DNA-METHYLASE IN LOACH EMBRYOS (*MISGURNUS FOSSILIS*)

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1. Introduction

The rare bases (MC* and others) discovered in various DNAs, are the products of enzymatic modification of the common bases at the polynucleotide level [1–4]. The functional role of this DNA modification is obscure, but it is believed to play an essential role in cellular differentiation [5]. Despite the fact that MC in DNA of higher organisms was found quite a long time ago [6], the data on the degree of methylation of animal DNA are still scanty [7, 8]. Nothing is yet known about cytoplasmic DNA methylation and DNA-methylases in cytoplasm of any cells, although at the early stages of embryogenesis it is this DNA (in addition to mitochondrial), but not nuclear, that makes up the bulk of the cellular DNA [9]. It is known that in differentiated animal cells of the DNA-methylase activity about 90% is localized in the nuclear fraction and only about 10% in the mitochondrial one, but it is practically absent in the soluble cytoplasmic fraction [2, 3]. There are no data at all pertaining to the properties and localization of DNA-methylase in embryonic cells.

This study is an attempt to detect DNA-MA in the developing embryos of loach (*Misgurnus fossilis*) and to investigate its localization in the cell. Special attention has been paid to the study of cytoplasmic DNA-MA. In particular, the ability of this enzyme to methylate the total DNA from the embryos, different somatic cells, sperm of loach and heterologous DNA of *E. coli* has been studied.

* *Abbreviations*:

MC	: 5-methylcytosine
T	: thymine
DNA-MA	: DNA-methylase activity
cDNA	: cytoplasmic DNA
nDNA	: nuclear DNA
$^{14}CH_3$-SAM	: [methyl-^{14}C]-*S*-adenosyl-L-methionine

2. Methods

For studying DNA-MA the loach embryos taken at the blastula and gastrula stages were used, i.e. when DNA replication in the cells is most intense [10, 11]. Fertilization of the eggs, preparation and incubation of blastoderms (embryos without coats) were carried out as described by Kostomarova and Neyfakh [12]. The blastoderms obtained were disrupted by ultrasonic treatment or by homogenization in 0.25 M sucrose, 0.05 M tris-HCl buffer pH 7.8 and 0.004 M K^+-acetate. The homogenates (samples containing about 2 mg of protein) were incubated with 1–2 μCi of [methyl-^{14}C] methionine (sp. activity 14 Ci/mole, Medimpex, Hungary) and 1.5 mg of ATP (Reanal) at 21° for 30–60 min. Extracts from different subcellular fractions (nuclei, mitochondria etc.) containing 0.1–1 mg of protein were incubated (with or without addition of exogeneous DNA) for 60 min at 30° in 1 ml of solution containing 0.2–1 μCi $^{14}CH_3$-SAM (sp. activity 52 Ci/mole, Radiochemical Centre, Amersham, UK). The incubation mixtures were deproteinized by chloroform treatment; DNA was precipitated by ethanol and treated with alkali (0.75 N NaOH, 18 hr, 37°) to remove RNA. DNA was hydrolyzed to acid-soluble products (5% $HClO_4$, 100°, 15 min) or to bases (72% $HClO_4$, 100°, 60 min). Bases were separated by paper chromatography and eluted with 0.1 N HCl [8]. Radioactivity of definite isolated bases or total DNA (acid-soluble products) was measured in a Mark I liquid scintillation counter (Nulcear Chicago).

3. Results and discussion

After incubation of the embryo homogenates with [methyl-^{14}C] methionine the label was found in all DNA bases. Radioactivity in MC was equal to 10% of the total DNA radioactivity (table 1). Thus, together with incorporation of ^{14}C into the rings of the bases, methylation of cytosine residues in DNA of loach embryos also takes place. After incubation of undisrupted blastoderms with [methyl-^{14}C] methionine in the presence of 20 mM K^+-formate the label was found both in nDNA and in cDNA. In both DNA only MC and T were radioactive (table 1).

The total radioactivities of MC and T in nDNA are almost equal (MC/T ratio of specific activities in this DNA is about 20) but in cDNA the total radioactivity of T is three times lower than that of MC (table 1). Thus, in the cells of loach embryos methylation goes on not only in nDNA but also in cDNA. Judging by the radioactivity in T it can be supposed that at the blastula stage both replication and methylation of nDNA occur, but it seems that at the same time cDNA is methylated without significant de novo synthesis. At any rate, cDNA of loach embryos is methylated (i.e. it contains MC) and its methylation seems to take place in the cytoplasm.

After incubation of the extracts obtained from different subcellular fractions of loach embryos with $^{14}CH_3$-SAM (see Methods) practically all the label (about 99%) incorporated in DNA (endogeneous or exogeneous) is localized in MC (table 1). There was no activity in N^6-methyladenine. This corresponds to the fact that in animal tissues methylation of adenine in DNA does not take place [8]. Also, it points to the absence of possible bacterial contamination in the incubation mixtures, as bacterial DNA are known to contain N^6-methyladenine [13].

Unlike in differentiated animal cells [2, 3], in loach-embryo cells almost all DNA-MA is localized in the cytoplasm; the nuclear fraction contains only about 10% of cellular DNA-MA (table 2). In the cytoplasmic fraction a small part of total cellular DNA-MA was detected in mitochondria and microsomes (10% in each). The major DNA-MA of the cells (more than 70%) is localized in the soluble cytoplasmic fraction (table 2). The presence of DNA-MA in the mitochon-

Table 1
Incorporation of the radioactivity from [methyl-^{14}C] methionine and $^{14}CH_3$-SAM into DNA of loach embryos.

Material	Donor of CH_3-groups	Radioactivity incorporated into DNA bases				
		G	C	MC (cpm)	A	T
Homogenates of blastoderms	[methyl-^{14}C] methionine, no K^+-formate	130	110	50	75	135
Undisrupted blastoderms	[methyl-^{14}C] methionine plus K^+-formate	0	0	200	0	115
a) nuclear fraction*		0	0	110	0	90
b) cytoplasmic (anucleate) fraction* (supernatant, 20,000 *g*, 30 min)		0	0	90	0	25
Homogenates of blastoderms	$^{14}CH_3$-SAM	0	0	580	0	5

* Isolated from blastoderms incubated with [methyl-^{14}C] methionine and K^+-formate.

Table 2
DNA-methylase activity in the different subcellular fractions of loach embryos.

Source of DNA-MA (subcellular fraction)	Methyl incorporated (pmoles) from $^{14}CH_3$-SAM	
	into DNA of total fraction	into endogeneous DNA per mg of protein of the same fraction
Nuclei (pellet, 2,000 *g*, 10 min)	21	4.6
Mitochondria (pellet, 20,000 *g*, 30 min)	14	5.8
Microsomes (pellet, 105,000 *g*, 60 min)	27	9.8
Soluble cytoplasmic fraction (supernatant, 105,000 *g*, 60 min)	138	2.6

drial fraction may indicate that loach embryo mitochondrial DNA is methylated and that it contains MC.

When the soluble cytoplasmic fraction of loach embryo cells was incubated with $^{14}CH_3$-SAM without addition ot exogeneous DNA, a marked labelling of endogeneous DNA took place (fig. 1). In other words, DNA-MA detected in the cytoplasm is able to methylate homologous DNA. This is the point of difference between this enzyme(s) and the bacterial ones and a feature making it similar to DNA-MA of rat and mouse spleen [3]. Thus, it could mean that in the loach-embryos cells DNA may be partially 'under-methylated'. In animal cells, unlike in bacterial ones [14, 15], there may exist a certain gap of time between the replication and modification of DNA.

Cytoplasmic DNA-methylase was active in tris-buffer over a rather wide pH range with the maximum activity at pH 7.8 (fig. 2). In K^+-phosphate buffer of the same molar concentration DNA-MA was essentially lower than in tris-buffer. The optimal temperature for cytoplasmic DNA-MA was 30° (fig. 3), that is much higher than that for the development of loach-embryos (21°). During the incubation of the soluble cytoplasmic fraction with $^{14}CH_3$-SAM under standard conditions the amount of incorporated label into endogeneous DNA had a linear dependence on time (at least during the first 60 min) (fig. 4).

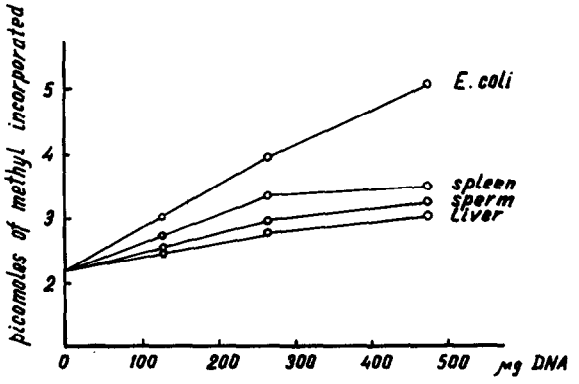

Fig. 1. Incorporation of CH_3-groups into DNA from *E. coli* and different loach tissues. Samples, containing the soluble cytoplasmic fraction (2 mg of protein) were incubated with exogeneous DNA (0–500 μg) and $^{14}CH_3SAM$ (0.5 μCi) in 0.05 mM tris-HCl buffer pH 7.8 at 30° for 60 min (total volume of reaction mixture, 1 ml).

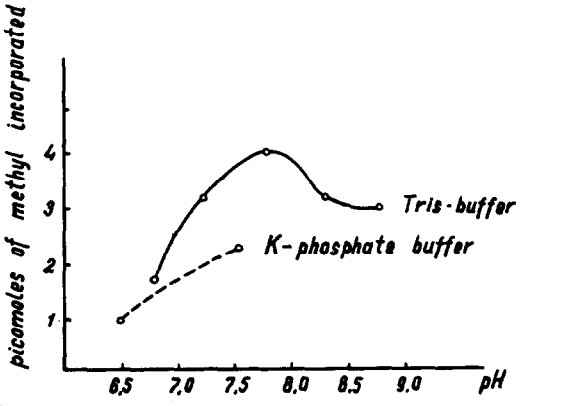

Fig. 2. The effect of pH on the activity of DNA-methylase in the soluble cytoplasmic fraction of loach embryos (incubation mixtures did not contain exogeneous DNA).

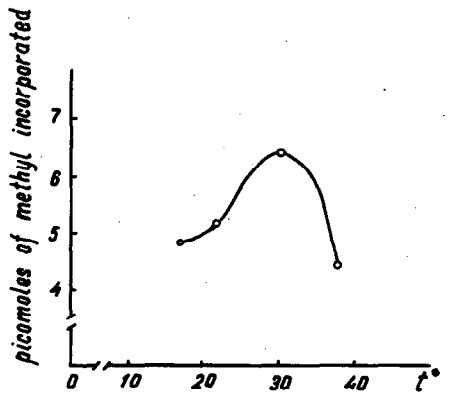

Fig. 3. The effect of temperature of the activity of DNA-methylase in the soluble cytoplasmic fraction of loach embryos.

The incorporation of label from $^{14}CH_3$-SAM was significantly stimulated by the addition of exogeneous DNA from sperm and different loach tissues and especially by foreign DNA of *E. coli* (fig. 1). In the presence of DNA from *E. coli* incorporation of the label increased by more than 300%. Thus, the enzyme from loach embryos, as well as other methylases [1–3] is able to methylate heterologous DNA. The fact that this enzyme methylates DNA from all tested somatic cells and sperm of loach points to the possible presence in the cytoplasmic of DNA-MA which is different from the nuclear only in its specificity. On the other hand, it may also reflect the existence of a different degree of methylation in the different cells or tissues of loach. Earlier we have established that DNA

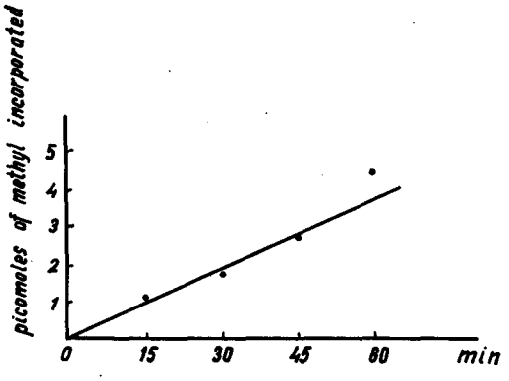

Fig. 4. Incorporation of $^{14}CH_3$-groups from $^{14}CH_3$-SAM into endogeneous DNA of soluble cytoplasmic fraction of loach-embryos (the dependence on time).

from sperm and spleen of different animals contain less MC than DNA from other somatic cells (liver, kidneys and others) [13]; the same is true for DNA from different cells (tissues) of loach. MC content ($M\pm\sigma$) in DNA from spleen, sperm, liver and kidneys of loach is, respectively, 1.24 ± 0.10, 1.35 ± 0.05, 1.42 ± 0.10 and 1.62 ± 0.11 mol.%. There is an inverse relationship between MC content in these DNAs and their ability to accept CH_3-groups from $^{14}CH_3$-SAM in the presence of cytoplasmic DNA-MA in vitro (fig. 1). The less methylated in vivo loach DNA (spleen, sperm) accept more CH_3-groups in vitro. We believe that the degree of DNA methylation in vivo positively correlates with the functional activity of the cell [13, 16], and as it seems it can reflect to some extent the activity level of the particular genes.

The presence of methylated DNA and DNA-MA in the soluble cytoplasmic fraction of loach-embryo cells show that DNA methylation can take place in the cytoplasm.

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