DNA methyltransferase activity in the early stages of a sea urchin embryo Evidence of differential control

Luisa Tosi[a], Francesco Aniello[a], Giuseppe Geraci[b], Margherita Branno[a,*]

[a]*Laboratory of Biochemistry and Molecular Biology, Zoological Station 'A. Dohrn', Villa Comunale, 80121 Naples, Italy*
[b]*Department of Genetics, General and Molecular Biology, University of Naples 'Federico II', Naples, Italy*

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Abstract The specific activity of DNA methyltransferase increases in the nuclei of *Sphaerechinus granularis* sea urchin embryos at increasing stages of development. The activity reaches maximal value at about 20 h of growth, when embryos are at the mesenchyme blastula stage, then abruptly decreases and is essentially zero at about 35 h of development, when embryos are at the early gastrula stage. Both the increase and the drop of the activity are faster than embryonic cell duplication indicating that the enzyme is under strict control during development and that, in the more advanced embryo, a mechanism is activated to specifically block its activity.

Key words: DNA methyltransferase; Enzyme control; Development (*Sphaerechinus granularis*); Proteolysis; DNA methylation

1. Introduction

DNA methylation is an important factor contributing to gene regulation in eukaryotes. In the mouse embryo changes in DNA methylation have been documented in the early stages of development [1,2] and DNA-MTase has been clearly shown to be crucial for embryo development and growth [3]. Changes in DNA methylation have been observed in the developing chick embryo [4]. In the sea urchin, evidence has been presented that DNA methylation is limited to non-transcribing DNA regions [5] but more recent studies show that changes in the DNA methylation patterns of genes occur during sea urchin embryo development [6] and that inducing DNA hypermethylation results in the arrest of embryo development [7]. Similarly, it has been found that exposure of sea urchin embryos to very low concentrations of 5-Azacytidine during one of the four initial cell division periods causes different and reproducible effects on development depending on the particular period of exposure to the perturbant [8]. Intriguingly, 5-Azacytidine has no effect on embryos that are at the 16-cell or at later stages of development. It has also been shown that, in the sea urchin, DNA-MTase is present both as a maternal enzyme in the unfertilized eggs [9] and in the embryos [10] and that the two partially purified activities show different catalytic properties [11].

We show here that in the sea urchin embryo the activity of DNA-MTase is under a strict and different control during the initial stages of development.

2. Materials and methods

2.1. Preparation of sea urchin embryo nuclei

Sphaerechinus granularis sea urchins were obtained from the bay of Naples. Collection of gametes and egg fertilization were performed as described [11]. Embryos were grown in Millipore (Millipore, Milano, Italy) filtered sea water at a temperature of 16°C at a concentration of about 6,000 embryos/ml under constant stirring. The earliest embryonic stage from which nuclei were prepared was at the 12 h of development. Nuclei were prepared free of cytoplasmic contaminants following the reported procedure [9].

2.2. DNA methylase assay

Nuclei corresponding to about 5 mg of proteins were suspended in 250 μl of 100 mM Tris-HCl, pH 8.2, containing 0.8 mM $MgCl_2$, 0.08 M D-Glucose. The mixture was brought to 25°C and the reaction started by adding [^{3}H]CH_3-AdoMet (15 Ci/mmol, Amersham, Milano, Italy) diluted to the specific activity of 1.5–3.0 Ci/mmol, at a final concentration of 25 μM, with unlabelled AdoMet (Boehringer, Mannheim, G). Mixtures were incubated for 30 min at 25 °C and then diluted with 0.5 ml of lysis solution containing 0.15 M NaCl, 0.1 M EDTA, pH 8.0, to which 0.2% SDS was added. The mixtures were heated for 10 min at 60°C and DNA was isolated and purified using standard procedures [12]. DNA-MTase activity was determined following the reported procedure [13]. In short, the purified DNA was hydrolyzed to free base in 90% formic acid in the presence of cold 5-methylcitosine as carrier and the 5-methylcitosine was isolated by paper chromatography. A minimum of 85% of the radioactivity present in the purified DNA samples was recovered in the 5-methylcitosine fraction. Radioactivity measurements were performed in 10 ml of scintillation cocktail (Insta-Gel Packard Instruments B.V., Groningen, NL).

2.3. Limited nuclei proteolysis

The effect of trypsin proteolysis on DNA-MTase activity was studied in the buffer conditions of enzymatic assay. Nuclei were pre-incubated for 10 min at 25°C at a concentration of 15 mg protein/ml. Proteolysis was started by adding different increasing concentrations of trypsin, as indicated in Fig. 1, incubating for additional 10 min and adding a five fold molar excess of soybean trypsin inhibitor (Sigma-Aldrich, Milano, Italy) to stop hydrolysis. The assay for DNA-MTase activity was immediately started by adding the substrate [^{3}H]CH_3-AdoMet. In the experiments performed for the determination of the time course of enzyme activity during development, limited proteolysis was carried out by incubating nuclei for 10 min at a concentration of about 20 mg protein/ml with 10 μg of trypsin/ml, corresponding to 30% maximal enzyme activation (Fig. 1).

2.4. Determination of the rate of DNA synthesis

The procedure to determine DNA Synthesis was essentially as reported [14] with the following modifications. 20 μCi of [^{3}H]thymidine (DuPont NEN, Milano, Italy), specific activity 0.2 mCi/mmol, were added, at the reported times, to 1.8 l of embryo cultures (6,000 embryo/ml). After 30 min of incubation in the presence of the labelled precursor, embryos were collected by centrifugation at 3,000 rpm for 10 min, nuclei were isolated, DNA purified and the incorporated [^{3}H]thymidine

*Corresponding author. Fax: (39) (81) 7641355.

Abbreviations: *S*-Adenosylmethionine, AdoMet; *S*-Adenosylmethionine DNA(cytosine-5) methyltransferase (E.C. 2.1.1.37), DNA-MTase.

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was determined by liquid scintillation counting as reported in the preceding paragraphs.

3. Results

3.1. Enhancement of DNA-MTase activity by trypsin hydrolysis

Conditions for the optimization of the known enhancing effect of trypsin hydrolysis on DNA-MTase activity [9,15] were investigated on nuclei of sea urchin embryos at the swimming blastula and at the gastrula stages. The first stage is that corresponding to the maximal DNA-MTase activity and the second to that in which activity is at the limit of reproducible determination. The effect of trypsin proteolysis on DNA-MTase activity in the sea urchin nuclei (Fig. 1) shows a sigmoidal correlation between concentration of trypsin used in the proteolytic step and the value of the activity determined in the treated nuclei. The effect on DNA-MTase activity caused by exposure of the nuclei to trypsin hydrolysis are so similar for the two investigated stages of development that they can be fitted with only one sigmoid (Fig. 1). It is apparent that at higher trypsin/protein ratios, similarly to what reported [9,15], the value of the enzyme activity detected in the nuclei levels off and, at still higher ratios, decreases. The conditions chosen to increase the sensitivity of the assay in the determination of the time course of enzyme activity during embryonic development were those corresponding to about 30% maximal enzyme activation, in which nuclei are exposed for a period of 10 min at 25°C to a trypsin/protein ratio of 1 to 2,000. This ratio is in the range of values where an almost linear correlation is observed between trypsin concentration and DNA-MTase activity. Electrophoretic analyses (not shown here) of the proteins extracted from nuclei after exposure to trypsin in these conditions, show minor alterations, with respect to control samples, only in the H1 histone bands. It is apparent that the limited proteolysis causes about a 12 fold increase in DNA-MTase activity in the nuclei of embryos both at swimming blastula and early gastrula stages (Table 1). The values of the enzyme activities present in the untreated nuclei of these two stages differ by almost a factor of 10. This shows that DNA-MTase activation by trypsin is not

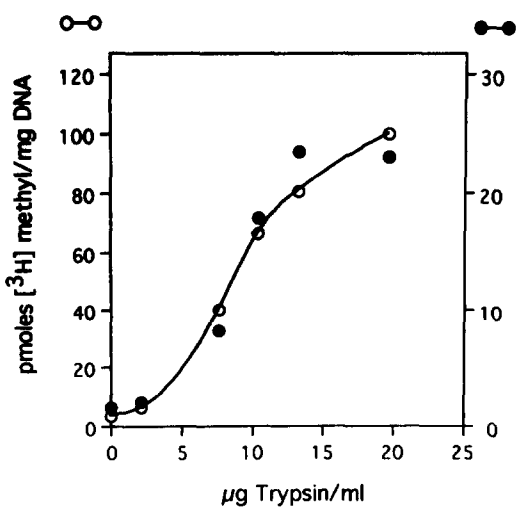

Fig. 1. Effect of trypsin proteolysis on DNA-MTase activity in sea urchin embryo nuclei at two stages of development. (○) Swimming blastula stage; (●) gastrula stage. Conditions as in section 2.

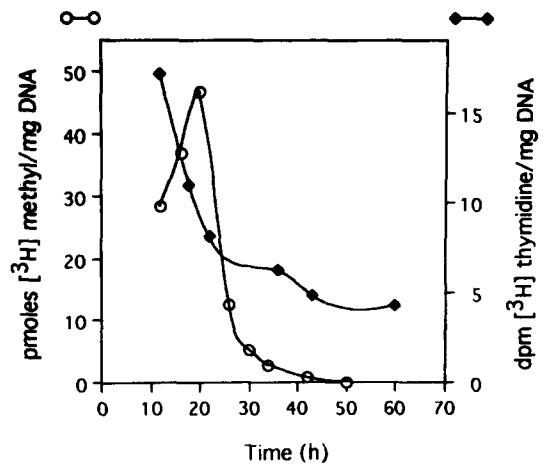

Fig. 2. Time courses of DNA-MTase activity (○) and of rate of DNA synthesis (◆) in the developing sea urchin embryo.

dependent on the initial level of enzyme activity present in the untreated nuclei. For this reason the activity values after trypsin hydrolysis are considered an enhanced and accurate representation of the enzyme activity present in the nuclei.

3.2. Time course of DNA-MTase

The time course of enzyme activity during embryo development, as observed on nuclei after exposure to trypsin proteolysis, is reported in Fig. 2. The enzyme activity is expressed as picomol of incorporated $[^3H]CH_3$- in cytosines/mg DNA to avoid possible alteration of data due to methyl groups deriving from trace amounts of proteins present as DNA contaminants. The value of DNA-MTase specific activity increases by a factor of about 2 from the initial determination on the 12 h embryo to the maximum value occurring at the 20 h of development, although this is the period of maximal rate of DNA synthesis (Fig. 2). After this stage, DNA-MTase activity abruptly decreases and in only 5 h, when the embryo shows initial gastrulation, is about 25% of the peak value. At the 35 h of development the activity is undetectable in the untreated nuclei and about 5% of the peak value in the trypsin-activated nuclei. At later stages, up to the 50 h of development, DNA methylase is zero under any assay condition.

Table 1
DNA-MTase activity in sea urchin embryo nuclei: effect of limited trypsin proteolysis

	Nuclei		Activity ratio treated/control
	untreated	Treated	
	pmol[^{3}H]-methyl/mg DNA		
Embryonic Stage			
Blastula	2.8	32.3	11.9
Early gastrula	0.4	4.7	11.8

Nuclei were exposed to trypsin proteolysis for 10 min at 25°C at a trypsin/protein ratio of 1 to 2,000, in the conditions for the determination of methylase activity. The reactions were stopped by addition of soybean trypsin inhibitor and enzyme activity immediately determined in the mixture by adding radioactive substrate. For other details see section 2.

4. Discussion

The relevance of DNA-MTase activity in the early stages of sea urchin embryo development, suggested by previous experiments [6,8,11], finds support in the results reported here. The most interesting aspect is that DNA-MTase is under a strict control with a minimum of two opposite mechanisms operating in the embryo. An initial one stimulating enzyme activity. Evidence of this is the *increase* of the specific activity of DNA-MTase observed in nuclei of cells up to the stage of blastula with primary mesenchyme in a period in which the rate of DNA synthesis is maximal increased (Fig. 2). If the enzyme activity in the newly produced embryonic cells had increased only proportional to DNA, its specific activity, referred to DNA, should have remained constant. While the enzyme specific activity increases faster than cell duplication before the 20 h of development, it suddenly *decreases* in the nuclei of more advanced embryos. This decrease is faster than expected assuming that the enzyme synthesis is stopped and its activity diluted in the dividing cells. In fact, while the number of cells in the embryo is less than doubled between blastula and prism stages [16], that differ in time by about 30 h, DNA-MTase activity drops, in a shorter period, from the maximum value of about 50 pmol/mg DNA/30 min to neglegible amounts. This shows that, after the blastula stage, the embryo activates a second mechanism to block DNA-MTase activity as if the enzyme had different relevance in the initial and in the later stages of development. In addition, the drop of activity appears to be specific of DNA-MTase because it occurs at a stage earlier than that at which maternally expressed genes change their patterns of expression [17]. The pattern of DNA-MTase activity in the developing sea urchin embryo is that of an enzyme required only at particular stages of the initial cleavage period to set signals in the genome. Once signals have been set, the enzyme activity seems no more required. However, the rapid drop of the activity occuring immediately after the blastula stage suggests that the embryo activates a mechanism to eliminate DNA-MTase activity as if the presence of the enzyme were deleterious for further development.

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