Increase of Soluble RNA Methylase Activities by Chemical Carcinogens

R. L. Hancock and P. I. Forrester
Division of Medical Biochemistry, Faculty of Medicine, The University of Calgary, Calgary, T2N 1N4, Alberta, Canada

SUMMARY

High levels of the hepatocarcinogen ethionine (1% of the DL isomer mixture) in diets were shown to increase soluble RNA methylase activity of rat and hamster liver cells. Enzyme increases were detected only after 4 days of ethionine feeding. Dimethylaminoazobenzene, another hepatocarcinogen, also increased soluble RNA methylase activity. Noncarcinogenic analogs of ethionine, norleucine and l-methionine, did not cause this increase in activity. Liver enzyme preparations from ethionine-treated rats methylated yeast phenylalanine transfer RNA more extensively than did enzymes from normal liver. The only nucleotides methylated were uridylic acid and cytidylic acid with the latter being 6 times more methylated. Ethionine treatment did not alter the amounts of soluble RNA methylase inhibitors. The same ethionine enhancement of soluble RNA methylase activity could be demonstrated in metabolizing yeast cells.

INTRODUCTION

Ethionine is an analog of the amino acid methionine, which is carcinogenic to the liver of rats (3) and mice (unpublished experiments), containing 1 additional carbon atom and 2 additional hydrogen atoms. Ethionine is in general terms an inhibitor of protein synthesis, although 1 exception appears to be its ability to increase RNA polymerase activity (16).

During studies with sRNA methylase activity of mouse liver, we discovered that ethionine increased the methylating capacities of liver extracts (5). Others have confirmed this finding (2, 14). We have endeavored to explore several aspects of this and will present evidence in this report on: (a) 3 species with respect to this increased activity; (b) effect of another hepatocarcinogen; (c) effect of l-methionine and the structurally related norleucine on sRNA methylase activity; (d) analysis of nucleotides methylated using 1 kind of tRNA molecule as substrate and enzyme preparations from ethionine-treated rats; and (e) in vivo effects of ethionine on sRNA methylase inhibitors.

MATERIALS AND METHODS

Chemicals. The chemicals used were purchased from the following commercial sources: S-adenosyl-L-methionine-methyl-14C (specific activity of 50 mCi/mmol), International Chemical and Nuclear, Irvine, Calif.; ethionine, Nutritional Biochemical Company, Cleveland, Ohio; l-methionine, puromycin, and dithiothreitol, Sigma Chemical Co., St. Louis, Mo.; glycine, Eastman Kodak Co., Rochester, N. Y.; ammonium chloride, Matheson, Coleman and Bell, Norwood, Ohio; and dimethylaminoazobenzene, Fisher Scientific Co., Fairlawn, N. J. All sRNA preparations were from General Biochemicals, Inc., Chagrin Falls, Ohio, except for yeast phenylalanine transfer RNA, Boehringer-Mannheim, Mannheim, Germany; and polyadenylic acid, Miles Chemical Co., Elkhart, Ind.

Enzyme Sources. Sprague-Dawley rats were purchased from Holtzman Farms, Madison, Wis. Syrian hamsters were obtained from a colony maintained by the Laboratory Animal Care of the University of Alberta. Female C57L/J mice and BW 7756 mouse hepatomas were obtained from the Laboratory Animal Care, Bar Harbor, Maine. The yeast used was Fleischmann's bakers' yeast.

Enzyme Preparations. Enzyme-active extracts were made according to previously described methods (10). The data as reported in the charts, unless otherwise stated, are the average of paired assays on single animals and are Escherichia coli K12 sRNA-dependent incorporations; i.e., the endogenous activity (also paired) has been subtracted. The yeast enzymes were obtained by the method of Cantoni and Richards (1). The electrophoresis procedure has been described previously (6). Twenty g of cake yeast were suspended and incubated at 35° for various times in 200 ml of a culture media containing 2 g NaNO3, 1 g KH2PO4, 0.5 g MgSO4, 1 g yeast extract, and 20 g sucrose, per liter with or without the addition of 1 g of DL-ethionine. Such cell suspensions were collected by centrifugation, and the enzyme preparation was prepared according to the above-stated methods (1). sRNA methylase assays were done as previously described (10). Glycine methyltransferase was assayed as follows: 20 μmoles Tris-HCl (pH 8.0), 0.4 μmole dithiothreitol, 4 μmoles glycine, 0.1 μCi S-adenosyl-L-methionine-methyl-14C, and aliquots of enzyme preparations in a total volume of 0.2 ml were incubated at 37° for various times. Adenosine (0.5 μmole) was added and the reaction was stopped by the addition of 0.1 ml of 20% (w/v) phosphotungstic acid. The mixture was made up to 1 ml by the addition of cold distilled water and was centrifuged at...
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5000 × g for 10 min. Aliquots (50 µl) of the supernatants were dried (100°), 10 ml of Liquifluor were added, and the radioactivity was determined in a Beckman LS-250 liquid scintillation counter.

The ethionine diets used were 1%. However, these were DL isomer mixtures for reasons of economy. There is no evidence that the D form is used or converted to the L form in animal systems. We have shown that extracts of mouse liver are incapable of forming S-adenosylmethionine from D-ethionine (4). This then effectively represents a 0.5% diet. For example, in the experiment described in Chart 1, an equivalent 0.5% L-methionine diet was used. The animals were maintained on Teklad animal meal (Rockland Laboratory, Monmouth, Ill.) for mice and rats. Protein and nucleic acid determinations used were those previously described (13, 17). All enzyme preparations used for sRNA methylation were dialyzed against 0.005 M Tris-HCl (pH 8.3) for approximately 4 hr unless otherwise indicated.

RESULTS

Earlier studies had shown that increased sRNA methylase activities could be induced in mouse liver after a 0.25% ethionine diet for 6 months (5). Such changes were made in only 10 days if a 1% diet was given (8). However, the critical control of feeding a 1% methionine diet had not been done. Chart 1 shows that a methionine control diet does not increase the sRNA methylase and that the ethionine diet will increase the enzyme activity in rats. Control rat liver was examined during this feeding period and no normally occurring periodic changes were noted. Furthermore, the L-carbon analog of L-ethionine, norleucine (Chart 2), also did not cause an increase in activity. It was deemed of interest to see whether another hepatocarcinogen besides ethionine would cause this increase in sRNA methylase. Dimethylaminoazobenzene was given as a 1% diet, and it too is seen to cause an increase in the enzyme activity (Chart 2). Hamsters have been reported to be unsusceptible to ethionine hepatocarcinogenesis (15). We have also attempted to induce hepatomas in Syrian hamsters (male and female) by placing them on 0.25% diets for about 4 months; however, many hamsters died. The females were especially sensitive; 82% (18 of 20) had died after 3 months on the diet yet none (0 of 6) of the males died.

With this failure to demonstrate ethionine as a hepatocarcinogen in hamsters we examined its response to ethionine
with respect to the sRNA methylase activity. The enzyme activity from hamster liver cells will increase in a manner similar to the activity of rat liver (Chart 3, "Discussion").

More controlled experiments were accomplished by using a single kind of yeast tRNA rather than unresolved E. coli sRNA preparations. Such studies also showed the superior methylating ability of ethionine-treated liver enzyme preparations over those of untreated preparations. In Chart 4 a difference in extent of methylation of yeast phenylalanine tRNA could be demonstrated. Upon analysis of the nucleotides these ethionine-treated liver extracts were able to methylate yeast phenylalanine tRNA bases with a uracil:cytosine ratio of 1:6 (Chart 5). A trace amount of cytidylate methylation may occur with rat liver nucleic acids present in the crude enzyme preparation, since the control received identical treatment but had no phenylalanine tRNA present. Experiments designed to study the increase in sRNA methylase activity before the 10-day period can be seen in Charts 6 and 7 for the rat and hamster, respectively. There is an initial large activity about 6 days before the primary increase occurs such as that depicted in Chart 1.

We have found, in general, that assays done with high ammonium concentrations showed parallel results but with increased methyl incorporation.

Experiments were extended to yeast cells for which, it was thought, a simpler biological system might be obtained for analysis of the ethionine effect. Yeast extracts were found to methylate E. coli sRNA proportional to the amount of protein used up to at least 2-mg protein equivalents of the supernatant fraction. However, one does see a large "endogenous" activity, i.e., which is not dependent upon exogenous introduced sRNA substrate. There was a great deal of variation in preparation to preparation with respect to this endogenous activity. Ethionine was found to increase the sRNA methylase activity by adding it to yeast cell incubating suspensions. Furthermore, the extent of methylation was found to be different for ethionine-treated preparations (Chart 8). Ammonium ions stimulated yeast enzyme ability to methylate the E. coli sRNA molecules in vitro and had a dramatic effect upon the ethionine-treated preparations. One interesting finding is depicted in Chart 9. Whereas control enzyme preparations, i.e., those which

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**Chart 3.** Effect of 1% DL-ethionine diet on hamster liver sRNA methylase activity. Reaction mixture contained 20 μmoles Tris-HCl (pH 8.3), with (A) or without (B) 10 μmoles ammonium acetate, 10 μg of E. coli K12 sRNA, 0.1 μCi S-adenosyl-L-methionine-methyl-14C (specific activity, 55.2 mCi/mmmole), and 600 μg protein equivalents of 100,000 x g supernatant cell fraction of liver tissue from adult female hamsters that had received 1% DL-ethionine diet for indicated times, in a total volume of 0.25 ml and incubated for 80 min at 45°C.

**Chart 4.** Yeast phenylalanine tRNA methylation by rat liver extracts. Reaction mixture contained 20 μmoles Tris-HCl (pH 8.3), 10 μg yeast phenylalanine tRNA, 0.1 μCi S-adenosyl-L-methionine-methyl-14C (specific activity, 55.2 mCi/mmmole), and 600 μg protein equivalents of 100,000 x g supernatant cell fraction of rat liver maintained for 16 days on a 1% DL-ethionine diet or 720 μg of protein equivalents from control liver tissue in a total volume of 0.25 ml and incubated for indicated times at 45°C.

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Chart 5. Nucleotide analysis of yeast phenylalanine tRNA methylation by liver extracts from ethionine-treated rats. Reaction mixtures contained 240 μmoles Tris-HCl buffer (pH 8.3), 600 μmoles ammonium acetate with (Exp.) or without (control) 720 μg of yeast phenylalanine tRNA, 1.2 μCi S-adenosyl-L-methionine-methyl-14C (specific activity, 55.2 mCi/mmole), and 1.056 mg protein equivalents of 100,000 x g supernatant cell fraction of liver tissue from rats maintained 28 days on a 1% DL-ethionine diet, in a total volume of 3 ml, and incubated for 80 min at 45°. The reaction was stopped by heating at 90° for 2 min. The methylated phenylalanine tRNA was isolated, hydrolyzed, and analyzed by electrophoresis as in "Materials and Methods." Paper electrophoresis was done at 4500 V and 30 ma for 2.75 hr at pH 4.5 on Whatman No. 3MM paper strips.

were incubated at 45° for 6 hr in the absence of ethionine, allowed maximal methylation rates, which were quite low, ethionine-treated preparations acted in a strict stoichiometric manner.

An experiment was devised to determine whether supernatants from liver tissue of rats fed an ethionine diet for 19 days had decreased amounts of sRNA methylase inhibitors. Whereas 2570 cpm/mg protein were obtained with control liver preparations and 6290 cpm/mg protein for the preparation from ethionine-treated rats, 7410 cpm/mg protein were obtained with a combination of ethionine and control extracts using 10 μg E. coli sRNA incubated for 80 min.

The effects of ethionine diet on one of the supposed sRNA methylase inhibitors, namely, glycine methyltransferase (12), shows that ethionine has no effect on this enzyme (Chart 10 A). The enzyme was shown to be lowered in fetal tissue and was almost entirely absent from a mouse hepatoma (Chart 10, B and C). Thus there is no evidence to support the theory that sRNA methylase inhibitors are lowered during ethionine carcinogenesis. In this laboratory E. coli sRNA has been the substrate used in our standard assays with liver extracts. Bacillus subtilis sRNA was the most efficient substrate for enzyme from yeast cells.

DISCUSSION

We have shown that 2 hepatocarcinogens, ethionine and dimethylaminobenzene, cause an increase in sRNA methylase activity, whereas 2 noncarcinogenic analogs, methionine and norleucine, do not. However, this increase in sRNA methylase activity can be demonstrated in hamsters, in which ethionine, thus far, has not been shown to be a hepatocarcinogen. This shows that (a) the increase in activity is not directly related to carcinogenesis; (b) the hepatomas have not been induced because of incorrect ethionine feeding regime and/or it is too toxic for this species, eliminating the opportunity for induction of hepatomas; or (c) ethionine is not required for induction per se but is a "corequirement" of the carcinogenic mechanism.

The parallelism of increased activity of various enzyme preparations in the presence or absence of ammonium ions suggests that certain tRNA’s in E. coli sRNA are altered in their conformation, which allows for a greater substrate efficiency with respect for potential methylating sites as has been previously suggested (7). Experiments have been designed with the idea that ethionine inhibits the synthesis of sRNA methylase inhibitor protein (11). From the "mixing" experiments, it is clear that increased methylating capacity of liver extracts from ethionine-treated rats cannot be altered by any inhibitor present only in the control. This is

Chart 6. Changes in sRNA methylase activity in rat liver during the 1st week of an ethionine diet. Reaction mixture contained 20 μmoles Tris-HCl (pH 8.3), with (A) or without (B) 50 μmoles ammonium acetate, with or without 10 μg E. coli K12 sRNA, 0.1 μCi S-adenosyl-L-methionine-methyl14C (specific activity, 55.2 mCi/mmole), 0.02 ml of 100,000 x g supernatant fraction containing from 560 to 680 μg protein, from liver of female rats maintained on a 1% DL-ethionine diet for the indicated days.
Chart 7. Changes in sRNA methylase activity on hamster liver during the 1st week of an ethionine diet. Reaction mixture is the same as described in Chart 9 except that the enzyme was 0.02 ml of 100,000 x g supernatant fraction containing from 420 to 640 µg protein from liver of female hamsters maintained on a 1% DL-ethionine diet for the indicated days.

Chart 8. E. coli sRNA methylation by yeast extracts. Reaction mixture contained 20 µmoles Tris-HCl (pH 8.3), with or without 10 µg E. coli K12 sRNA, 0.1 µCi S-adenosyl-l-methionine-methyl-14C (specific activity, 55.2 µCi/m mole), 1.4 mg protein from various yeast cell supernatant fractions (as in "Materials and Methods") in a total volume of 0.25 ml and incubated for indicated times at 45°. Data are averages of 4 separate assays. A, untreated yeast cell extracts assayed without E. coli sRNA in assay mixture; B, yeast cell extracts incubated for 6 hr with ethionine (see "Materials and Methods") without E. coli sRNA in assay mixture; C, untreated yeast cell extracts assayed with E. coli sRNA in assay mixture; D, yeast cell extracts incubated for 6 hr with ethionine with E. coli sRNA in assay mixture.
Chart 9. sRNA methylase activity of extracts from yeast cells cultured in ethionine. Reaction mixture contained 20 μmoles Tris-HCl (pH 8.3), 10 μg E. coli K12 sRNA, 40 μmoles ammonium acetate, 0.1 μCi S-adenosyl-L-methionine-methyl-14C (specific activity, 55.2 mCi/mmole), with varying amounts of the enzyme preparation as indicated, in a total volume of 0.33 ml and incubated for 120 min at 45°. E, incubated (for 6 hr in the presence of ethionine); C, (incubated for 6 hr in the absence of ethionine) (see “Materials and Methods”).

Chart 10. Enzymatic methylation of glycine using various extracts. A, control rat liver (⊙) and ethionine-treated rat liver (□); B, control rat liver (⊙) and 20-day fetal rat liver (△); C, normal C57L/J mouse liver (⊙) and mouse hepatoma BW 7756 (●).
further substantiated by the experiments showing that glycine methyltransferase activity (12) is not altered by ethionine treatment. The difference in extent of methylation of yeast phenylalanine tRNA may indicate a change in specific kinds of sRNA methylase activity. Upon analysis the increase methylation was found to be due to cytidine methylase activity. We have found a similar high cytidine methylase activity towards yeast phenylalanine tRNA in previous studies (9).

ACKNOWLEDGMENTS

We wish to thank Y. Urvet for her expert assistance during the course of this work.

REFERENCES


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