The Methylation of Transfer RNA in Vitro by Extracts of Normal and Malignant Tissue

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SUMMARY

The transfer RNA (tRNA) methylase activities of normal and regenerating rat liver, liver of newborn rats, and a minimum deviation hepatoma have been studied in vitro with Escherichia coli tRNA as substrate. The levels of activity are higher in extracts of malignant, newborn, and regenerating tissue than in extracts of normal tissue, when comparison is made at the optimum concentration of ammonium ions. With liver tRNA as substrate there is no incorporation of methyl groups by the various liver extracts, but a small amount of incorporation was consistently observed under the action of the ethionine tumor extracts.

Sequential incubation of tRNA with extracts from different tissues showed that the ethionine tumor preparation can effect supermethylation of samples previously exposed to enzymes from liver. The reverse experiment gave no incorporation. The addition of tumor enzyme preparation to an assay mixture previously saturated with respect to liver enzyme can bring about an increase in the extent of methylation of E. coli tRNA. These observations suggest that there are some differences in the complement of tRNA methylases in the malignant tissue compared with the normal.

INTRODUCTION

The field of methylation of transfer RNA (tRNA) by mammalian tissue extracts has been recently reviewed (2, 10). The experimental work reported below was carried out in an attempt to clarify the claims that the differential methylation of tRNA and DNA was in some way connected with species specificity. It had also been suggested that this specificity was lost in malignant tissue, extracts of which showed patterns of methylase activity differing markedly from that of the tissue of origin (10).

Initial experiments with extracts from normal and malignant liver tissue from the rat gave results suggesting that the levels of methylase activity in the malignant tissue were much higher than those in normal liver when tRNA from Escherichia coli was used as acceptor for the methyl groups. These results seemed to confirm the findings of Bergquist and Matthews (1), who had shown that the tRNA extracted from several types of tumor tissue contained a very high proportion of methylated bases compared with tRNA from the corresponding normal tissues. The extracts used for these studies were essentially similar to those of Cantoni (3) which were used for the study of many cytoplasmic enzymes and contained a system for the generation of ATP from phosphoenolpyruvate in order to conserve a high concentration of S-adenosylmethionine, the methyl donor in the reaction (7).

More recent publications (6, 8) have been concerned with the study of these enzymes in rat liver and a variety of normal and malignant tissues. The conditions used in these experiments were more carefully controlled than those from Borek's laboratory (12), the most important differences being the absence of an ATP generating system and the addition of NH₄⁺ ions at a concentration of 0.25–0.36 M as an essential for optimum enzyme activity.

Kaye and Leboy (6) showed that under these conditions the levels of tRNA methylase activity in extracts of normal and malignant tissue were not significantly different and that extracts of neonatal tissue gave levels almost as high as those obtained with tumor tissue. The tacit assumption from these findings is that the level of methylase activity is a reflection of the rate of growth of the tissue, rather than a specific change due to the onset of malignancy.

The present report deals with studies carried out on the methylation in vitro of E. coli tRNA by extracts from rat liver and a transplantable secondary hepatocarcinoma in an attempt to clarify the conflicting opinions expressed by the earlier groups of workers.

MATERIALS AND METHODS

The hepatocarcinoma was an ethionine-induced "minimum deviation" variety which had been initiated at the Chester Beatty Research Institute, London, England, and was kindly supplied by Dr. Eric Reid, of the University of Surrey. The tumors were transplanted at approximately 21-day intervals into rats of the Norwegian hooded strain PVG/C, which were obtained from the Department of Surgical Science at the University of Edinburgh. Regenerating liver tissue was obtained from rats which had been subjected to partial hepatectomy under ether anesthesia 24 hours previously.

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E. coli tRNA was obtained from Calbiochem Ltd., London. Dithiothreitol (Cleland's reagent) was also obtained from Calbiochem. 2,5-diphenyloxoazole, 1,4-di-2,5-phenyloxazolobenzene, and tolune (scintillation grade) were obtained from Koch-Light Ltd., Colnbrook, Bucks., England. Hyamine hydroxide was purchased from Nuclear Enterprises (GB) Ltd., Sighthill, Edinburgh, Scotland. Pyruvate kinase (crystalline suspension in ammonium sulphate) was obtained from Sigma Ltd., London, England.

**S-Adenosylmethionine-methyl-3H** was obtained from the Radiochemical Centre, Amersham, Bucks., England. All other chemicals were AnalA-Re grade from BDH Ltd., Poole, Dorset, England.

### Preparation of Extracts with tRNA Methylase Activity

The tissue was homogenized in 4–6 volumes 0.25 M sucrose: 0.01 M MgCl₂ in a glass/Teflon homogenizer for 10 min at half speed on a Multispeed stirrer. The homogenate was filtered through four layers of muslin and then centrifuged at 105,000 × g for 1 hr at 4°C. The supernatant solution was poured through a plug of glass wool to remove the fatty layer, and the final solution was diluted to the appropriate protein concentration with 0.25 M sucrose: 0.01 M MgCl₂.

In the second method the tissues were homogenized in 4 volumes chilled 0.25 M sucrose: 0.01 M MgCl₂: 0.01 M Tris-HCl buffer, pH 8.8: 0.001 M dithiothreitol. The homogenate was centrifuged for 1 hr at 105,000 × g, poured through a plug of glass wool, and dialyzed for 2 hr three times against 7 liters 0.01 M Tris-HCl buffer, pH 7.9. After dialysis, dithiothreitol was added to the solution to a final concentration of 5 mM.

### Assay of the tRNA Methylase Activity of the Extracts

The reaction mixture had a total volume of 2 ml and contained 100 μmoles Tris-HCl buffer, pH 8.2, 100 μmoles reduced glutathione, 100 μmoles MgCl₂, 15 μmoles phosphoenolpyruvate (sodium salt), 2 μmoles ATP, 0.1 μmole of a 1-in-10 dilution of the pyruvic kinase solution (in water), 1.5 μC S-adenosylmethionine-methyl-3H, 1.0 ml enzyme preparation, and varying amounts of tRNA in water.

Incubation was for 45–90 min at 30°C. At its completion the tRNA was unloaded by incubation with 1 ml freshly prepared 3 M hydroxylamino hydrochloride (adjusted to pH 7.5 with NaOH) for 10 min at 37°C. The protein and RNA were precipitated with 0.6 ml 5 M HCl and 1 ml 10 percent (w/v) trichloracetic acid. The precipitate was sedimented by centrifugation at 800 × g for 10 min, washed with 10 percent (w/v) trichloracetic acid, ethanol, and ether and dried. The dry residue was dissolved in 0.2 ml 1 M hyamine, and 5 ml toluene-based scintillation fluid were added for counting in a Nuclear Chicago scintillation spectrometer No. 725.

In the second method the assay mixture contained 0.03 M triethanolamine buffer, pH 8.9, 9.4 mM mercaptoethanol, 0.36 M ammonium acetate, 0.4 mM MgCl₂, 100 μg E. coli tRNA, 22.4 μmoles S-adenosylmethionine (labeled or unlabelled), and 600 μg protein in a final volume of 0.25 ml.

Incubation was for 40 min at 37°C, after which the tRNA was unloaded with 3 M hydroxylamine as above. The reaction was stopped by the addition of 0.3 ml 5 N HCl and 5 ml 5 percent (w/v) trichloracetic acid. The precipitate was sedimented by centrifugation for 5 min at 500 × g and washed twice in 5 percent (w/v) trichloracetic acid, once in ethanol, and once in ether, and dried. The material was deproteinized by dissolving in 1 ml 10 percent (w/v) trichloracetic acid and incubating for 10 min at 60°C. The remaining precipitate was removed by centrifugation at 500 × g for 5 min, and 0.5-ml samples of the supernatant solution were added to scintillation vials containing 10 ml ether. The vials were capped and agitated vigorously for 1 min to promote extraction of the trichloracetic acid. The ether was removed by suction and the remaining traces driven off by heating the vials in an oven for 10 min at 80°C. The samples were cooled, and to each was added 5 ml dioxane-based scintillation fluid.

### Preparation of Rat Liver tRNA

The method was essentially that of Hoagland et al. (5). The livers were finely minced with scissors and homogenized in a solution containing 0.35 M sucrose, 0.004 M MgCl₂, 0.025 M KCl, 0.35 M KHCO₃. The final pH was 7.0. The homogenate was centrifuged at 15,000 × g for 10 min at 4°C, and the supernatant fluid was diluted with 3 volumes of an unbuffered medium containing 0.9 M sucrose, 0.004 M MgCl₂, and 0.025 M KCl. The diluted extract was centrifuged at 105,000 × g for 1 hr, and the clear supernatant solution was withdrawn and added to 1 volume of 90 percent (w/v) phenol containing 0.1 percent (w/v) 8-hydroxyguanine. The mixture was stirred for 1 hr at 20°C and then centrifuged for 10 min at 15,000 × g. The aqueous phase, which formed the lower layer owing to the high concentration of sucrose, was removed and shaken three times with an equal volume of ether at 4°C. The RNA was precipitated by the addition of solid sodium acetate to 2 percent (w/v) and ethanol to 70 percent (w/v). It was reprecipitated twice in a similar manner from 0.1 M Tris-HCl buffer, pH 7.5.

### Purification on Diethylaminoethyl (DEAE) Cellulose

DEAE cellulose was equilibrated overnight in 0.1 M Tris-HCl buffer, pH 7.5, at 4°C. Approximately 2 gm resin were equilibrated in 2 liters buffer. A thin slurry was added to a glass column of 1 cm internal diameter fitted at the bottom with a Joymar chromatographic disc. The column was then washed with 1 liter 0.1 M Tris-HCl buffer, pH 7.5, and stored at 4°C until required.

The tRNA solution, containing up to 10 mg tRNA in 10 ml 0.1 M Tris-HCl buffer, pH 7.5, was added slowly to the column which was then washed with the same buffer until the effluent showed zero extinction at 260 μM. Elution of the tRNA was affected by passing a solution of 1 M NaCl in the same buffer through the column at a rate of 5 ml/min. The first 10 ml of the effluent were discarded, and the bottom of the column was then connected up to an LKB Uvicord II flow cell and monitored at 254 μM. Samples were collected using an ISCO fraction collector modified so as to activate the event recorder on the LKB chart recorder. The sample size was 5 ml. Samples containing RNA were pooled and the RNA precipi-
tated by the addition of 3 volumes 95 percent (w/v) ethanol. The solution was left overnight at 4°C to ensure complete precipitation, and the tRNA was collected by centrifugation, washed in 95 percent ethanol, and dried.

Samples of tRNA were stored at −10°C as dry powder in boxes containing silica gel as desiccant.

RESULTS

In our initial studies, the method of Tsuitsui et al. (12) was used. The relative levels of tRNA methylase activity in crude extracts from liver, regenerating liver, ethionine tumor, and the liver of a tumor-bearing rat with E. coli tRNA as substrate are shown in Table 1a.

It can be seen that the level of activity in the tumor tissue was about 6–10 times higher than that in normal liver tissue when limiting amounts of tRNA were present. Regenerating liver also showed a higher level. This was not always significant in experiments carried out using the crude system, but later work with the improved reaction conditions gave results for regenerating liver which were at least three times as high as those for normal liver, and the values for tumor extracts were approximately ten times as high. In some cases the preparations from the liver of tumor-bearing rats showed an elevated level of tRNA methylase activity, but this was small and variable. There was a large variation in the values for results obtained in different experiments, a fact also noted by Kaye and Leboy (6). Earlier experiments with the crude system were each repeated five times, but those employing the improved conditions of Kaye and Leboy (6) were only repeated three occasions.

When the effect of storage on the activity of the extracts was investigated, it was found that the activity was stable at 4°C for up to a week. Freezing to −60°C followed by thawing led to the loss of approximately 70 percent of the activity.

In order to check the effect of malignancy on the specificity of the methylase enzymes, the extracts were assayed with liver tRNA as substrate (Table 1b). All the assays were carried out with limiting amounts of tRNA, and in this case the incubation was extended to 90 min in order to detect any low level of activity. The results consistently showed that although all samples of liver tissue were inactive in incorporating methyl groups into liver tRNA, there was always a low amount of incorporation by the tumor tissue. When the experiments were repeated using the improved conditions of Rodeh et al. (8), the same results were obtained (Table 2).

A series of sequential incubation experiments was then carried out in order to determine whether the observed effects were a true indication of increased methylase levels in malignant tissue. In these the tRNA was first incubated with an extract in the presence of unlabeled S-adenosylmethionine to ensure that it was fully methylated with respect to that enzyme complex. The tRNA was then isolated by trichloracetic acid precipitation, washed with alcohol, dried, and added to a fresh batch of incubation medium containing labeled S-adenosylmethionine and the second tissue extract.

As can be seen from Table 3, with a limiting amount of tRNA there is an incorporation of methyl-3H groups into E. coli tRNA by an ethionine tumor extract even after a prior incubation with liver extract in the presence of unlabeled S-adenosylmethionine. Samples treated first with tumor extract and then with liver extract showed no incorporation, and neither did samples treated with the same extract on both occasions.

It was also found that samples of E. coli tRNA exposed first to an extract from normal liver and then to one from regenerating liver showed no incorporation of methyl-3H groups despite the fact that levels of activity in this extract had been shown earlier to be much higher than those in the normal liver (Chart 1).

Following reports by Kaye and Leboy (6) that the levels of activity in tumor tissue were not elevated if the assays were

<table>
<thead>
<tr>
<th>Source of extract</th>
<th>E. coli tRNA</th>
<th>Liver tRNA</th>
<th>Control</th>
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<tr>
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<td>6</td>
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<td>Liver of a tumor-bearing rat</td>
<td>52</td>
<td>6</td>
<td>6</td>
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<tr>
<td>Regenerating liver</td>
<td>104</td>
<td>10</td>
<td>4</td>
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<td>Ethionine tumor</td>
<td>306</td>
<td>53</td>
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Table 2

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<tr>
<td>Ethionine tumor</td>
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<tr>
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<tr>
<td>b, Liver tRNA as substrate</td>
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<td>Ethionine tumor</td>
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<tr>
<td>Liver of tumor-bearing rat</td>
<td>14</td>
</tr>
<tr>
<td>Regenerating liver</td>
<td>7</td>
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</tbody>
</table>

Table 1

Levels of tRNA methylase activity in extracts from normal liver, regenerating liver, ethionine tumor, and the liver of a tumor-bearing rat. Each assay contained 100 μmoles Tris-HCl buffer, pH 8.2, 100 μmoles reduced glutathione, 200 μmoles MgCl2, 15 μmoles phosphoenolpyruvate, 2 μmoles ATP, 0.1 ml pyruvate kinase suspension, 5 μl S-adenosylmethionine-methyl-3H, 80 μg tRNA (liver or E. coli), and 1.0 ml enzyme preparation containing 1.5 mg protein in a total volume of 2.5 ml. Controls were incubated without tRNA which was added after the trichloracetic acid. a, incubation time, 45 min at 40°C; b, incubation time, 90 min at 40°C.
Methylation of tRNA in Malignant Cells

Table 3

<table>
<thead>
<tr>
<th>Sample</th>
<th>µmoles methyl-3H groups incorporated</th>
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<tbody>
<tr>
<td>A</td>
<td>74</td>
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<tr>
<td>B</td>
<td>146</td>
</tr>
<tr>
<td>C</td>
<td>42</td>
</tr>
<tr>
<td>D</td>
<td>5</td>
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<tr>
<td>E</td>
<td>8</td>
</tr>
<tr>
<td>F</td>
<td>7</td>
</tr>
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The effect of sequential incubation of E. coli transfer RNA (tRNA) with extracts from liver and ethionine tumor tissue. The constituents of the assay were 75 µmoles Tris-HCl buffer, pH 8.8, 250 µmoles ammonium acetate, 75 µg dithiothreitol, 800 µg tRNA, 1.5 mg protein, 3 µC S-adenosylmethionine-methyl-3H in a total volume of 1 ml. A, single incubation, rat liver preparation, in the presence of S-adenosylmethionine-methyl-3H; B, single incubation, ethionine tumor preparation in the presence of S-adenosylmethionine-methyl-3H; C, tRNA incubated with unlabeled S-adenosylmethionine-methyl-3H in the presence of liver extract and then with S-adenosylmethionine-methyl-3H in the presence of an extract from the ethionine tumor; D, tRNA incubated with unlabeled S-adenosylmethionine-methyl-3H in the presence of liver extract and then with S-adenosylmethionine-methyl-3H in the presence of an extract of neonatal liver; E, tRNA incubated with ethionine tumor extract in the presence of unlabeled S-adenosylmethionine and then with labeled S-adenosylmethionine in the presence of the same extract; F, tRNA incubated with ethionine tumor extract in both cases.

Chart 1. The effect of addition of further tissue extracts to a system already saturated with respect to liver enzyme. Each assay contained 75 µmoles Tris-HCl buffer, pH 8.8, 250 µmoles ammonium acetate, 75 µg dithiothreitol, 800 µg E. coli tRNA, 1.5 mg protein in normal liver extract, 3 µC S-adenosylmethionine-methyl-3H, and extra protein from extracts of normal liver, regenerating liver, or ethionine tumor as indicated in a final volume of 1 ml water. Incubation was for 45 min at 37°C.

Chart 2. Effect of enzyme concentration on the transfer RNA methylase activity of extracts from ethionine tumor tissue and normal and neonatal liver. Each assay contained 0.03 M triethanolamine buffer, pH 8.9, 4 mM mercaptoethanol, 0.36 M ammonium acetate, 0.4 mM MgCl2, 100 µg E. coli transfer RNA, 22.4 µM S-adenosylmethionine-methyl-3H, protein as shown in a final volume of 0.25 ml. Incubation was at 37°C for 45 min.

DISCUSSION

There are two fundamental difficulties which at present constrain the study of the mammalian tRNA methylase enzymes. First, the function of the methyl groups is not known although several theories have been put forward (2, 12); second, it has been shown by Gold and Hurwitz (4) that the RNA methylase activity in E. coli is a combination of the effects of several enzymes, each of which methylates a different position on a particular base. Studies on crude extracts which may contain all, or a variable selection, of these enzymes are therefore limited, and the results must be interpreted with caution.
An analogous situation is present if liver tRNA is used as substrate since in this case the tRNA is presumably fully methylated with respect to liver enzymes in vivo. The results showed that the ethionine tumor extract was able to promote the incorporation of labeled methyl groups into liver tRNA although the levels were very low. They were, however, consistently reproducible. No incorporation was observed with extracts from normal or regenerating liver, or with liver from a tumor-bearing rat.

These observations suggest that there is not only a generalized increase in the levels of total tRNA methylase activity in tumor tissue, which is also found in regenerating tissue or in livers from newborn animals, but in addition there appears to be an increase in the variety of enzymes present in the malignant tissue which enables extracts to supermethylate tRNA already saturated with respect to the liver enzyme.

More meaningful results will have to await the fractionation of the methylase complex in mammalian tissues.

ACKNOWLEDGMENTS

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REFERENCES

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