Time Dependence of Ethionine-induced Changes in Rat Liver Transfer RNA Methylation

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SUMMARY

Methyl-deficient transfer RNA (tRNA) and subnormal levels of tRNA-methylating enzymes were found in the livers of female rats that had received injections of 250 mg Dl-ethionine per kg body weight per day and 120 mg adenine per kg body weight per day for 2 days. Adenine alone had no effect. When the ethionine plus adenine injections were continued for longer periods of time, liver tRNA-methylating enzyme activity measured in vitro gradually increased and exceeded that of the controls. Concurrently, the relative methyl deficiency of liver tRNA decreased. The latter was evident because of the decreased ability of the tRNA to accept methyl groups during in vitro methylation catalyzed by homologous enzymes. Liver tRNA from animals that were treated with ethionine for 7 days could accept only about 40% as many methyl groups as could tRNA from animals that had received ethionine for only 2 days. No further significant change in methyl deficiency of the tRNA was seen when ethionine administration was extended to a total of 14 days. Enzyme preparations from ethionine-treated, but not control, rat livers contained dialyzable substances that inhibited the tRNA methylases and altered the base specificity of these enzymes. Although S-adenosylhomocysteine and S-adenosylmethionine were found to be present in the liver preparations, neither of these substances could account for the observed changes in specificity.

INTRODUCTION

Ethionine, which is a liver carcinogen (4), inhibits tRNA methylation in vivo in bacteria and rats, and incompletely methylated tRNA can be isolated from these sources (8, 13, 20, 22, 23). At first, these observations appeared to be inconsistent with earlier findings by other investigators (6, 7) that liver from ethionine-treated rats had higher than normal tRNA-methylating enzyme activity. However, in earlier reports (21, 22) we presented evidence that these observations are not really contradictory but reflect alterations in the process of tRNA methylation that coincide with different sampling times during the period of ethionine administration. A time course study of the ethionine-induced changes in liver tRNA methylation in vivo and in the enzymes that carry out these reactions seemed to be a necessary first step in a meaningful investigation of these phenomena.

We report now the results of such experiments, in which ethionine was given daily to rats during a 2-week period, and the sequential changes in the methyl acceptor capacity of the liver tRNA and the activities of the liver tRNA-methylating enzymes were studied. This system offers a means for examining the early effects of this liver carcinogen on 1 aspect of the processing of tRNA.

MATERIALS AND METHODS

Animals. Female CFN rats of the Wistar strain, obtained from Carworth Farms, New City, N.Y., were used for all experiments. The rats were maintained on a diet of commercial laboratory rat chow and given food and water ad libitum. Both control and experimental animals were given sodium pentobarbital (50 mg/100 ml) in their drinking water for 2 days prior to sacrifice, because pentobarbital has been reported to minimize RNase activity in liver (9). For periods of up to 14 days, experimental rats received daily i.p. injections of Dl-ethionine (250 mg/kg body weight) and adenine (120 mg/kg body weight) suspended in carboxymethylcellulose. The animals were killed by decapitation on the morning after the last injection. Some animals were given adenine alone, whereas control rats did not receive any injections.

tRNA Preparations. Low-molecular-weight RNA was prepared by a modification of the method of Fliessner and Borek (5) described earlier (17). The sources of the RNA were: (a) Escherichia coli B cells harvested in midlogarithmic growth phase (frozen cells purchased from Miles Laboratories, Inc., Elkhart, Ind.); (b) cells of methionine-starved E. coli K12 W6, kindly provided by Dr. T. Staudt of Merck & Co., Inc., Rahway, N. J.; and (c) livers from control and ethionine-injected rats.

Enzyme Preparations. Liver tRNA methylase extracts were prepared as described earlier (22). These preparations are essentially a 100,000 x g supernatant from a liver homogenate and are designated S-100. In some instances enzymes were dialyzed at 4° against large volumes of 0.01 M Tris:0.005 MgCl2:0.005 M mercaptoethanol, pH 8, for 3 hr and then assayed immediately.

Enzyme Assays. The assay system consisted of tRNA from any of the various sources listed above, methyl-labeled AdoMet2 as methyl donor, and enzyme incubated in 0.01 M

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2 The abbreviations used are: AdoMet, S-adenosylmethionine; AdoEth, S-adenosylmethionine; AdoHcy, S-adenosylhomocysteine.
Tris:0.005 M MgCl₂:0.005 M mercaptoethanol buffer, pH 8, containing approximately 0.25 M ammonium acetate. Incorporation of radioactive methyl groups into tRNA was measured.

A typical incubation tube contained about 100 μg of tRNA, 0.1 to 0.15 μCi [methyl-¹⁴C]AdoMet (specific activity, 52 mCi/mmmole; International Chemical and Nuclear Corp., Irvine, Calif.), and enzyme extract in a final concentration of 0.25 to 0.35 M ammonium acetate:0.010 M Tris:0.005 M MgCl₂:0.005 M mercaptoethanol, pH 8, in a volume of about 0.5 ml. The mixture was incubated at 35° for 40 to 50 min. At the end of the incubation period, 0.2 ml of 1.5 M hydroxylamine, pH 7.5, was added to each tube, and the samples were maintained at 35° for 10 min longer. Samples were chilled; 2 mg carrier RNA followed by 4 ml of 0.5 M NaCl in 75% ethanol were added. After 2 hr at −15°, precipitates were collected by centrifugation in the cold and then extracted with 2 M NaCl. RNA was reprecipitated from the extracts by addition of 60% trichloroacetic acid solution. The precipitates were washed and assayed for radioactivity as described earlier (22).

Base Specificity of tRNA Methylation. A typical incubation mixture for the isolation of [methyl-¹⁴C]-tRNA contained: 0.15 to 0.3 mg of tRNA; 0.15 to 0.4 μCi [methyl-¹⁴C]AdoMet (specific activity, 52 mCi/mmmole); 0.01 M Tris buffer, pH 8, 0.005 M MgCl₂; 0.005 M mercaptoethanol; and a saturating level of S-100 enzyme preparation. The volume was 1 ml or less. After incubation at 35° for 50 to 60 min, 0.3 ml of 2 M hydroxylamine, pH 8, was added and the mixture was incubated for an additional 10 min. The samples were then chilled, precipitated, and washed as described above. The RNA was subjected to alkaline hydrolysis, and methylated nucleotide content was determined by a combination of high-voltage electrophoresis and chromatography. Details of these procedures have been described in an earlier publication (17).

RESULTS

tRNA was isolated from the livers of animals killed after various time intervals during which they had received daily injections of ethionine and adenine. The ability of tRNA's to accept methyl groups in vitro from [methyl-¹⁴C]AdoMet in the reaction catalyzed by enzyme preparations from livers of untreated (control) rats was measured. The availability of methyl acceptor sites for in vitro methylation with homologous enzymes indicates that these sites have not been methylated in vivo. The methyl acceptor capacity of this tRNA is thus a measure of how complete the methylation of tRNA has been in vivo. The results of these experiments are shown in Chart 1. As expected, tRNA from control rats was able to accept only a very low level of ¹⁴CH₃ in this in vitro reaction (22). No significant change was found after 1 day of treatment, but after 2 days a large increase in the ability of the tRNA to accept methyl groups in the reaction catalyzed by homologous enzymes was evident. The methyl-accepting capacity of the tRNA decreased during the succeeding 5 days of ethionine administration. Liver tRNA from rats treated for 7 to 14 days could accept 40% as many methyl groups as could liver tRNA from rats that had received ethionine for only 2 days.

The base-specific distribution of methyl groups that had been accepted by this same group of liver tRNA during in vitro methylation was determined. The results of these analyses are shown in Table 1. Most of the sites available for methylation by homologous enzymes were in guanine moieties of the tRNA. When total ¹⁴CH₃ incorporated into a fixed quantity of tRNA was measured, relatively minor changes occurred in the total number of sites available for methylation of tRNA bases other than guanine, while the methyl deficiency in tRNA guanine moieties seen after 2 days of treatment was greatly diminished as the period of ethionine treatment was prolonged.

The specific activities of liver tRNA methylases of rats that had received daily injections of ethionine for various intervals over this same 14-day period were measured in vitro with heterologous tRNA from E. coli as substrate. The very limited quantities of methyl-deficient liver tRNA available and the comparatively low capacity of this tRNA to accept methyl groups from homologous enzymes (Table 1) made it more practical to use the heterologous E. coli tRNA for these experiments. As shown in Chart 2, specific activity was depressed below normal at Day 2 and then increased steadily thereafter.

Dialysis of S-100 preparations from ethionine-treated rats...
caused significant increases in the maximum methyl transfer capacity (level of methyl transfer to 100 μg of E. coli tRNA) and changed the shape of the specific activity curve. As the amount of undialyzed S-100 preparations from ethionine-treated animals was increased in excess of 1 mg of protein per sample, progressive decreases in the amount of methyl transfer to RNA were seen. These are typical specific activity curves for enzyme preparations that contain inhibitors. The results obtained with enzymes from livers of rats given ethionine for 2 or 3 days are shown in Chart 3. Substantially the same results were obtained with S-100 preparations from livers of animals that had received ethionine for longer periods. We have shown previously that, in contrast to effects observed on S-100 preparations from ethionine-treated animals, dialysis of control rat liver S-100 preparations did not increase significantly the maximum methyl transfer capacity or change the shape of the specific activity curves of these enzymes (22). The results suggest that low-molecular-weight inhibitors of tRNA methylases, which do not occur in control preparations in significant quantities, are present in livers of ethionine-treated rats.

To determine whether the separate base-specific tRNA-methylating enzymes were inhibited equally by these dialyzable materials, methylated base composition of tRNA was assayed after in vitro methylation, catalyzed by enzymes in dialyzed and undialyzed preparations. The dialyzable inhibitors present in liver preparations from ethionine-treated animals inhibited more strongly the enzymes that catalyzed the methylation of guanine in tRNA than those specific for the other bases (Table 2).

Experiments were then performed to determine whether these base specific effects could be attributed primarily to AdoEth or AdoHcy. Both of these compounds are inhibitors of tRNA-methylating enzymes, have been reported to be present in the livers of rats receiving ethionine (2, 12, 14), and were detected in our preparations by means of paper chromatography. Methyl-deficient tRNA from E. coli K12W6 (λ) was methylated in vitro, using S-100 enzyme preparations from control rat livers, with and without added AdoEth or AdoHcy. This tRNA is an excellent substrate for study of base specificity of the methylating enzymes and of inhibitors, since it is able to accept methyl groups at virtually all sites recognized by the enzymes (5). The distribution of methyl groups in tRNA that had been methylated in the presence of sufficient AdoEth to cause 60% inhibition of the reaction was compared with the distribution in samples incubated without the inhibitor. The results of these experiments appear in Table 3. The effect observed here differed from that observed with dialysis. The methylation of ade-
nine and cytosine was more strongly inhibited by AdoEth than was the methylation of the other bases. These data agree with the pattern reported by Moore (11) for inhibition of bacterial enzymes by AdoEth. In similar experiments, AdoHcy inhibited all of the base-specific liver tRNA methylases equally, so that a decrease in total methyl transfer but no change in methylated base pattern was caused by this inhibitor.

Ethionine sulfoxide, reported by Brada et al. (2) to be present in livers of rats given injections of ethionine, was tested over a concentration range of 70 to 415 μg/ml, in the presence of \( 5 \times 10^{-4} \) M AdoMet. This compound did not significantly inhibit the methyl transfer reaction.

**DISCUSSION**

Study of the mechanisms that regulate the methylation of tRNA in vivo has been hampered by the close coupling of tRNA synthesis and its methylation. As a consequence of this coupling, tRNA isolated from normal tissues has already been methylated in vivo and cannot act as a substrate for homologous enzymes in vitro.

The recent findings that ethionine can cause accumulation of incompletely methylated tRNA in animals (8, 13, 16, 22) and in bacteria (20) have demonstrated the availability of systems that can be used to study the factors that control tRNA methylation. Earlier work in these laboratories showed that reported observations of ethionine-induced inhibition of tRNA methylation in rat liver and of ethionine-induced increases in the activity of rat liver tRNA-methylating enzymes were the same as described for Table 1, except that E. coli K12 tRNA was used.

### Table 2

*Effect of dialysis on methyl group transfer to E. coli tRNA by enzymes from livers of rats treated with ethionine for 7 days*

Conditions were the same as described for Table 1, except that E. coli K12 tRNA was used.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Total</th>
<th>MeA + MeC</th>
<th>MeG</th>
<th>MeU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dialeyzed</td>
<td>2136</td>
<td>460</td>
<td>1580</td>
<td>95</td>
</tr>
<tr>
<td>Undialeyzed</td>
<td>1000</td>
<td>360</td>
<td>610</td>
<td>84</td>
</tr>
<tr>
<td>Dialeyzed: undialeyzed</td>
<td>2.1</td>
<td>1.5</td>
<td>2.6</td>
<td>1.0</td>
</tr>
</tbody>
</table>

*a Abbreviations as in Table 1.*

### Table 3

*Inhibition of liver tRNA-methylating enzymes by AdoEth*

Conditions were the same as described for Table 2, except that the AdoEth was included in the incubation mixture. AdoMet concentration is \( 2.45 \times 10^{-4} \) M; inhibition of MeC + MeA > MeG > MeU.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Total</th>
<th>MeC + MeA</th>
<th>MeG</th>
<th>MeU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control rat liver</td>
<td>2140</td>
<td>258</td>
<td>1850</td>
<td>32</td>
</tr>
<tr>
<td>Control rat liver + 1.2 ( 10^{-4} ) M AdoEth</td>
<td>840</td>
<td>49</td>
<td>765</td>
<td>26</td>
</tr>
<tr>
<td>CONTROL: AdoEth-inhibited</td>
<td>2.5</td>
<td>5.3</td>
<td>2.4</td>
<td>1.2</td>
</tr>
</tbody>
</table>

- AdoEth inhibited
this laboratory, a number of compounds related to adenosine are preferential inhibitors of guanine tRNA methylation in vitro (16, 18, 19). In order to be effective in mammalian systems, however, these substances must be resistant to adenosine deaminase (16, 19).

From the results of these experiments, it is now possible to describe the early changes in liver tRNA methylation that occur during the course of ethionine-induced carcinogenesis.

During the 1st 7 days, as ethionine is given to rats, there is a sequence of continuous changes in the activity of the tRNA-methylating enzymes and in the resultant methylation of tRNA in liver. Early in the sequence (at 2 days) the tRNA-methylating enzyme activity is decreased below normal levels, and undermethylated tRNA accumulates in the liver. The principal methyl deficiency occurs in the guanine bases. This is followed by an increase in the level of tRNA methylease activity. The increased enzyme activities can overcome, in part, the effects of the inhibitors that accumulate during ethionine administration, and the tRNA, while not completely methylated, becomes successively less methyl deficient as the period of ethionine treatment is extended. It is not clear from information presently available whether the observed increases in tRNA-methylating enzyme activity are due primarily to increased enzyme synthesis, altered enzymes, or changes in the levels of substances that modulate the activity of these enzymes. It seems very possible that interplay of several of these factors may be involved.

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REFERENCES

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