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Transfer Ribonucleic Acids in Rat Liver and Morris 5123 Minimal Deviation Hepatoma*

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ABSTRACT: Twenty aminoacyl-tRNAs from the "minimal deviation" hepatoma Morris 5123 have been compared on RPC-II chromatography with normal rat liver aminoacyl-tRNA.

The analysis of the chromatographic profiles of individual aminoacyl-tRNAs showed a complete similarity between tRNAs for 17 amino acids. Of the remaining three, one, $tRNA^{Asn}_{5123}$ was displaced with respect to the corresponding one of rat liver, $tRNA^{Glu}_{5123}$ showed only one peak of acceptor activity, while $tRNA^{Glu}_{rat\ liver}$ had a second peak which eluted at a higher salt concentration. The most interesting feature was a new $tRNA^{Phe}$ present in Morris 5123 hepatoma. Its coding properties have been extensively tested Using random polynucleotides and oligonucleotide triplets as templates for ribosome binding measurements, no differences were found between the two $tRNA^{Phe}_{5123}$, rat liver $tRNA^{Phe}$, and *E. coli* $tRNA^{Phe}$. Hybridization experiments using the $tRNA^{Phe}$ specific for Morris 5123 hepatoma and rat liver DNA indicate that there is a specific complementarity between their base sequences. The reasons for the chromatographic differences between tRNAs of hepatoma and rat liver are discussed in terms of differences in methylation of some bases which are not involved in the codon or aminoacyl-tRNA synthetase recognition site.

The attention of many authors has been focused recently on the possible role of tRNA in the regulatory processes of the cell. The chromatographic behavior of all species of tRNAs has been analyzed by different methods. Comparisons have been made between species, organs, and tissues (Holland *et al.*, 1967; Taylor *et al.*, 1967, 1968), between cytoplasm and organelles (Buck and Nass, 1968, 1969; Fournier and Simpson, 1968), during mammalian virus infection (Subak-Sharpe *et al.*, 1966), and after bacterial virus infection (Sueoka *et al.*, 1966; Pollack, 1966; Waters and Novelli, 1967; Hung and Overby, 1968), at various stages of differentiation (Lee and Ingram, 1967; Yang and Comb, 1968; Vold and Sypherd, 1968; Anderson and Cherry, 1969), in different growth conditions (Doi *et al.*, 1968; Heyman *et al.*, 1967; Wettstein and Stent, 1968; Yang *et al.*, 1969), and under hormonal action (Agarwal *et al.*, 1969). Many different tumors have been examined: leukemia P-388 (Morton and Rogers, 1965), ethionine-induced hepatomas (Axel *et al.*, 1967; Ortwerth *et al.*, 1968), plasma cell tumors (Yang and Novelli, 1968a,b; Mushinsky and Potter, 1968, 1969), and Novikoff ascites tumors (Baliga *et al.*, 1968, 1969; Goldman *et al.*, 1969).

In most of the above-mentioned studies, both quantitative and qualitative differences were observed between some of the aminoacyl-tRNAs.

Many hypotheses have been postulated about the significance of the experimental data, but without clear correlations between chromatographic patterns, coding properties, and functional conditions of the cells, no single hypothesis has been firmly substantiated.

In this paper we compared, by reverse phase chromatography (Weiss and Kelmers, 1967), aminoacyl-tRNAs obtained from rat liver and so-called "minimal deviation hepatoma," Morris 5123 (Wu, 1967). As we pointed out in a preliminary report (Gonano and Chiarugi, 1969), by direct comparison with the normal tissue from which the tumor has been originated, this system potentially can give us information about relatively early modifications of a malignant tissue. Whether the tRNA plays any role in the malignant transformation of the hepatic cell or the tumor transformation affects the properties of the normal cell tRNA, it is possible to detect alterations in the chromatographic pattern of the tRNAs.

In this report we present evidence that a difference has been observed for asparaginyl-, glutamyl-, and phenylalanyl-tRNA, between Morris 5123 hepatoma and rat liver. The coding properties and the origin of the new $tRNA^{Phe}$ Morris have been studied by oligonucleotide triplet-induced binding to ribosomes and by DNA–RNA hybridization techniques. No significant differences have been found between the two $tRNA^{Phe}_{Morris}$ species.

Materials and Methods

Animals and Tissues. Morris 5123 and 5123c were obtained by serial transplantations in the inbred Buffalo strain of rats. The tumor was in a solid form, encapsulated, and was

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removed 25 days after transplantation in the rat thigh. The tumor was freed of necrosis and muscular tissue; liver was obtained from normal adult male rats of the same strain.

tRNAs and Aminoacyl-tRNA Synthetases. One volume by weight of fresh tumor or liver was homogenized in 3 volumes of buffer containing Tris-HCl, 0.1 M, pH 7.5–$MgCl_2$, 0.001 M–KCl, 0.015 M–β-mercaptoethanol, 0.02M–washed Bentonite, 1 mg/ml. Two successive centrifugations at 15,000*g* for 1 hr resulted in a supernatant that was used to prepare both tRNA and aminoacyl-tRNA synthetases.

tRNA. The supernatant was shaken with phenol and the tRNA prepared according to the method of Holley (1963).

Aminoacyl-tRNA synthetases were purified as follows: 5 ml of $MnCl_2$, 1 M, was added to 100 ml of the 150,000*g* supernatant; the precipitate obtained after 15,000*g* centrifugation was dissolved in 0.1 M Tris-HCl, pH 7.4, containing 0.02 M β-mercaptoethanol, dialyzed overnight, and passed over a DEAE-52 (Whatman) column equilibrated, with the same buffer. The initial colored material was collected, precipitated with 50% ammonium sulfate, dissolved in Tris, 0.01 M, pH 7.4–$MgCl_2$, 0.001 M–KCl, 0.015 M–β-mercaptoethanol, 0.02 M, dialyzed overnight against the same buffer, and stored in small volumes under liquid N_2.

Acylation of tRNA. The incubation mixture for the acylation of tRNA contained the following, in μmoles/ml: Tris-HCl, 7.5, 100; KCl, 10; $MgCl_2$, 10; ATP, 4; β-mercaptoethanol, 1; 5–10 mCi of [^{14}C]amino acid or 25–50 mCi of [^{3}H]amino acid; 0.2 of each cold amino acid except the labeled one; 1–2 mg of tRNA and excess aminoacyl-tRNA synthetase such that all the tRNA present was acylated (the amount was pretested for each enzyme preparation). After 15 min of incubation the reaction was stopped by adding an equal volume of Na acetate, 1 M, pH 4.5, followed by phenol extraction and ethanol precipitation at −20° overnight. The precipitate was dissolved in an appropriate buffer depending on the chromatographic conditions just before use.

Specific Activity of the Amino Acids. The radioactive amino acids were all purchased from New England Nuclear Corp., Boston, Mass. The specific activities for the ^{14}C-labeled amino acids were the following, expressed in mCi/mmole; L-alanine, 123; L-arginine, 246; L-asparagine, 207; L-aspartic acid, 164; L-cystine, 265, L-glutamic acid, 208; L-glutamine, 95; L-glycine, 116; L-histidine, 222; L-isoleucine, 222; L-leucine, 273; L-lysine, 247; L-phenylalanine, 370; L-proline, 200; L-threonine, 158; DL-tryptophan, 9; L-tyrosine, 468; L-serine, 118; L-valine, 200.

The specific activities for the ^{3}H-labeled amino acids were the following, expressed in Ci/mmole: L-alanine, 1.5; L-arginine, 1.3; L-asparagine, 0.15; L-aspartic acid, 2.14; L-glutamic acid 2.06; L-glycine, 0.062; L-histidine, 1.1; L-isoleucine, 1.5; L-leucine, 50; L-lysine, 0.3; L-methionine, 0.15; L-phenylalanine, 1.5; L-proline, 1.15; L-serine, 3.8; L-threonine, 2.23; DL-tryptophan, 0.17; L-tyrosine, 0.5; L-valine, 1.7.

For the Freon reverse phase chromatography (RPC-II) essentially the method described by Weiss and Kelmers (1967) and Yang and Novelli (1968a,b) was used. The column (1 cm × 240 cm) was charged with aminoacylated tRNA dissolved in starting buffer (adding 3–4 mg of *Escherichia coli* tRNA uncharged as a carrier) and was eluted with a linear gradient (1000 ml × 1000 ml) from 0.3 to 0.75 M NaCl, at room temperature (20°) and a flow rate of 0.6 ml/min. The radioactivity in each tube was determined by precipitation with 5% trichloroacetic acid followed by collection of the precipitate on Whatman GFA/2.5-cm glass fiber disks. The filters were dried under an infrared lamp and counted in a Packard 2000 liquid scintillation counter, using standard toluene scintillation mixture. The recovery from the columns varied for the different amino acids, for different runs and different batches of Chromosorb, but never exceeded 50–60% of the total input.

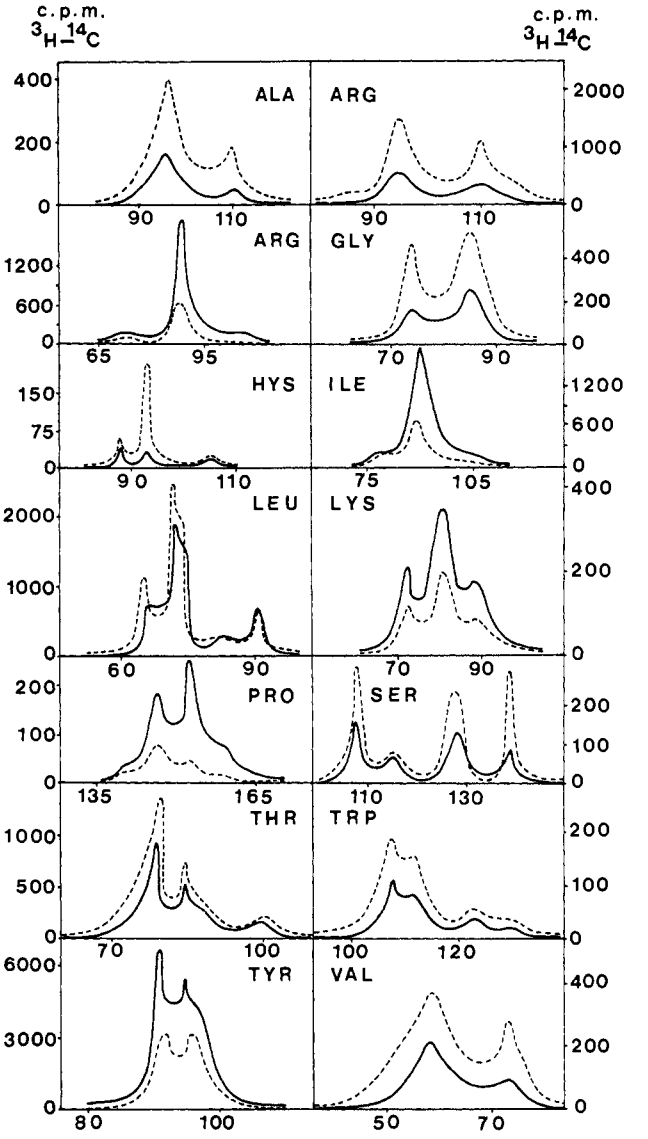

FIGURE 1: Elution profiles obtained from RPC-II columns with aminoacyl-tRNA from Morris 5123 hepatoma and normal rat liver. Linear gradients were from 0.3 to 0.7 M NaCl. In all the figures the continuous line represents Morris 5123 and the dotted line represents the rat liver tRNA. The lines connect points that were omitted for drawing clearness and represent ^{3}H and ^{14}C precipitated by cold 5% trichloroacetic acid in a 10-ml fraction.

For preparative purposes, [^{3}H]- or [^{14}C]phenylalanyl-tRNA was purified on a benzoylated-DEAE column, following the method of Wimmer *et al.* (1968). The radioactive phenylalanyl-tRNA, 10- to 15-fold purified by this method, was subsequently applied to a RPC-II column and eluted as previously described. Only aliquots from each tube were counted and the acceptor activity peaks pooled, concentrated with a Diaflo UM-10 filter (Amicon Co.), dialyzed against 0.001 M Na acetate, pH 4.5, buffer, and stored in small volumes at −20°.

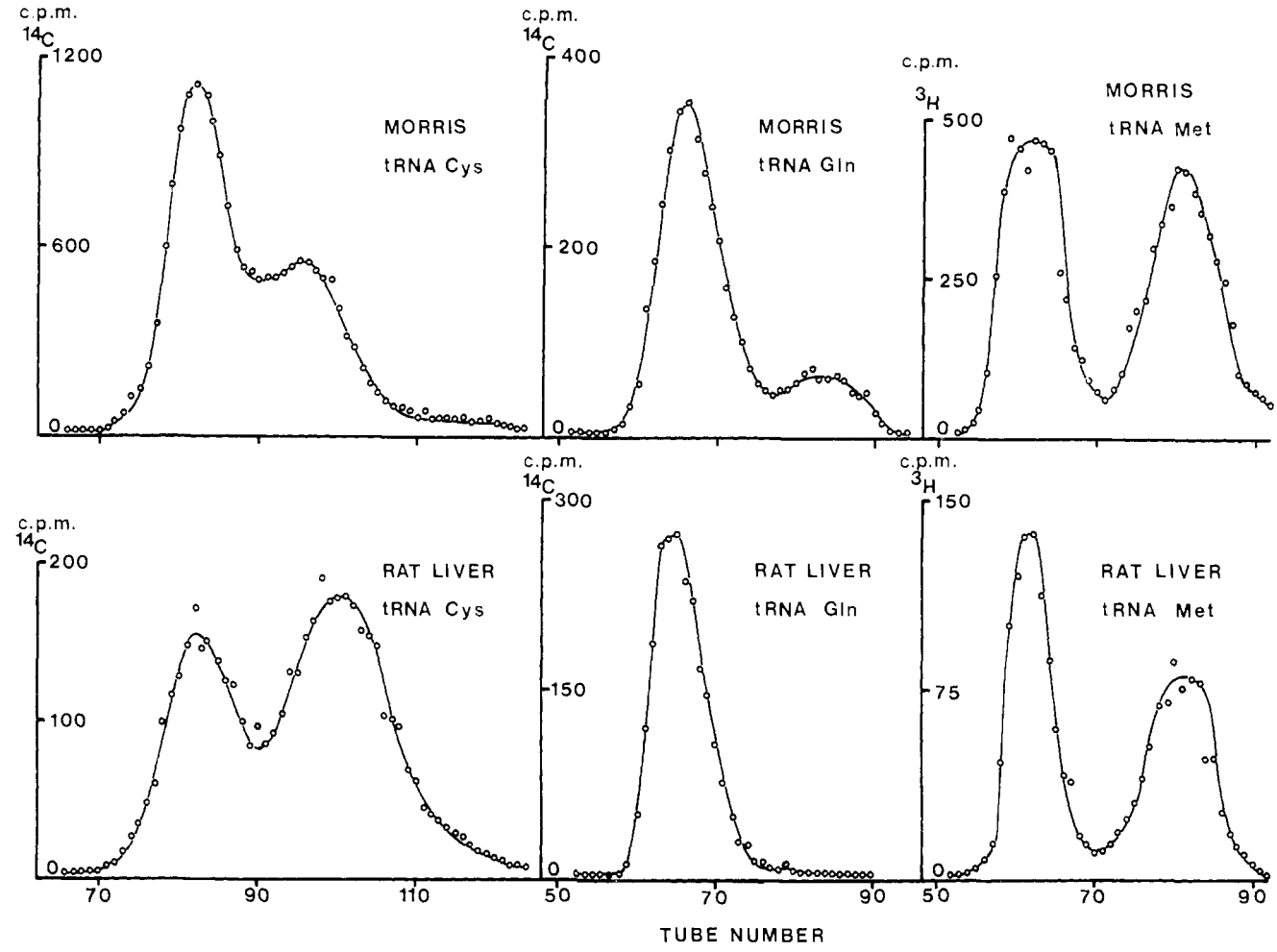

FIGURE 2: Elution profiles obtained from RPC-II columns with aminoacyl-tRNA from Morris 5123 hepatoma and normal rat liver. Two different chromatographies were necessary for the two species of tRNA, respectively, [^{14}C]cysteinyl-tRNA [^{14}C]glutaminyl-tRNA, and [^{3}H]methionyl-tRNA, due to the unavailability of both ^{3}H-and ^{14}C-labeled amino acid. The gradients were all from 0.3 to 0.7 M NaCl and superimposable profiles were obtained for each amino acid.

Polynucleotide Incorporation System. E. coli strain MRE 600 (provided by Dr. M. Di Girolamo, LIGB, Naples) was grown in Pennassay broth and harvested in early log phase. S-30 was prepared according to Nirenberg and Matthaei (1961). All the conditions for polynucleotide-dependent incorporation in the cell-free system were the same as described by Von Ehrenstein and Dais (1963). Polynucleotides were prepared according to Steiner and Beers (1961). The nucleotide ratios are expressed (in the tables) as input of the nucleoside diphosphates.

Polynucleotide and Trinucleotide Binding Assay. The binding assay was performed as described by Nirenberg and Leder (1964). Ribosomes were prepared from *E. coli* MRE 600 according to the method of Kurland (1966). They were stored in liquid N_2 until used. The triplets used in these experiments were prepared by the method of Leder *et al.* (1965).

For the hybridization of $tRNA^{Phe}_{Morris}$ *with various DNA* the method of Nass and Buck (1969) was used. DNA (from Morris 5123 hepatoma, rat liver, calf thymus, and *E. coli*) was immobilized on nitrocellulose filters (Schleicher and Schuell, type B-6). The DNA filters were annealed in single vials containing: 0.4 ml of 4 times standard sodium citrate (0.15 M NaCl–0.015 M Na citrate, pH 4.2) and 0.4 ml of formamide (Merk). [^{3}H]Phenylalanyl-$tRNA_{Morris}$, peaks Phe 1 and Phe 2, had 4000 cpm and 5000 cpm, respectively, for each vial. The incubation proceeded for 15 hr at 33° and each filter was finally washed with 2 times standard sodium citrate, pH 6.0, treated with pancreatic RNase (2 μg/ml) for 20 min at room temperature. After a final wash the filters were dried and counted.

Results

Chromatographic Profiles of Rat Liver and Morris 5123 tRNAs. The profiles of individual aminoacyl-tRNAs obtained with the reverse phase chromatography (RPC-II of Yang and Novelli, 1968b) are shown in Figure 1. Rat liver tRNA and Morris 5123 hepatoma tRNA ($tRNA_{Morris}$ = $tRNA_{5123}$) were all analyzed as aminoacyl-tRNA, precharged with radioactive amino acids (^{14}C or ^{3}H labeled) using homologous synthetases. Rechromatography using the reverse labeled amino acid was performed only when differences were found between the elution profiles of the two species of tRNA examined. All the amino acids showed multiple peaks, except $tRNA^{Glu}_{Morris}$ and $tRNA^{Phe}_{rat\ liver}$ (Figures 3a and 4), which will be discussed in detail later. It is well known that a correlation can be made for some amino acids between chromatographically different tRNA molecules and multiple code words for that amino acid (Weisblum *et al.*, 1962; Caskey *et al.*, 1968). However, it appears difficult to compare the profiles

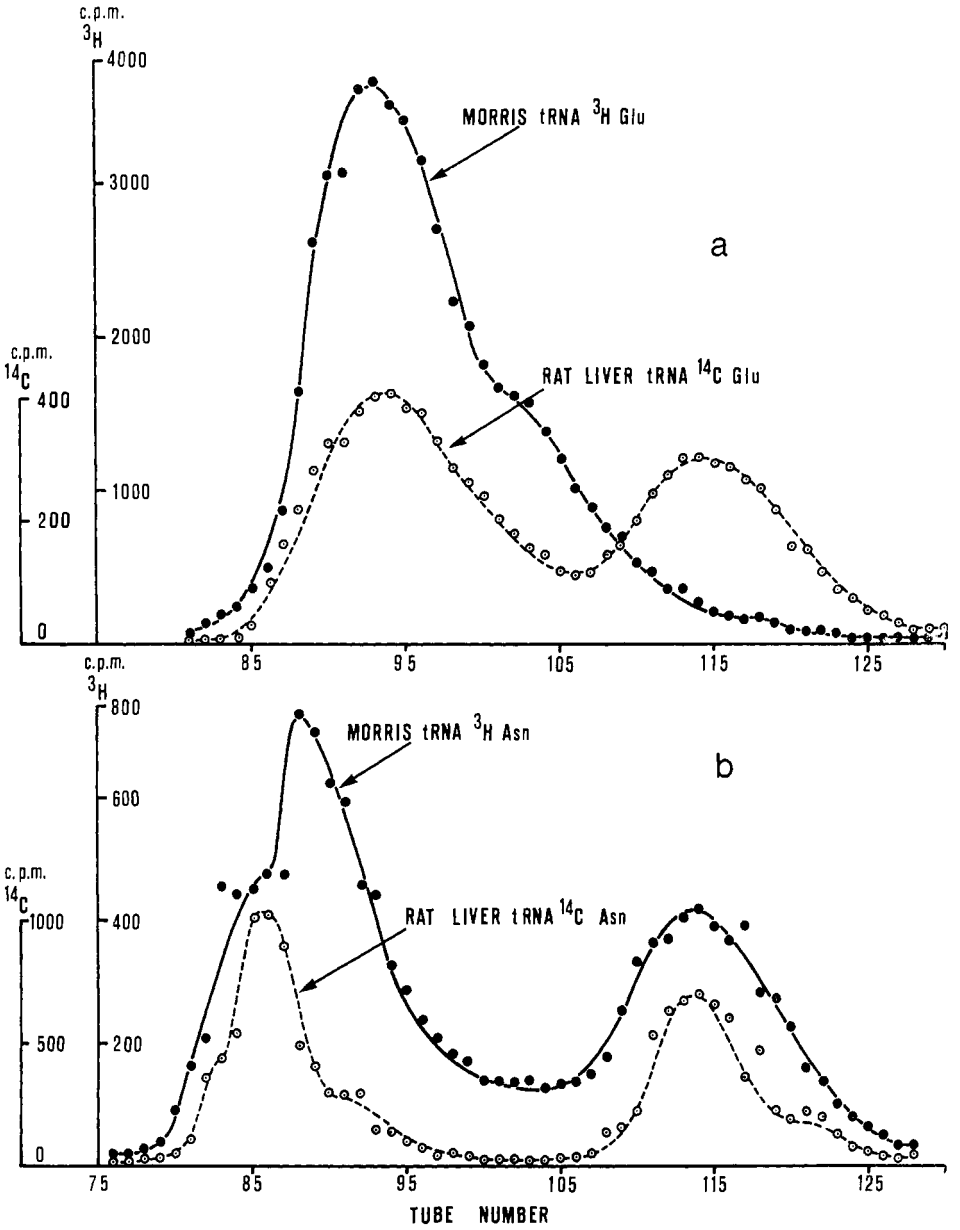

FIGURE 3: Elution profiles obtained from RPC-II columns with aminoacyl-tRNA from Morris 5123 hepatoma and normal rat liver. Linear gradients were from 0.3 to 0.7 M NaCl. In both figures the symbols ●——● and O- - - - -O represent the ^3H and ^{14}C cpm precipitated by cold 5% trichloroacetic acid in a 10-ml fraction.

obtained with different chromatographic methods, since each method may differ in its capacity to resolve the various tRNAs for an amino acid.

The resolution achieved is variable from laboratory to laboratory. Muench and Safille (1968) and Littauer *et al.* (1966) demonstrated that a completely different pattern can be seen also for various tRNA preparations of the same organism (*E. coli*). Therefore for a critical comparison of various species of tRNA we consider it essential to use a preparation of tRNA from different tissues that have been prepared and stored identically and to use double label techniques for the column analyses. With these precautions 17 out of 20 amino acids have shown on RPC-II exactly the same chromatographic profiles for both rat liver tRNA and Morris 5123 hepatoma tRNA (Figures 1 and 2). We do not have a clear explanation for the small differences in the height of the peaks, varing from one species to another, shoulders, or any other chromatographic asymmetry that appear in some of the figures.

Asparaginyl-tRNA from Morris hepatoma (Figure 3b) was eluted at a slightly higher salt concentration and in relatively different proportions with respect to rat liver $tRNA^{Asn}$. Glutamyl-tRNA from Morris hepatoma showed only one peak of acceptor activity, where two $tRNA^{Glu}$ were present in rat liver (Figure 3a).

Exactly the contrary is true for glutaminyl-tRNA (Figure 2): only one peak is present in $tRNA^{Gln}_{rat\ liver}$ where for $tRNA^{Gln}_{Morris}$ a minor peak is eluted at higher salt concentration.

In this case, the lack of [^3H]Gln made it impossible to examine the two species of tRNA by cochromatography. Therefore the evidence for this finding is less convincing even if the result was repeatedly consistent using different $tRNA_{5123}$ preparations and different batches of Chromosorb.

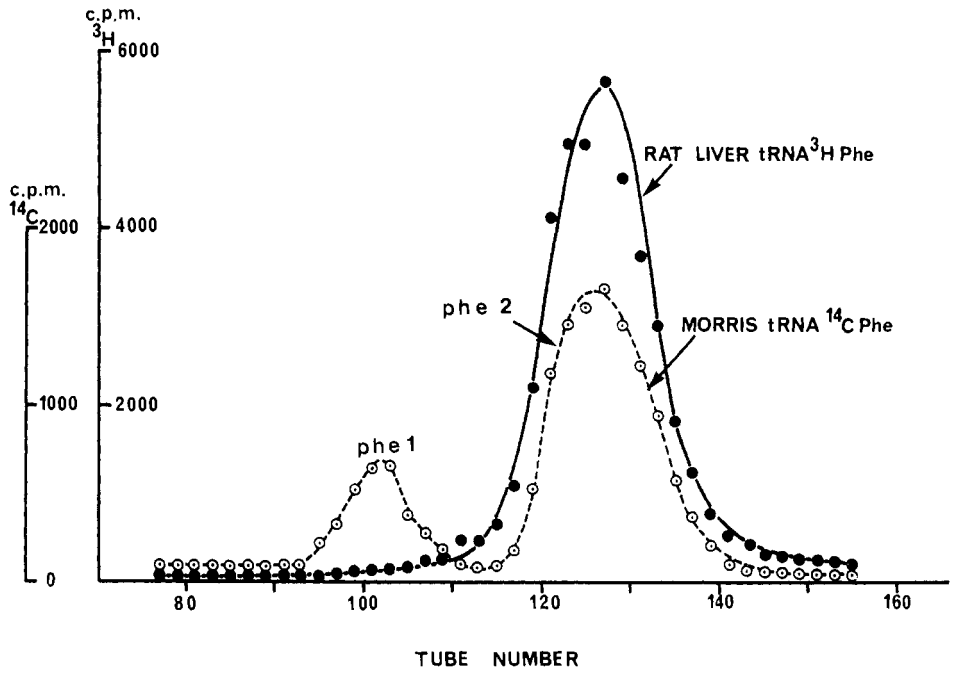

FIGURE 4: Elution profiles obtained from an RPC-II column with aminoacyl-tRNA from 5123 Morris hepatoma and normal rat liver. Linear gradients were from 0.3 to 0.7 M NaCl. The symbols ●——● and ⊙- - - -⊙ represent the ^{3}H and ^{14}C cpm precipitated by cold 5% trichloroacetic acid in a 10-ml fraction.

Phenylalanyl-tRNA is the most interesting amino acid we have examined, which differs between the two species. Figure 4 describes the direct comparison on RPC-II of $tRNA^{Phe}$ from rat liver and Morris 5123 hepatoma. Only one major peak of phenylalanine acceptor activity is present in $tRNA_{rat\ liver}$, whereas at lower NaCl concentrations a second species of $tRNA^{Phe}$ is eluted with the Morris hepatoma tRNA. The main $tRNA^{Phe\ 2}_{Morris}$ is symmetrical with the unique $tRNA^{Phe}_{rat\ liver}$. Inverse labeling did not affect the chromatographic behavior of $tRNA^{Phe}_{Morris}$. As we have previously reported (Gonano and Chiarugi, 1969), the inversion of the aminoacyl-tRNA synthetases did not alter the chromatographic profiles: $tRNA^{Phe\ 1}_{Morris}$ was always present when charged with rat liver synthetase. Figure 5 illustrates the chromatography of Morris $tRNA^{Phe}$ charged with *E. coli* aminoacyl-tRNA synthetase, compared to Morris tRNA and *E. coli* tRNA charged with their homologous synthetases.

As can be seen, the *E. coli* synthetase is able to recognize $tRNA^{Phe\ 1}_{Morris}$. The charging efficiency with the *E. coli* synthetase for Morris 5123 tRNA was 1.3 nmoles/mg. The only $tRNA^{Phe}$ species present in *E. coli* cochromatographs exactly as the main $tRNA^{Phe}$ species present in Morris hepatoma.

To study further this new $tRNA^{Phe\ 1}_{Morris}$ a preparative RPC-II chromatography was done with $tRNA^{Phe}_{Morris}$ purified on a benzoylated-DEAE column as described in Materials and Methods. The separated peaks were used for an analysis of their coding properties. A classical Nirenberg and Matthaei (1961) polynucleotide-dependent, cell-free system was used and the two $tRNA^{Phe}_{Morris}$ were compared with $tRNA^{Phe}_{rat\ liver}$ and with $tRNA^{Phe}_{E.\ coli}$. Since only one species of phenylalanine is present in both *E. coli* and rat liver tRNAs, unfractionated tRNA was used in all the experiments. Table I summarizes the results.

As expected, the maximum response was obtained with poly(U) for all the $tRNA^{Phe}$ tested.

The 74% incorporation with respect to the input from $tRNA^{Phe\ 1}_{Morris}$ indicates that the peak of phenylalanine acceptor activity present only in Morris 5123 hepatoma is not an artefact: the tRNA has completely maintained its capacity to read an artificial messenger like poly(U). No significant differences were observed among the various tRNAs in their capacity to synthesize polyphenylalanine in response to poly(U). Poly(UC) 1:1 is also able to stimulate the synthesis of polyphenylalanine with all the species of tRNA tested. The codeword UUC is well represented in this polynucleotide and it is specific for phenylalanine both in mammalian and bacterial cells (Marshall *et al.*, 1967). Some incorporation was also achieved with all $tRNA^{Phe}$ in the presence of poly(UA) 5:1. It is clear that this response is due to the high content of U that probably far exceeds the input ratio of 5U for 1A. All the other copolymers tested were unable to stimulate incorporation of phenylalanine.

Essentially the same data were obtained when the polynucleotides were used in a binding test according to Nirenberg and Leder (1964) (Table II). The maximum of binding efficiency with phenylalanyl-tRNA was obtained when poly(U) or poly(UC) (1:1) was attached on washed ribosomes. Poly(UA) (5:1) with its high U content is also a good code word for the binding of $tRNA^{Phe}$.

Of the six triplets used, UpUpC was the most effective in promoting the binding of all the $tRNA^{Phe}$ tested. UpCpG gives also 34% binding with respect to the 100% obtained with poly(U), in the case of $tRNA^{Phe}_{E.\ coli}$. No differences were observed between the two $tRNA^{Phe}_{Morris}$ with all the triplets and polynucleotides tested.

In Figure 6a,b another type of experiment is illustrated. $tRNA^{Phe\ 1}_{Morris}$ and $tRNA^{Phe\ 2}_{Morris}$ were used in a ribosome-dependent binding system. The binding efficiency with increasing polynucleotide concentrations, was practically the same for both poly(U) and poly(UC) (1:1) with the two $tRNA^{Phe}_{Morris}$.

TABLE I: Polynucleotide-Dependent Incorporation of [^{14}C]-Phenylalanine Bound to Rat Liver, *E. coli*, and Morris 5123 Heptoma tRNAs.

In Response to[a]	[^{14}C]Phenylalanyl-tRNAs, Cpm Transferred			
	Unfractionated		Morris 5123	
	E. coli	Rat Liver	Phe 1	Phe 2
Input	1081	1210	315	1850
No polymer	10	17	2	15
Poly(U)	738	939	233	1121
Poly(UC) 1:1	189	127	14	87
Poly(UA) 5:1	270	192	62	488
Poly(UG) 5:1	46	23	6	23
Poly(UC) 1:5	20	30		9
	% of [^{14}C]Phe Incorporated[b]			
Input	100	100	100	100
No polymer	1.0	1.4	0.6	0.8
Poly(U)	68.2	77.6	73.9	60.6
Poly(UC) 1:1	17.5	10.5	4.4	4.7
Poly(UA) 5:1	24.9	15.9	19.7	26.4
Poly(UG) 5:1	4.2	1.9	1.9	1.2
Poly(UC) 1:5	1.8	2.5		0.5

[a] Total counts per minute precipitable by hot 5% trichloroacetic acid after 15 min in a polynucleoide-dependent S-30 assay using [^{14}C]phenylalanyl-tRNA from various sources. $tRNA^{Phe\ 1}_{Morris}$ and $tRNA^{Phe\ 2}_{Morris}$ were taken from the RPC-II column described in Figure 4. Conditions as described in Materials and Methods. [b] The same results as in *a* are expressed in per cent of the input.

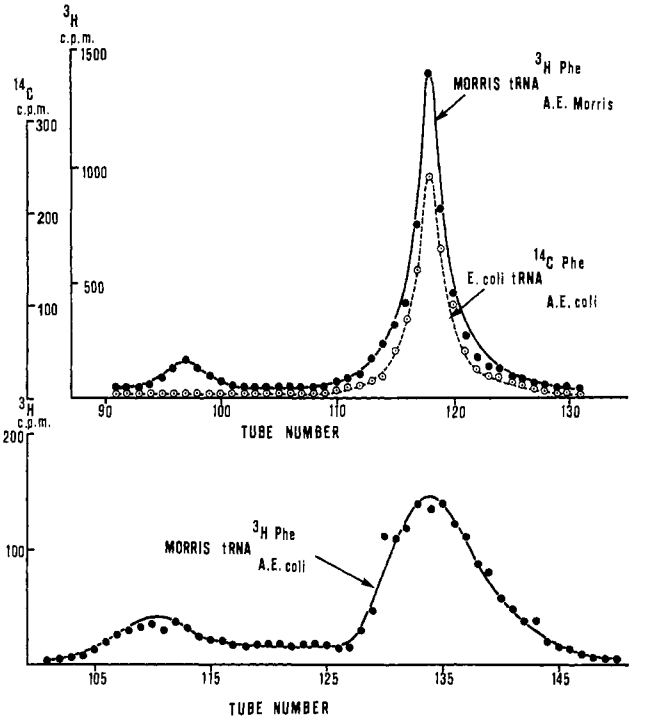

FIGURE 5: Elution profiles obtained from RPC-II columns with aminoacyl-tRNA from Morris 5123 hepatoma and *E. coli*. In the first chromatography Morris $tRNA^{Phe}$ aminoacylated with homologous charged *E. coli* $tRNA^{Phe}$, $tRNA^{Phe}_{Morris}$ acylated with *E. coli* synthetase was eluted in the second column. Linear gradients were from 0.3 to 0.7 M NaCl. In both figures the symbols ●——● and ⊙- - - -⊙ represent ^{3}H and ^{14}C cpm precipitated by cold 5% trichloroacetic acid in a 10-ml fraction.

At the concentrations of poly(U) used in these experiments, the binding of $tRNA^{Phe\ 2}_{Morris}$ was linear, whereas $tRNA^{Phe\ 1}_{Morris}$ reached the plateau, indicating a tendency of Phe 2 to have more affinity for poly(U). The kinetics for poly(UC) (1:1) were identical for both $tRNA^{Phe}_{Morris}$ (Figure 6b).

The results seem to indicate that the $tRNA^{Phe}_{Morris}$ are identical as far as coding properties are concerned. With hybridization experiments we further tested their properties, using various DNAs. The data obtained are summarized in Table III. The experiments were done with $tRNA^{Phe}_{Morris}$, highly purified by two chromatographic procedures; a benzoylated-DEAE column according to Wimmer *et al.* (1968) enriched the aromatic aminoacyl-tRNAs (mostly phenylalanine, since the charging was done in the absence of other cold amino acids); subsequently the two $tRNA^{Phe}_{Morris}$ were separated with an RPC-II column and found to have a specific activity between 30 and 40 nmoles of Phe/mg of tRNA. With these $tRNA^{Phe}$ free from all the other tRNAs, there should be no competition for the tRNA cistrons on the DNA molecules by species of tRNA different from the ones accepting phenylalanine. The maximum level of hybridization, as expected, was obtained with homologous DNA and we referred all the others to this as 100%. With rat liver DNA, both $tRNA^{Phe\ 1}_{Morris}$ and $tRNA^{Phe\ 2}_{Morris}$ have a very high affinity (91 and 99%). The capability to form hybrid molecules drops significantly with calf thymus DNA (31 and 54%) and it is almost negligible with *E. coli* DNA (16 and 29%). No differences between the two $tRNA^{Phe}_{Morris}$ were observed.

Discussion

The search for deviations from the normal cell at the biochemical level in various experimental tumors is a major goal of many pathologists. The working hypothesis of our experiments was to examine deviations from the normal pattern in tRNAs we extracted from "minimal deviation" hepatomas. These tumors are so-called "minimal deviation" because they strictly derive from hepatocytes and their morphology is similar to that of the cells of origin. Despite their morphology and the possibility of growing encapsulated for a relatively long time in the leg muscle of the rat, Morris hepatomas are very malignant by other parameters. The differences found especially at the biochemical level between the liver and the hepatoma have led to incomplete agreement about the maintenance of the name "minimal deviation." From the comparative work of Wu (1967) it is not possible to give a defined number of parameters to establish the minimum of deviation between the normal hepatocyte and the hepatoma cell. For the purpose of this work, it is enough to know that even if for some markers the deviations are not so minimal, the similarites between the hepatoma cell and the normal liver cell are so strong that it is possible to compare the two cells as far as tRNA patterns are concerned. Alterations at the level of tRNA may in fact occur at all stages of the oncogenic process. The aim is of course to evaluate differences that occur at very early stages.

A good model has been proposed with ethionine-induced hepatomas by Axel *et al.* (1967) and Ortwerth *et al.* (1968). The differences they found in the tRNA between the ethionine-induced tumors and normal liver disappeared when

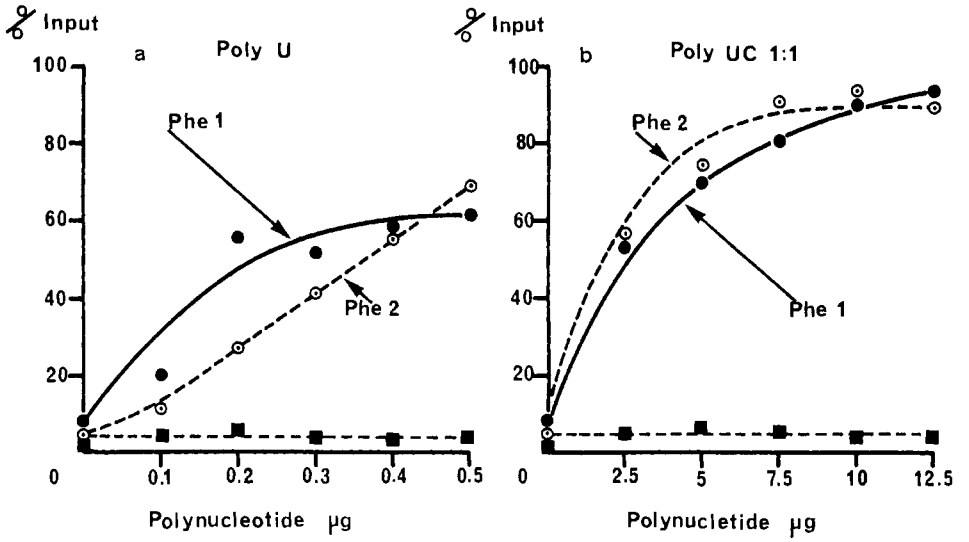

FIGURE 6: Binding assays on Millipore filter with $tRNA^{Phe}_{Morris}$ fractions taken from the RPC-II column illustrated in Figure 4. The results are indicated as per cent of the input of $tRNA^{Phe1}_{Morris}$ (●——●) and of $tRNA^{Phe2}_{Morris}$ (⊙- - - - -⊙) bound to ribosomes as a function of concentration of poly(U) and poly(UC) 1:1, respectively. The magnesium concentration was 0.01 M. Negative binding is represented by the ■- - -■ line and was obtained using a tRNA without any affinity for UUU or UUC.

TABLE II: Binding Efficiency of Different $tRNA^{Phe}$.[a]

Source of tRNA	Polynucleotides				Triplets					
	No	U	UC 1:1	UA 5:1	UCU	UUC	ACG	UCG	AUG	UAG
$tRNA^{Phe\ 1}_{Morris}$	6	86	[100]	87	3	58	4	2	3	5
$tRNA^{Phe\ 2}_{Morris}$	7	[100]	93	97	8	70	3	12	5	5
$tRNA^{Phe}_{E.\ coli}$	11	[100]	83	95	12	78	10	34	11	11
$tRNA^{Phe}_{rat\ liver}$	4	[100]	33			36		7		

[a] Millipore filter binding assays with various [^{14}C]phenylalanyl-tRNAs. The results are expressed as % of binding with respect to the maximum obtained with one polynucleotide (in brackets). Each polynucleotide (1 μg of each) and 0.2–0.4 mμmole of each triplet were used in the assays at 0.01 M magnesium.

TABLE III: Hybridization of Morris 5123c Hepatoma $tRNA^{Phe}$ with Homologous and Heterologous DNA.[a]

	Cpm of [^{3}H]Phenylalanyl-tRNA Bound to DNA	
	$tRNA^{Phe\ 1}$	$tRNA^{Phe\ 2}$
Rat liver	468 (91)	236 (99)
Hepatoma	513 (100)	240 (100)
Calf thymus	162 (31)	129 (59)
E. coli	85 (16)	70 (29)
No DNA	40 (8)	33 (13)

[a] [^{3}H]Phenylalanyl-$tRNA^{Phe\ 1}_{Morris}$ (4000 cpm) and 5000 cpm of [^{3}H]phenylalanyl-$tRNA^{Phe\ 2}_{Morris}$ taken from a RPC-II column, as described in Figure 4, were incubated with various DNA-coated nitrocellulose filters (see Materials and Methods). The results are expressed as total cpm attached to the filters and in parentheses as per cent with respect to the maximum annealing obtained with the homologous DNA.

ethionine was not present in the diet of the rats. Different other tumors have been examined (quoted in the introduction) and indeed differences have been found in the tRNA pattern for some amino acids. Since various techniques have been used, it is not possible to compare all the results, but it seems clear that some of the tRNAs, in Novikoff tumor ascites cells, have a different chromatographic behavior from the one of rat liver. It seems reasonable to think that very undifferentiated cells, like the Novikoff ascites cells, have no more relation to the liver cells than any other tissue of the same organism. Many differences in tRNA profiles were in fact observed by Holland *et al.* (1967) and Taylor *et al.* (1967, 1968) in comparing various tissues: embryonic, adult, and in culture. From all the data, therefore, it is only possible to say that different tissues can show, for some amino acids and with particular chromatographic techniques, differences in tRNA acceptor activity profiles.

It is now an open question, since the paper of Yang *et al.* (1969), as to whether the differences are due only to nutritional conditions. The presence or absence of serum in the growing medium of the cells changed drastically the tRNA

pattern for a few amino acids. Some changes have been also found in different stages of differentiation (Lee and Ingram, 1967; Yang and Comb, 1968; Vold and Sypherd, 1968; and Anderson and Cherry, 1969) and between compartments of the cell (Buck and Nass, 1968, 1969). This study presents evidence for the presence in the minimal deviation hepatoma of a new $tRNA^{Phe}$, and one glutamyl-$tRNA_{Morris}$ instead of the two $tRNA^{Glu}_{rat\ liver}$. Essentially the same results were obtained by Volkers and Taylor (1971) for phenylalanine in Morris 5123 D. This is in accordance with a similar finding in a different 5123 strain and in a 7777 hepatoma from Dr. Morris (F. Mushinsky, personal communication). Dr. R. Quist also working with an hepatoma induced by Dr. Zajdela in Paris with dimethylaminoazobenzene found a phenylalanine tRNA specific of the tumor (R. Quist, personal communication). Our $tRNA^{Phe\ 1}$ was present both in Morris 5123 and 5123c hepatomas. The differences that have been found by Volkers and Taylor (1971) for serine and hystidine can be presumably ascribed to a different strain of tumor (they used 5123 d Morris hepatoma). The conclusions, from a pathological point of view, are that it is reasonable to compare the hepatoma with the tissue from which it originated. The fact that the tRNAs of 17, out of 20, amino acids are absolutely identical is strong evidence in favor of the hypothesis that the few differences are early events in the tumor deviation of the hepatocyte: they can be a consequence of the oncogenic process or one of the possible reasons for the pathological alteration of the cell. The last hypothesis can be derived from the many observations on lower organisms of the regulatory functions of tRNAs (Garen, 1968).

We can argue, then, that also in mammalian cells a particular tRNA may function as a suppressor molecule since it appears able to recognize some key triplets along mRNAs (Weisblum *et al.*, 1965, 1967; Gonano, 1967; and Sekya *et al.*, 1969). The absence or presence of these tRNAs could influence the synthesis of specific proteins. In the tumor cell, the appearance of a new tRNA, like $tRNA^{Phe\ 1}_{Morris}$, or the disappearance of the $tRNA^{Glu}_{Morris}$ might influence the translation of some preexisting mRNA, in which at fixed points the amino acids have to be inserted by those tRNAs.

This hypothesis is an extension of the modulation theory of Ames and Hartmann (1963) and Stent (1964) to mammalian cells. In order to prove this, we ought to be able to demonstrate not only the existence of a new tRNA in the modified cell, but also that the coding properties of the molecule are drastically changed. All our efforts have been made to test the coding properties of the $tRNA^{Phe}$, from both normal liver and Morris 5123 hepatoma. The data presented in Tables I and II and Figure 6 are a clear demonstration that indeed the tRNAs were able to support the synthesis of polyphenylalanine in the presence of synthetic polynucleotides, but exactly the same response was obtained with all the tRNAs tested. Only a limited number of triplets have been available and more emphasis was, therefore, given to the initiation and termination codons (ApUpG and UpApG). Not one of those triplets was active in promoting the binding the $tRNA^{Phe\ 1}_{Morris}$, indicating thus that the new tRNA has nothing to do with initiation or termination processes. The possibility that $tRNA^{Phe\ 1}_{Morris}$ is an aggregate of $tRNA^{Phe\ 2}_{Morris}$ has been tested by gel filtration on Sephadex G-100. The results indicating that, by this method, the molecular weights of $tRNA^{Phe\ 1}_{Morris}$ and $tRNA^{Phe\ 2}_{Morris}$ are identical, will be presented elsewhere (F. Gonano and G. Pirro, manuscript in preparation).

One of the hypotheses that we previously proposed (Gonano and Chiarugi, 1969) concerned the viral origin of this tRNA as did Subak-Sharpe *et al.* (1966) in their studies. It seems reasonable, now, from the hybridization data (Table III) to rule out this hypothesis because the two species of $tRNA^{Phe}_{Morris}$ are able to make specific hybrids with both hepatoma DNA and liver DNA. This indicates that the sites of synthesis of $tRNA^{Phe\ 1}_{Morris}$ and $tRNA^{Phe\ 2}_{Morris}$ are present both on liver and Morris hepatoma DNA. For $tRNA^{Glu}_{Morris}$ (Figure 3a) work is now in progress synthesizing GpApA and GpApG in order to test whether the two codons correspond in rat liver to $tRNA^{Glu}$ different and if one of those disappeared in the tumor cell.

From our data therefore we can draw some conclusions: (1) a new chromatographically different $tRNA^{Phe}$ molecule appears in Morris hepatoma 5123; (2) no coding differences can be detected between the normal and the hepatoma-specific $tRNA^{Phe}$; (3) the molecule hybridizes with rat liver DNA indicating an overall complementary sequence of nucleotides with normal rat liver DNA.

The differences so far obtained between tissue of origin and tumor tRNA do not necessarily mean modification in the anticodon portion of the molecule.

The sequence of nucleotides of the aminoacyl-tRNA synthetase recognition site can in some case be modified, as in the tRNA from Novikoff ascites and rat liver cells (Goldman, *et al.*, 1969). This is not the case for the $tRNA^{Phe\ 1}_{Morris}$ since, reversing the aminoacyl-tRNA synthetase from liver to tumor, the chromatographic pattern was not altered (Gonano and Chiarugi, 1969). Moreover, *E. coli* aminoacyl-tRNA synthetase recognizes both $tRNA^{Phe\ 1}_{Morris}$ and $tRNA^{Phe\ 2}_{Morris}$ (Figure 5), indicating a strong similarity in the aminoacyl-tRNA synthetase recognition site of the two species of tRNA.

We considered that the different rates of growth and the different nutritional conditions of tumor and liver cells might explain our results for phenylalanine, especially after the work of Wettstein and Stent (1968) and Yang *et al.* (1969). In this respect, J. F. Mushinsky (personal communication) found that regenerating liver of the same strain of rats presents no variations in the chromatographic profile of $tRNA^{Phe}$ compared to normal rat liver.

At this point the most reasonable hypothesis to explain the new $tRNA^{Phe}$ involves base-specific modifications occurring on the molecule after its synthesis and not influencing the coding and chargeability properties.

Modifications involving methyl groups have been demonstrated for many tumor tRNAs (Tsutsui *et al.*, 1966; Hancock, 1968; Viale, 1969). It is impossible, however, to decide now whether some particular bases are methylated in $tRNA^{Phe\ 1}_{Morris}$ and not in $tRNA^{Phe\ 2}_{Morris}$, or if we are dealing with a hypomethylated $tRNA^{Phe\ 1}_{Morris}$, like the one described by Biezunski *et al.* (1970) for *E. coli* with very similar behavior on RPC-II. This possibly can be the case since it has been proven that methyl groups do not modify the coding properties of $tRNA^{Phe}_{E.\ coli}$ even when the chromatographic profiles of the normally and hypomethylated species are different (Stern *et al.*, 1970).

Work is now in progress in this direction with large amounts of highly purified $tRNA^{Phe}_{Morris}$. Base analysis and fingerprints will probably tell us the structural differences between the two molecules and possibly also give us information about their functions.

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