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Variations in Leucine Transfer Ribonucleic Acid in Mouse Plasma Cell Tumors Producing κ-Type Immunoglobin Light Chains*

J. Frederic Mushinski and Michael Potter

ABSTRACT: A comparison was made of the chromatographic profiles of leucyl transfer ribonucleic acid from normal mouse liver and from several murine plasma cell tumors all of which secrete a unique example of the same antigenic class (κ) of immunoglobulin light chain. Using the Freon–Aliquat 336 reversed-phase column at least five leucyl transfer ribonucleic acid peaks are seen in profiles from liver and several tumors, with stable, reproducible differences among them in the relative proportion of each isoaccepting species. Certain other tumors producing the same class of light-chain protein appear to be very deficient in peaks 3 and/or 5 suggesting that these tumors do not have significant amounts of these components normally found in mammalian transfer ribonucleic acid. It is suggested that these quantitative and qualitative variations in leucyl transfer ribonucleic acid within a very closely related group of similarly differentiated tissues indicate that the different leucyl transfer ribonucleic acid genes are under independent control.

Multiplicity of tRNAs has been established in bacteria and higher organisms (Doctor *et al.*, 1961; Weiss and Kelmers, 1967; Caskey *et al.*, 1968; Yang and Novelli, 1968a). In only a few cases so far has a correspondence been shown between the several isoaccepting tRNAs and the multiple codons for an amino acid (Weisblum *et al.*, 1962; von Ehrenstein and Dais, 1963; Kano-Sueoka *et al.*, 1968; Caskey *et al.*, 1968). Compared with a system having only a single translational equivalent (codon and adaptor) for each amino acid the existence of several code words and multiple tRNAs for a single amino acid could permit additional versatility in protein synthesis. Evolution has not diminished this degeneracy and, instead, may have found some special values and uses for alternate codon–anticodon combinations specifying a single amino acid in regulating processes in more complex higher organisms such as mammals. Manipulation of this degenerate adaptor system may play a role in differentiation of complex genomes (Holland *et al.*, 1967) or in regulation of intracellular protein synthesis (Kano-Sueoka and Sueoka, 1966; von Ehrenstein, 1966). It has been speculated that a flexible, but regulated, translational system for immunoglobulin molecules could, with genetic economy, greatly increase the number and variability of immunoglobulins an organism could generate (Potter *et al.*, 1965; Campbell, 1967; Mach *et al.*, 1967).

Owing to the greater complexity in higher organisms, we would not consider the translational machinery of a whole organism as is usually done in yeast or bacteria, but we have the opportunity to look at individual tissues

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from this point of view. For example, we can compare the tRNA of a highly specialized mammalian tissue with that of an organ such as the liver whose synthetic capabilities and other biological functions are many and complex. As a model tissue to which differentiation has conferred a more restricted function we selected that small group of mineral oil induced mouse plasma cell tumors which secrete an excess of the light chain portion of immunoglobulin molecules. These tumors are examples of specialized tissues which are nearly identical in many respects including the genetically inbred nature of the host mouse strain (BALB/c), the morphologic tissue type (plasma cell neoplasm) and the protein secretory product (κ-type light chains). With so many similarities, any differences found in the translational machinery may be related to that detail in which all these tumors differ from one another, the amino acid sequence of the protein product. The light chains secreted by several of the tumors selected for study have been studied chemically (Hood *et al.*, 1966) and have been used as raw material for the generation of theories of immunoglobulin variation. Thus, this system has clear advantages for correlating translational events with end products of protein synthesis because these products are well characterized and vary not in class of protein but only in certain amino acid residues.

We investigated the tRNA portion of the translational apparatus, not forgetting that there are many alterations made on tRNA molecules which might be responsible for the appearance of chromatographic differences, and that decoding properties of tRNA owe much of the specificity to the aminoacylating enzymes. Since leucine has a large number of code words assigned to it (Marshall *et al.*, 1967) and, presumably, anticodon-containing adaptor molecules, our efforts were directed toward separating the mammalian tRNA species specific for this amino acid. Countercurrent distribution appears to offer the most powerful separatory potential for tRNA subspecies (Weisblum *et al.*, 1965), though its use with mammalian Leu-tRNA has not been reported. Reversed-phase column chromatography is another powerful means of fractionating these tRNAs and has been successful with mammalian preparations (Weiss and Kelmers, 1967; Yang and Novelli, 1968a). Moreover, this type of fractionation permits direct comparison between two different tRNA preparations distinguishable by charging with amino acids bearing different radioactive labels and then fractionating the aminoacyl-tRNA. In *Escherichia coli* six code words have been assigned to leucine; in this report we have demonstrated at least five components in mammalian Leu-tRNA. Comparison of the chromatographic profiles of the Leu-tRNA prepared from light-chain-secreting tumors reveals reproducible patterns for each tumor with certain qualitative and quantitative differences among them.

Materials and Methods

Plasma Cell Tumors. Mineral oil (MOPC) and adjuvant-induced (Adj. PC) plasma cell tumors were maintained by continuous transplantation in BALB/c mice as described elsewhere (Potter, 1966).

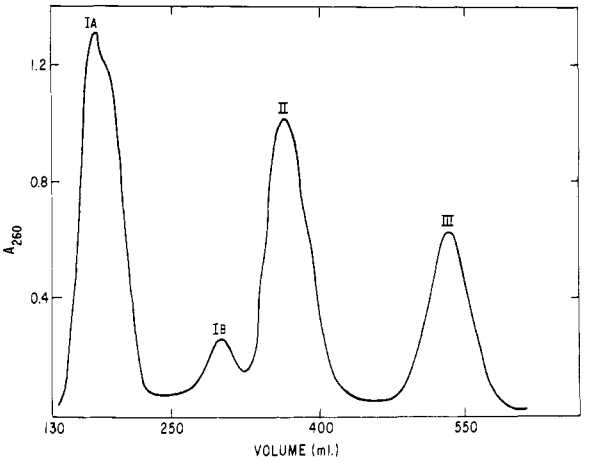

FIGURE 1: Chromatographic pattern of 1 M NaCl–0.001 M $MgCl_2$ extracts of total ethanol precipitate following phenol extraction of MOPC 315 tumors; 203 A_{260} units in 3.5 ml was applied to a 1 × 36 in. column of Sephadex G-100 equilibrated with 1 M NaCl–0.001 M $MgCl_2$ and eluted at 4° with this same buffer at a rate of about 10 ml/hr. Four peaks of material absorbing light at 260 mμ were routinely recovered and named, as indicated, IA, IB, II, and III. Peak IB frequently merges with peak IA or II if excessive material or excessive volume is applied to the column.

RNA Preparations. Tumors were harvested from mice before extensive necrosis occurred. The animals were killed by cervical dislocation, and the tumor nodules were removed, trimmed of necrotic tissue, and plunged immediately into preweighed beakers of ice-cold medium E (0.01 M Tris-HCl (pH 7.5), 0.001 M $MgCl_2$, and 0.0001 M Na_2EDTA). The initial medium E was decanted from the pooled, weighed tumors which were then placed in a chilled Waring Blendor with 1.5 volumes (v/w) of medium E plus 10 mg/ml of bentonite. After a preliminary 5-sec homogenization, an equal volume of redistilled phenol which had been saturated with distilled water or medium E was immediately added and homogenization continued for 2 min. The phenol mixture was transferred to a plastic bottle and shaken at room temperature for 30 min. After centrifugation for 10 min at 13,000*g* in a refrigerated centrifuge, the aqueous layer was aspirated and kept in ice. The phenol layer was reextracted by shaking with one-half the original volume of medium E for 30 min at room temperature followed by centrifugation and aspiration of aqueous phase. The pooled aqueous phases were reextracted with 50-ml portions of aqueous phenol two or three times. The RNA was precipitated at −20° by the addition of 0.1 volume of 20% potassium acetate (pH 5.5) and 2.5 volumes of absolute ethanol. After standing at −20° overnight, the precipitate was collected by centrifugation, drained free of ethanol in the cold, and extracted in the cold sequentially with three 10-ml portions of 1 M NaCl–0.001 M $MgCl_2$. About 450 A_{260} units of RNA in less than 10 ml of NaCl–$MgCl_2$ extract was introduced onto a G-100 Sephadex column (1 × 36 in.) and

TABLE I: Sephadex G-100 Fractionation of 203 A_{260} Units of 1 M NaCl–0.001 M $MgCl_2$ Extract from MOPC 315 Tumor (see Figure 1).

	Recovery				After Water Dialysis					
Fraction	Vol (ml)	A_{260} units	A_{280} units	A_{260} units	Vol (ml)	A_{260} units	A_{280} units	A_{260} units	Lyophilized Dry Wt (mg)	[^{14}C]Leu Acceptance[a] ($\mu\mu$moles/A_{260} unit)
IA	83	0.630	0.330	52.3	115	0.435	0.249	50.0	3.9	0
IB	59	0.219	0.097	12.9	94	0.113	0.064	10.6	<0.5[b]	4.7
II	117	0.460	0.220	53.8	152	0.321	0.170	48.8	3.1	50.5
III	121	0.327	0.169	39.6	182	0.039	0.024	7.1	<0.5[b]	0
Total				158.6				116.5	7.1	

[a] Reaction conditions described in text. Total volume of 250 μl includes 100 μl of the RNA fraction dissolved in water (approximately 1 mg/ml), 25 μl of liver enzyme (8 mg/ml of protein) and 760,300 cpm [^{14}C]leucine (263 mCi/mmole). After 10 min at 37° 50 μl was trichloroacetic acid precipitated on filter paper disks; background counts per minute without added RNA were subtracted. [b] Too small to be weighed.

eluted with 1 M NaCl–0.001 M $MgCl_2$ (Schleich and Goldstein, 1964) at 5–10 ml/hr (Figure 1). The absorbance of each fraction at 260 mμ was measured, and individual peaks were pooled, dialyzed exhaustively against distilled water, and lyophilized. Typical recovery is seen in Table I. Peak III was dialyzable and the other peaks' abilities to accept leucine can be ranked II > IB > IA. Peak II was used as tRNA in all cases.

Stripping and Charging. To remove any amino acids endogenously acylated to tRNA, solutions of tRNA were brought to pH 8–9 by addition of 0.1 volume of 1 M Tris-HCl (pH 8) and incubated at 37° for 30 min (von Ehrenstein and Lipmann, 1961). The RNA was then precipitated with potassium acetate and ethanol as above or dialyzed against an appropriate buffer. On some occasions the pH 8 step was performed before the Sephadex G-100 fractionation.

A DEAE-cellulose fraction of supernatant enzymes was used for aminoacylation reactions. Solid plasma cell tumor (*ca.* 5 g) was excised from the mouse and plunged immediately into an ice-cold, tared beaker of medium A (0.25 M sucrose, 0.006 M $MgCl_2$, 0.08 M KCl, and 0.03 M Tris-HCl, pH 7.5). After being weighed the tumor was removed from the beaker and minced with scissors for 30 sec. The mince was then homogenized by hand in a Teflon-glass homogenizer for 3 min with 1.5 volumes (v/w) of cold medium A. The homogenate was centrifuged 20 min at 30,000*g* in a refrigerated centrifuge. The supernatant was subsequently centrifuged 2 hr in the 40 head of a Beckman-Spinco Model L ultracentrifuge at 39,000 rpm. Avoiding the upper one-tenth and lower one-third of the tube, the ribosome-free supernatant was removed with a Pasteur pipet. The supernatant was applied to a 1 × 9 in. column of DEAE-cellulose (Serva) and eluted as described by Muench and Berg (1966). The enzyme fraction was concentrated approximately tenfold by positive pressure dialysis and stored in 50% glycerol at −15°.

The aminoacylating reaction mixture contained 0.01 M $MgCl_2$, 0.1 M Tris-HCl (pH 7.4), 0.01 M reduced glutathione, 0.001 M ATP, 0.01 M phosphoenolpyruvate, 0.02 mg/ml of pyruvate kinase, 2×10^{-5} M [^{14}C uniformly labeled]- or [4,5 ^{3}H]leucine (New England Nuclear), 2×10^{-4} M 19 natural [^{12}C]amino acids (omitting leucine), activating enzyme between 0.01 and 0.1 mg per ml of protein and RNA from Sephadex G-100 peak II between 0.1 and 2.0 mg per ml. The reaction proceeded at 37° and was assayed periodically by withdrawing 5–100-μl aliquots which were dried onto 1 in. filter paper disks, precipitated in cold 10% trichloroacetic acid, washed with 5% trichloroacetic acid, ethanol, and ether, dried, and counted in a liquid scintillation counter in vials containing 10 ml of toluene containing 0.6% diphenyloxazole and 0.006% *p*-bis[2-(5-phenyloxazolyl)]benzene. The reaction plateaued at 15 min (see Figure 2) at all tRNA and enzyme concentrations employed at which time the charged tRNA was shaken with a pinch of bentonite and an equal volume of water-equilibrated phenol. The phases were separated by centrifuging at 5000*g* for 5 min in the cold. The phenol phase was reextracted with an equal volume of medium E and the aqueous layers were pooled. After two to three additional phenol extractions, the aqueous phase was mixed with 0.1 volume of 20% potassium acetate (pH 5.5) and precipitated at −20° with three volumes of absolute ethanol. The precipitate was collected by centrifugation and drained in the cold, after which it was dissolved in a convenient volume of 0.001 M $MgCl_2$ and 0.001 M Na_2EDTA or initial chromatography buffer and stored frozen. Alternatively the precipitate was dissolved in water and lyophilized.

Figure 2 shows the kinetics of the aminoacylation reaction with several enzyme preparations and demonstrates that the final amount of radioactive material is dependent upon the amount of tRNA used. Liver enzyme preparations showed slightly more specific activity than similar preparations from tumor tissue, although enzyme preparations from different tumors

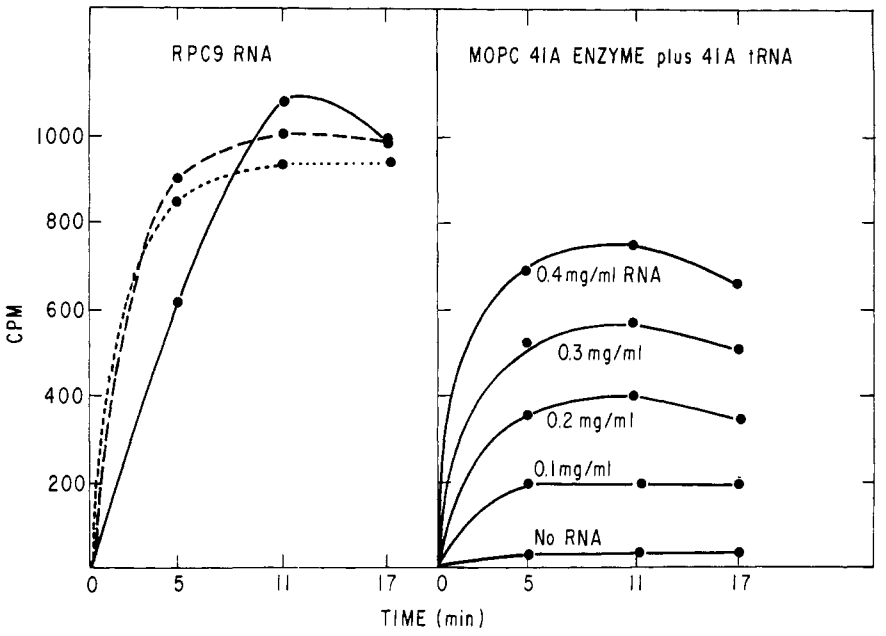

FIGURE 2: Kinetic studies. Left panel: similar plateauing of incorporation of [^{14}C]leucine into cold trichloroacetic acid insoluble material using G-100 peak II RNA (0.4 mg/ml) from Adj. PC-9 tumor tissue and DEAE enzyme preparations from three different light-chain tumors: (—) MOPC 41A (1.2 mg/ml of protein), (---) MOPC 46B (1.8 mg/ml), and (····) Adj. PC-9 (3.0 mg/ml). See text for reaction conditions. Right panel: G-100 peak II RNA from MOPC 41A tumor in different concentrations plateaus at different levels using the same amount of DEAE enzyme from MOPC 41A (1.2 mg/ml of protein). Each point represents cpm/5 μl of aminoacylating reaction mixture precipitated on a filter paper disk (Mans and Novelli, 1961).

demonstrated similar ability to charge leucine onto any RNA preparation, whether from their particular tumor or another. The DEAE-cellulose step served to remove all tRNA from the enzyme preparation as evidenced by absence of incorporation into trichloroacetic acid insoluble material in the absence of added tRNA. Protein concentration was measured by method of Lowry *et al.* (1951).

Fractionation of Charged tRNA. To examine the several species of Leu-tRNA in each tissue, the reversed-phase chromatographic system of Weiss and Kelmers (1967) was employed. This column was 240 cm high and 1 cm in diameter and packed with Chromosorb W (Johns-Manville Co.) to which an organic phase of 5% Aliquat 336 (a kind gift from General Mills, Inc.) in Freon 214 (purchased from du Pont de Nemours Co.) had been adsorbed. The mobile, aqueous phase was a linear, 2-l. NaCl gradient between 0.30 or 0.35 and 0.55 M followed by a flush at 2 M NaCl (for details of gradient, see each figure) in a buffer consisting of 0.01 M sodium acetate and 0.01 M $MgCl_2$ or magnesium acetate (pH 4.5). Tritiated Leu-tRNA (7000–60,000 cpm) from one tissue was mixed with a similar number of cpm from ^{14}C-labeled Leu-tRNA from the other tissue and applied in 1–2 ml of initial buffer to the column. No variation in profile was observed over a wide range of applied aminoacyl-tRNA, therefore, no attempt was made to apply the same amount of tRNA in milligrams or A_{260} units from each tissue nor in all column runs. The column was water jacketed and maintained at 15°. Elution rate was 1.5 ml/min, and fractions of 10 ml were collected.

The refractive index of collected fractions was used to follow the salt gradient and absorbance was measured at 260 mμ. RNA was precipitated by adding a drop of carrier (1 mg/ml of bovine serum albumin and 0.1 mg/ml of DNA in normal saline) and 0.1 volume of 50% trichloroacetic acid and mixing. Precipitation continued at 4° for 30 min after which time the precipitate was collected on membrane filters (Millipore HAWP) under suction. The filters were washed with additional ice-cold 5% trichloroacetic acid, placed in glass scintillation vials, and dried in a 100° oven for 20 min. Acid hydrolysis of the precipitate was effected with 0.2 ml of N HCl in the same vials after capping and heating in the 100° oven for 20 min.[1] Absolute ethanol (3 ml) scintillant; 0.6% diphenyloxazole and 0.0006% *p*-bis-[2-(5-phenyloxazolyl)]benzene in toluene were added to the vials, and the samples were counted in a Packard Tri-Carb liquid scintillation spectrometer equipped with automatic external standardization and punched tape output. Replicate counts were averaged and absolute disintegrations per minute of ^{3}H and ^{14}C computed in an IBM 1620 computer using a standard quench curve generated with this solvent mixture. Alternatively replicate counts were averaged and reported as counts per minute, with the ^{14}C contribution to the ^{3}H channel subtracted.

The patterns described in this report are due to trichloroacetic acid precipitable radioactivity eluting during the course of the shallow NaCl gradient, where

[1] The hydrolysis was essential to dissolving the precipitate in scintillation fluid so that automatic external standardization procedures would be valid. Indeed, it was found that at least 95% of all radioactivity remained behind if the membrane was removed after the hydrolysis step, but nearly all the radioactivity remained on the filter if the hot HCl hydrolysis was omitted.

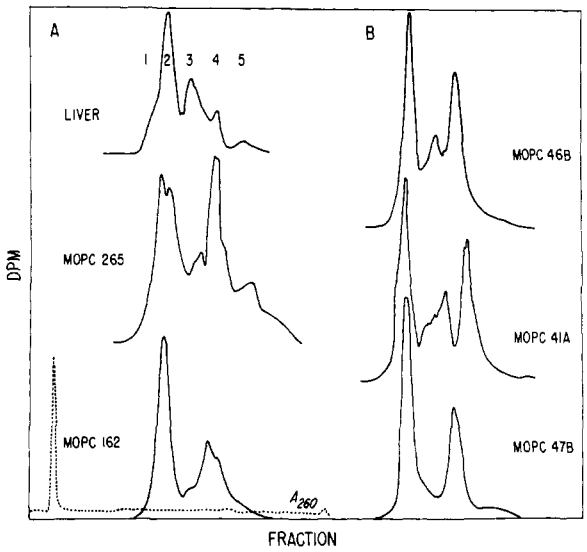

FIGURE 3: Comparison of Leu-tRNA profiles from liver and κ-chain-producing tumors. All patterns resulted from 15° chromatography of the precharged tRNA on the Freon reversed-phase column. Peak 1 (an ascending shoulder) and peaks 2–5 are named as indicated. An A_{260} pattern typical of all 15° Freon column runs is indicated by the broken line accompanying the Leu-tRNA pattern of MOPC 162. The tall A_{260} peak around fraction 13 represents nucleotides not bound to the column under these conditions. No trichloroacetic acid precipitable isotope elutes with this "fall-through" peak at column volume, but considerable nonprecipitable radioactivity can be found in this fraction as the result of deacylation of charged tRNA. All the patterns in 3A resulted from tRNA charged by enzyme preparations from the same tumor. Those in 3B were charged with liver enzyme. The NaCl gradients were from 0.30 or 0.35 to 0.55 M in pH 4.5 acetate buffer, 0.01 M in sodium and magnesium.

all the ultraviolet-absorbing material from Sephadex G-100 peak II (Figure 1) appears when eluted from the Freon column. In addition, a peak of isotope always appeared during the 2 M NaCl flush of each column run. While this material could represent an additional species of Leu-tRNA, it probably represents aggregation of tRNA which was not completely removed by the gel filtration step or which may have occurred after charging with the radioactive leucine. Material from peak IB of the G-100 Sephadex chromatography may represent such tRNA dimers or aggregates, and all of its ultraviolet-absorbing material elutes from the Freon column only when the column is flushed with the high molarity of NaCl.

Results

Chromatographic Patterns of Leu-tRNA from Liver and Plasma Cell Tumors. The number of chromatographic peaks may reflect the total number of species of Leu-tRNA that an organism is capable of generating. Since all of the Leu-tRNAs elutes close together, and the resolution depends upon optimal conditions, it is difficult to be certain what the maximum number of species is for each pattern. It appears that mouse tissues have one predominating species seen as the highest peak in the liver Leu-tRNA profile shown in Figure 3. A minor species elutes before it, but it is poorly resolved by the Freon column at 15°. Three additional smaller peaks are observed in the liver profile. For descriptive purposes, we will refer to the major peak as peak 2 and the minor ones as peaks 1, 3, 4, and 5. Figure 3 also shows Leu-tRNA profiles from five κ-chain-producing plasma cell tumors. Some of these, *e.g.*, MOPC 265, have five chromatographic peaks as seen in liver, while others have fewer.

Chromatographic Comparisons of tRNA from Different Tumors. Reproducibility of general patterns from each tissue source is consistent although one column run may differ from another in resolution of peaks (see below). For detailed comparisons of two tRNA preparations cochromatography was employed so that the same precise conditions are brought to bear on both RNA preparations. Isotope effects are ruled out by the repetition of a comparative run after reversal of [^{3}H]- and [^{14}C]leucine labels for the different tumor tRNAs as seen in Figure 4A,B where the Leu-tRNAs from MOPC 46B and MOPC 47B are compared in reversed isotope studies. In both runs the MOPC 46B pattern contains a large amount of peak 3 in comparison with the near absence of this peak in MOPC 47B.

Like MOPC 47B, MOPC 162 is very deficient in peak 3, as seen in Figure 3C in comparison with MOPC 46B. In Figure 4D still another qualitative difference is revealed by cochromatography with MOPC 46B tRNA. In this comparison MOPC 265 is seen to contain a substantial amount of peak 5 which seems totally absent in MOPC 46B as well as in several of the other tumor patterns shown in Figure 3.

Charging Enzyme Effects. To determine if the differences seen in the Freon column patterns were due to tRNA and not to the charging enzymes, the MOPC 265 tRNA (containing at least five chromatographic Leu-tRNA peaks) was charged with enzymes derived from MOPC 47B and MOPC 46B whose Leu-tRNA chromatographic profiles after charging with homologous enzymes were deficient in peaks 3 and 5, respectively. The resulting charged MOPC 265 tRNAs were cochromatographed using different isotopes (Figure 5A), and a similar pattern with five Leu-tRNA peaks was obtained from each. This result demonstrates that the MOPC 47B and MOPC 46B cells are not deficient in charging enzymes for Leu-tRNA peaks 3 and 5.

In a second experiment to rule out enzyme effects liver tRNA and MOPC 265 tRNA were charged with the same liver enzyme. These two tRNAs both contain five Leu-tRNA peaks, but they differ in the relative amounts of peaks 2–4. In MOPC 265 peaks 2 and 4 are of similar peak height while peak 3 is about 60–70% of this height. In liver, peak 2 is the largest, and peaks 3 and 4 decrease progressively in size. As may be seen by comparing the MOPC 265 chromatograms in Figure 5A,B, the same relative heights of peaks 2–4 are found in all, although each of these three preparations of MOPC 265 tRNA was charged by a different enzyme. The three enzymes were from the tumors whose homologously charged tRNAs show striking losses of certain peaks (see above) as well as from liver whose

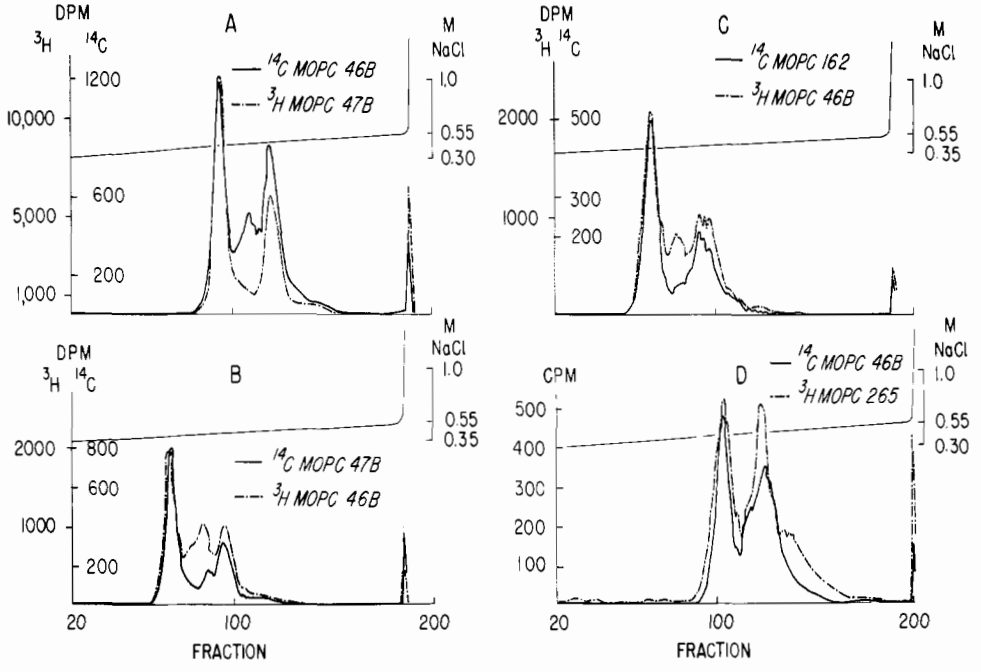

FIGURE 4: Cochromatographic Freon column profiles of Leu-tRNA from four κ-chain producers as indicated. The NaCl gradients are linear from 0.3 to 0.55 M in 0.01 M sodium acetate and 0.01 M magnesium acetate (pH 4.5) in A and D, but in B and C the gradients are shallower, from 0.35 to 0.55 M NaCl in acetate buffer as above.

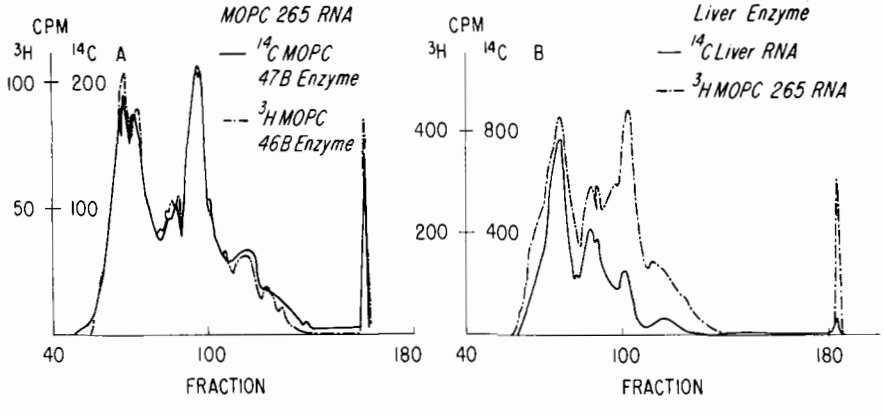

FIGURE 5: Freon column comparisons of Leu-tRNA patterns after charging with heterologous enzyme preparations. In part A the same MOPC 265 tRNA is charged by enzymes from MOPC 46B and MOPC 47B using different isotopes as indicated. In part B the same enzyme preparation from BALB/c liver was used to charge two isotopes of leucine onto different tRNAs, as indicated. In both cases the gradient was linear from 0.35 to 0.55 M NaCl in 0.01 M sodium and magnesium acetate, just as in Figure 4C, D.

profile contains all five peaks. Thus, relative differences in peak heights cannot be ascribed to enzyme effects but are due to differences in amounts of the species of Leu-tRNA present in each tumor.

Stability of tRNA Patterns. tRNAs isolated from the same tumor transplant line (MOPC 47B) but at different transfer generations were compared. tRNA preparations isolated 21 months (24 transfer generations) apart were cochromatographed after charging with the same homologous enzyme preparation. During the period of time separating these two preparations from this tumor line, the method of tRNA isolation had been modified in our laboratory. The generation 64 tRNA was isolated April 15, 1966 by DEAE-cellulose chromatography according to the method of von Ehrenstein (1967), and the generation 88 tRNA was isolated on G-100 Sephadex (Figure 1), January 6, 1968. Despite the differences in isolation procedure and transplant generation, both tRNA preparations produced very similar patterns (Figure 6) consisting prin-

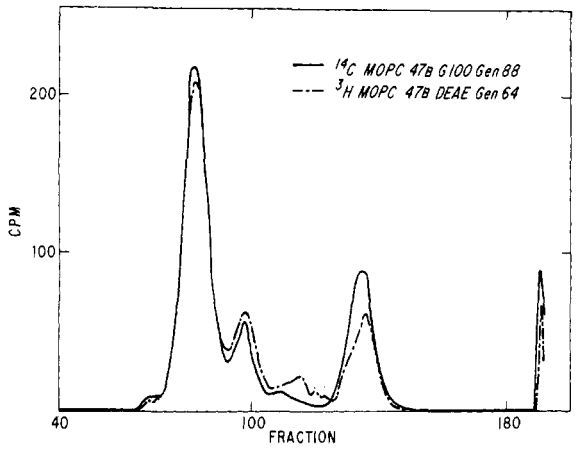

FIGURE 6: Leu-tRNA patterns of MOPC 47B comparing tRNA preparations made from different transplant generations and by different methods of tRNA isolation (see text). The gradient was linear from 0.35 to 0.55 M NaCl in 0.01 M sodium and magnesium acetate as in Figure 4C, D. During the last half of the elution of this chromatogram, the column temperature rose from 15 to 22° (rate of change not monitored).

cipally of the two major peaks, 2 and 4. This finding suggests that the Leu-tRNA pattern in the cells of a tumor is stabilized, and apparently not subject to changes during long-term syngeneic transplantation.

Chromatogram Constancy. Although the three patterns from MOPC 265 Leu-tRNA seen in Figure 5 and the four MOPC 46B patterns in Figure 4 are very similar, variations in patterns have been observed. For example, the MOPC 47B patterns in Figure 6 show much greater separation between peaks 2 and 4 than the patterns seen in Figures 3 and 4. Further, the emergence of a well-defined peak from the descending limb of peak 2 is clear in Figure 6 but only hinted at by a shoulder in Figures 3 and 4. The increased resolution was due to a gradual rise in temperature from 15–22° during the chromatographic run shown in Figure 6; the other MOPC 47B patterns had a close control of temperature at 15°. Weiss and Kelmers (1967) reported that tRNA components elute at slightly lower salt concentrations when the column temperature is lowered, so in the case of Figure 6, the elution of peak 4 apparently was retarded as the temperature slowly rose.

Rigid temperature control is essential to achieving reproducible patterns from this type of reversed-phase chromatography, but, in addition, the degree of resolution is also dependent upon the chosen temperature even if it is kept constant. Many chromatographic runs of Leu-tRNAs from the above-mentioned tissues as well as from several other κ-chain-producing plasma cell tumors were made at 30° but not illustrated here. At this higher temperature the resolution between peaks 2 and 4 was very poor, but the resolution of peak 1 from the ascending limb of peak 2 was better than at 15°.

Posttranscriptional alteration of tRNA such as enzymatic methylation (Peterkofsky *et al.*, 1966) and dimerization (Zachau, 1968; Loehr and Keller, 1968) have been shown to influence the chromatographic patterns of aminoacyl-tRNA. The enzymes used in the present study did contain methylases (to be described elsewhere) and did not cause alteration in the chromatographic pattern of tRNA isolated from different sources (Figure 5). Further, chromatographic patterns from the same tRNA preparation charged at different times did not show alterations attributable to configurational changes during storage, nor were appreciable differences seen between tRNA purified by DEAE-chromatography and that purified on Sephadex with peak IB, probably representing tRNA dimers, removed from the preparation (see Figure 6). Thus it is unlikely that such chemical and conformational changes contributed significantly to the tRNA variations reported here.

Discussion

Qualitative and quantitative differences have been found in the Leu-tRNAs isolated from tissues representing very similar end stages of cell differentiation (κ-chain-producing plasma cell tumors of BALB/c mice). These differences consist of the nearly total loss of certain peaks or increase in relative amounts of other peaks compared with similar chromatographic patterns of normal liver tRNA.

It is of interest in this connection that Mach *et al.* (1967) reported methylated albumin kieselguhr profile differences for Leu-tRNA from three murine plasmacytomas, and Yang and Novelli (1968b) have found striking qualitative and quantitative serine-tRNA differences in mouse plasma cell tumors producing different heavy-chain types using Freon column chromatography. The present results indicate that such differences can exist within a group of tumors producing only κ-type immunoglobulin light chains.

These results, the finding of different responses to triplet binding for chromatographic fractions in analogous systems (Kano-Sueoka *et al.*, 1968; Caskey *et al.*, 1968), and the description of different nucleotide sequences for different chromatographic fractions (Goodman *et al.*, 1968) suggest that each of the chromatographic peaks of tRNA represents a special biological product. The synthesis of each tRNA depends upon transcription of a corresponding tRNA structural gene, and the levels of the individual tRNAs might plausibly be ascribed to the individual and independent regulation of these genes.

Ritossa *et al.*, (1966) have recently studied the tRNA genes in Drosophila by DNA–RNA hybridization. Assuming a total of 60 different tRNAs and corresponding genes (tDNAs), they found that there was about a 13-fold multiplicity or redundancy for all these genes. The redundancy is probably in the form of tandem, linear repeats within some or all of the 60 loci, but these loci may be found on different chromosomes. Assuming that a similar redundancy exists in the mouse, a possible explanation for the relative amounts of each Leu-tRNA in normal mouse liver is that the amount of tRNA produced is a function of the multiplicity of the genes within individual loci. However, the relative amounts of Leu-tRNAs in the plasma cell tumors reported here were strikingly different from liver. In some tumors, species of Leu-tRNA seen in liver

were missing (*e.g.*, peaks 3 and 5 in MOPC 47B and MOPC 46B, respectively) and in others there was a relative superabundance of certain species (*e.g.*, peaks 4 and 5 in MOPC 265). It is known that mouse plasma cell tumors are highly aneuploid, and that most of the tumors have near tetraploid chromosome numbers (Cohn, 1967; Yosida *et al.*, 1968). Assuming a uniform transcription of all tDNA, increases in relative quantities of certain Leu-tRNAs would be seen if the DNA loci carrying their genes were on chromosomes which were polysomic in individual tumors. However, if this were true, all of the known species of Leu-tRNA should be demonstrable in all tumors despite a particular preponderance of one or another peak. This was not the case, for in three of the tumors reported here, MOPC 46B, MOPC 47B, and MOPC 162, marked or almost complete deficiencies were observed for certain Leu-tRNA peaks. With the existing information, it is unlikely that the Leu-tRNA patterns are a function of aneuploidy.

On the other hand, different rates of transcription operating on individual tDNA sites would also yield chromatographic peaks of different sizes even if the gene dose for each Leu-tRNA subspecies were the same. Other kinds of genes including mammalian structural cistrons (Wilt, 1966), bacterial operons (Jacob and Monod, 1961; Martin, 1963) and amphibian rRNA genes (Brown and Gurdon, 1964) have been shown to be under individual regulation and intermittently transcribed. A similar sort of independent regulation acting on the individual tRNA gene loci would be compatible with the absence and the superabundance of various species of Leu-tRNA reported here in plasma cell tumors. Apparently the genes controlling the reduced or missing tRNA species are "turned off" or are operating at very low levels. This possibility suggests that cells, *e.g.*, MOPC 47B and MOPC 46B, can replicate and synthesize constituent proteins as well as immunoglobulins without utilizing all of the tRNA genes in the genome. These nonessential tRNAs might be available for regulatory activities or fine adjustments of protein structure (amino acid sequence) during translation of genetic messages. The data presented here lead us to believe that at least some of the genes for mammalian Leu-tRNA species are under independent regulation and the stable levels of these species seen in the unique chromatographic profiles of different tumors reflect the status of these controls which vary from tumor to tumor.

The biological significance of the tRNA variations seen in these highly specialized cells, cells making very similar but not identical end products, is not yet clear. Deficiencies in certain tRNA species in plasma cell tumors may be related to immunoglobulin variability. It is of particular interest that these have been found among κ-chain-producing tumors, since κ-chain variability has been implicated in immunoglobulin specificity (Potter and Leon, 1968). It is now apparent from amino acid sequence studies that there are more than one κ-chain genes in the mouse (Gray *et al.*, 1967). Individual κ-chain messages from any one of these structural genes could be subject to translational variation if the available tRNA molecules could be independently regulated, *e.g.*, at the transcriptional level, as they appear to be in the present studies. Certain codons in this genetic message, in the absence of one of the specific Leu-tRNAs, might be translated as another amino acid if a species of tRNA bearing this amino acid could be bound, albeit less strongly, to the codons which would ordinarily be apparently specific for the missing Leu-tRNA subspecies. This possibility can only be established by definitive protein synthesis studies in which myeloma protein light-chain sequences can be studied relative to their dependence upon these Leu-tRNA subspecies. These studies are under way in our laboratory.

Alternatively the tRNA differences reported here might be due to a message-dependent control of tRNA transcription or tRNA degradation such that the levels of tRNA are regulated by the number of codons requiring these specific adaptors in the cell's collection of genetic messages. This hypothesis may be approached in the future when more coding data for individual Leu-tRNAs and more detailed amino acid sequences of the κ chains become available.

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Enzymatic Hydrolysis of Sphingolipids. VIII. Further Purification and Properties of Rat Brain Ceramidase*

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ABSTRACT: Rat brain ceramidase, the enzyme that catalyzes both the hydrolysis and synthesis of ceramide (*N*-acylsphingosine), was purified over 200-fold. Advantage was taken of the fact that the enzyme withstands prolonged treatment with trypsin and chymotrypsin. This treatment digests 80% of the protein and decreases the molecular weight of the enzyme as determined by gel filtration through Bio-Gel but does not impair the enzymatic activity. Evidence is presented that fatty acyl coenzyme A is not a direct substrate for ceramide synthesis.

The acyl portion can be utilized only after hydrolysis by an accompanying hydrolase to coenzyme A and a free fatty acid. Trials were made to separate the hydrolytic and synthetic activities by subjecting the enzyme to ammonium sulfate fractionation, sonic irradiation, adjustment to acid pH, heating, treatment with proteolytic enzymes or with SH reagents, and chromatography on TEAE-cellulose. None of these procedures resulted in a separation of the two activities from each other. Reaction mixtures were taken to an apparent equilibrium, starting with either ceramide or a mixture of sphingosine and fatty acid. The calculated equilibrium constant, defined as K_{equil} = (sphingosine) × (fatty acid)/(ceramide), depended upon the substrate employed. It was about 10^{-4} M when determined in the direction of ceramide synthesis, but only 5×10^{-6} M when measured in the direction of hydrolysis. The meaning and possible significance of these findings are discussed.

Ceramide is the trivial name of the *N*-acyl derivative of sphingosine or a related long-chain base. All sphingolipids can be considered as derivatives of ceramide; *e.g.*, sphingomyelin is ceramide phosphorylcholine and cerebroside is galactosylceramide. The biosynthesis and degradation of this compound are therefore important steps in the metabolism of all sphingolipids. An enzyme that catalyzes the hydrolysis of ceramide to sphingosine and fatty acid was purified from rat brain (Gatt, 1963, 1966). This same enzyme also catalyzes the reverse reaction, namely, the synthesis of ceramide from sphingosine and a free fatty acid.

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