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Effect of Carcinogen Ethionine on Enzymatic Methylation of DNA Sequences with Various Degrees of Repetitiveness*

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Abstract—*When P815 mastocytoma cells are cultivated in McCoy 5a medium in the presence of ethionine (0.0075–6 mM), neither inhibition of cell proliferation nor of DNA synthesis was observed. In order to study the effect of this carcinogen on enzymatic DNA methylation the cells were labelled simultaneously with L-(methyl-^{3}H)-methionine and (^{14}C) deoxycytidine. The fractionation of isolated DNA was based on the reassociation kinetics of inverted repetitive sequences of type ABC... CBA, ordinary repetitive sequences of type ABC... ABC... ABC, intermediary sequences and the unique sequences. The individual fractions were then hydrolyzed in 96% formic acid and the DNA bases were separated by paper chromatography. The relative rates of enzymatic DNA methylation were computed on the basis of (^{3}H) radioactivity in 5-methylcytosine and (^{14}C) radioactivity in cytosine. They showed that the inverted repetitive sequences are approximately 50% higher methylated than the ordinary repetitive sequences, and about 300% higher than the intermediary and unique ones. In cells grown in presence of ethionine, this pattern changes dramatically. The enzymatic methylation of inverted repetitive sequences decreases to the level of methylation of ordinary repetitive ones, at a concentration as low as 0.01 mM. Although the other sequence classes are lower methylated in cells grown in presence of ethionine than in controls, the effect of this compound here is less profound. Because of a possible regulatory role of the inverted repetitive sequences in mammalian genome their hypomethylation may be related to ethionine induced re-expression of certain genes.*

INTRODUCTION

ETHIONINE, an ethyl analog of the essential amino acid methionine, induces morphological changes in the liver and other organs after a prolonged administration at sublethal doses. These lesions eventually give rise to a carcinoma [1, 2]. Though the mechanism of ethionine carcinogenesis was not yet clarified, various effects of this compound have been considered: (a) the incorporation of ethionine instead of methionine into proteins [3]; (b) the post-synthetic ethylation of cellular macromolecules, such as RNA, DNA and proteins via *S*-adenosyl-L-ethionine [4–6] and (c) the inhibition of enzymatic methylation of RNA, DNA and proteins via *S*-adenosyl-L-ethionine [7–10].

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As ethionine, in form of *S*-adenosylethionine, was recently shown to inhibit the methylation reaction of DNA methylase activity from Novikoff hepatoma [9], we became interested, and began a study into the effects of this compound on the enzymatic methylation of various DNA sequence classes in P815 mastocytoma cells. We describe here that the ethionine in subtoxic doses inhibits the methylation of inverted repetitive sequences of type ABC...CBA to a higher extent than the methylation of other sequence classes.

MATERIALS AND METHODS

P815 mastocytoma is a highly malignant cell line which, after an intraperitoneal (i.p.) transplantation, rapidly disseminates in the breast and other tissues and kills the tumor-bearing animals within 9–10 days. In McCoy 5a medium supplemented with 20% fetal calf serum these cells grow in a suspension and

divide rapidly with an average generation time of 13–14 hr. The cells were isolated from ascites fluid on the 7th day after i.p. inoculation of about 10^6 cells in $BD2F_1$ mice [$(C57BL/6 \times DBA/2)F_1$], washed three times in physiological saline and resuspended in McCoy's 5a medium supplemented with 20% fetal calf serum ($1-2 \times 10^5$ cells/ml).

The following precursors were added: 5 μCi (^{14}C) deoxycytidine (429 mCi/mmole. The Radiochemical Centre Amersham, G.B.) and 150 μCi L-(methyl-^{3}H)-methionine (50 Ci/mmole, New England Nuclear) per 100 ml culture medium. To prevent the incorporation of the methyl-groups from L-methionine via "one-carbon" pool, the medium was supplemented with 0.02 M sodium formate [11–13]. Under these conditions, only 5-methylcytosine contained the (^{3}H) radioactivity. The (^{14}C) radioactivity of deoxycytidine was incorporated into all DNA pyrimidines (Fig. 1). After 48 hr of cultivation (2–3 cell divisions), DNA was isolated by a modified method of Marmur [14] including repeated RNAase and pronase treatment, and sheared in a Branson sonifier (Branson Sonic Power, Danbury, Conn., USA) to a fragment length of about 450 base pairs, as measured by acrylamide-gel electrophoresis using DNA of standard lengths (Boehringer, Mannheim, West Germany). After heat denaturation, DNA was allowed to reassociate at 60°C to appropriate Cots (mole × sec × liter^{-1}) and fractionated on hydroxylapatite (Fig. 2).

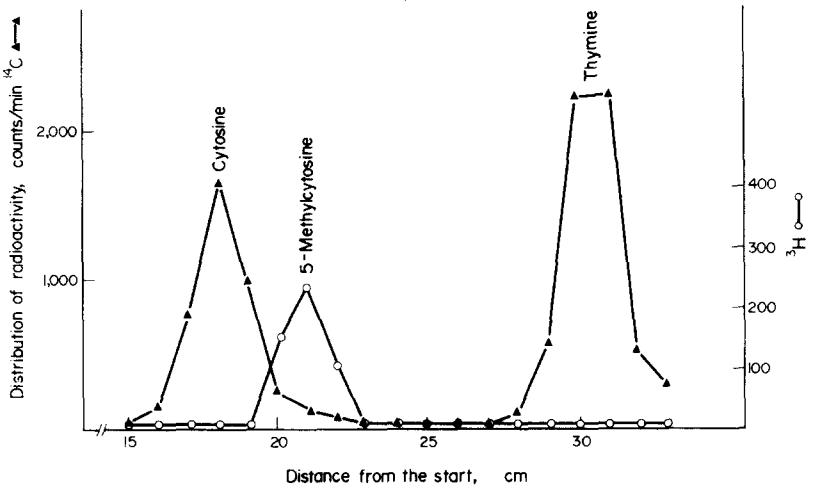

Fig. 1. Distribution pattern of radioactivity in DNA bases of unique sequences after a labelling under conditions described in Materials and Methods. About 96% of (^{3}H) radioactivity of L-(methyl-^{3}H) methionine was recovered in 5-methylcytosine, and about 93% of (^{14}C) radioactivity of (^{14}C) deoxycytidine in thymine, cytosine, and 5-methylcytosine residues, respectively.

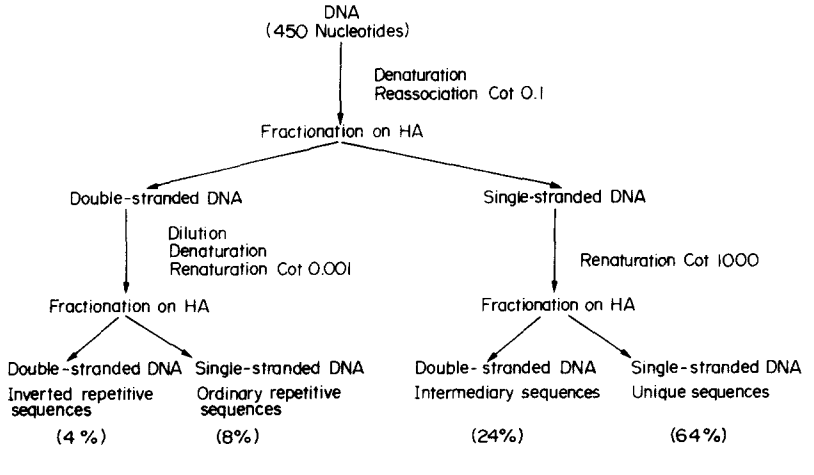

Fig. 2. Flow diagram of DNA processing.

Single-stranded fraction was eluted with 0.15 M sodium phosphate buffer (pH 6.8), and the double-stranded structures with 0.4 M sodium phosphate buffer. Separation of inverted and ordinary repetitive DNA sequences was achieved after a denaturation and restoration to Cot lower than 0.001. Under these conditions it is unlikely that a bimolecular reaction ($c/c_0 = 1/1 + kCot$) takes place and thus only the inverted repetitive sequences, capable to form a double-stranded structure within a single-polynucleotide chain, reassociate at a rate of unimolecular reaction ($c/c_0 = e^{-kt}$). This DNA retains on hydroxylapatite in 0.15 M buffer.

For base analysis DNA fractions of different degrees of repetitiveness were hydrolyzed in presence of 1 mg of unlabelled carrier DNA in 96% formic acid at 170°C for 90 min. Individual bases were separated by paper chromatography in isopropanol:HCl:H_2O (68:6:26 v/v) on MN 214 paper (Macherey & Nagel, Düren, West Germany) for 30 hr. The dried papers were cut into strips (0.5–1.0 cm), the radioactivity was eluted with H_2O and measured in dioxan-based scintillation cocktail.

RESULTS

Christman *et al.* [10] have observed that DNA of erythroleukemic cells grown in presence of ethionine is undermethylated. In an *in vitro*-system this DNA is a better methyl-acceptor for the homologous DNA methylase than DNA of the same cells grown in absence of this compound. Although the authors did not correlate the degree of DNA hypomethylation to mitotic activity they observed that the cells with hypomethylated DNA transcribed the information for hemoglobin. This circumstantial correlation led us to analyze the effect of ethionine on the pattern of enzymatic DNA methylation in sequences of various function and degrees of repetitiveness. For purpose of this analysis, a concentration of ethionine which does not affect cell proliferation had to be chosen, since the enzymatic methylation of DNA involves primarily the newly replicated DNA [15, 16]. In contrast to Friend erythroleukemia cells, whose proliferative activity is inhibited by ethionine in concentrations above 1.5 mM [10], the P815 cells appear to be more resistant to this compound; the concentration of ethionine up to 6 mM did not affect the growth of these cells in McCoy 5a medium supplemented with 20% fetal calf serum (Fig. 3).

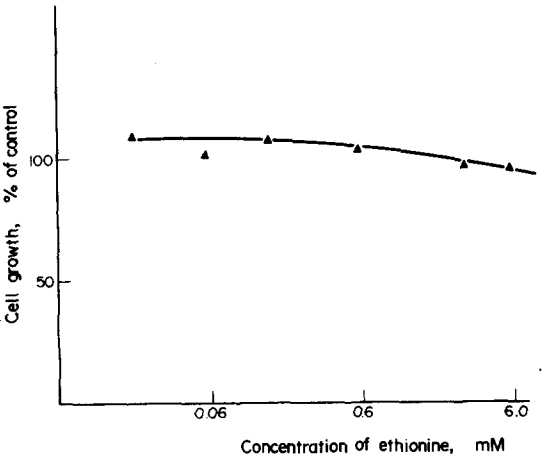

Fig. 3. Growth of P815 cells in presence of different concentrations of ethionine. The values are mean of three independent experiments and given as a percentage of the control.

DNA was isolated and fractionated as it is outlined in Fig. 2. When the sonicated DNA with an average length of 450 base pairs was denatured and reassociated to Cot 0.1, about 12% of the starting material reassociated and retained on hydroxylapatite in 0.15 M sodium phosphate buffer, pH 6.8. this fraction was eluted with 0.4 M buffer, diluted and carried through the second step which consisted of heat denaturation for 10 min, adjustment to 60°C and chromatographing on hydroxylapatite. Taking the dilution and time between denaturation and loading on the hydroxylapatite column into account the Cot of the second step is below 0.001. The DNA reassociated in the second step represents about 4% of the total genome and corresponds to the inverted repetitive sequences of type ABC...CBA. This was proved in two different experiments. In neutral CsCl density gradient centrifugation, about 17% of this fraction banded in the density area of satellite DNA [17], while the remaining 83% banded in a broader peak in a density area corresponding to main band DNA [17]. Second, the Cot lower 0.001 reannealed DNA was treated with single-strand-specific nuclease S1. This treatment abolished to about 70% the capacity of this fraction to reanneal at Cot lower than 0.001 and bind to hydroxylapatite after denaturation. This suggests that the fraction isolated by the above procedure (Fig. 2) represents the inverted repetitive sequences of type ABC...CBA. The fraction which did not bind to hydroxylapatite in the second step represents the ordinary repetitive sequences of type ABC...ABC...ABC [17]. This fraction, about 8.5% of the genome, is likely to contain satellite DNA sequences which are

known to be arranged in simple repeating units [18, 19]. No attempt was made to separate the ordinary repetitive sequences of the satellite and non-satellite fraction in this study. The DNA non-reassociated in the first hydroxylapatite step was allowed to reassociate in 0.15 M sodium phosphate buffer at 60°C for a time period corresponding to Cot 1000. Subsequent fractionation on hydroxylapatite yielded about 24% of total genome as double-stranded structures which correspond to intermediary sequences and about 64% as single-stranded DNA or so-called "unique" DNA sequences.

To monitor the enzymatic methylation of DNA the P815 cells were simultaneously labelled with (^{14}C) deoxycytidine and L-(methyl-^{3}H)-methionine for 2–3 cell cycles. Isolated DNA was hydrolyzed in 96% formic acid and individual bases were separated by paper chromatography [17, 20]. the relative rates of enzymatic DNA methylation were computed from (^{3}H) radioactivity in 5-methylcytosine and (^{14}C) radioactivity either in cytosine or in thymine.

We have described earlier [17] that the relative rates of enzymatic DNA methylation, if monitored by the above described method, vary significantly in different DNA sequence classes. The inverted repetitive sequences show the highest methylation rate and are methylated 50% higher than the ordinary repetitive sequences and approximately 300% higher than the intermediary and unique sequences. In cells, grown in presence of ethionine these methylation rates changed dramatically (Fig. 4). At an ethionine concentration as low as 0.01 mM the methylation of inverted repetitive sequences drops to a level of enzymatic methylation of the ordinary repetitive sequences and remains unchanged at high concentrations of ethionine up to 0.3 mM. Although the other sequence classes are also lower methylated in cells grown in the presence of ethionine than in controls, the effect of this compound here was less profound.

DISCUSSION

During the replication of mammalian genome about 10^{7} specific methyl group transfers take place. The process occurs after the ligation of the shortest replication intermediates [15, 16], and the only product, 5-methylcytosine, was found by means of pyrimidine isoplith analysis in a large variety of nucleotide sequences [9, 21, 22]. The methyl groups involved originate from the activated L-methionine, *S*-adenosyl-L-methionine, and the enzymes catalyzing the methylation of mammalian DNA were isolated from different mammalian tissues and cells [23–26]. The high fidelity by which some of these enzymes select the nucleotide specific methylation sites [27–30] may be compatible with the original suggestions of Borek and Srinivasan [31] that the enzymatic methylation of DNA plays a role in differentiation and that aberrant process may be involved in oncogenesis. Among chemical carcinogens, known to interfere with the enzymatic methylation of mammalian DNA, L-ethionine has a unique feature. Compared to non-specific inhibition of DNA methylating enzymes of mammals by *N*-methyl-*N*-nitro-*N*-nitroso-guanidine [32], L-ethionine inhibits such enzymes competitively, via *S*-adenosyl-L-ethionine [9]. This type of inhibition may eventually lead to an aberrant methylation of the newly replicated genome, if such a replication takes place in the presence of L-ethionine. Although a causual relation of enzymatic DNA methylation to DNA replication was not established L-ethionine (2–4 mM) inhibits completely DNA synthesis in mitogen-stimulated lymphocytes [33]. In the present paper we have studied enzymatic methylation of DNA in P815 mastocytoma cells grown in the presence of ethionine at very low concentrations (0.0075–0.3 mM). Under these conditions L-ethionine affects neither DNA synthesis nor proliferating activity of the cells. The relative rates of enzymatic methylation of DNA, if measured by the above-described double-labelling method differ in various sequence classes and the inverted repetitive sequences of type ABC... CBA clearly show the highest rate of methylation [17]. The data show that the methylation of these sequences is inhibited to a higher extent than the methylation of other sequences, if the cells were grown in the presence of L-ethionine (Fig. 4).

The reason for the decreased methylation of inverted DNA repeats in cells grown in the presence of L-ethionine cannot be clarified on the basis of presented data. Two possibilities have to be taken into account: (a) The enzymes responsible for the methylation of inverted repeats are more sensitive towards *S*-adenosyl-L-ethionine than the enzymes for the methylation of other sequence classes. However, an existence of various DNA methylating enzymes has not been proved as yet; (b) In liver DNA of rats fed with L-ethionine, 7-ethylguanine has been found. This alkylation may eventually affect the en-

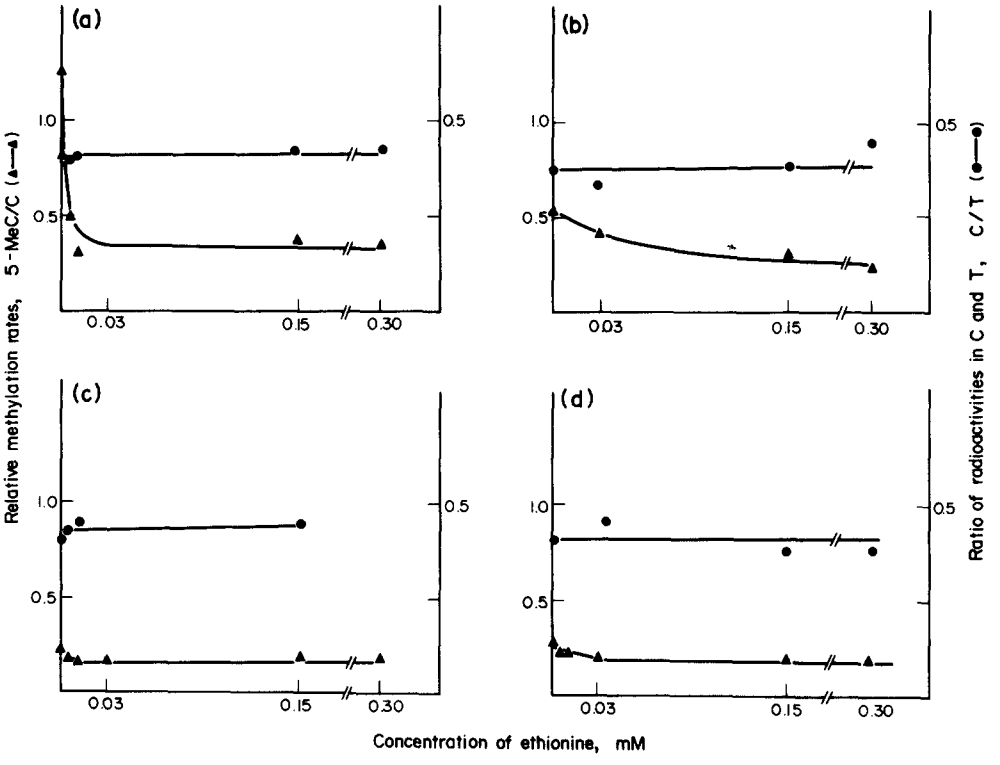

Fig. 4. Enzymatic DNA methylation in P815 cells grown in presence of different concentrations of ethionine. DNA was isolated by a modified method of Marmur [14] and processed as it is outlined in Fig. 2. The relative rates of enzymatic DNA methylation and labelling of DNA–cytosine and DNA–thymine was computed as it is indicated in Materials and Methods. (A) represent inverted repetitive sequences, (B) ordinary repetitive sequences, (C) intermediary sequences, and (D) unique sequences.

zymatic methylation of DNA as it was shown that DNA containing *N*-7 alkylated guanine does not serve as a substrate for DNA methylating enzymes of mammals [29]. It is likely, however, that alkylation of DNA with ethionine will modify guanine residues all over the genome and not only in inverted repeats.

In mouse P815 cells the inverted repetitive sequences comprise about 4% of the genome and are interspersed within sequences of other degrees of repetitiveness [17]. This is compatible with an incidence and distribution of these sequences in various mammalian cells as it was observed in different laboratories [34, 35]. Although the function of these sequences is unknown, it is speculated that the inverted repetitive sequences might represent acceptor sites for regulatory proteins [36–38]. Indeed, most of the binding sites for regulatory proteins and restriction enzymes have a two-fold axis of rotational symmetry [39], a feature also of the inverted repetitive sequences. We have previously shown, that the inverted repetitive sequences are significantly higher methylated than the sequences of other degrees of repetitiveness [17]. One can speculate that if the enzymatic methylation of these sequences negatively controls the recognition process, similar as it is in the case of restriction enzymes [40, 41], then the change in enzymatic methylation of these sequences caused by L-ethionine, may lead to the expression of certain genes. This is exemplified by the ethionine-induced re-expression of embryonic genes in rats [42] and globin synthesis in erythroleukemic cells [10].

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