A Comparison of Phenylalanyl-tRNA Synthetase from Rat Liver and a Minimal Deviation Hepatoma

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SUMMARY

Phenylalanyl-transfer RNA synthetase has been purified from rat liver and Morris hepatoma 5123D, and the properties of the enzymes have been compared. With the use of ammonium sulfate fractionation, and chromatography on DEAE-cellulose and brushite, the liver and hepatoma enzymes were purified 240- and 275-fold, respectively. The two enzymes were chromatographically indistinguishable, and only one peak of enzyme activity was evident in either tissue. The liver and hepatoma enzymes exhibited similar heat-inactivation kinetics at 50°, and the apparent Michaelis constants for the substrates of the enzymes were identical. All parameters measured in comparing the enzymes failed to reveal functional differences. The inference drawn is that the liver enzyme has been conserved during the neoplastic transformation to the hepatoma. This study represents the first direct comparison of aminoacyl-transfer RNA synthetases from a minimal deviation hepatoma and liver.

INTRODUCTION

Systems undergoing differentiation or alterations in metabolic control processes have been examined for chromatographic differences in their isoaccepting aminoacyl-tRNA's, and there is a considerable body of existing literature that describes such differences in a number of neoplastic tissues. Advances in this area have recently been reviewed (6, 25). Examples of apparently tumor-specific isoaccepting tRNA's have been reported in several systems, including mouse plasma cell tumors (18, 31), leukemic lymphoblasts (5), SV40-infected and-transformed 3T3 cells (22), and Novikoff hepatoma (1, 8). Chromatographic comparisons have also been made of aminoacyl-tRNA's from the minimal deviation Morris hepatomas and adult rat liver (9, 23, 30) and, in each of these studies, the elution patterns of certain tumor and liver tRNA's were found to be significantly different. In particular, these studies revealed the presence of altered phenylalanyl-tRNA's in sublines of Morris hepatoma 5123. Despite the rather widespread observation of such tumor-specific isoaccepting tRNA's, the biological significance of these molecules remains open to question.

In 1967, Strehler et al. (24) presented data in support of a codon restriction hypothesis, and it was suggested that the aminoacyl-tRNA synthetases played a regulatory role during differentiation. Iain et al. (11) later presented evidence in support of this view; their data implied that new tRNA's and aminoacylating enzymes appeared during morphogenesis of the yellow mealworm Tenebrio molitor. Similarly, changes in synthetase properties during senescence (3) and during soybean development (12) have been described. These findings and the work of Tidwell et al. (27) on normal and regenerating liver suggested to us that tumorigenesis could also be accompanied by the expression of tumor-specific aminoacyl-tRNA synthetases which might exhibit selectivity with regard to their interaction with tumor-specific tRNA's.

Since we have reported a phenylalanyl-tRNA unique to Morris hepatoma 5123D (30), it was felt that a comparative study of phenylalanyl-tRNA synthetase from this tumor and liver would be valuable in examining the relative specificities of the synthetases for their homologous and heterologous phenylalanine tRNA's. An investigation of this nature would determine whether the enzyme was altered during oncogenic transformation and whether this alteration resulted in a change in substrate specificity.

MATERIALS AND METHODS

All rats used in this study were Buffalo female retired breeders purchased from Microbiological Associates (Bethesda, Md.).

Rats bearing Morris hepatoma 5123D were originally obtained from Dr. Harold P. Morris of Howard University, Washington, D. C.

The hepatomas were maintained by serial transplantation into the thigh muscle of 200-g rats. Six weeks from the date of inoculation, the rats were sacrificed by decapitation, and the tumors were excised and trimmed to remove all nontumorous and necrotic material. Tissues were either used immediately upon removal from the animal or were frozen at -70° for future use.

Livers were removed from uninoculated 200-g rats that had been maintained on a regimen identical to that of the tumor-bearing animals.
Preparation of Crude Aminoacyl-tRNA Synthetases

All steps, unless noted, were conducted at 4°. The freshly excised or thawed tissue was finely minced and homogenized in 2 volumes of buffer containing 0.01 M Tris-HCl, pH 7.5:0.15 M NaCl, 0.01 M β-mercaptoethanol: 15% (v/v) glycerol. After 3 min at medium speed in a VirTis homogenizer, a postribosomal supernatant was obtained by sequential centrifugation of this homogenate at 20,000 × gav for 30 min and 78,480 × gav for 90 min. Lipid material was removed by filtration through glass wool.

The filtrate was subjected to differential streptomycin sulfate precipitation in order to eliminate high-molecular-weight RNA's. Streptomycin sulfate (Eli Lilly and Co., Indianapolis, Ind.) was dissolved in 0.05 M Tris-HCl, pH 7.5, to a final concentration of 1 g/ml. This chilled solution was then added dropwise to the filtrate with stirring until the final streptomycin sulfate concentration was 20 mg/ml. The suspension was stirred an additional 30 min, and the precipitated nucleic acids were removed by centrifugation at 20,000 × gav for 30 min.

A 50 to 75% ammonium sulfate fraction of the streptomycin sulfate supernatant was prepared as follows. Solid ammonium sulfate [(NH₄)₂SO₄] (ultrapure enzyme grade, Mann Research Laboratories, Orangeburg, N. Y.) was added with stirring to a final concentration of 29.1 g/100 ml of supernatant. Following final addition of the ammonium sulfate, the slurry was stirred for 30 min, after which it was centrifuged at 20,000 × gav for 30 min. The pellet was discarded, and the ammonium sulfate concentration of the supernatant was increased to 75% saturation by the further addition of 15.9 g (NH₄)₂SO₄/100 ml of solution. The precipitated proteins were collected by centrifugation as described above, and the 50 to 75% ammonium sulfate was resuspended in a minimal volume of TMG₄ buffer.

Sephadex G-50 Chromatography

Sephadex G-50 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) was swollen in TMG, and broken beads were removed by aspiration. A 40-ml sample of the 50 to 75% ammonium sulfate-saturated fraction was desalted on a 2- × 90-cm Sephadex G-50 column as previously described (21). No phenylalanyl-tRNA synthetase activity was found in either the 0 to 50% or the 75 to 100% ammonium sulfate-saturated fractions.

Preparation of tRNA

The tRNA's were prepared from both tissues by a modification of the procedures of Volkers and Taylor (30) and Taylor et al. (26). The minced tissues were homogenized in 0.05 M Tris-HCl, pH 7.0, containing 50 μg bentonite per ml to which an equal volume of Tris buffer-saturated phenol was added. After blending, the homogenate was made 0.5% with respect to sodium dodecyl sulfate, and the suspension was vigorously stirred 25° for 1 hr. The aqueous layer was separated from the phenolic phase by centrifugation, and it was repeatedly extracted with phenol until protein was no longer precipitated at the solvent interface. After similar chloroform extraction, the nucleic acid solution was adjusted to a final concentration of 0.20 M NaCl; the nucleic acids were then precipitated overnight at −20° with 2.5 volumes of 95% ethanol. The precipitate was collected by centrifugation, re-dissolved in 0.01 M Tris-HCl, pH 7.5:0.01 M MgCl₂, and incubated at 37° for 60 min with electrophoretically pure DNase (DNase EP, Sigma Chemical Company, St. Louis, Mo.) at a final DNase concentration of 2 μg/ml. The tRNA's were then phenol extracted, reprecipitated with ethanol, suspended in 1.0 M NaCl, and stirred vigorously for 3 hr at 4°. The 10 M NaCl-soluble material was dissolved in 0.10 M Tris-HCl, pH 8.5, and incubated at 37° for 90 min to remove amino acids esterified to the tRNA's, and the tRNA's were again alcohol precipitated. The properties of these tRNA's have been described elsewhere (19, 30).

BDC Chromatography

BDC was purchased from Schwarz/Mann, Orangeburg, N. Y. The columns were eluted by a modification of the procedure of Gillam et al. (7). BDC columns were washed with 3-column volumes of 0.01 M sodium acetate buffer, pH 5.0, containing 2.0 M NaCl and 20% (v/v) ethanol, and then were equilibrated with an equal volume of 0.01 M sodium acetate buffer, pH 5.0, containing 0.45 M NaCl. The deacylated tRNA's were dissolved in a minimal volume of equilibrating buffer and loaded onto the column. The column was washed with 1.5 column volumes of equilibrating buffer in order to remove glycogen from the column-bound tRNA's. The tRNA was eluted from the column with 1.5 column volumes of 0.01 M acetate buffer, pH 5.0, containing 1.0 M NaCl and 15% ethanol. The tRNA's were ethanol precipitated, dissolved in sterile distilled water, and stored at −70°.

Aminoacylation Assay

All reaction mixtures contained the following buffer: 20 μmoles sodium cacodylate, pH 7.4:2 μmoles magnesium acetate: 1 μmole KCl.

Since the amino acid, ATP, and tRNA substrates, and the reaction volumes varied with the experiment, these are specified in the corresponding sections.

Radioactive aminoacyl-tRNA was measured by a modification of the tRNA esterification assay of Mans and Novelli (17). Sample aliquots (50 μl) were pipetted onto a 2.3 cm in diameter Whatman No. 3MM filter paper discs. Discs were placed in at least 20 ml of ice-cold 10% TCA per disc, washed twice with similar volumes of both 5% TCA and 95% ethanol, dried, and counted in a Beckman LS-150 liquid scintillation counter.

Liver and Hepatoma Phenylalanyl-tRNA Synthetase
Radiochemicals

L-Phenylalanine-$^{14}$C, 384 mCi/m mole, and L-phenylalanine-$^{3}$H, 5.02 Ci/m mole, were purchased from New England Nuclear, Boston, Mass.

DEAE-Cellulose Chromatography of Phenylalanyl-tRNA Synthetase from Buffalo Rat Liver and Morris Hepatoma 5123D

DEAE-cellulose, medium mesh, 0.89 mEq/g, was purchased from Sigma. The DEAE-cellulose was pre-cycled as described by Peterson and Sober (20), and the prepared slurry was equilibrated with TMG buffer. A 200-mg protein sample of desalted 50 to 75% ammonium sulfate fraction was applied to a 2 x 60-cm column, and the column was washed with 1 column volume of TMG. After the washing, a 1000-ml linear gradient from 0.005 M to 0.075 M NaCl in TMG was eluted from the column. Each fraction was assayed for 15 min at 37° for the ability to catalyze the formation of acid-insoluble phenylalanyl-tRNA. Each reaction mixture contained the following in 190 μl: 20 μmoles sodium cacodylate, pH 7.4: 2 μmoles magnesium acetate; 1 μmole KCl: 0.2 μmole ATP: 40 μg rat liver tRNA: 250 pmoles L-phenylalanine-$^{3}$H: 50 μl column fraction. Fractions containing phenylalanyl-tRNA synthetase activity were pooled and concentrated 10-20-fold by dialysis against the TMG buffer containing 10% (w/v) Carbowax 6000. Each column was then washed with PMG until the eluate no longer absorbed light at 260 nm. No phenylalanyl-tRNA synthetase activity was detected in this fraction. The enzyme was eluted with a linear PMG gradient from 0.10 to 0.20 KH$_2$PO$_4$, pH 7.0. The reaction mixture and assay described above for DEAE-cellulose chromatography was used. Active fractions were pooled and concentrated by dialysis, as described above. After concentration, 1.0-ml aliquots were stored at -70°.

Protein Determinations

The protein content of homogenates and low-speed supernatants was determined by the biuret method of Koch and Putnam (13). Protein determinations of nonturbid solutions were done by the method of Lowry et al. (16). In both cases, protein concentration values were determined by extrapolation from standard curves, with bovine serum albumin (Sigma) as the standard.

Heat Inactivation Kinetics

One ml of purified phenylalanyl-tRNA synthetase (320 μg/ml) from both tissues was dialyzed against three 2-liter changes of 0.01 M Tris-HCl, pH 7.5, 0.01 M β-mercaptoethanol, 7% (v/v) glycerol for 36 hr at 4°. Enzyme preparations that had been equilibrated at 37° were incubated at 50° by pipetting into a preheated tube. At 1-min intervals, 50-μl aliquots of the heated enzyme were removed and pipetted into reaction mixtures containing 20 μmoles sodium cacodylate, pH 7.4: 2 μmoles sodium acetate; 1 μmole KCl: 0.20 μmole ATP: 1.3 nmole L-phenylalanine-$^{14}$C: 0.68 nmole liver tRNA in a total volume of 130 μl. After 90-sec of incubation at 37°, 50-μl samples were placed on Whatman discs and prepared for counting as described above.

Determination of Apparent K_m Values

Phenylalanine. Reaction mixtures contained 20 μmoles sodium cacodylate, pH 7.4: 2 μmoles sodium acetate; 1 μmole KCl, plus the following levels of fixed substrates in 130 μl: 0.20 μmole ATP: 0.68 nmole hepatoma 5123D tRNA. L-Phenylalanine-$^{14}$C was variable from 0.26 to 1.30 nmole/reaction. Reactions were initiated by the addition of 50 μl (20 μg) of purified phenylalanyl-tRNA synthetase. Reactions were terminated after 90 sec at 37° by pipetting 50-μl aliquots onto Whatman discs that had been prespotted with 10% TCA. Discs were then prepared for counting in the usual manner.

ATP. Reaction mixtures contained 20 μmoles sodium cacodylate, pH 7.4: 2 μmoles sodium acetate; 1 μmole KCl: 1.03 nmole L-phenylalanine-$^{14}$C; and 0.68 nmole of hepatoma 5123D tRNA. ATP was varied from 10 to 500 μmoles/reaction.

tRNA. Total tRNA from Morris hepatoma 5123D and from Buffalo rat liver varied from 0.288 to 1.44 nmole/reaction, while ATP and phenylalanine-$^{14}$C were kept constant at 20 μmoles/reaction and 1.03 nmole/reaction, respectively.

Determination of the Extent of Acylation with Homologous and Heterologous tRNA Substrates

Reaction mixtures containing 20 μmoles sodium cacodylate, pH 7.4: 2 μmoles sodium acetate; 1 μmole KCl: 0.20 μmole ATP: 1.3 nmole L-phenylalanine-$^{14}$C; and 0.68 nmole of either liver tRNA or hepatoma 5123D tRNA were incubated in the presence of either purified liver or hepatoma phenylalanyl-tRNA synthetase. Reactions were incubated 20 min at 37°, at which time 50-μl aliquots were removed and acid-insoluble phenylalanyl-tRNA was measured.

RESULTS

Purification of Phenylalanyl-tRNA Synthetases. The scheme used in the partial purification of the respective synthetases is presented in Table 1. The procedures applied to the preparation of phenylalanyl-tRNA synthetase were reproducible for purification of the enzyme from either liver or hepatoma 5123D. Comparable recoveries and levels of purification were obtained from both tissues, and the specific activity of this enzyme was the same in liver and hepatoma homogenates.
Table 1

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total units</th>
<th>% recovery</th>
<th>Protein/ml (mg)</th>
<th>Phenylalanine incorporated/mg protein/10 min (pmoles)</th>
<th>Purification (±fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Hepatoma</em> 5123D phenylalanyl-tRNA ligase</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Crude homogenate</td>
<td>16,000</td>
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<td>10.6</td>
<td>2.6</td>
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<tr>
<td>50-75% (NH₄)₂SO₄ fraction</td>
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<td>7.5</td>
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<td>5.0</td>
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<td>DEAE-cellulose</td>
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<td>41</td>
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<td>220.0</td>
<td>85</td>
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<tr>
<td>Brushite</td>
<td>4,100</td>
<td>25</td>
<td>0.32</td>
<td>720</td>
<td>275</td>
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<tr>
<td><em>Buffalo rat liver phenylalanyl-tRNA ligase</em></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Crude homogenate</td>
<td>10,600</td>
<td></td>
<td>10.5</td>
<td>2.5</td>
<td></td>
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<tr>
<td>50-75% (NH₄)₂SO₄ fraction</td>
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<td>58</td>
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<td>44</td>
<td>1.1</td>
<td>200</td>
<td>80</td>
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<td>2,120</td>
<td>20</td>
<td>0.38</td>
<td>600</td>
<td>240</td>
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</table>

DEAE-cellulose chromatography of liver and hepatoma 5123D phenylalanyl-tRNA synthetase is illustrated in Chart 1, A and B. In each case, only 1 peak of enzyme activity was detectable, and the liver and the hepatoma synthetases eluted from DEAE-cellulose over the same concentration range of 0.025 to 0.038 M NaCl. Upon completion of the gradients, columns were washed with 0.35 M NaCl in TMG; no phenylalanyl-tRNA synthetase activity was found in this high-salt fraction.

Brushite chromatography (Chart 2, A and B) of these partially purified enzyme preparations also revealed only 1 peak of synthetase activity. The 2 enzymes were chromatographically indistinguishable and eluted at the same ionic strength, between 0.16 and 0.174 M phosphate.

The kinetics of heat inactivation at 50° of the liver and hepatoma synthetases was studied and, as seen in Chart 3, found to be very similar; the half-lives of the liver and tumor synthetases were 1.7 and 1.9 min, respectively. The initial shoulder seen during the 1st min at 50° is probably indicative of a lag in raising the temperature of the enzyme solutions from 37° to 50°.

The Michaelis constants for the substrates of phenylalanyl-tRNA synthetase were also determined. Initial velocities of reactions were determined at varying concentrations of the substrate under study, and these initial velocity data are expressed in double-reciprocal form. Chart 4, A through D, indicates that for the 4 substrates examined, the liver and hepatoma synthetases have identical apparent Kₘ values. The calculated Michaelis constants for these substrates are expressed in Table 2. The values for phenylalanine and ATP are well within the range of previously published values for these substrates (10, 14, 15).

The data in Table 3 indicate that both enzymes are able to acylate the same tRNA species, and that there are no tRNA's in either liver or hepatoma 5123D that cannot be aminoacylated by either enzyme. The striking feature of these data is the apparent difference in the amount of phenylalanine tRNA present in these 2 tissues. This observation has since been elaborated, and we have found that, in general, this hepatoma contains significantly greater amounts of acylatable tRNA per μg tRNA and that the differences in amounts of acylatable tRNA are independent of the source of enzyme (19).

**DISCUSSION**

One of the major factors motivating us to investigate these aminoacylating enzymes was the suggestion by several laboratories (3, 11, 12, 24) that the properties of aminoacyl-tRNA synthetases may undergo changes during events in differentiation. It was felt, therefore, that the neoplastic transformation could elicit a similar response, resulting in the presence of what might be described as a tumor-specific aminoacyl-tRNA synthetase. However, we found no evidence to this effect in our system, and we report no indication of any differences between the phenylalanyl-tRNA synthetases of liver and Morris hepatoma 5123D. By every criterion employed in this study, the 2 enzymes appear functionally indistinguishable.

While all the data presented here indicate that the liver enzyme was conserved during dedifferentiation of the liver cell to the hepatoma, the possibility exists that the liver and hepatoma enzymes may not be structurally identical. Quite possibly, the 2 enzymes may be isozymes or a class of isozymes, and the techniques applied in this study may not have been sensitive enough to discriminate between them. In addition, a number of laboratories have described a particulate aminoacyl-tRNA synthetase complex in rat liver (2, 28, 29). Since this study has dealt only with the enzymes of the soluble fraction, the existence of tissue differences among the microsomal phenylalanyl-tRNA synthetases remains the subject for future research. It can be stated, however, that within the accuracy of the methods used, all efforts to find significant differences between the liver and hepatoma phenylalanyl-tRNA synthetases were unsuccessful.
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Chart 1. DEAE-cellulose column chromatography of rat liver and hepatoma phenylalanyl-tRNA synthetase. Proteins were eluted with a 1000-ml linear gradient from 0.00 to 0.075 M NaCl in TMG. Active fractions were determined by the tRNA esterification assay as described in "Materials and Methods," and the data are expressed as cpm of acid-insoluble, radioactive phenylalanyl-tRNA.

We have described a reproducible method for preparing phenylalanyl-tRNA synthetase from either liver or hepatoma 5123D that results in enzyme preparations of reasonable purity with moderate yield. The chromatographic behavior of the 2 enzymes is the same, and polyacrylamide disc-gel electrophoretic analysis of the purified enzyme preparations resulted in gels with similar staining patterns (data not shown).

The specific activity of phenylalanyl-tRNA synthetase was essentially the same in liver and hepatoma homogenates. This finding is in agreement with the data of Del Monte and Cini (4) who examined the specific activities of all 20 aminoacyl-tRNA synthetases from adult liver and Morris hepatoma 5123C. In nearly each case, these workers found that the enzymes of liver and hepatoma have very similar specific activities.

The apparent KM values for the substrates of phenylalanyl-tRNA synthetase are the same for the liver and hepatoma enzyme, and these Michaelis constants agree very closely with previously published data (10, 14) for rat liver aminoacyl-tRNA synthetases. Since KM may be defined as the substrate concentration required to achieve one-half the maximal velocity, it is clear that the 2 synthetases are functionally very similar, if not identical. These kinetic data were of particular interest to us, because it was felt that a particular synthetase would demonstrate some type of selectivity for its homologous tRNA's. Such specificity was not encountered, since both enzymes have near identical KM's for liver and hepatoma tRNA. This finding was further supported by the observation that tRNA's from either tissue are acylated to the same extent, irrespective of source of synthetase (Table 3). In addition, Volkers and Taylor (30) demonstrated that reversed phase II chromatographic profiles of phenylalanyl-tRNA's from these tissues were unaffected when the heterologous synthetase was used to acylate the tRNA's in preparation for the columns.

Chart 2. A and B, Brushite chromatography of rat liver and hepatoma phenylalanyl-tRNA synthetase. Proteins were eluted from a 1.0- x 22-cm column in 3.3-ml fractions with a 150-ml, 0.10 to 0.20 M KH₂PO₄ gradient, pH 7.0, in 0.01 M β-mercaptoethanol, 10% glycerol (v/v). In B, the fraction volume was 5.0 ml. The peak fractions were located as described in the legend to Chart 1.

Chart 3. Heat-inactivation kinetics at 50° of purified phenylalanyl-tRNA synthetase from liver and hepatoma 5123D. Enzyme samples were heated at 50° for the indicated times and assayed for 90 sec at 37°. Data are expressed as activity of the heated fraction relative to an unheated control under the same assay conditions.
Liver and Hepatoma Phenylalanyl-tRNA Synthetase

Table 3
Phenylalanyl-tRNA formation: comparison of liver and hepatoma 5123D synthetase with homologous and heterologous tRNA

<table>
<thead>
<tr>
<th>Enzyme source/tRNA source</th>
<th>cpm of phenylalanine-14C incorporated/A260 unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver/liver</td>
<td>4,865 ± 3</td>
</tr>
<tr>
<td>Hepatoma/liver</td>
<td>4,666 ± 3</td>
</tr>
<tr>
<td>Liver/hepatoma</td>
<td>12,410 ± 2</td>
</tr>
<tr>
<td>Hepatoma/hepatoma</td>
<td>12,295 ± 2</td>
</tr>
</tbody>
</table>

It seems clear that differences exist between the phenylalanyl-tRNA's of liver and those of sublines of Morris hepatoma 5123 (9, 23, 30). However, our results indicate that the derivation of this tumor was not correspondingly accompanied by the expression of a tumor-specific phenylalanyl-tRNA synthetase but, rather, that the liver enzyme was conserved.

REFERENCES

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