Poly(A)-dependent incorporation of [^{3}H]lysine from [^{3}H]Lys-$tRNA_I$ (solid lines) or [^{3}H]Lys-$tRNA_{II}$ (dashed lines). Conditions were the same as those in Figure 2 except that the [^{14}C]-lysine and $tRNA^{Lys}$ were replaced by precharged [^{3}H]Lys-tRNA (4.38 Ci/mmole). Each reaction mixture contained 4200 cpm of [^{3}H]Lys-$tRNA_I$ or [^{3}H]Lys-$tRNA_{II}$. Samples of 0.1 ml were taken at the times shown.

of [^{14}C]lysine whereas $tRNA_I^{Lys}$ exhibited only a slight stimulation. The slight stimulation observed with $tRNA_I^{Lys}$ is probably due to a combination of the incomplete tRNA separation mentioned earlier and the presence of small amounts of endogenous tRNA on the rat liver ribosomes. In the presence of poly(AG) (3:2) both Lys-tRNA peaks stimulated [^{14}C]lysine incorporation (Figure 3). This was expected, since the codons AAA and AAG are both present. The random poly(AG) (3:2) message contains codons for four different amino acids. Table III lists the incorporation into protein of all of these amino acids in response to poly(AG) (3:2). The incorporation of glutamic and arginine were found to be only slightly stimulated in the presence of $tRNA_I^{Lys}$ or $tRNA_{II}^{Lys}$, however, lysine incorporation was found to be stimulated 2- to 3-fold. Glycine incorporation was also stimulated but only with $tRNA_{II}^{Lys}$.

Poly(A)- and Poly(AG)- (3:2) Dependent Incorporation of [^{3}H]Lysine from [^{3}H]Lys-$tRNA_I$ and [^{3}H]Lys-$tRNA_{II}$. Yang and Novelli (1968) reported that reversed-phase chromatography of charged tRNA gave better separation than uncharged tRNA. In an effort to obtain better separations and to eliminate the problem of endogenous tRNA, [^{3}H]Lys-tRNA was prepared and chromatographed on a reversed-phase Freon column. The isolated [^{3}H]Lys-$tRNA_I$ and [^{3}H]-Lys-$tRNA_{II}$ were then used directly in the rat liver protein-synthesizing system. A 200-fold excess of cold lysine was added to prevent any recycling of [^{3}H]lysine. [^{3}H]Lys-$tRNA_I$ or [^{3}H]Lys-$tRNA_{II}$ (4200 cpm or 4.8 pmoles) was added to each reaction mixture. The addition of [^{3}H]Lys-$tRNA_{II}$ resulted in a marked stimulation of [^{3}H]lysine incorporation in a poly(A)-dependent system as shown in Figure 4. No significant [^{3}H]lysine incorporation was observed with the addition of [^{3}H]Lys-$tRNA_I$. In the poly(AG)- (3:2) dependent reaction, both peaks stimulated [^{3}H]lysine incorporation (Figure 5). The ratio of Lys-$tRNA_{II}$:Lys-$tRNA_I$ incorporation was found to be close to the ratio of AAA:AAG codons present in the random poly(AG) (3:2) copolymer.

Rabbit Reticulocyte Lysate Incorporation of [^{3}H]Lysine from [^{3}H]Lys-$tRNA_I$ and [^{3}H]Lys-$tRNA_{II}$. In order to show that both lysine-isoaccepting tRNA species are functionally active with a natural message, 60 pmoles of [^{3}H]Lys-$tRNA_I$ or [^{3}H]Lys-$tRNA_{II}$ was added to a reticulocyte lysate incorporating system. A 100-fold excess of cold lysine was added to prevent recycling of the [^{3}H]Lys-tRNA. Both Lys-tRNAs were found to be active in this system, however the amount of

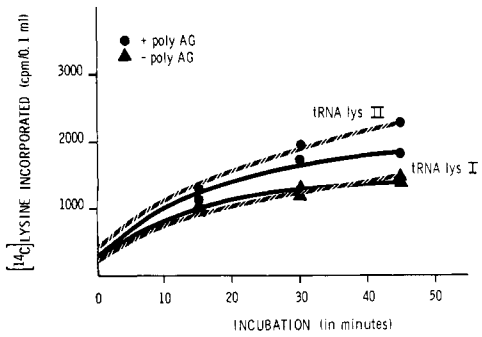

FIGURE 3: Poly(AG)-dependent incorporation of [^{14}C]lysine stimulated by the addition of $tRNA_I^{Lys}$ (solid lines) or $tRNA_{II}^{Lys}$ (dashed lines). Conditions were the same as those in Figure 2 except that 50 μg of poly(AG) (3:2) was used in place of poly(A).

TABLE III: Effect of $tRNA_I^{Lys}$ and $tRNA_{II}^{Lys}$ on Poly(AG)-Directed Amino Acid Incorporation.[a]

tRNA Added	pmoles of Amino Acid Incorporated in Response to Poly(AG) (3:2)			
	Gly	Lys	Glu	Arg
None	21.0	25.0	9.9	13.5
$tRNA_I^{Lys}$	22.6	64.20	12.0	15.4
$tRNA_{II}^{Lys}$	56.5	80.05	4.8	17.7

[a] Each reaction mixture contained 0.075 M Tris-HCl (pH 7.5), 0.001 M ATP, 0.0002 M GTP, 0.04 M KCl, 0.08 M NH_4Cl, 0.01 M β-mercaptoethanol, 0.01 M phosphoenolpyruvate, 40 μg of pyruvate kinase, 25 μg of poly(AG) (3:2), 0.2 mg of rat liver ribosomal protein, 1.6 mg of rat liver soluble enzyme, 0.6 A_{260} unit of $tRNA_I^{Lys}$ or $tRNA_{II}^{Lys}$, 0.5 μCi of [^{14}C]-glycine (116 mCi/mmole), [^{14}C]lysine (269 mCi/mmole), [^{14}C]glutamic acid (197 mCi/mmole) or [^{14}C]arginine (237 mCi/mmole), and 0.25 μmole of the other 19 amino acids in a final volume of 0.5 ml.

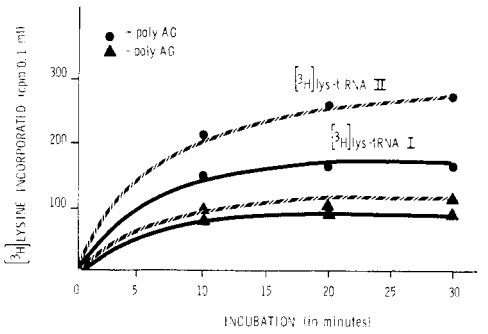

FIGURE 5: Poly(AG)-dependent incorporation of [^{3}H]lysine from [^{3}H]Lys-tRNA$_I$ (solid lines) or [^{3}H]Lys-tRNA$_{II}$ (dashed lines). Conditions were the same as those in Figure 4 except that 50 μg of poly(AG) (3:2) was used in place of poly(A).

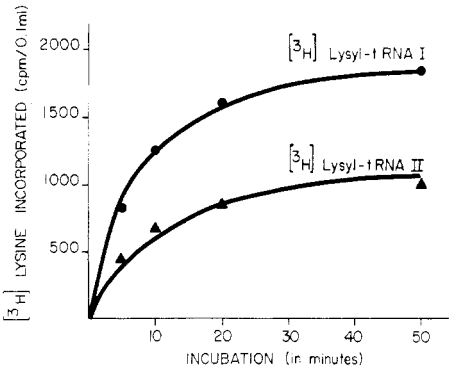

FIGURE 6: Incorporation of [^{3}H]lysine from [^{3}H]Lys-tRNA$_I$ and [^{3}H]Lys-tRNA$_{II}$ in a reticulocyte lysate system. Conditions were those described in Materials and Methods. Each reaction mixture contained 40,000 cpm of either [^{3}H]Lys-tRNA$_I$ or [^{3}H]Lys-tRNA$_{II}$. Samples of 0.1 ml were taken at the times shown.

[^{3}H]lysine incorporated from Lys-tRNA$_I$ was 2-fold greater than that incorporated from Lys-tRNA$_{II}$ (Figure 6).

Discussion

The present study indicates that the two isoaccepting species of lysine tRNA differ in their coding specificity as shown by both ribosomal-binding and amino acid incorporation studies. Our ribosomal-binding results agree with those of Carbon and Hung (1966), namely, that Lys-tRNA$_I$ binds only in response to ApApG while Lys-tRNA$_{II}$ binds only with ApApA.

In order to determine whether the specificity observed with the ribosomal-binding assay was also true for cell-free protein synthesis, a poly(A)- and poly(AG)-stimulated amino acid incorporating system from rat liver was established. In this system we were able to obtain a 2- to 4-fold stimulation by the addition of poly(A), however, considerable endogenous incorporation was still present. Our soluble enzyme preparation was found to be tRNA free since treatment of this fraction with high levels of ribonuclease did not release any acid-soluble, 260-nm-absorbing material. Sufficient tRNA was present in the ribosomal preparation, however, so that the incorporation system was not completely tRNA dependent. The results obtained by adding the $tRNA_I^{Lys}$ and $tRNA_{II}^{Lys}$ to this system agreed with our ribosomal-binding data. The incorporation of [^{14}C]lysine with poly(A) was stimulated 2- to 4-fold by $tRNA_{II}^{Lys}$, but only slightly by $tRNA_I^{Lys}$, whereas both $tRNA^{Lys}$ species were active with poly(AG). The stimulation with the poly(AG) (3:2) message was low in both cases since only 35% of the codons are specific for lysine.

The problem of endogenous tRNA contamination was overcome by the use of [^{3}H]Lys-tRNA as the only source of radioactivity in the incorporating system. Also precharging of the tRNA with lysine allowed a better separation in the reversed-phase Freon system. We found that the addition of [^{3}H]Lys-tRNA$_I$ failed to stimulate [^{3}H]lysine incorporation in the presence of poly(A), whereas [^{3}H]Lys-tRNA$_{II}$ exhibited a marked stimulation of [^{3}H]lysine incorporation. In the poly(AG)-dependent assay, both [^{3}H]lysyl-tRNA$_I$ and [^{3}H]Lys-tRNA$_{II}$-stimulated [^{3}H]lysine incorporation. Since the poly(AG) used has a ratio of 3A:2G, then on a random basis, there would be three AAA triplets for every two AAG triplets. Since Lys-tRNA$_I$ was shown to be inactive with poly(A) in both the ribosomal-binding and the amino acid incorporation assays, the [^{3}H]lysine incorporated from [^{3}H]lysine tRNA$_I$ in the poly(AG)-dependent assay must be due to the recognition of the AAG codon alone. Since the ratio of Lys-tRNA$_{II}$:Lys-tRNA$_I$ is approximately 3:2, this incorporation suggests that Lys-tRNA$_{II}$ responds to AAA only. The codon specificity is further substantiated by the results from the reticulocyte lysate synthesis of hemoglobin. [^{3}H]Lysine from Lys-tRNA$_I$ was found to incorporate 2-fold better than [^{3}H]Lys-tRNA$_{II}$, in spite of the fact that an equal amount of [^{3}H]Lys-tRNA was added to each reaction mixture. If lysine tRNA$_{II}$ recognizes both AAA and AAG, then one would expect better [^{3}H]lysine incorporation from Lys-tRNA$_{II}$ than from Lys-tRNA$_I$. Since just the opposite is true (see Figure 6), the data is best explained by a specificity of $tRNA_{II}^{Lys}$ for the AAA codon alone. Similar results were also reported recently by Woodward and Herbert (1971) for reticulocyte $tRNA^{Lys}$. In addition they showed that reticulocyte $tRNA_I^{Lys}$ and $tRNA_{II}^{Lys}$ were incorporated into different positions in the hemoglobin product.

Since our amino acid incorporation results agree with our ribosomal binding results, it may be concluded that $tRNA_I^{Lys}$ recognizes only the codon AAG, while $tRNA_{II}^{Lys}$ recognizes only the codon AAA. According to the "Wobble Hypothesis" of Crick (1966), the anticodon UUC should recognize the codon AAG specifically, whereas the codon UUU should recognize both AAA and AAG codons. Our results with $tRNA_I^{Lys}$ suggest that it has the anticodon sequence, UUC. However, our results with $tRNA_{II}^{Lys}$ suggest that this species specifically recognizes a codon which contains an adenosine residue in the third position of the codon without any wobbling effect. Similar coding specificity has also been reported for $tRNA^{Glu}$ from yeast by Sekiya *et al.* (1969). Their results showed that $tRNA_I^{Glu}$ coded for GAG and $tRNA_{III}^{Glu}$ coded for GAA only. They proposed that a thiolated uridine might be present at the first position of the anticodon, thereby allowing the specific recognition of GAA by $tRNA_{III}^{Glu}$. Recently, Kimura-Hareda *et al.* (1971) reported the isolation of a 5-methyl-2-thiouridine from rat liver $tRNA_{II}^{Lys}$ and yeast $tRNA_{III}^{Glu}$. This minor constituent, if present at the anticodon region, would explain the unusual specificity exerted by these two tRNA species. The replacement of an oxygen atom by a sulfur in uridine as in 5-methyl-2-thiouridine could prevent complete hydrogen bonding with guanine while at the same time, permitting hydrogen bonding with adenine. The proposal that this situation exists is further substantiated by the loss of the ribosomal binding of $tRNA_{II}^{Lys}$ upon iodine oxidation, since this procedure appears to be specific for 2-thiouridine.

References

Adamson, S. D., Herbert, E., and Godchaux, W. (1968), *Arch. Biochem. Biophys. 125*, 671.
Carbon, J. A., and Hung, L. (1966), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 25*, 403.
Carbon, J. A., Hung, L., and Jones, D. (1965), *Proc. Nat. Acad. Sci. U. S. 53*, 979.
Caskey, C. T., Beaudet, A., and Nirenberg, M. (1968), *J. Mol. Biol. 37*, 99.
Crick, F. (1966), *J. Mol. Biol. 19*, 548.
Gardner, R. S., Wahba, A. J., Basilio, C., Miller, R. S., Lengyel, P., and Speyer, J. F. (1962), *Proc. Nat. Acad. Sci. U. S. 48*, 2087.
Hatfield, D., and Portugal, F. H. (1970), *Proc. Nat. Acad. Sci. U. S. 67*, 1200.
Kimura-Hareda, F., Saneyoshi, M., and Nishimura, S. (1971), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 13*, 335.
Liu, L. P., and Ortwerth, B. J. (1971), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 30*, 1217.
Manns, R. J., and Novelli, G. D. (1961), *Arch. Biochem. Biophys. 94*, 48.
Nirenberg, M., and Leder, P. (1964), *Science 145*, 1399.
Nishimura, S., and Weinstein, I. B. (1969), *Biochemistry 8*, 832.
Sekiya, T., Takeishi, K., and Wkita, T. (1969), *Biochim. Biophys. Acta 182*, 411.
Söll, D., and RajBhandary, U. L. (1967), *J. Mol. Biol. 29*, 113.
Weiss, J. F., and Kelmers, A. D. (1967), *Biochemistry 6*, 2507.
Woodward, W. R., and Herbert, E. (1971), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 30*, 1217.
Yang, W. K., and Novelli, G. D. (1968), *Proc. Nat. Acad. Sci. U. S. 59*, 208.

Investigation of the Transfer of Amino Acid from a Transfer Ribonucleic Acid Synthetase–Aminoacyl Adenylate Complex to Transfer Ribonucleic Acid*

Emmett W. Eldred† and Paul R. Schimmel‡

ABSTRACT: The kinetics of transfer of isoleucine (Ile) from Ile-tRNA synthetase (IRS)–aminoacyl adenylate complex to $tRNA^{Ile}$ was investigated. Complex of enzyme with isoleucyl adenylate (Ile∼AMP) was isolated on a Sephadex column and reacted with $tRNA^{Ile}$ under a variety of conditions. It is shown that the reaction proceeds *via* a rapid initial formation of IRS-bound Ile—$tRNA^{Ile}$ followed by a slow release of the charged $tRNA^{Ile}$ from IRS. Neither step is significantly altered by the absence or presence (up to 5 mM) of added Mg^{2+}. Experiments employing an excess of Ile∼AMP over IRS demonstrate that a new molecule of Ile∼AMP binds to IRS prior to the dissociation of the just synthesized Ile—$tRNA^{Ile}$. Furthermore, the Ile∼AMP stimulates the release of enzyme-bound Ile—$tRNA^{Ile}$. It is also shown that synthesis of Ile—$tRNA^{Ile}$ starting from ATP and isoleucine proceeds with a rapid initial production of IRS-bound Ile—$tRNA^{Ile}$, followed by a much slower production of additional molecules of Ile—$tRNA^{Ile}$ in subsequent catalytic cycles of the enyzme. The slow phase results from the necessity of completing release of bound Ile—$tRNA^{Ile}$ before new Ile—$tRNA^{Ile}$ can be made. The results obtained support and extend the evidence from nitrocellulose filtration studies (Yarus, M., and Berg, P. (1969), *J. Mol. Biol. 42*, 171) that release of aminoacyl-tRNA is the rate-determining step in the aminoacylation of tRNA and that this release is promoted by Ile∼AMP. In addition, an examination of various kinetic data provides strong evidence against the hypothesis advanced by others that aminoacylation of tRNA proceeds *via* a concerted mechanism in which the aminoacyl adenylate is not an intermediate. A slightly modified purification procedure for IRS is given which improves the yield of enzyme by about 250% over an earlier published procedure.

The AA-tRNA[1] synthetase reaction involves the specific attachment of an amino acid to its cognate tRNA, the reaction involving a distinct tRNA synthetase for each amino acid. It is customarily written as a two-step process in which an enzyme–aminoacyl adenylate complex is first formed, followed by reaction of the complex with tRNA (Berg, 1961; Novelli, 1967). The two steps are

$$E + AA + ATP \overset{Mg^{2+}}{\rightleftharpoons} E{\cdot}AA{\sim}AMP + PP_i \quad (1)$$

$$E{\cdot}AA{\sim}AMP + tRNA \rightleftharpoons AA\text{-}tRNA + AMP + E \quad (2)$$

Although the amino acid specificity of eq 1 can be somewhat irregular (Baldwin and Berg, 1966a; Mitra and Mehler, 1967; Calendar and Berg, 1967), the error in the transfer step (eq 2) is negligibly small (Berg *et al.*, 1961. Loftfield *et al.*, 1963).

The kinetic and mechanistic features of eq 1 have recently been investigated (Cole and Schimmel, 1970a,b) for IRS from

* Contribution from the Departments of Biology and Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139. *Received August 19, 1971.* This work was supported by Grant No. GM-15539 from the National Institutes of Health, and by a grant from the Alfred P. Sloan Foundation.

† Predoctoral Fellow of the National Institutes of Health, 1968–1972.
‡ Alfred P. Sloan Fellow, 1970–1972; to whom to address correspondence.

[1] Abbreviations used are: AA, amino acid; AA∼AMP, aminoacyl adenylate; AA-tRNA, aminoacyl-tRNA; BSA, bovine serum albumin; E, enzyme; IRS, isoleucyl-tRNA synthetase; Ile∼AMP, isoleucyl adenylate; Ile—tRNA, isoleucine esterified to tRNA at the 2′- or 3′-terminal ribose hydroxyl; Val-ol-AMP, L-valinyl adenylate.