Transfer RNA Base Composition Studies in Morris Hepatomas and Rat Liver

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SUMMARY

The major and modified base composition of cytoplasmic tRNA from Morris hepatomas 7777 and 5123D and from rat liver has been determined for 16 constituents using a new chemical tritium derivative method. Analysis of the total base composition of the tumor tRNA's did not reveal a general overmodification or overmethylation, but the total modified and methylated base content was found to be slightly lower than in tRNA from normal liver and host liver. As shown by analysis of the modified base composition, hepatoma 7777 tRNA contains less 5-methyluridine, pseudouridine, 3-methylcytidine, and 5-methylcytidine and more dihydrouridine and 1-methylguanosine than does liver, and hepatoma 5123D contains less dihydrouridine and 5-methylcytidine and more N²,N²-dimethylguanosine and X (an unidentified modified nucleoside) than does liver. Differences ranged from 2 to 17%, N²,N²-dimethylguanosine and X showing the greatest deviations in hepatoma 5123D. For the major nucleosides, differences of 2 to 4% were observed. Both tumor RNA's contain more uridine and less cytidine, and hepatoma 7777 has in addition less adenosine and more guanosine. No unusual modified bases were detected in these tumors by the chromatographic procedures used. A comparison of the two tumor tRNA's does not provide evidence for a distinct correlation between degrees of modification and methylation of tRNA and growth rates and histological characteristics of the tumors.

The following possibilities exist for explaining the observed base composition differences: (a) changes in concentrations of specific tRNA's; (b) presence in the tumors of tRNA's with altered sequences; and (c) aberrant modification.

INTRODUCTION

Although the general mechanism of protein synthesis in cancer cells appears not to differ from that in normal cells, specific components of the protein-synthesizing system may conceivably be altered and such alterations may be involved in carcinogenesis. A component of the protein-synthesizing system particularly likely to be affected is tRNA because of the multitude and complexity of processes leading to the formation of a mature tRNA population. Changes in the structure of tRNA can be expected to have a profound influence on protein synthesis and structure, since tRNA probably is more responsible than any other macromolecule for the correct translation of the genetic code into the amino acid sequence of proteins.

During the past decade a considerable amount of indirect evidence has been accumulated in an effort to support the assumption that tRNA may be altered in tumor cells. In particular, the activity of tRNA methyltransferases (for reviews, see Refs. 7, 22, and 52) has been shown in many laboratories to be increased severalfold in extracts from a variety of neoplastic tissues including virally transformed cells and chemically induced tumors (for a recent review, see Ref. 6). Elevated methylase levels have been assumed to lead to overmethylation of RNA and to be associated with carcinogenesis (48, 49). This hypothesis was supported by the fact that the alkylating carcinogen dimethylnitrosamine leads to a methylation of nucleic acids in vivo (27). Indirect evidence for changed tRNA species in various neoplastic cells has also been provided by comparative investigations of the column chromatographic profiles of aminoacylated tRNA's (for reviews, see Refs. 6 and 53).

Although there is considerable indirect evidence for abnormal tRNA in tumors, little is known about chemistry and structure of tRNA in normal and neoplastic mammalian cells. The question of whether there is excessive methylation of tumor tRNA in vivo has been studied to a much lesser extent than the levels of the tRNA methylases have been, a few reports claiming substantial overmethylation of tRNA in tumor tissues (4, 31, 55–57) and others finding no overmethylation (1, 18, 33, 37).

Direct investigation of mammalian tRNA structures has been impeded by the multitude of relatively similar species present in the cell and the necessity to work with large amounts of tissue because of the lack of sufficiently sensitive analytical methods. Most of the structural work has been carried out with bacterial tRNA thus far, using procedures based on in vivo ³²P-labeling of nucleic acids as described by Sanger et al. (46) as reviewed by Barrell (2). This approach, however, is not feasible in mammalian cells, unless they can be grown in culture. In vivo labeling of tRNA with methionine-¹⁴C has been used to analyze methylated bases (12, 17), but this approach does not allow one to determine
RNA constituents other than methylated bases.

A recently developed method (34, 35, 39–42) for base analysis of RNA, which is based solely on chemical postlabeling of nonradioactive RNA derivatives, thus not requiring in vivo labeling, is suitable for assaying the 4 major and most modified bases in mammalian tRNA. This tritium derivative method has since been used for base composition analysis of various RNA's, e.g., tRNA from human brain and brain tumors (33, 37) and other normal and neoplastic mammalian tissues,3 tRNA from avian liver and avian leukemic cells and the avian myeloblastosis virus (43), tRNA and rRNA from Mycoplasma hominis (19), and various nuclear and nucleolar RNA's from Novikoff hepatoma cells (44).

In this communication we report tRNA base composition data for Morris hepatomas 5123D and 7777 and rat liver obtained by the tritium derivative method. Part of this work has been reported in a preliminary form (32). Since the tRNA methyltransferase activity has been described to be 3.7 and 5 times higher in extracts from hepatomas 5123D and 7777, respectively, than in those from normal liver (47) and since alterations of column chromatographic profiles of aminocytated tRNA's have been reported for several hepatomas, including hepatoma 5123D (50, 58), it was of particular interest to compare the degree of methylation of tRNA from normal liver and these tumors. It was also of interest to look for differences in terms of individual bases or the presence of unusual bases in the tumors, which had to be regarded as a distinct possibility in view of the fact that bacterial tRNA's contain modified bases that do not occur in mammalian tRNA (14, 35).

MATERIALS AND METHODS

Materials. Morris hepatomas 7777 and 5123D were originally induced by Dr. H. P. Morris and continuously transplanted i.m. Female Buffalo rats (150 to 250 g) were used for transplants and as a source for normal liver. Hepatomas 7777 and 5123D have growth rates of 5.0 and 3.7 cm/month and are histologically "poorly differentiated" and "intermediate between well differentiated and poorly differentiated," respectively (47). Apparatus used for tissue and gel homogenization included a Heidolph "Inframo" stirrer (Will Scientific, Cambridge, Mass.) and Teflon pestle homogenizers (A. H. Thomas Co., Philadelphia, Pa.). Triton X-100™ was from Beckman Instruments, Fullerton, Calif. Chemicals for gel electrophoresis and a Model EC-474 standard vertical gel apparatus were from EC-Apparatus Corp., St. Petersburg, Fla. Enzymes and chemicals used for digestion of tRNA and labeling of the digests, as well as materials for chromatography and liquid scintillation counting, have been described elsewhere (35, 40, 42).

Isolation of tRNA from Tissues. Tissue was excised quickly from etherized rats immediately after bleeding the animals and used immediately or stored frozen at -70 to -80°. tRNA was isolated from 2 to 3 g of tissue taken from 1 liver and from 2 tumors of the same animal. Supernatant solutions (100,000 x g) of tissue homogenates were prepared, from which tRNA was isolated by precipitation at pH 5 and subsequent phenol treatment. Preparation of the high-speed supernatant solution and precipitation at pH 5 were done by minor modifications of procedures originally described by Keller and Zamecnik (21) and Blobel and Potter (5). The complete procedure has been described in detail previously (10).

Purification of RNA by Polyacrylamide Gel Electrophoresis. For purification, the crude tRNA was subjected to polyacrylamide gel electrophoresis on 10% gels at pH 7.8 using a Tris/sodium acetate/EDTA buffer similar to that described by Loening (26). Staining of the gels was with methylene blue essentially according to the procedure of Peacock and Dingman (30). The 4 S RNA band was excised from the gel and subjected to homogenization and phenol treatment. The RNA was finally isolated from the aqueous phase, which had been concentrated under vacuum, by precipitation with acetonitrile/ethanol. The details of the procedure have been described (10).

Preparation of tRNA from 15,000 x g Pellets. Pellets (15,000 x g) were obtained in the course of preparing 100,000 x g supernatant solutions of tissue homogenates (10). Nucleic acids including tRNA were isolated from the low-speed pellets by phenol extraction following essentially the same procedure as described for the extraction of tRNA from pH 5 precipitates (10). In order to separate tRNA from rRNA's and DNA, the nucleic acid preparation was subjected to polyacrylamide gel electrophoresis using stacked gels (10 and 3.5% gels) and a buffer similar to that described by Loening (26). Isolation of 4 S RNA from the gels was performed as described for cytoplasmic tRNA (10).

Enzymatic Digestion of RNA and Trifluoracil Labeling of Digests. The purified 4 S RNA was digested to nucleosides using a mixture of RNase A, snake venom phosphodiesterase, and alkaline phosphatase as described previously (35, 40). The solution of N,N-bis(2-hydroxyethyl)glycine buffer used for preparing the enzyme digest (40) was freshly prepared, since storage of this buffer in the frozen state (−20°) led to changes in its properties that interfered with the subsequent trifluoracil-labeling procedure. Incubation of the digests was for 6 hr at 37°. After appropriate dilution, the enzymatic digests were directly subjected to periodate oxidation and subsequent reduction with KBH₄-3H (about 2 Ci/mmol) as described, which results in the formation of trifluoracil-labeled nucleoside trihalochols (39, 40). Labeled digests were stored at −70 to −90° and analyzed within 2 weeks. All operations involving KBH₄-3H were carried out under a well-ventilated hood. It was important to use a potassium borotrifluoride solution not older than about 4 months (storage at −70 to −90°). The use of older borotritide solutions or of solutions that had been subjected to repeated freezing and thawing may result in incomplete reduction of the nucleoside dialdehydes to trifluoracil-labeled "monoaialdehydes" (40), particularly in the case of dialde-
tRNA Base Composition in Morris Hepatomas and Rat Liver

The abbreviations used are: hU, dihydrouridine; m°C, 3-methylcytidine; m'G, 7-methylguanosine; ψ, pseudouridine; tA, N-9-(β-D-ribofuranosyl)purin-6-y1 carbamoyljthreonine; m"U, 5-methyluridine; m"C, 5-methylcytidine; m"A, 1-methyladenosine; m"G, N°,N°-dimethylguanosine; m"G, 1-methylguanosine; m"A, N°-methyladenosine.

Requirements for Comparative Investigations on tRNA. For accurate determination of degrees of modification and methylation of tRNA preparations from various tissues, it was necessary to free the tRNA preparation of any contaminating RNA [such as 5 S RNA, 4.5 S RNA, various low-molecular-weight rRNA's (45), and high-molecular-weight tRNA]. As described earlier (10), purification of the RNA by polyacrylamide slab gel electrophoresis, followed by extraction of the 4 S band, leads to RNA preparations that are pure in terms of molecular weight and completely free of contaminating RNA. It was also advantageous to isolate the RNA by precipitation at pH 5 (21) rather than by phenol extraction of the tissues (9), since the former procedure gave cleaner preparations from the tissues investigated as judged by analytical gel electrophoresis (10). tRNA purified by phenol extraction contained varying amounts of background material which overlapped the 4 S RNA band on polyacrylamide gels and consisted of degradation products of high-molecular-weight RNA, whereas tRNA isolated by pH 5 precipitation was completely free of such background material. The 4 S RNA band of tRNA isolated by pH 5 precipitation usually represented about 85 to 90% of the total RNA (10). The residual material consisted of several faint bands of low-molecular-weight RNA, e.g., 5 S RNA and 4.5 S RNA, and traces of rRNA (10). Thus polyacrylamide gel electrophoresis proved to be a valuable means to remove all impurities contained in the crude tRNA preparation. As shown previously (10), polyacrylamide gel electrophoresis may be successfully combined with subsequent base analysis by the tritium derivative method.

Another important factor in comparative investigations is the integrity of the RNA to be analyzed. As judged by polyacrylamide gel electrophoresis, tRNA prepared by pH 5 precipitation from Morris hepatomas 7777 and 5123D and liver contained <3% polynucleotide material moving ahead of 4 S RNA. However, even under strictest precautions, undegraded tRNA cannot be isolated from certain Morris hepatomas, e.g., hepatoma 9121. The tRNA derived from such hepatomas is thus unsuitable for comparative investigations. As a control, tRNA was also isolated directly from low-speed supernatant solutions of hepatomas 7777 and 5123D and liver by phenol treatment, precipitation of the RNA from the aqueous phase, and gel electrophoresis in order to exclude the possibility of degradation and other artifacts during the pH 5 precipitation procedure. Identical base compositions were obtained irrespective of the mode of preparation.

The 4 S RNA isolated from pH 5 precipitates was found to represent about 50% of total cellular 4 S RNA, the rest being present in the low-speed (15,000 × g) pellets and consisting of membrane and ribosome-bound tRNA, as well as mitochondrial and nuclear tRNA. We have also carried out base composition studies on tRNA isolated from such low-speed pellets of the same tissues as well as rat liver mitochondria and shall refer to these results in connection with the base composition data for tRNA's from pH 5 precipitates.

During the course of our work it was found that experiments must be conducted in parallel if the 2 RNA preparations to be compared exhibit only small differences. In particular, the labeling reactions must be carried out simultaneously using the same borotritide preparation, and conditions leading to the formation of monoaldehydes must be avoided (see "Materials and Methods"). It is also essential to cut the nucleoside trialcohol spots from the chromatograms in an identical manner. This could best be

achieved if the same person did the particular experiment.

Chromatography of Tritium-labeled tRNA Digests. Fig. 1 shows fluorograms (36) of 2-dimensional thin-layer chromatograms of tritium-labeled nucleoside trialcohols (35, 40) obtained by digestion and labeling of tRNA of Morris hepatoma 5123D (top) and host liver (bottom). The RNA was prepared by extracting the 4 S band from polyacrylamide gels following isolation by pH 5 precipitation (21) (see "Materials and Methods"). Both maps (Fig. 1) show the same spots, spot intensities closely resembling each other, thus indicating an overall similarity of the base compositions. There is no evidence for the presence in the tumor of any additional, unusual bases. Very similar patterns had been observed previously for tRNA from human brain and brain tumors (33, 37), chicken liver and avian myeloblasts (43), and various mammalian tissues, whereas labeled digests of tRNA from the avian myeloblastosis virus (43) and from bacteria (35) showed considerably different spot patterns and quantitative results. Fluorograms obtained from hepatoma 7777 and host liver tRNA were also very similar to each other and to those depicted in Fig. 1, again indicating an overall similarity of the base compositions.

Comparison between tRNA Base Composition of Hepatoma 7777 and Liver. In Table 1 the total base composition of tRNA from Morris hepatoma 7777 is compared with that of host liver. Sixteen compounds were analyzed as trialcohol derivatives by the tritium derivative method (35, 40) including most base-modified and all base-methylated nucleosides known to occur in mammalian tRNA. 2'-O-Methylated nucleosides, thio derivatives, and N6- (Δ2-isopentenyl)adenosine have not been assayed for reasons discussed elsewhere (35). tA (16, 29) (nucleoside trialcohol t'A' on Fig. 1) was not analyzed, since only after completion of the work reported here was the nucleoside trialcohol of this compound prepared by derivatization of an authentic sample of tA. We have, however, analyzed the radioactive derivative of an unidentified modified nucleoside X (Fig. 1), which appears to be identical with a nucleoside occurring next to m5G in several Escherichia coli tRNA species of known sequence (3, 11, 28, 59, 60). This was shown by subjecting several purified E. coli tRNA's known to contain X (tRNA^met, tRNA^phe, tRNA^ala) to tritium base composition analysis. Since some other tRNA species from E. coli as well as from yeast contain uridine or hU instead of X in the same position, X is assumed to be a derivative of uridine (60).

As can be seen from Table 1, the base composition of hepatoma 7777 tRNA is similar to that of host liver tRNA. There are, however, small but statistically significant differences in the 4 major and 5 modified constituents ranging from 2 to 10%, the greatest differences being in the content of m8U (10%), m8C (8%), and m4C (7%). All modified constituents significantly altered in the tumor tRNA, i.e., m8U, ψ, m5A, m8C, and m8C, were found to be lower than in liver tRNA. A slight undermodification of the tumor tRNA is reflected by the ratios Σmodified nucleosides/Σmajor nucleosides and Σmodified nucleosides/Σtotal nucleosides which are about 3% lower for the tumor tRNA. The hepatoma tRNA is also slightly undermethylated, the corresponding ratios being again about 3% lower. Regarding the major constituents, the tumor tRNA contains more cytidine and guanosine and less adenosine and uridine than does liver tRNA, the differences being 2 to 4%.

We have also investigated the total base composition of tRNA from livers of normal Buffalo rats. This analysis
showed statistically significant differences for the same major and modified nucleosides as the comparison between hepatoma 7777 and host liver tRNA. A comparison between tRNA from normal liver and host liver (hepatoma 7777) showed great similarity in terms of base composition, m^5U, m^5C, m^2G, and X being 5 to 8% lower in host liver tRNA than in normal liver tRNA. No significant differences were found for any other nucleoside.

By excluding the major constituents from the calculations, one is able to compare the modified base composition of tRNA from various samples. Table 2 shows such a comparison for hepatoma 7777 and host liver tRNA. The values for 4 modified pyrimidines, including \( \psi \) and the 3 base-methylated nucleosides m^5U, m^5C, and m^6C, are slightly but significantly lower in the tumor tRNA, whereas m^G and hU are slightly elevated. Differences are again relatively small, the greatest difference being 11% for m^G. Similar results with regard to the modified base composition were obtained with normal liver tRNA.

Comparison between tRNA Base Compositions of Hepatoma 5123D and Liver. Table 3 compares the total base composition of hepatoma 5123D and host liver tRNA. Again, there is great overall similarity in the 2 base compositions with differences ranging from 1 to 16%, being for the most part < 10%. Two major nucleosides (uridine and cytidine) and 6 modified nucleosides (hU, m^A, m^3C, m^5C, m^2G, and X) show statistically significant differences. Four modified constituents (m^5U, m^A, m^3C, and m^5C) are lower and 2 modified constituents (m^2G and X) are higher in the tumor tRNA, the greatest differences observed being 13 and 16% in m^2G and X, respectively. In spite of the relatively high values for these 2 nucleosides in the tumor tRNA, the ratios \( \Sigma \text{modified nucleosides} / \Sigma \text{major nucleosides} \) are relatively low (about 3 and 2%, respectively) for the tumor tRNA. The ratios \( \Sigma \text{methylated nucleosides} / \Sigma \text{major nucleosides} \) are also about 3% lower. The tumor tRNA contains more cytidine and less adenosine than does liver tRNA, these differences being about 1 to 2%.

In Table 4 the modified base compositions of Morris...
Modified base composition of tRNA from Morris hepatoma 7777 and host liver

For explanatory text, consult Table 1.

| Nucleosides | Hepatoma 7777 (x) | | Host liver (x) | | Hepatoma 7777 compared with host liver | | X<sub>hepatoma 7777</sub>/X<sub>host liver</sub> |
|-------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|
| m<sup>5</sup>U | 3.587 | 0.1108 | 3.868 | 0.0794 | 0.001 | 0.927 | 0.0927 |
| hU | 19.970 | 0.2600 | 19.533 | 0.1076 | 0.01 | 1.022 | 0.984 |
| h | 24.017 | 0.1889 | 24.410 | 0.3325 | 0.05 | 0.993 | 0.984 |
| m<sup>1</sup>A<sup>'</sup> | 8.767 | 0.1027 | 8.830 | 0.1121 | 0.4 | 1.044 | 1.044 |
| Inosine | 2.302 | 0.2343 | 2.205 | 0.2061 | 0.6 | 0.941 | 0.941 |
| m<sup>1</sup>C<sup>'</sup> | 2.202 | 0.0422 | 2.340 | 0.0358 | 0.001 | 0.941 | 0.941 |
| m<sup>2</sup>C | 12.548 | 0.1167 | 13.193 | 0.3451 | 0.01 | 0.951 | 0.951 |
| m<sup>2</sup>G | 4.055 | 0.1783 | 3.870 | 0.0920 | 0.1 | 1.048 | 1.048 |
| m<sup>5</sup>G | 8.517 | 0.1560 | 8.433 | 0.2091 | 0.8 | 0.996 | 0.996 |
| m<sup>5</sup>G<sup>*</sup> | 5.505 | 0.3185 | 5.275 | 0.2978 | 0.02 | 1.110 | 1.110 |
| X | 2.700 | 0.1521 | 2.705 | 0.2112 | >0.9 | 0.998 | 0.998 |
| Total | 100.003 | 99.990 | |

*See Table 1, Footnotes a to h.

A comparison of total and modified base compositions of normal Buffalo rat liver tRNA<sup>4</sup> with hepatoma 5123D tRNA gave essentially similar results, again indicating great similarity between normal and host liver tRNA. Only X was found to be slightly (6%) but significantly lower in host liver when host liver and normal liver tRNA were compared. In terms of base composition normal liver tRNA resembles more closely host liver tRNA from ani-

Table 3

Total base composition of tRNA from Morris hepatoma 5123D and host liver

For explanatory text, consult Table 1.

<table>
<thead>
<tr>
<th>Nucleosides</th>
<th>Hepatoma 5123D (x)</th>
<th>Host liver (x)</th>
<th>Hepatoma 5123D compared with host liver</th>
<th>X&lt;sub&gt;hepatoma 5123D&lt;/sub&gt;/X&lt;sub&gt;host liver&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uridine</td>
<td>14.025</td>
<td>0.0818</td>
<td>14.200</td>
<td>0.0616</td>
</tr>
<tr>
<td>Adenosine</td>
<td>16.928</td>
<td>0.1767</td>
<td>17.093</td>
<td>0.1776</td>
</tr>
<tr>
<td>Cytidine</td>
<td>26.720</td>
<td>0.1568</td>
<td>26.280</td>
<td>0.2503</td>
</tr>
<tr>
<td>Guanosine</td>
<td>27.908</td>
<td>0.0714</td>
<td>27.675</td>
<td>0.2562</td>
</tr>
<tr>
<td>m&lt;sup&gt;5&lt;/sup&gt;U</td>
<td>0.585</td>
<td>0.0311</td>
<td>0.615</td>
<td>0.0100</td>
</tr>
<tr>
<td>hU</td>
<td>2.543</td>
<td>0.0427</td>
<td>2.673</td>
<td>0.0206</td>
</tr>
<tr>
<td>h</td>
<td>3.525</td>
<td>0.0507</td>
<td>3.595</td>
<td>0.0208</td>
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<td>m&lt;sup&gt;1&lt;/sup&gt;A&lt;sup&gt;'&lt;/sup&gt;</td>
<td>1.243</td>
<td>0.0096</td>
<td>1.270</td>
<td>0.0115</td>
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<tr>
<td>Inosine</td>
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<td>0.0299</td>
<td>0.290</td>
<td>0.0082</td>
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<td>m&lt;sup&gt;1&lt;/sup&gt;C&lt;sup&gt;'&lt;/sup&gt;</td>
<td>0.308</td>
<td>0.0126</td>
<td>0.330</td>
<td>0.0082</td>
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<tr>
<td>m&lt;sup&gt;2&lt;/sup&gt;C</td>
<td>2.010</td>
<td>0.0548</td>
<td>2.178</td>
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<tr>
<td>m&lt;sup&gt;5&lt;/sup&gt;G</td>
<td>0.675</td>
<td>0.0332</td>
<td>0.598</td>
<td>0.0096</td>
</tr>
<tr>
<td>m&lt;sup&gt;5&lt;/sup&gt;G&lt;sup&gt;*&lt;/sup&gt;</td>
<td>1.265</td>
<td>0.0100</td>
<td>1.265</td>
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<td>m&lt;sup&gt;5&lt;/sup&gt;G&lt;sup&gt;*&lt;/sup&gt;</td>
<td>0.735</td>
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<td>X</td>
<td>0.478</td>
<td>0.0096</td>
<td>0.413</td>
<td>0.0050</td>
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<tr>
<td>Total</td>
<td>99.999</td>
<td>100.005</td>
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<td></td>
</tr>
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*<sup>x</sup> = mean of 4 chromatographic analyses; for calculation, consult "Materials and Methods."

*<sup>a</sup> See Table 1, Footnotes b to h.
Table 4

Modified base composition of tRNA from Morris hepatoma 5123D and host liver

For explanatory text, consult Table 1.

<table>
<thead>
<tr>
<th>Nucleosides</th>
<th>Hepatoma 5123D</th>
<th>Host liver</th>
<th>Host liver compared with Hepatoma 5123D</th>
<th>tRNA hepatoma 5123D</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>x (%)</td>
<td>y (%)</td>
<td>p (%)</td>
<td>Xhepatoma 5123D/Xhost liver</td>
</tr>
<tr>
<td>m5U</td>
<td>4.053</td>
<td>4.165</td>
<td>0.4</td>
<td>0.973</td>
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<tr>
<td>hU</td>
<td>17.633</td>
<td>18.108</td>
<td>0.0689</td>
<td>0.974</td>
</tr>
<tr>
<td>ψ</td>
<td>24.483</td>
<td>24.390</td>
<td>0.1252</td>
<td>1.004</td>
</tr>
<tr>
<td>m'A</td>
<td>8.618</td>
<td>8.598</td>
<td>0.0685</td>
<td>1.002</td>
</tr>
<tr>
<td>Inosine</td>
<td>1.920</td>
<td>1.955</td>
<td>0.0311</td>
<td>0.982</td>
</tr>
<tr>
<td>m3C</td>
<td>2.133</td>
<td>2.220</td>
<td>0.0548</td>
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<td>m5C</td>
<td>13.945</td>
<td>14.758</td>
<td>0.2941</td>
<td>0.945</td>
</tr>
<tr>
<td>m22G</td>
<td>4.675</td>
<td>4.050</td>
<td>0.0762</td>
<td>1.154</td>
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<td>m5G</td>
<td>8.773</td>
<td>8.575</td>
<td>0.1363</td>
<td>1.023</td>
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<tr>
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<td>5.385</td>
<td>0.0881</td>
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<tr>
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<td>3.310</td>
<td>2.823</td>
<td>0.0250</td>
<td>1.173</td>
</tr>
</tbody>
</table>

* x = mean of 4 chromatographic analyses; for calculation, consult “Materials and Methods.”

Many bearing the slowly growing hepatoma 5123D than it does host liver tRNA from those bearing the rapidly growing hepatoma 7777.

Comparison of Hepatoma 7777 and 5123D Data. Base composition of the 2 tumor tRNA’s are quite similar. A comparison of the tumor tRNA’s with the respective host liver tRNA’s shows, however, that, with respect to individual nucleosides, there are differences. Thus, in hepatoma 7777 tRNA, uridine, adenosine, m5U, ψ, m'A, m3C, and m5C are lower and cytidine and guanosine are higher when compared with host liver tRNA (Table 1); and in hepatoma 5123D, uridine, hU, m'A, m3C, and m5C are lower and cytidine and guanosine are higher (Table 3). Both tumor tRNA’s are slightly undermodified and undermethylated to about the same degree. Differences between the 2 tumor tRNA’s are also apparent when the data for the modified base compositions are compared (Tables 2 and 4). Hepatoma 7777 tRNA contains less m5U, ψ, m3C, and m5C and more hU and m5G when compared with host liver tRNA, whereas hepatoma 5123D tRNA is poorer in hU and m5C and richer in m22G and X. The comparison of the data for the 2 tumors does not provide evidence for a distinct correlation between degrees of modification and methylation of tRNA and growth rates (and histological characteristics) of the tumors. tRNA of the fast-growing hepatoma 7777 is not more modified or methylated than tRNA of hepatoma 5123D, which has a much slower growth rate.

Reproducibility of Base Composition Data. tRNA from hepatomas 7777 and 5123D as well as from livers has been prepared and subjected repeatedly to enzymatic digestion and tritium labeling. In each instance significant differences between hepatoma and liver tRNA were obtained for the same nucleosides, and the ratios of Xhepatoma 7777/Xhost liver and Xhepatoma 5123D/Xhost liver (see Tables 1 to 4) were found to be essentially the same. No differences were found between tRNA preparations obtained from frozen and fresh tissues. In general, data are well reproducible if the guidelines described under “Requirements for Comparative Investigations on tRNA” are followed.

The observed differences in base composition between the tumor and host liver tRNA appear to be stable characteristics of each tumor cell line. For hepatoma 7777, the alterations were observed for transplant generations 62 and 88, which were about 2.5 years apart. The characteristic base pattern for hepatoma 5123D was also found in different generations of the tumor.

tRNA from 15,000 x g Pellets of Liver and Hepatomas 7777 and 5123D. The data presented (Tables 1 to 4) pertain to cytoplasmic tRNA obtained from pH 5 precipitates only, which represents about 50% of the total cellular tRNA. Since the possibility existed that the residual tRNA of the tumors might be overmodified or overmethylated, we have also investigated the base constituency of tRNA preparations from low-speed (15,000 x g) pellets, which contain most of the residual cellular tRNA (about 45%). A comparison of tumor tRNA with liver tRNA isolated from the respective low-speed pellets revealed the following characteristics.

1. The pellet tRNA of the tumors is considerably more undermodified and undermethylated than the corresponding cytoplasmic tRNA. The degrees of modification and methylation for hepatoma 7777 and 5123D pellet tRNA are both about 11 and 14% lower, respectively, than those for pellet tRNA from liver, whereas the cytoplasmic tRNA from the tumors is only about 2 to 3% undermodified and undermethylated when compared with liver cytoplasmic tRNA (Tables 1 and 3).

2. In terms of the total base composition, differences for individual modified nucleosides between the pellet tRNA’s of the tumors and liver are generally somewhat greater than those between the respective cytoplasmic tRNA’s. Some differences amount to about 20 to 25%; for example, in pellet tRNA of hepatoma 7777, m5C was
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DISCUSSION

In view of the conflicting reports in the literature on
tRNA methylation in tumor tissues, it appeared desirable
to investigate the chemistry of tRNA in cancer cells in a
systematic fashion. Since the usual spectrophotometric
methods are not sensitive enough for the analysis of the
small amounts of material available in many instances
from mammalian sources and in vivo labeling of mamma
lian tRNA does not yield the high specific radioactivity
necessary for structural studies (8), the development of new
methods for base composition (35, 39-42) and sequential
analysis was required. These methods are based on the
chemical introduction of tritium label into derivatives of
RNA. The results presented in this communication were
obtained by applying such a method to a study of the base
constituency of tRNA in liver and 2 Morris hepatomas.
These tumors are well suited for comparative studies be
cause the cell of origin (hepatocyte) is known and availa
ble in adequate amounts for control purposes. We have
chosen the particular tumors (hepatomas 7777 and 5123D)
for 2 main reasons: (a) of several Morris hepatomas in
vestigated, these tumors exhibited the highest activities of
tRNA methylases (47); (b) in contrast to other tumors,
including certain Morris hepatomas, undegraded tRNA
could be readily obtained.

In first attempt to study the overmethylation question,
our laboratory concentrated on the reinvestigation of tRNA
from human brain and brain tumors (33, 37), in an effort
to reproduce and extend data published by Viale et al. (31,
55, 57). Our results, however, did not confirm the substan
tial elevation of various methylated nucleosides in tumor
tRNA [e.g., an 80-fold increase in m'G for glioblastoma
multiforme tRNA (56)] reported by these authors. Instead,
our data revealed only slight differences between the over
all modified base composition of human brain tRNA and
brain tumor tRNA, and no evidence was obtained for a
substantial increase in the ratios $\Sigma_{\text{modified nucleosides}} / \Sigma_{\text{major nucleosides}}$. Our
base composition data were, however, similar to the data
reported by Iwanami and Brown (18) and Baguley and
Staehelin (1) for tRNA from other malignant sources. The
results reported in the present communication on cytoplasm
ic tRNA from Morris hepatomas and liver are very simi
lar to our earlier data obtained for cytoplasmic tRNA
from human brain and brain tumors.

During the course of these earlier investigations, it be
came apparent that it was of great importance to isolate
tRNA in a pure and undegraded form in order to exclude
artifacts caused by contamination of the preparations with
other RNA species and degradation products of high-
molecular-weight RNA or by tRNA loss as a result of
nuclease degradation. These points were carefully evalua
ted in the present investigation. The results of investiga
tions on the chemistry of tRNA in which purity and
integrity of the preparation were not carefully evaluated
appear to be of doubtful value. The substantial overmethyla
tion of tumor tRNA reported for mouse tumors (4) and
human brain tumors (31, 55, 57) may thus conceivably be
artifacts of preparation. This appears to be particularly
likely in view of the difficulties in obtaining undegraded
RNA from many malignant tissues.

Our data regarding the overall similarity of hepatoma
and liver tRNA are consistent with results reported by
Sheid et al. (47) showing a general similarity of in vitro
methylation patterns obtained with E. coli B tRNA sub
strate and tRNA methylase preparations from rat liver and
4 Morris hepatomas (7777, 5123D, 7316B, and 8995).

Klagsbrun (23), in a recent report on the methylation of
tRNA in normal and SV40-transformed 3T3 cells, also
finds a contrast between the in vivo and in vitro situation
insofar as tRNA methylase activity was found to be greater
in extracts from transformed cells than in extracts from
control cells, but the degree of methylation and methylated
base composition of normal and transformed 3T3 cell
tRNA (determined by methylation in vivo) appeared not to
differ measurably.

Interestingly, as pointed out under "Results," tRNA
isolated from 15,000 x g pellets of the hepatomas was
found to be undermodified and undermethylated to an
even greater extent than cytoplasmic tRNA from the
tumors. The lack of modified and methylated nucleosides
in pellet tRNA of liver when compared with cytoplasmic
tRNA of liver can for the most part be accounted for by
the presence in the pellet tRNA preparations of mitochon
drial tRNA. The base constituency of mitochondrial tRNA
of rat liver, which represents 20 to 30% of the pellet tRNA,
was found to be quite different from that of cytoplasmic
tRNA. For example, the degree of modification of mito
chondrial tRNA is 0.10, and that of cytoplasmic tRNA is
0.17. The corresponding values for the degree of methyla
tion are 0.052 and 0.088, respectively. At the present time
we are, however, unable to explain why the pellet tRNA
of the tumors is considerable more undermodified and
undermethylated than its cytoplasmic counterpart. The
question of whether this may be caused by a higher content
or specific characteristics of mitochondrial tRNA in these
hepatomas or by some other factors is currently being investigated.

The relatively small differences between tumor and liver tRNA observed by us are consistent with differences in column chromatographic profiles from various Morris hepatomas including hepatoma 5123D and rat liver (50, 58). tRNA from hepatoma 5123D was shown to contain 2 new species of tRNA\textsuperscript{ser} and 1 new species of tRNA\textsuperscript{ser}, whereas 2 of the 3 tRNA\textsuperscript{Met} species found in Buffalo rat liver could not be detected in the tumor (58). Since the structures of only 2 mammalian tRNA's in normal tissue, \textit{i.e.}, of tRNA\textsuperscript{ser} from rat liver (51) and of tRNA\textsuperscript{Met} from rabbit liver (20), are known and no sequence of a tRNA in tumor tissue has been elucidated, it is not possible at this time to correlate differences in base composition directly with the changes observed in the tRNA column chromatographic profiles.

If one assumes that there are about 60 tRNA species (15) in approximately equal amounts (chain length, 80 nucleotides), a calculation shows that the insertion of 1 additional modified base into 1 chain of the total tRNA population would increase the base composition value of this particular base by about 0.02% in terms of the total base composition (see Tables 1 and 3) or by about 0.15% in terms of the modified base composition (see Tables 2 and 4). For example, the increase in X and m\textsubscript{2}G observed in hepatoma 5123D tRNA (Table 3) would correspond to the addition of about 3 and 4 nucleosides, respectively, and the decreases in hU and m\textsuperscript{5}C to a deletion of about 6 and 8 nucleosides, respectively. The values for hepatoma 7777 tRNA (Table 1) indicate similar changes. It thus appears possible that the differences in individual modified nucleosides observed by us may be the result of the presence or absence of several tRNA species in hepatomas.

The presence of new tRNA species in tumor tRNA may be explained on the basis of either one or a combination of the following mechanisms: the presence of new modifying enzymes with specificities different from the enzymes present in normal tissue may cause modification of tRNA precursors in unusual positions (Mechanism 1); the absence or decreased activity of some modifying enzymes may lead to undermodified species (Mechanism 2); an alteration of the specificities of the modifying enzymes may result in overmodified species if the altered enzymes recognize additional sites in the tRNA (Mechanism 3); new precursor tRNA species may be transcribed from DNA (Mechanism 4); partially altered tRNA sequences may be transcribed from DNA (Mechanism 5).

Mechanisms 2, 3, and 5 may also lead to the absence of certain tRNA species in tumor tissues. At the present time none of these mechanisms can be ruled out completely on the basis of experimental evidence. Mechanism 3 was shown not to be operative in certain instances in which purified enzymes from tumor and normal tissues were compared. Thus, Kuchino \textit{et al.} (25) showed for instance 2 guanylate residue-specific tRNA methylases to incorporate methyl groups only into guanosine residues in certain positions of purified \textit{E. coli} tRNA\textsuperscript{Met} and tRNA\textsuperscript{Val}, regardless of whether the enzymes originated from tumors, \textit{i.e.}, ascites hepatoma and 3'-methyl-4-dimethylamino-azobenzene-induced hepatoma, or from normal rat liver, although the extent of incorporation was higher for the tumor enzymes. In our view, it would be premature to generalize these observations to mean that no aberrant modification of tumor tRNA occurs as a result of changes at the enzyme level.

The presence of new modifying enzymes with different specificities (Mechanism 1), in addition to those present in normal tissue, would be reflected by an increased activity of modifying enzymes in \textit{in vitro} assays. Alteration of the specificities of the normal modifying enzymes (Mechanism 3) will lead to an overall increase in enzyme activity when assayed \textit{in vitro}. As shown in Tables 1 and 3, statistically significant elevations of modified nucleosides in the tRNA's of the 2 hepatomas have been observed for 2 nucleosides only, namely m\textsubscript{2}G and X in hepatoma 5123D, whereas a significant decrease was found for 9 modified nucleosides. The overmodification in terms of these 2 nucleosides may possibly be the result of aberrant modification. In this connection the report by Craddock (13) on increased activity of tRNA N\textsubscript{2}-guanine dimethylase in certain chemically induced rat liver and kidney tumors should be noted. It is difficult, however, to explain the observed undermodification on the basis of Mechanisms 1 and 3 unless the tumor tRNA population contains sequences that are poor substrates for the modifying enzymes (\textit{cf.} Mechanisms 4 and 5). If this were the case the increased methylase activities in extracts from tumor tissues may be the consequence of some regulatory response to the presence of new precursor tRNA species and/or new sequences in precursor tRNA. Other possibilities of explaining the increased activities of the tRNA methylases are higher synthesis rates and higher turnover of tRNA in rapidly growing tumors as evidenced by the elevated urinary excretion of methylated tRNA constituents by tumor-bearing hosts (reviewed in Ref. 6).

As reported elsewhere (42), the pattern of modification of vertebrate tRNA has been preserved over a period of considerable evolutionary change. Using different methods, Taylor \textit{et al.} (54) and Klagsbrun (24) have reached a similar conclusion. Against this background, even slight alterations of tRNA modification and structure in tumors will have to be regarded as highly significant deviations from the normal situation. The data reported here demonstrate that there are characteristic changes in tRNA modification patterns for 2 Morris hepatomas. It is not yet possible to decide to which extent each of the mechanisms discussed above is involved in establishing the observed alterations. We intend to carry out comparative chemical studies on purified tRNA's from normal and tumor tissues in order to provide answers to these questions.

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Transfer RNA Base Composition Studies in Morris Hepatomas and Rat Liver

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