Properties of $tRNA_4^{Lys}$ from Various Tissues†

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ABSTRACT: Since the presence of one specific isoaccepting tRNA, $tRNA_4^{Lys}$, has been shown to correlate with the ability of a cell to divide, further studies on the properties of this tRNA were pursued. Preparations of lysyl-tRNA from several tissues were cochromatographed on reversed-phase Plaskon columns and in every case the Lys-$tRNA_4$ peaks eluted in the same position suggesting that this tRNA has similar composition regardless of tissue source. Also, $tRNA_4^{Lys}$ from rat liver, calf liver, Morris Hepatoma 7800, and mouse leukemic cells were all resistant to iodine oxidation. Ribosomal-binding studies with $tRNA_4^{Lys}$ from three sources showed that Lys-$tRNA_4$ binding was stimulated by the triplet ApApG but not by ApApA. In this respect $tRNA_4^{Lys}$ had the same coding properties as $tRNA_2^{Lys}$. In fact, the relative affinities of Lys-$tRNA_4$ and Lys-$tRNA_2$ were identical in this assay and distinctly different from Lys-$tRNA_5$. The isoaccepting lysyl-tRNAs of Morris Hepatoma 7800 were also tested in a rat liver, cell-free, protein synthesizing system with poly(A) and poly(AG) messages. Both $tRNA_2^{Lys}$ and $tRNA_4^{Lys}$ incorporated lysine into protein in response to poly(AG) but not in response to poly(A). It was also suggested that $tRNA_4^{Lys}$ functions in the synthesis of rat liver proteins *in vivo* since the tRNA could be isolated from a preparation of rat liver ribosomes. The tRNA fraction apparently represents those tRNA molecules which were actively transferring amino acids at the time the ribosomes were isolated.

$tRNA_4^{Lys}$ does not appear to be identical with $tRNA_2^{Lys}$, however, because $tRNA_4^{Lys}$ elutes at a different position during RPC-5 chromatography and because the chemical stability of the aminoacyl bond of Lys-$tRNA_4$ appears to be slightly greater than Lys-$tRNA_2$ under the same conditions. All these data are consistent with the idea that $tRNA_4^{Lys}$ may be a modified form of $tRNA_2^{Lys}$.

Many reports have appeared in the literature which show that changes in tRNA profiles can be seen when comparing normal and tumor tissues. We have extended these observations by showing that at least one species of tRNA, $tRNA_4^{Lys}$, is not only increased in tumor tissues, but is also present in normal tissues which undergo cell division (Ortwerth and Liu, 1973). More specifically it was proposed that $tRNA_4^{Lys}$ is present in dividing cells and cells which could be stimulated to divide, but is absent in cells which could no longer divide. This proposal was based solely on chromatographic evidence. This evidence was gathered from a wide variety of tissues and from tissues under different conditions. It was, therefore, necessary to show that $tRNA_4^{Lys}$ represented the same tRNA species with similar properties regardless of the biological source of the tRNA preparation. We also hoped that the properties of this tRNA species would suggest the manner in which it functions in the process of cell division.

Methods

All tRNAs were isolated by phenol extraction and DEAE-cellulose chromatography as described previously (Ortwerth and Liu, 1973). The tRNA synthetase used throughout this work was a crude tRNA synthetase isolated by Sephadex G-75 filtration of a 160,000*g* supernatant from rat liver. Aminoacylation of tRNA samples was carried out in a reaction mixture containing 0.16 M Tris-HCl (pH 7.5), 0.032 M ATP, 0.08 M KCl, 0.032 M $MgCl_2$, 0.0016 M β-mercaptoethanol, 5 μCi of [^{14}C]lysine or 25 μCi of [^{3}H]lysine, 2.0–10.0 A_{260} units of tRNA, and 0.25–0.50 ml of the rat liver tRNA synthetase fraction in a total volume of 5.0 ml. In larger reaction mixtures, each component was increased proportionately. Aminoacyl-tRNAs were isolated by DEAE-cellulose chromatography which is described by Yang and Novelli (1968). The aminoacyl-tRNA fraction was diluted to 0.5 M in NaCl and made pH 4.5 by the addition of acetate buffer. Samples prepared in this manner and stored at $-70°$ suffered little hydrolysis over many months and could be thawed and applied directly to an RPC-5 column. tRNA chromatography was performed on 2.5 × 100 cm reversed-phase Plaskon columns (RPC-5). The column packing was prepared as described by Pearson *et al.* (1971).

All columns were developed with a 2-l. linear gradient from 0.5 to 0.8 M or 0.5 to 1.0 M NaCl. Each gradient contained 0.01 M $MgCl_2$, 0.01 M sodium acetate (pH 4.5), and 0.03 M β-mercaptoethanol in addition to the NaCl. A constant flow rate of 1.5–2.0 ml/min was maintained with a pump and about 180 fractions of 10 ml each were collected. A portion of the gradient (70–100 tubes) was removed and the tRNA precipitated by the addition of 2.0 ml of 50% Cl_3CCOOH. Each tube was stirred vigorously and kept on ice for at least 30 min. The contents of each tube was then filtered on Millipore filters (0.45 μ pore size), dried, and counted. The distribution of $tRNA^{Lys}$ following reversed-phase chromatography of uncharged tRNA was obtained by assaying aliquots from every tube by the filter paper disc method of Mans and Novelli (1961).

The rat liver [^{3}H]Lys-$tRNA_2$ and [^{3}H]Lys-$tRNA_5$ samples were prepared by RPC-5 chromatography of a single precharged sample. The rat liver $tRNA_4^{Lys}$ preparation was first isolated by RPC-5 chromatography at pH 4.5 and then aminoacylated with [^{3}H]lysine. This separation is presented in the Results section. Calf liver $tRNA_2^{Lys}$, $tRNA_4^{Lys}$, and $tRNA_5^{Lys}$

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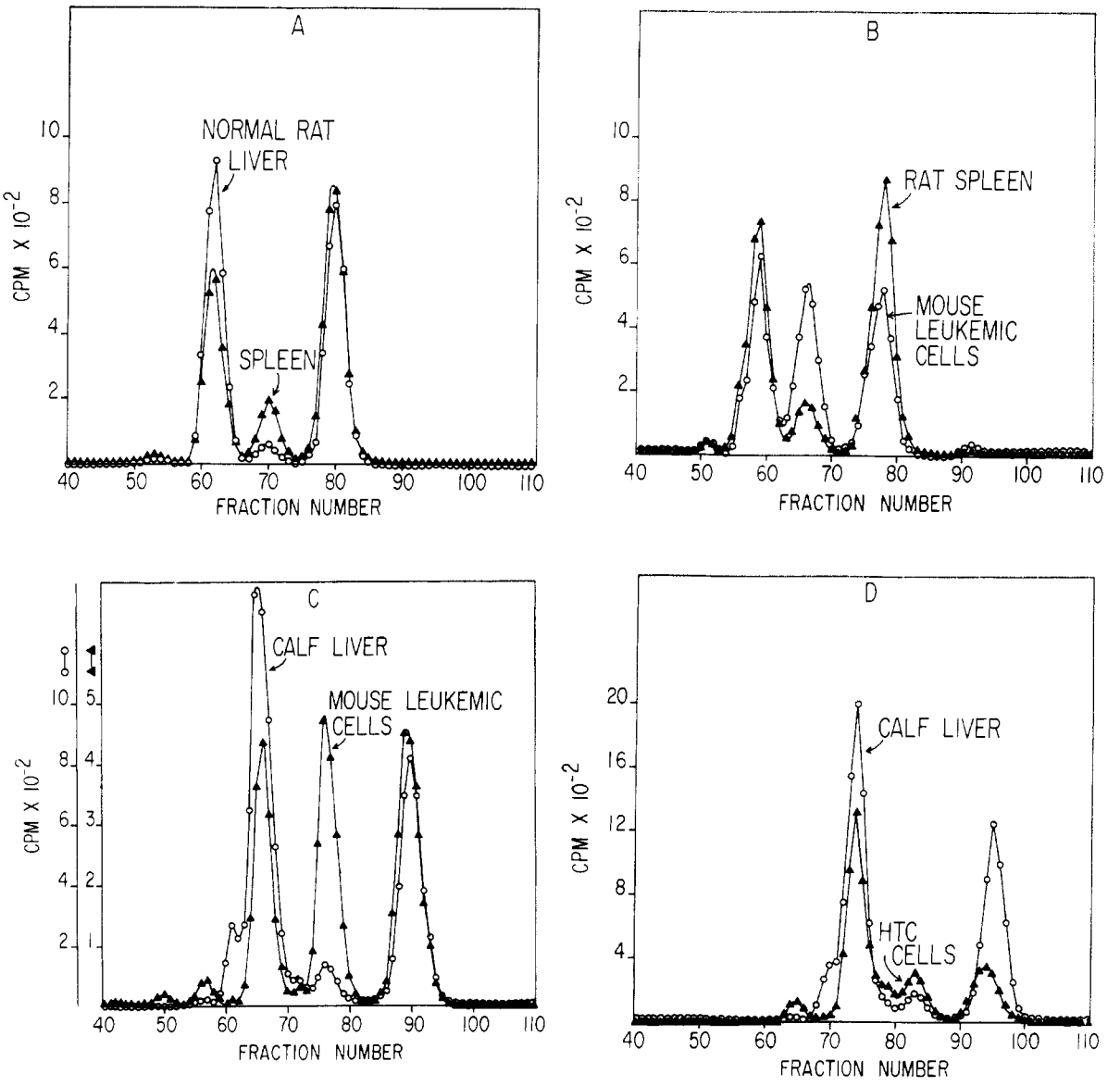

FIGURE 1: Cochromatography of Lys-tRNA from several different sources. (A) Cochromatography of [^{3}H]Lys-tRNA from rat spleen with [^{14}C]Lys-tRNA from rat liver. (B) Cochromatography of [^{3}H]Lys-tRNA from rat spleen with [^{14}C]Lys-tRNA from mouse leukemic cells. (C) Cochromatography of [^{3}H]Lys-tRNA from mouse leukemic cells with [^{14}C]Lys-tRNA from 2-day old calf liver. (D) Cochromatography of [^{3}H]Lys-tRNA from HTC cells with [^{14}C]Lys-tRNA from 2-day old calf liver. All separations were carried out using RPC-5 chromatography with a 0.5–1.0 M NaCl gradient.

were isolated by RPC-5 chromatography at pH 4.5 and aminoacylated with [^{14}C]lysine. Each [^{14}C]Lys-tRNA peak was rechromatographed with crude calf liver [^{3}H]Lys-tRNA. Both Lys-tRNA$_2$ and Lys-tRNA$_5$ eluted as single peaks, whereas Lys-tRNA$_4$ contained a 15% impurity of Lys-tRNA$_2$. The Morris Hepatoma Lys-tRNAs used in the protein synthesis experiment were separated by RPC-5 chromatography of precharged [^{3}H]Lys-tRNA. Mouse leukemic cell tRNA was processed in the same manner; however, this separation was not as sharp as usual. Lys-tRNA$_2$ accounted for only 15%, while Lys-tRNA$_4$ was over 50% of the total Lys-tRNA.

Iodine oxidation was carried out according to the method of Carbon *et al.* (1965). Ribosomal binding studies were carried out using the method of Nirenberg and Leder (1964) as described previously (Liu and Ortwerth, 1972). All protein synthesis procedures were carried out exactly as described previously for rat liver Lys-tRNA (Liu and Ortwerth, 1972). Ribonuclease T$_1$ treatments of Lys-tRNA were accomplished using the method of Berg *et al.* (1962) as described by Ishida and Miura (1963).

For stability studies, samples of charged Lys-tRNAs (20-50,000 cpm) were placed in 5.0 ml of water containing a drop of 0.1 M sodium acetate (pH 4.5). To this mixture 5.0 ml of 0.1 M Tris-HCl (pH 7.5) was added with vigorous mixing. This was placed in a constant-temperature water bath at 25° and samples of 1.0 ml were removed every 20 min for 3 hr. Each sample was pipetted directly into 1.0 ml of cold 10% Cl_3-CCOOH, mixed, and placed in ice. At the end of the experiment, the samples were filtered on Millipore filters, dried, and counted.

Rat liver ribosomes were isolated as described previously (Liu and Ortwerth, 1972). After sedimenting the ribosomes through 0.9 M sucrose, the pellets were resuspended in 0.35 M sucrose, 0.025 M KCl, 0.01 M $MgCl_2$, 0.035 M Tris-HCl (pH 7.5), 0.01 M β-mercaptoethanol, and 0.25% sodium dodecyl sulfate. Total RNA was isolated by phenol extraction and

tRNA was isolated by DEAE-cellulose chromatography. This tRNA was charged with [^{3}H]lysine and separated by RPC-5 chromatography.

Results

Before we could study the properties of $tRNA_4^{Lys}$ in detail it was necessary to determine whether this tRNA was indeed the same peak in the various tissues where it has been shown to be present (Ortwerth and Liu, 1973). Chromatographic evidence presented in the previous paper showed that this tRNA eluted between the two major Lys-tRNA species, $tRNA_2^{Lys}$ and $tRNA_5^{Lys}$. To establish that these tRNAs were chromatographically the same, two tRNA samples were charged with either [^{3}H]- or [^{14}C]lysine and chromatographed on the same column. Four of these cochromatography experiments are shown in Figure 1. In keeping with the numbering system presented in the previous paper (Ortwerth and Liu, 1973), the two main $tRNA^{Lys}$ peaks are $tRNA_2^{Lys}$ and $tRNA_5^{Lys}$. The $tRNA_4^{Lys}$ peaks elutes about midway between the two main peaks and appears to elute at the same salt concentration in each comparison. In the comparison of calf liver and mouse leukemic cell tRNA, the peaks are unusually distinct and it can be seen that $tRNA_4^{Lys}$ is well separated from another minor peak, $tRNA_3^{Lys}$, which is only occasionally seen. This shows that the major peak in mouse leukemic cell tRNA is indeed $tRNA_4^{Lys}$. In general, the peaks are symmetrical with the peak tube being the same in every case.

The possibility that $tRNA_4^{Lys}$ was an artifact produced by our procedures was also considered. This seemed unlikely, however, due to the reproducibility of our chromatographic profiles when several tRNA preparations from the same tissue were run. The amount of $tRNA_4^{Lys}$ was constant if either [^{3}H]- or [^{14}C]lysine was used or if the rat liver synthetase preparation was freshly prepared or stored for 6 months at $-20°$. The presence of ribonuclease-damaged molecules was determined by heating a preparation of calf liver tRNA to 80° and allowing it to cool slowly before aminoacylation as suggested by Nishimina and Novelli (1965). When this tRNA was chromatographed, the profile was similar to that for untreated calf liver tRNA. Misrecognition was also eliminated as a possible explanation, since the inclusion of 19 other amino acids in the aminoacylation reaction did not change the profile of calf liver tRNA. Since $tRNA_4^{Lys}$ is present in precharged profiles of Lys-tRNA, it cannot represent a tRNA molecule which has an incomplete CpCpA sequence at the 3′ end. The possibility that $tRNA_4^{Lys}$ was an inactive configuration of a major $tRNA^{Lys}$, however, had not been eliminated. Such a tRNA has been previously reported by Ishida and Sueoka (1967) for tryptophanyl-tRNA from *E. coli*. Panels A and B of Figure 2 show the elution profiles of rat liver tRNA when the tRNA was precharged with lysine and when the column was run with uncharged tRNA, and the lysine acceptance was determined on each fraction. In both cases $tRNA_4^{Lys}$ accounts for about 3% of the total lysine acceptance activity. These data show that $tRNA_4^{Lys}$ is not produced during the aminoacylation reaction and that this tRNA is active in accepting lysine after chromatography. We have also shown that precharged $tRNA_4^{Lys}$ can be chromatographed, discharged, and again recharged without any apparent loss in activity. Rechromatography of $tRNA_4^{Lys}$ shows that it elutes in the same position as it had originally. While all the data presented in Figure 2 were obtained using RPC-5, similar results were obtained with RPC-2; however, RPC-2 cannot separate

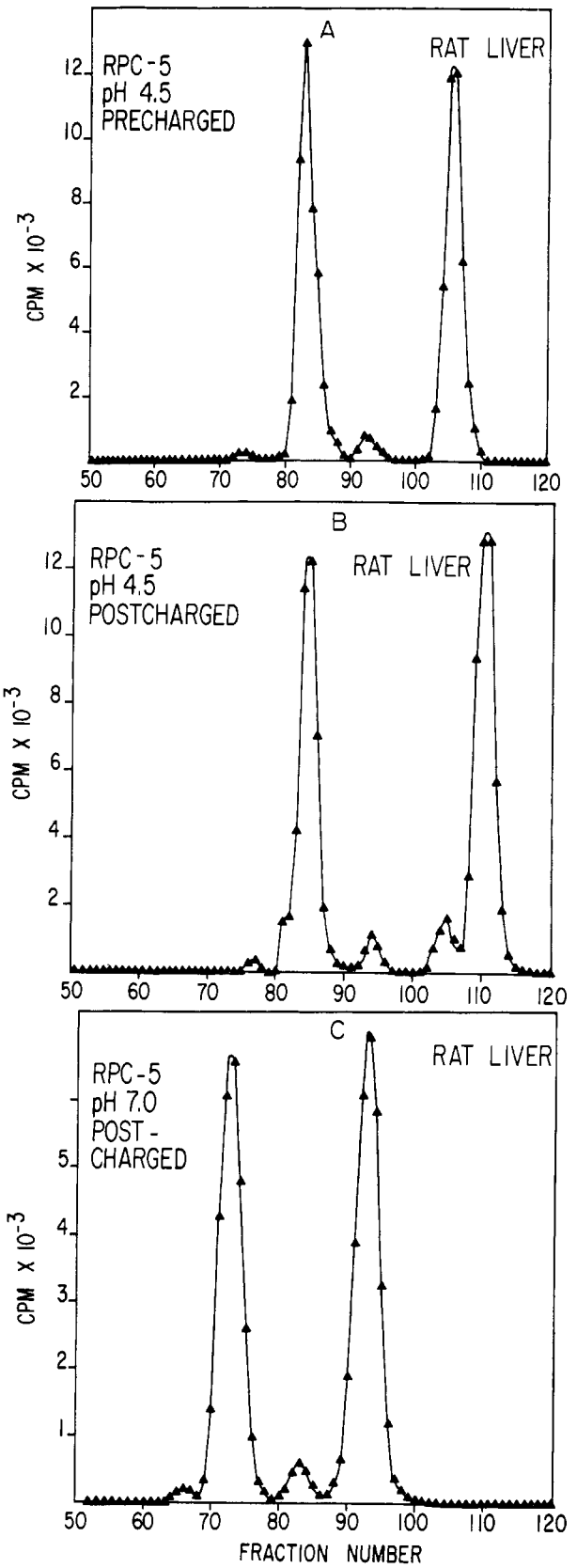

FIGURE 2: Chromatography of rat liver Lys-tRNA under various conditions. (A) Chromatography of 180 A_{260} units of rat liver tRNA precharged with [^{3}H]lysine. The ordinate shows Cl_3CCOOH precipitable counts present in a 0.05-ml aliquot from each tube. (B) Chromatography of 180 A_{260} units of rat liver tRNA at pH 4.5. The ordinate represents the [^{3}H]lysine acceptance activity of a 0.05-ml aliquot from each tube. (C) Chromatography of 180 A_{260} units of rat liver tRNA at pH 7.0. The ordinate represents the [^{3}H]lysine acceptance activity of a 0.05-ml aliquot from each tube.

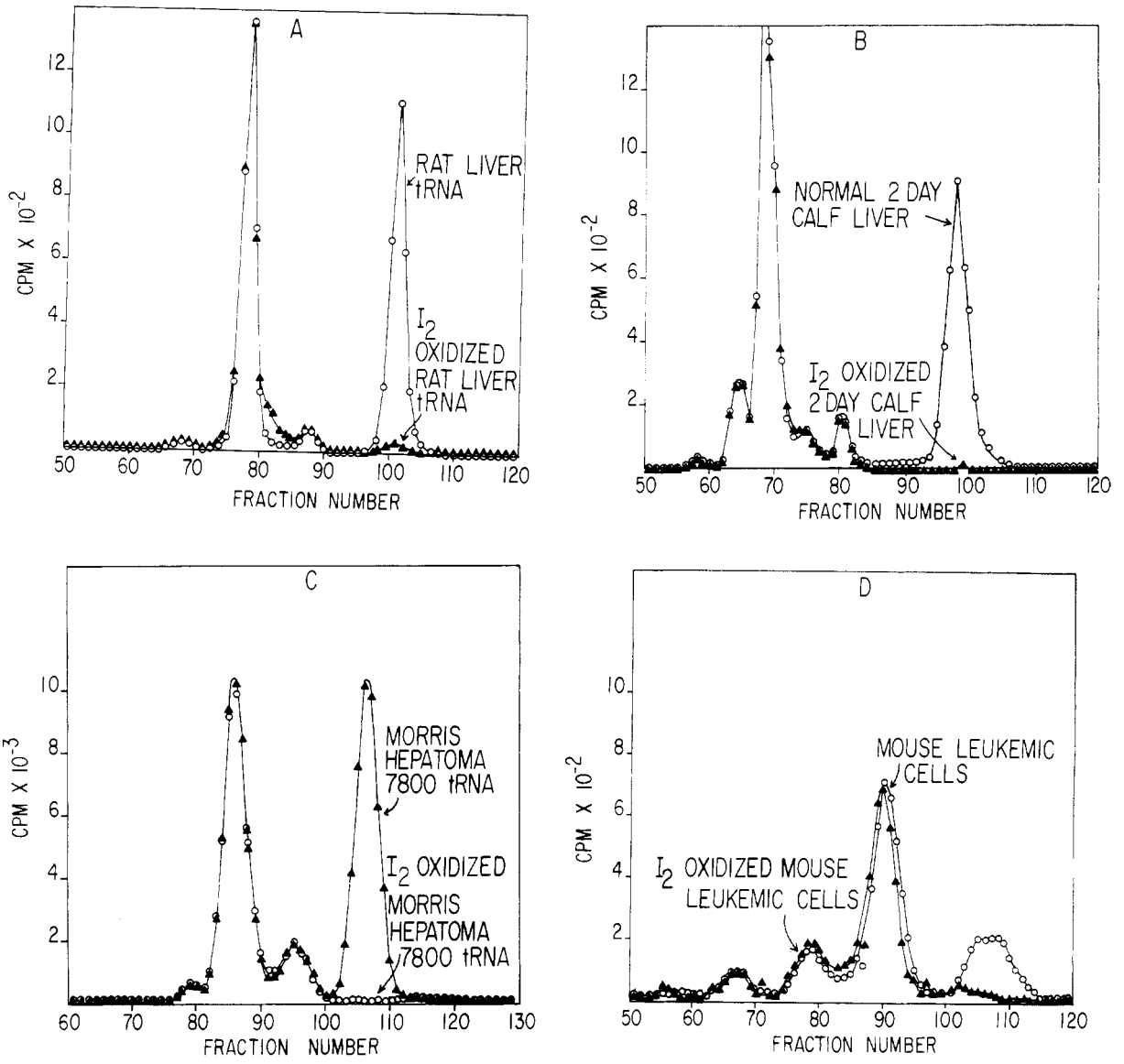

FIGURE 3: Effect of iodine oxidation on the cochromatography of the Lys-tRNA from several different sources. (A) Cochromatography of [^{14}C]normal and [^{3}H]iodine-oxidized Lys-tRNA from rat liver. (B) Cochromatography of [^{14}C]normal and [^{3}H]iodine-oxidized Lys-tRNA from 2-day old calf liver. (C) Cochromatography of [^{3}H]normal and [^{14}C]iodine-oxidized Lys-tRNA from Morris Hepatoma 7800. (D) Cochromatography of [^{14}C]normal and [^{3}H]iodine-oxidized Lys-tRNA from mouse leukemic cells. All separations were carried out using RPC-5 chromatography with a 0.5–1.0 M NaCl gradient.

$tRNA_3^{Lys}$ and $tRNA_4^{Lys}$ and was, therefore, abandoned. Panel C of Figure 2 shows the separation of rat liver $tRNA^{Lys}$ at pH 7.0 instead of pH 4.5 which is normally used. When the lysine acceptance of each tube was measured, it was apparent that $tRNA_4^{Lys}$ was cleanly separated from the major Lys-tRNAs and that it accounted for about 3% of the total. In fact, the distribution of rat liver $tRNA^{Lys}$ at pH 7.0 was exactly the same as the precharged Lys-tRNA at pH 4.5. The distribution of uncharged $tRNA^{Lys}$ at pH 4.5, however, indicated the presence of another peak eluting between $tRNA_4^{Lys}$ and $tRNA_5^{Lys}$. When the percentage of Lys-tRNA in each peak was calculated and compared to the precharged profile, it appears that this new $tRNA^{Lys}$ elutes with Lys-$tRNA_2$ in the precharged profile. The fact that charged Lys-tRNA chromatographs differently from uncharged $tRNA^{Lys}$ has been reported previously (Chatterjee and Kaji, 1970; Yang and Novelli, 1968). This new tRNA species appears to be the only variable species and accounts for slightly more than 5% of the total lysine acceptance in rat liver. The properties of this tRNA are currently being investigated in our laboratory. It should be noted that separations of $tRNA^{Lys}$ have also been carried out with BD-cellulose. While the separation is much poorer than RPC-5, a difference in the precharged and post-charged profiles can also be seen.

As a result of the above data, it is apparent that $tRNA_4^{Lys}$ represents a distinct tRNA species which is present in a wide variety of tissues. While this tRNA may represent a new gene product, it could also be a modified form of one of the major species of Lys-tRNA. Due to the work of Carbon *et al.* (1965), it is an easy task to distinguish between the two major species of Lys-tRNA in mammalian tissues. Oxidation of mammalian $tRNA^{Lys}$ by I_2–KI will completely inactivate $tRNA_5^{Lys}$, while $tRNA_2^{Lys}$ is resistant as judged by aminoacylation and subsequent chromatography. To determine the effect of iodine oxidation on $tRNA_4^{Lys}$, four samples of tRNA were treated, aminoacylated, and chromatographed with con-

TABLE I: Binding of Fractionated Lys-tRNAs to *E. coli* Ribosomes.

Input	Triplet	cpm Bound	Δcpm Bound	Δpmoles Bound
	Rat Liver tRNA			
[^{3}H]Lys-$tRNA_2$	ApApA	837	0	0
6500 cpm[a]	ApApG	1904	975	1.30
	None	929		
[^{3}H]Lys-$tRNA_4$	ApApA	1364	24	0.02
5870 cpm[b]	ApApG	2324	984	0.79
	None	1340		
[^{3}H]Lys-$tRNA_5$	ApApA	3173	1334	1.92
7000 cpm[a]	ApApG	2810	981	1.40
	None	1829		
	Calf Liver tRNA			
[^{14}C]Lys-$tRNA_2$	ApApA	600	0	0
2160 cpm[c]	ApApG	1268	599	1.71
	None	669		
[^{14}C]Lys-$tRNA_4$	ApApA	354	0	0
1540 cpm[c]	ApApG	792	415	2.69
	None	377		
[^{14}C]Lys-$tRNA_5$	ApApA	1148	395	1.13
2100 cpm[c]	ApApG	1051	298	0.85
	None	753		
	Mouse Leukemic Cell tRNA			
[^{3}H]Lys-$tRNA_2$	ApApA	1730	128	0.10
3520 cpm[b]	ApApG	2482	880	0.70
	None	1602		
[^{3}H]Lys-$tRNA_4$	ApApA	3260	265	0.21
8120 cpm[b]	ApApG	5780	2785	2.22
	None	2995		
[^{3}H]Lys-$tRNA_5$	ApApA	4212	659	0.53
7140 cpm[b]	ApApG	4089	495	0.40
	None	3553		

[a] 3.0 Ci/mmol. [b] 5.0 Ci/mmol. [c] 341 mCi/mmol.

trol samples treated in a similar manner with KI only. In each case $tRNA_5^{Lys}$ was completely inactivated, whereas $tRNA_2^{Lys}$ and $tRNA_4^{Lys}$ were resistant. These profiles are shown in Figure 3. In general, each set of profiles are superimposable except for the loss of $tRNA_5^{Lys}$. In the rat liver profile, however, a species appears to be present which elutes as a shoulder on the $tRNA_2^{Lys}$ peak. Preliminary evidence suggests that this is a species of tRNA which normally chromatographs with peak 5, but is iodine resistant. Oxidation with iodine, however, does change its chromatographic position. $tRNA_4^{Lys}$, however, does not appear to be changed in any manner by the iodine treatment. In this regard, it is similar to $tRNA_2^{Lys}$.

If $tRNA_4^{Lys}$ is a modified $tRNA_2^{Lys}$, we could expect the anticodon sequence to be CUU and read the codon AAG only (Liu and Ortwerth, 1972). To test this possibility, ribosomal-binding studies were carried out with the triplets ApApA and ApApG. Samples of $tRNA_4^{Lys}$ and the two major $tRNA^{Lys}$ species, $tRNA_2^{Lys}$ and $tRNA_5^{Lys}$, were tested for their binding ability. Also, tRNA species from three different sources were tested in order to confirm that the $tRNA_4^{Lys}$ in each tissue has the same biological activity. These ribosomal-binding experiments are shown in Table I. As can be seen in every case, $tRNA_4^{Lys}$ read only one codon, AAG. It is, therefore, similar to $tRNA_2^{Lys}$ and distinct from

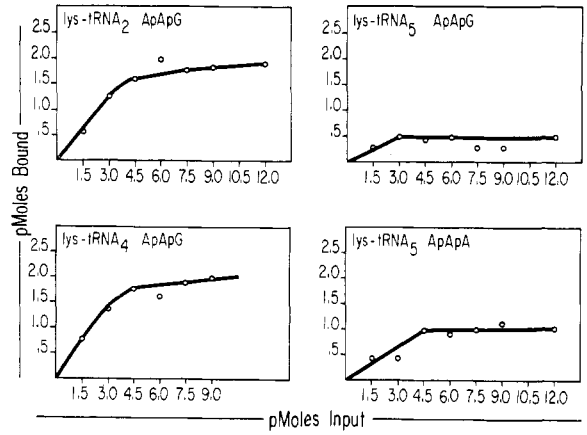

FIGURE 4: Effect of increasing levels of Lys-tRNA on the binding of Lys-tRNA to *E. coli* ribosomes. Two-day old calf liver tRNA was separated by RPC-5 chromatography. The lysine acceptance activity of each fraction was determined and the major peaks were pooled. Each isoaccepting $tRNA^{Lys}$ was charged with [^{14}C]lysine (342 mCi/mmol) and tested for ribosomal-binding activity in the input amounts shown.

$tRNA_5^{Lys}$, which appears to read both AAA and AAG. Actually, the coding response of $tRNA_5^{Lys}$ is not completely clear, since $tRNA_5^{Lys}$ is composed of at least two tRNA species, one of which is resistant to iodine oxidation. This is true of rat liver and calf liver $tRNA_5^{Lys}$ but was not tested with mouse leukemic cell $tRNA_5^{Lys}$.

Since $tRNA_4^{Lys}$ has the same coding properties as $tRNA_2^{Lys}$, we wanted to see if these two tRNAs differed in their affinities for the ApApG–ribosome complex. Due to the different inputs and different radiolabels, the data in Table I would not accurately reflect differences in relative affinity. In Figure 4, the effect of increased Lys-tRNA levels was measured on the binding of Lys-$tRNA_2$, Lys-$tRNA_4$, and Lys-$tRNA_5$ to a constant amount of *E. coli* ribosomes and ApApG triplet. The results in panels A and B show that $tRNA_2^{Lys}$ and $tRNA_4^{Lys}$ are identical in their binding affinity. On the other hand, $tRNA_5^{Lys}$ binds only half as well as $tRNA_2^{Lys}$ or $tRNA_4^{Lys}$ even at saturating levels (panel C). Also $tRNA_5^{Lys}$ preferentially binds to ApApA (panel D) whereas, $tRNA_2^{Lys}$ and $tRNA_4^{Lys}$ showed no significant binding even at saturating levels. The $tRNA_4^{Lys}$ used in this experiment was obtained from an RPC-5 column run with 2000 A_{260} units of calf liver tRNA. In this case, the separation was not as sharp as with small samples and $tRNA_4^{Lys}$ contained about 15% of $tRNA_2^{Lys}$ as judged by rechromatography of a small precharged sample. This amount of $tRNA_2^{Lys}$, however, is insufficient to account for the amount of tRNA bound to ribosomes in panel B, and since saturation point is reached at the same input, we conclude that $tRNA_4^{Lys}$ has the same affinity as $tRNA_2^{Lys}$. Whether differences exist in the affinity of these two tRNAs for mammalian ribosomes is not clear. Attempts to do these experiments with washed rat liver ribosomes were unsuccessful.

We have demonstrated, however, that $tRNA_2^{Lys}$ and $tRNA_4^{Lys}$ do have the same qualitative coding properties in a mammalian system. This was done using a cell-free, protein-synthesizing system from rat liver. Precharged samples of tRNA were added and the incorporation was measured into Cl_3CCOOH precipitable material both in the presence and absence of added synthetic polynucleotides. Previous work with rat liver tRNA showed that $tRNA_2^{Lys}$ incorporated only in response to poly(AG) whereas, $tRNA_5^{Lys}$ incorporates in

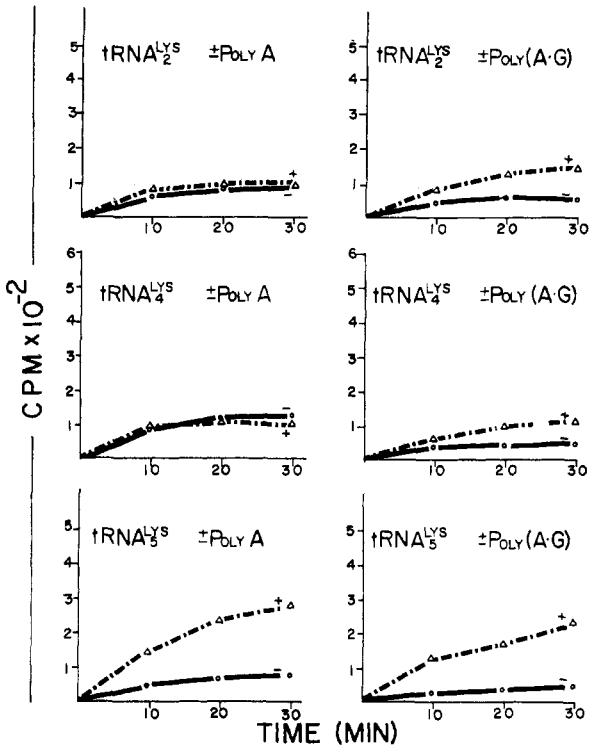

FIGURE 5: Incorporation of [^{3}H]lysine from the isoaccepting Lys-tRNAs of Morris Hepatoma 7800 into protein both in the presence and absence of synthetic polynucleotides. Crude tRNA from Morris Hepatoma 7800 was charged with [^{3}H]lysine and separated by RPC-5 chromatography. Each peak was pooled, precipitated with ethanol, filtered, and eluted with water. Aliquots were added to a cell-free, protein-synthesizing system from rat liver. Incorporation was measured both in the presence and absence of either poly(A) or poly(AG) (3:2).

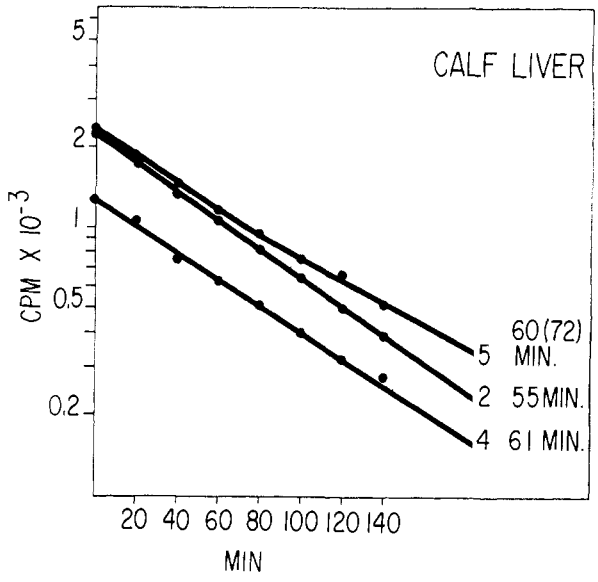

FIGURE 7: Stability of the isoaccepting Lys-tRNAs from calf liver to chemical hydrolysis of the aminoacyl-tRNA bond. Times shown are half-lives in minutes. Where a break was present in the decay curve, two separate half-lives are shown. The curves in each panel were run simultaneously.

response to both poly(A) and poly(AG) (Liu and Ortwerth, 1972). In the data presented in Figure 5, Morris Hepatoma 7800 tRNA was used since this tRNA had not been previously studied in the ribosomal binding assay. As can be seen, tRNA$_2^{Lys}$ and tRNA$_5^{Lys}$ had the same activity as the tRNAs from rat liver. In this system tRNA$_4^{Lys}$ was incorporated in response to poly(AG) only and was, therefore, similar to tRNA$_2^{Lys}$. These data agree with the previous ribosomal binding data.

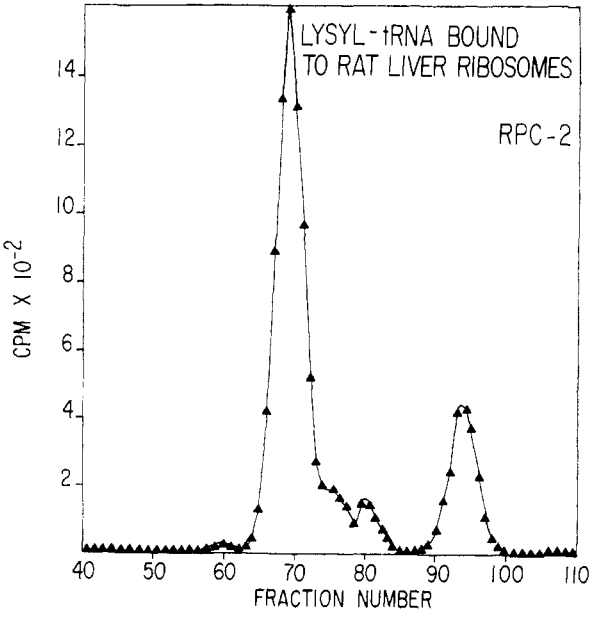

FIGURE 6: [^{3}H]Lys-tRNA profile for tRNA isolated from a rat liver ribosomal preparation. Separation was carried out using RPC-2 chromatography with a 0.3–0.6 M NaCl gradient.

Even though we have demonstrated that tRNA$_4^{Lys}$ will function both in ribosomal binding and in *in vitro* protein synthesis, we wanted to be able to get some indication that this tRNA was being used in protein synthesis *in vivo*. We felt that this could be shown indirectly by looking for the presence of tRNA$_4^{Lys}$ bound to isolated ribosomes. We had previously demonstrated that rat liver ribosomes contain bound tRNAs which cannot be removed with low salt washes (Liu and Ortwerth, 1972). This tRNA fraction is presumably the tRNA which is in the act of synthesizing protein. This tRNA fraction was, therefore, extracted from the ribosomes, isolated, aminoacylated with lysine, and chromatographed on RPC-5. This profile is shown in Figure 6. This profile differs from the profile for rat liver cytoplasmic tRNAs and, therefore, does not appear to result from nonspecific binding of tRNA to ribosomes. It is clear from this profile that Lys-tRNA$_4$ is bound to isolated ribosomes and presumably is functional in the synthesis of rat liver proteins.

All the data presented, so far, suggest that tRNA$_4^{Lys}$ is a modified form of tRNA$_2^{Lys}$. We attempted to gain further support for this idea, by studying the 3′ end of these two tRNAs. In this procedure the individual, isoaccepting tRNAs were aminoacylated with lysine and treated with ribonuclease T$_1$. After the enzymatic hydrolysis was complete, the 3′-terminal fragment was left with the radioactive amino acid attached. The digest was chromatographed on a DEAE-cellulose column with an ammonium formate gradient. The elution position of the radioactivity is determined mainly by the size of the 3′ fragment released.

Using this system, rat liver Lys-tRNA$_2$, Lys-tRNA$_4$, and Lys-tRNA$_5$ all produced an oligonucleotide which passed directly through the DEAE-cellulose column. Subsequent paper chromatography indicated that the lysine was still

attached to a nucleotide fragment and was not free lysine. This suggests that the T_1 fragment is very short and may be ApCpCpG. Since all the Lys-tRNAs produced the same fragment, it can only be said that the data are not inconsistent with $tRNA_4^{Lys}$ being a modified form of $tRNA_2^{Lys}$.

If, indeed, the only difference between $tRNA_2^{Lys}$ and $tRNA_4^{Lys}$ is the presence or absence of a modified base, we would expect these tRNAs to have similar properties and they do. However, we would also expect these tRNAs to exhibit some differences as a result of the modification. One important difference has been presented. This is the fact that $tRNA_4^{Lys}$ chromatographs as a discrete peak which is cleanly separated from $tRNA_2^{Lys}$. This difference persists on rechromatography and suggests a structural difference between these two tRNAs. This idea was supported by measurements of the stability of the lysine to tRNA bond with Lys-$tRNA_2$ and Lys-$tRNA_4$. The data obtained in one comparison with calf liver Lys-tRNAs are shown in Figure 7. As can be seen from the calculated half-lives, Lys-$tRNA_4$ appears to be more stable than Lys-$tRNA_2$. The stability of Lys-$tRNA_4$ was closer to that of Lys-$tRNA_5$. Similar differences were obtained in other measurements with calf liver Lys-tRNAs and with rat liver Lys-tRNAs; however, no difference was seen when comparing Lys-$tRNA_2$ and Lys-$tRNA_4$ from mouse leukemic cells. In this case both tRNAs had half-lives which were comparable to $tRNA_4^{Lys}$ from calf liver and rat liver.

Discussion

In the previous paper we have reported that the presence of $tRNA_4^{Lys}$ appears to correlate with the ability of a cell to undergo division. As a result of these observations, we have been interested in the properties of this tRNA. This information may suggest the mechanism by which this tRNA is produced and whether we are dealing with a nuclear or a cytoplasmic event. In other words, is $tRNA_4^{Lys}$ a new gene product or does it arise from one of the major species of $tRNA^{Lys}$ due to the presence or absence of a particular modified base.

Our first concern in these studies was to demonstrate that the chromatographic peak, which is present in tRNA preparations from a variety of tissues, is indeed the same tRNA molecule. As a result of cochromatography studies, the Lys-$tRNA_4$ from several tissues eluted in identical positions suggesting homogeneity. In addition, $tRNA_4^{Lys}$ from four different sources were all resistant to iodine oxidation. This suggests that $tRNA_4^{Lys}$ is similar to $tRNA_2^{Lys}$ and distinct from $tRNA_5^{Lys}$. Several experiments were also carried out to try to eliminate the possibility that $tRNA_4^{Lys}$ might be an artifact of our procedures. It was shown that $tRNA_4^{Lys}$ does not represent a ribonuclease-damaged molecule, nor is it an inactive form of Lys-tRNA produced during chromatography or the aminoacylation reaction. This tRNA does not arise as a result of misrecognition by the rat liver synthetase, neither could it be due to differences in the synthetase preparation or the radiolabeled amino acid used. The presence of $tRNA_4^{Lys}$ can be detected with both charged and uncharged samples in the same amounts. This is also true for RPC-2 chromatography as well as RPC-5 chromatography at both pH 4.5 and 7.0. Isolated $tRNA_4^{Lys}$ is still active in accepting amino acids and rechromatographs in its original position.

The properties of $tRNA_4^{Lys}$ as determined by ribosomal binding and protein synthesis with synthetic polynucleotides suggest that it functions in the same way as $tRNA_2^{Lys}$. Also, $tRNA_2^{Lys}$ and $tRNA_4^{Lys}$ were found bound to isolated rat liver ribosomes in the same relative amounts as found in the cytoplasm. If this tRNA fraction represents tRNA which is actively synthesizing protein, then it is possible that $tRNA_4^{Lys}$ is indistinguishable from $tRNA_2^{Lys}$ in the synthesis of rat liver proteins *in vivo*.

In the previous paper it was postulated that $tRNA_4^{Lys}$ is normally present in both dividing cells and cells which can be stimulated to divide. This means that $tRNA_4^{Lys}$ is present in a potentially dividing cell no matter where the cell is in the cell cycle. Preliminary evidence in this laboratory with synchronized mouse leukemic cells supports this idea since no changes were seen in $tRNA_4^{Lys}$ at four different times during the cell cycle. We, therefore, conclude that $tRNA_4^{Lys}$ functions in normal protein synthesis in the same manner as $tRNA_2^{Lys}$ during most of the cell cycle; however, at the time when the cell is stimulated to divide, the presence of $tRNA_4^{Lys}$ is a necessary requirement for cell division to continue. It is at this time that $tRNA_4^{Lys}$ must have a specific function or activity which cannot be carried out by $tRNA_2^{Lys}$. This could involve the synthesis of certain proteins required for cell division, or it could involve $tRNA_4^{Lys}$ acting as an enzyme modulator or as a corepressor.

$tRNA_4^{Lys}$ while similar to $tRNA_2^{Lys}$ in biological activity, appears to be distinct from $tRNA_2^{Lys}$ since it chromatographs differently in RPC-5 and seems to be slightly more stable to hydrolysis of the Lys-tRNA bond. The exact difference between these tRNAs will require nucleotide analysis and sequence determination; however, all the data presented here and in the previous paper (Ortwerth and Liu, 1973) seem consistant with the idea that $tRNA_4^{Lys}$ is a modified form of $tRNA_2^{Lys}$.

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