*PATTERNS OF TRANSFER RNA IN NORMAL RAT LIVER AND DURING HEPATIC CARCINOGENESIS**

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Because of the central role of transfer RNA in the translation of the genetic code, it is possible that in higher organisms changes in the abundance of specific types of tRNA are associated with metabolic regulation, cellular differentiation, and neoplastic transformation.[1-3] Evidence is accumulating to support the theory that in bacteria the pattern of tRNA's can change during T2 phage infection,[2] sporulation,[4, 5] and changes in growth conditions.[6] Whereas extensive studies have been done on the fractionation of bacterial and yeast tRNA, there are few published studies on the fractionation of mammalian tRNA's. In the present study, we have used a modification of the methylated albumin kieselguhr (MAK) column chromatography technique of Sueoka and Yamane[7] to examine the types of tRNA's present in normal rat liver and the alterations which occur during feeding of the hepatic carcinogen ethionine.[8]

Materials and Methods.—In initial studies, we used a commercial preparation (from General Biochemicals) of tRNA that had been extracted from the livers of albino Wistar rats by a modification of the procedure of Brunngraber.[9] To study the effects of ethionine feeding, tRNA was prepared from the livers of both normal and ethionine-fed male Wistar rats (200–250 gm) by phenol extraction of the microsomal supernatant fraction.[10] Preparations of tRNA were stripped of endogenous amino acids[11] prior to *in vitro* charging.

Radioactive amino acids were purchased from Schwarz BioResearch and had the following specific activities in mc/mmole: C^{14}-lysine (240), C^{14}-arginine (116), H^3-leucine (4500), H^3-isoleucine (4500), H^3-valine (290), C^{14}-phenylalanine (355), and C^{14}-leucine (200). L-ethionine-ethyl-1-C^{14} (20 mc/mmole) was purchased from Tracerlab. The sources of the remaining materials have been described previously.[12]

In all of the present experiments tRNA's were charged with a crude mixture of amino acid-activating enzymes obtained from normal rat liver. Three male Wistar rats (each weighing 200–250 gm and starved overnight) were decapitated, the livers were excised, and the microsomal supernatant fraction (S-122) was prepared as previously described.[12] The amino acid-activating enzymes were then prepared from the S-122 fraction by adding a sufficient volume of saturated $(NH_4)_2SO_4$ (previously adjusted to pH 7.5 with KOH) to yield a 70 per cent saturated solution. After ten minutes, the precipitate was harvested by centrifugation at 11,000 × *g* for 20 minutes, suspended in 2 ml of charging buffer (Tris-HCl pH 7.2, 0.1 *M;* magnesium acetate 0.005 *M*), dialyzed four hours against 1000 volumes of charging buffer, and the insoluble residue removed by centrifugation. The entire procedure was performed at 4°C. The enzyme fraction could be stored at −20°C for two weeks without appreciable loss of activity.

Optimal conditions for the charging of rat liver tRNA were established in preliminary experiments. A one-ml reaction system contained the following components (in micromoles/ml unless otherwise specified): Tris-HCl buffer, pH 7.2, 100; magnesium chloride, 5; adenosine 5′-triphosphate, 10; an L-(C^{14}) or -(H^3) amino acid, 0.025; 1–1.6 mg of rat liver tRNA; and enzyme fraction, approximately 25 A_{260} units. Samples were incubated at 37° for 19 minutes, immediately extracted with phenol,[7] and the entire aqueous phase was applied directly to a MAK column. The column (3.0 cm diam. × 3.5 cm) was prepared by previously described methods.[7] Following sample application, the column was washed with 80 ml of 0.2 *M* NaCl, 0.05 *M* Na-phosphate buffer, pH 6.7, to remove residual phenol, ATP, and free amino acids. The RNA was then eluted with 200 ml of a linear gradient of 0.2–1.1 *M* NaCl (in 0.05 *M* Na-phosphate buffer, pH 6.7) at a flow rate of 1.25 ml/min. The effluent was monitored at 260 mμ in the flow-through cuvette of a Gilford model 2000 recorder. Fractions (2.5 ml) were precipitated by addition of 2.5 ml of cold 10 per cent trichloroacetic acid and 200 μg of carrier DNA, deposited on membrane filters, and washed with 5 per cent TCA. The filters were dissolved in 15 ml of Bray's solution and counted in a Packard liquid scintillation spectrometer, model 3375. Double isotope counting was done by standard procedures.

Results.—Normal rat liver tRNA was charged with six different radioactive amino acids and fractionated by MAK column chromatography (Fig. 1). The profiles revealed two components for arginine, one for isoleucine, three for valine, two for lysine, one for phenylalanine, and three for leucine. The number of peaks

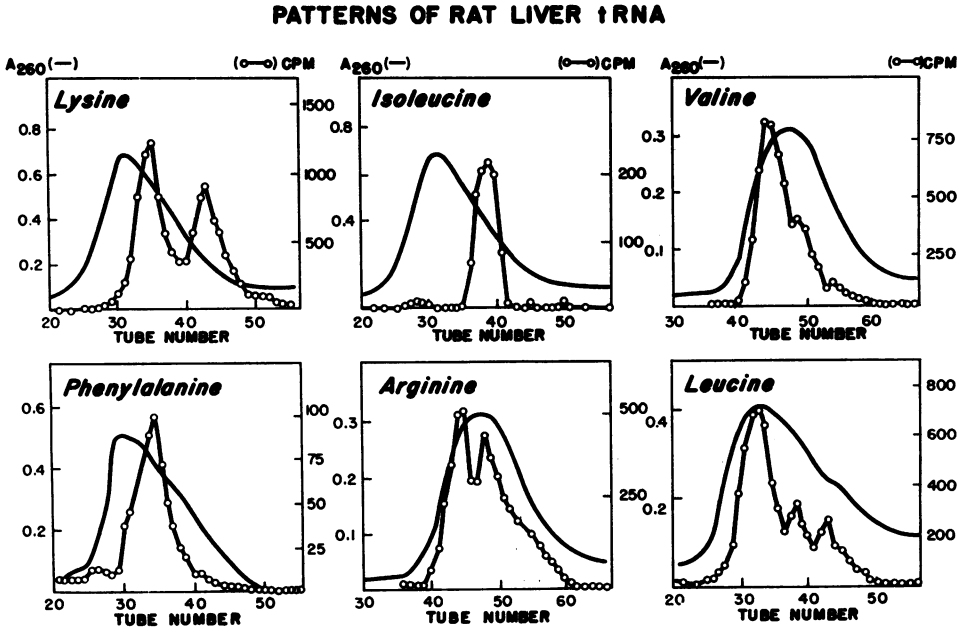

FIG. 1.—Elution patterns on methylated albumin kieselguhr (MAK) columns of radioactive aminoacyl-tRNA's from normal rat liver. Radioactive aminoacyl-tRNA's were prepared and chromatographed as described under *Materials and Methods*.

described probably represents a minimum estimate of individual tRNA's, because of the limited resolution of the MAK column. These results indicated that in mammalian cells, as in *Escherichia coli* and yeast,[13] there exist multiple forms of tRNA for the same amino acid, a finding consistent with previous evidence for degeneracy of the genetic code in higher organisms.[14] Since the multiple forms of tRNA obtained in *E. coli* differ in their codon response during translation,[13] it is likely that this is also the case with rat liver tRNA's and this is being examined in current studies.

Having established certain tRNA patterns in normal rat liver, we then examined whether these patterns change during feeding of the hepatic carcinogen ethionine. This was of interest since it is known that ethionine results in ethylation of rat liver tRNA.[15] In all subsequent studies, tRNA was prepared in parallel from both normal and ethionine-fed rats using identical isolation procedures (see *Materials and Methods*). To determine whether ethylation is restricted to one or several types of tRNA, rats which had been on an ethionine diet[10] for one month were injected with 28.3 μc of L-ethionine-ethyl-1-C^{14} daily for four days and then sacrificed. The C^{14}-ethyl-labeled tRNA was extracted from liver and cochromatoraphed with a six-fold excess (on an A_{260} basis) of normal liver tRNA (Fig. 2).

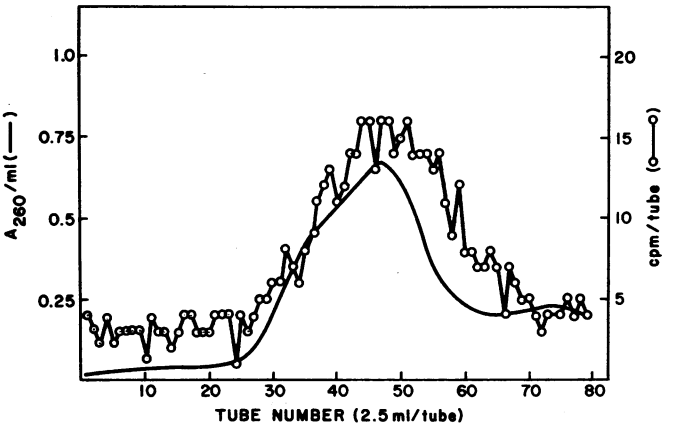

FIG. 2.—Elution profiles of normal versus ethylated liver tRNA. Five A_{260} units of ethylated liver tRNA, labeled *in vivo* with L-ethionine-ethyl-1-C^{14}, were mixed with 30 A_{260} units of normal liver tRNA and cochromatographed on a MAK column. Fractions were precipitated with trichloroacetic acid and assayed for radioactivity. Additional details are described in the text and *Materials and Methods*. The elution profile of the ethylated tRNA is represented by the radioactivity whereas the A_{260} curve mainly reflects the elution profile of normal tRNA.

The Et-tRNA is represented by the radioactivity whereas the A_{260} profile mainly reflects the pattern of the normal tRNA. The elution of the Et-tRNA was slightly delayed when compared to normal tRNA. In contrast to the sharp peaks obtained with tRNA's specific for individual amino acids (Fig. 1), the profile of ethyl-labeled tRNA revealed a broad peak which was similar in contour to the A_{260} profile of normal tRNA, thus indicating that many, if not all, species of tRNA were ethylated.

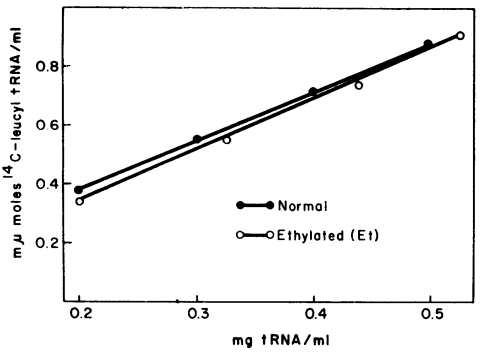

FIG. 3.—Leucine-acceptance capacity of normal and ethylated liver tRNA. Increasing amounts of normal rat liver tRNA or ethylated tRNA were incubated with C^{14}-leucine in the standard charging system (see *Materials and Methods*). After 19 min at 37° the nucleic acid was precipitated and washed with cold 5% trichloroacetic acid and assayed for radioactivity.

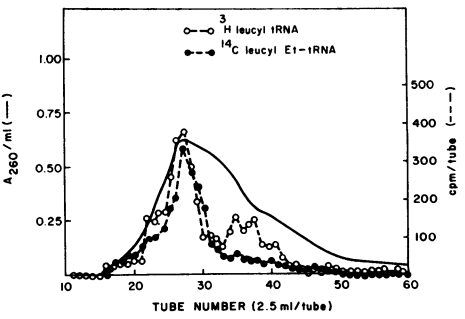

FIG. 4.—A comparison of the elution profiles of normal rat liver leucyl-tRNA and ethylated leucyl-tRNA. Normal rat liver tRNA (38 A_{260} units) was charged with H^3-leucine, and an equivalent amount of ethylated tRNA (Et-tRNA) was charged with C^{14}-leucine in the standard charging system. The aqueous phases obtained after phenol extraction were pooled and cochromatographed on a MAK column. The details of the procedure are described under *Methods*.

The functional capacity of Et-tRNA was examined by comparing the leucine-acceptance capacity of Et-tRNA to that of normal rat liver tRNA (Fig. 3). Over a concentration range of RNA from 0.2 to 0.5 mg/ml, the acceptance capacity of the ethylated tRNA was equivalent to that of normal tRNA. The Et-tRNA was also similar to normal liver tRNA when assayed for valine-acceptance capacity.

To determine whether the types of leucyl-tRNA's were altered after ethionine feeding, Et-tRNA and normal tRNA were charged with C^{14}- and H^3-leucine, respectively, and were cochromatographed on a MAK column. Figure 4 indicates that whereas the normal tRNA contained at least three leucine components, the Et-tRNA revealed only one. The single component obtained with Et-tRNA eluted in a region which corresponded to the major component of leucyl-tRNA present in the normal material. This difference in the elution profiles of liver leucyl-tRNA's obtained from normal and ethionine-fed rats was confirmed when these two types of tRNA were prepared a second time in parallel by identical procedures. In preliminary experiments the coding properties of unfractionated normal rat liver and Et-tRNA were tested in the Nirenberg and Leder ribosomal-binding assay.[16] Normal tRNA charged with leucine responded to both poly UC and poly UG, whereas the Et-tRNA responded only to poly UC. These results suggest that the Et-tRNA is deficient in a species of leucyl-tRNA which normally recognizes the UUG codon. When normal and Et-tRNA were charged with radioactive valine, the elution profiles of both preparations were identical and similar to the pattern of valyl-tRNA given in Figure 1.

The Et-tRNA used in the preceding study was obtained from rats fed on ethionine for one month, which is prior to the gross appearance of hepatomas. In view of the results obtained, it was of interest to study the tRNA of an ethionine-induced hepatoma.[17] The tRNA was extracted from a hepatoma which had been induced by ethionine approximately one year before and maintained by serial transplantation in Wistar rats fed a normal diet. This material (T-tRNA) was charged with C^{14}-leucine and cochromatographed with normal liver tRNA charged with H^3-

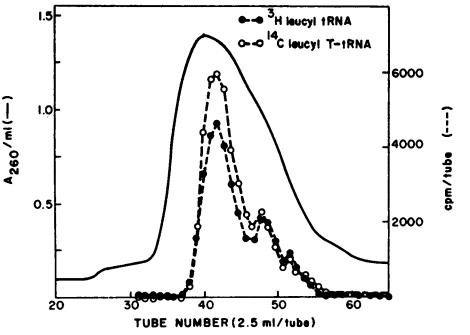

FIG. 5.—A comparison of the elution profiles of normal rat liver leucyl-tRNA and leucyl-tRNA obtained from an ethionine-induced hepatoma (T-tRNA). The procedure was similar to that described in the legend to Fig. 4 with the exception that T-tRNA was obtained from an ethionine-induced hepatoma maintained by serial transplantation in rats fed a normal diet, and this was used in place of the Et-tRNA.

leucine. The T-tRNA revealed three leucine components which were identical in their elution profile to those present in normal tRNA (Fig. 5). It appears, therefore, that the changes in leucyl-tRNA seen during ethionine feeding are not maintained in the tumor itself, presumably because ethionine is no longer present in the diet, and ethylation of tRNA cannot occur.

Discussion.—The relevance of our findings to the causation of cancer remains to be determined. It is possible that alterations in tRNA play a role in the initiation of hepatomas by ethionine and that maintenance of the neoplastic state is due to secondary charges which persist even when the tRNA pattern returns to normal. This hypothesis is consistent with other evidence from the field of carcinogenesis indicating that initiation, promotion, and maintenance of cancer may occur by separate mechanisms.[18] Evidence which favors the possibility that ethylation of tRNA is a key event in the carcinogenic effect of ethionone includes the following: (1) The feeding of ethionine results in the ethylation of liver tRNA to a considerably greater extent than its incorporation into liver protein and causes little or no ethylation of liver DNA.[15] (2) Appreciable ethylation of tRNA occurs only in the liver, the only organ in which ethionine induces tumors.[15] (3) Supplementation of the diet with methionine prevents ethionine carcinogenesis, markedly diminishes ethylation of liver tRNA,[15] and prevents the changes in leucyl-tRNA described in the present study (and unpublished studies). Studies indicating differences between the patterns of methylation of normal and tumor tRNA's[3, 19] also suggest that changes in tRNA play an important role in abnormal growth.

At this time we can only speculate about the mechanism by which changes in tRNA might lead to the defects in cell regulation which characterize tumor cells. The loss of a given tRNA could prevent the translation of messenger RNA's containing codons specific for that tRNA and thereby block the synthesis of one or more proteins normally required for cell regulation. Alternatively, since there is some evidence that in bacteria aminoacyl tRNA's may play a role in enzyme repression,[20] the loss of a given tRNA might prevent the function of a specific repressor system. In addition, there is increasing evidence that transcription and translation are more tightly coupled than previously suspected;[21] therefore agents which act on tRNA might indirectly influence rates of synthesis of specific messenger RNA's and thereby alter patterns of gene expression.

Our results do not, however, exclude the possibility that changes in tRNA seen during ethionine feeding represent a side reaction which is unrelated to the carcinogenic process. In addition recent evidence that certain tRNA's may be denatured during isolation[22] introduces a note of caution in the interpretation of tRNA

profiles. Studies designed to exclude this possibility, as well as a more detailed examination of tRNA profiles during the time course of ethionine feeding, are in progress. These studies are required to test the hypothesis that changes in the abundance of specific types of tRNA play an important role in carcinogenesis.

Note added in proof: After the completion of this manuscript we learned of additional studies on the fractionation of mammalian tRNA's by M. W. Taylor, G. A. Granger, C. A. Buck, and J. J. Holland (these PROCEEDINGS, **57**, 1712 (1967)) and by B. L. Strehler, D. D. Hendley, and G. P. Hirsch (these PROCEEDINGS, **57**, 1751 (1967)). The MAK column elution profiles of leucyl-tRNA's of normal rabbit tissues, obtained by these authors, are similar to those we obtained with normal rat liver. The authors of these two studies also discuss the possible role of aminoacyl tRNA's in differentiation and dedifferentiation.

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