EFFECT OF TRYPSIN ON DNA METHYLATION IN ISOLATED NUCLEI FROM DEVELOPING SEA URCHIN EMBRYOS[+]

by

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Summary: Nuclei isolated from the developing sea urchin embryo Paracentrotus lividus and incubated in the presence of [^{3}H-methyl] S-adenosylmethionine methylate their own DNA. Addition of small amounts of trypsin produces a 20-fold increase in DNA methylation. The time kinetics and the specificity of the trypsin activation of DNA methylation are described. The only products of the reaction are 5-methylcytosine and thymine. DNA adenine, guanine and cytosine are not labeled. The distribution of the counts between 5-methylcytosine and thymine is variable. While 5-methylcytosine originates by enzymatic methylation of DNA cytosines, the origin of the labeled thymine cannot be inferred with certainty.

Isolated nuclei from developing sea urchin embryos incubated in the presence of SAM[1] methylate their own DNA (1). The products of the reaction are 5-methylcytosine and thymine. We have called the thymine, which appears to originate at the DNA level, DNA minor thymine or DNA methylase thymine to distinguish it from the major thymine or polymerase thymine (1-5). From our previous *in vivo* (6,7) and *in vitro* (1) work it is clear that in the developing sea urchin embryos DNA 5-methylcytosine is synthesized at the polymer level by direct transfer of the methyl group of SAM to specific DNA cytosines. More complex, however, is the problem of the mechanism by which the intact methyl group of SAM is found in thymine of DNA isolated from developing sea urchin embryos *in vivo* (5) and *in vitro* (1) experiments. In the *in vitro* work the distribution of the methyl label of SAM between 5-methylcytosine and thymine varies in different preparations of nuclei. The per cent of the methyl label in thymine over that in 5-methyl cytosine ranges from 2% to 50% with nuclei isolated from developing *Sphaerechinus granularis* embryos.

[+]These findings were presented at the EMBO Workshop 'Biological Role of S-Adenosylmethionine, Methyl Transfer and Polyamine Biosynthesis', June 1973, Gif-sur-Yvette, France, and at the 9th International Congress of Biochemistry, July 1973, Stockholm, Sweden.

[1]The abbreviations used are: SAM, S-adenosyl-L-methionine; ^{3}H-SAM, [^{3}H-methyl] S-adenosyl-L-methionine; SDS, Sodium dodecyl sulfate.

The lack of reproducibility of the results in vitro prompted us to search for other experimental approaches. Because extensive attempts at enzyme purification failed, we thought that DNA modifications in eukaryotes probably should not be considered as a simple interaction between DNA and an enzyme and that a complex DNA modification apparatus might be involved in the DNA enzyme modifications of the eukaryote chromosome. To probe the putative DNA modification apparatus we decided to study the effect of proteolytic enzymes on the DNA methylation by isolated nuclei.

The aim of this paper is to report on a very striking activation by small amounts of trypsin of the DNA methylation in isolated nuclei of the developing sea urchin embryo Paracentrotus lividus. Moreover, data on the distribution of the label from the methyl group of SAM between DNA 5-methylcytosine and DNA thymine in the same system will be included.

MATERIALS AND METHODS

The materials used were obtained as indicated: ^{3}H-SAM from New England Nuclear Corp. Glucose, sucrose, Tris and EDTA from Merck A.G., Darmstadt. SDS from K & K Laboratories, N.Y. Insta-Gel Emulsifier from Packard Instrument Corp. Trypsin from bovine pancreas, cytochrome c from heart, papain and pronase from Sigma. Pancreatic deoxyribonuclease, ribonuclease, chymotrypsin and pepsin from Worthington. Subtilisin and thermolysin from Nutritional Biochem. Corp. and Calbiochem, respectively. All the enzymes were of the highest purity available.

Pronase was heated at pH 5 and 80°C for 10 min before use. Ribonuclease was heated in 0.15 M NaCl at 80°C for 10 min before use.

All other chemicals were reagent grade of the highest purity available.

Sea urchins. Paracentrotus lividus was obtained from the Zoological Station, Naples. Egg collection, fertilization and embryo cultures were performed as previously described (7).

Isolation of nuclei. The nuclei were isolated as previously described (1). The contamination of the nuclei by cytoplasmic components was evaluated by measuring the cytochrome-oxidase activity. The isolated nuclei contained from 1/15 to 1/30 of the cytochrome-oxidase activity of the cytoplasm. In the experiments were used those nuclei preparations that had at most 1/20 the cytochrome-oxidase activity of the cytoplasm.

The cytochrome-oxidase activity was measured as previously described (1). Protein was estimated according to Lowry et al. (8).

DNA was determined from the absorbancy at 260 nm. One mg of DNA/ml was considered to have an absorbancy of 21. In a few instances the spectrophotometric determination was checked with the diphenylamine method described by

TABLE I

Effect of Trypsin on Methylation of DNA and RNA in Nuclei Isolated from Paracentrotus lividus Embryos

Trypsin µg/ 0.3 ml	DNA mg	mg DNA / mg protein	DNA ^{3}H-DPM	^{3}H DPM / mg protein	^{3}H DPM / mg DNA	Activation of DNA methylation	RNA mg	^{3}H DPM / mg RNA
0	1.3	.19	10,259	1,466	7,820	1	1	74,345
3	1.2	.17	110,254	15,751	90,150	11.5	1	80,689
4	1.2	.17	148,885	21,269	128,018	16.4	1.1	61,109
5	1.1	.16	153,233	21,890	144,016	18.4	1	78,611
6	1.4	.2	217,857	31,122	160,189	20.5	1.2	60,103
8	1.3	.19	199,045	28,435	148,986	19.0	1.2	76,670

The nuclei were prepared from Paracentrotus lividus embryos at the blastula stage. Each reaction mixture contained in a final volume of 0.3 ml: 40 µmoles Tris; 5 µmoles KCl; 7.5 nmoles ^{3}H-SAM, 4.5 µCi·nmole^{-1}; nuclei containing 7 mg protein; trypsin as listed. pH, 8.2; temperature 26°C; incubation time 30 min. At the end of the incubation 0.6 ml of 0.15 M NaCl, 0.1 M EDTA pH 8.0 and 30 µl of SDS were added. After 10 min at 60°C, the samples were cooled to 4°C and brought to 1 M final concentration of $NaClO_4$ with a 2 M solution of the salt. An equal volume of chloroform-isoamyl alcohol (24:1, v/v) was added and the suspension shaken for 40 min. After centrifugation the water phase was collected and an equal volume of isopropylalcohol was added to it. The samples were kept overnight at -20°C. The precipitate collected by centrifugation was dissolved in 1 ml of 0.1 M Tris-HCl, 10 mM $CaCl_2$, pH 8.2. 200 µg of pronase were added and the samples were incubated for 5 hours at 37°C. 1 ml of cold 10% $HClO_4$ was added and the samples were kept for 1 hour at 4°C. The precipitate collected by centrifugation was dissolved in 1 ml of 0.5 M NaOH and kept at 60°C for 20 min. After cooling 30 µl of concentrated HCl and 1 ml of cold 10% $HClO_4$ were added. After standing for 1 hour at 4°C the precipitate was collected by centrifugation. The NaOH hydrolysis was repeated once. The two supernatants were neutralized and pooled together. They contained the hydrolyzed RNA which was determined by the A_{260} nm. The precipitate was washed twice with cold 5% $HClO_4$ and was dissolved in 1 ml of water by adjusting the pH to 6-7 with NaOH. The DNA was determined by the A_{260} nm. Aliquots of the solutions of the two nucleic acids were hydrolyzed in 0.5 M $HClO_4$ at 90°C for 60 min. Radioactivity was measured with a Packard Tri-Carb liquid scintillation spectrometer Model 3375. 1 ml of the DNA or of the RNA solutions was added to 10 ml of Insta-Gel Emulsifier Packard. Quenching corrections were made by the channel ratio method.

Burton (9). The determination of RNA was performed spectrophotometrically at 260 nm. One mg of RNA per ml was considered to have an absorbancy of 28.

The assay of DNA methylase activity is based on the incorporation of ^{3}H from ^{3}H-SAM into DNA of the isolated nuclei incubated in vitro. The assay is described in the legend of Table I.

Hydrolysis of DNA to free bases and paper chromatography. The water solution of DNA prepared as described in Table I was dried under reduced pressure at room temperature. The dried DNA was dissolved in 85% HCOOH, 0.5 ml per 1 mg DNA, and heated in a sealed vial at 175°C for 30 min. The solution was dried as above and the residue taken up with a small amount of 0.01 N

TABLE II

Specificity of the Trypsin Effect on DNA Methylation

Addition µg/0.3 ml	^{3}H DPM / mg DNA	Activation of DNA methylation	Addition µg/0.3 ml	^{3}H DPM / mg DNA	Activation of DNA methylation
None	4,472	1	Papain		
Trypsin			2	12,521	2.8
6	107,335	24	5	16,099	3.6
Chymotrypsin			10	17,890	4
6	8,940	2	13	19,232	4.3
14	9,843	2.2	19	20,125	4.5
26	13,420	3	Subtilisin		
54	21,022	4.7	2.7	13,418	3
108	7,155	1.6	5.4	16,995	3.8
Pepsin			10.8	19,676	4.4
6	4,919	1.1	Thermolysin		
14	5,366	1.2	3.3	16,090	3.6
56	7,155	1.6	19.2	19,677	4.4
112	4,920	1.1	38.4	32,646	7.3
			57.6	37,118	8.3

The DNA methylase activity was measured as described in Table I. The amount of proteolytic enzymes added per incubation mixture, final volume 0.3 ml, is listed.

HCl. Paper chromatography was performed as previously described (7). The bases were separated with solvent 1 and brought to constant specific activity by rechromatography in solvent 2 and solvent 4. In a few instances the thymine spot was rechromatographed in solvent 5 and no change in its specific activity resulted.

RESULTS

Action of Trypsin. The data in Table I show that addition of trypsin to nuclei of Paracentrotus Lividus embryos incubated in the presence of SAM activates DNA methylation up to 20-fold, while the methylation of RNA is not affected. The maximal activation is obtained with 6 µg of trypsin per 0.3 ml of incubation mixture. At 3 µg, 54% of the maximal activation is obtained.

The time dependence of DNA methylation in the presence of trypsin is not linear. The rate of the reaction from 5 to 10 min is twice that from 0 to 5 min; it is almost constant for the next 10 min and then declines. 79% of the whole reaction in 60 min occurs in the first 20 min.

DNA is the product of the reaction because the product is hydrolyzed by pancreatic DNA-ase and it is not hydrolyzed by RNA-ase and by pronase. Moreover, the ^{3}H-DNA, analyzed by CsCl equilibrium density gradient centrifugation, sediments as one band and the same band is obtained after RNA-ase digestion and after pronase digestion.

TABLE III

Distribution of the Label into ^{3}H-DNA between 5-methylcytosine and thymine

Trypsin µg/0.3 ml	Thymine		5-methylcytosine	
	DPM	%	DPM	%
0	2,900	30	6,900	70
6	3,000	3	100,000	97
6	4,400	4	105,600	96
6	4,600	6	72,100	94
6	13,000	19.4	54,000	80.6
6	11,000	12.8	75,000	87.2

The incubation mixtures were as in Table I. Incubation time 20 min. All the experiments, except for the first two, were performed with different preparations of nuclei.

Specificity of the activation of DNA methylation. The data in Table II show that trypsin is by far the most active among the proteolytic enzymes that were tested. Pancreatic deoxyribonuclease tested at concentrations from 0.3 ng up to 8 µg per ml of incubation mixture both in the presence and in the absence of trypsin has no effect.

Distribution of the label into DNA bases. The bases were obtained from the ^{3}H-DNA as described under Methods.

In experiments performed as in Table I in the presence of 6 µg of trypsin per 0.3 ml of incubation mixture from 90 to 100% of the label in ^{3}H-DNA is found in 5-methylcytosine and in thymine. Adenine, guanine and cytosine are not labeled.

The distribution of the radioactivity between 5-methylcytosine and thymine is variable. In many experiments from 98 to 99% of the label was found in 5-methylcytosine and only from 1 to 2% in thymine. However, the results in Table III show that in some experiments a higher amount of label is found in thymine. The results might depend on the batch of the sea urchin embryos and on the preparation of nuclei.

DISCUSSION

The experiments performed with nuclei isolated from the developing sea urchin embryo Paracentrotus Lividus reported in the present paper will be briefly discussed under two headings: 1) the striking activation of DNA methylation by trypsin, which by using the terminology of enzyme chemistry could be referred to as a 20-fold increase in the total units of DNA methylase activity (Table I), 2) the distribution of the label of the methyl group of SAM between DNA 5-methylcytosine and DNA thymine.

Activation of DNA methylation by trypsin. No change in permeability of the nuclear membrane to SAM appears to play a role in the activation of DNA methylation because the methylation of RNA is not affected (Table I). The same argument suggests that no change occurs in the internal pool of SAM in the nuclei caused by trypsin.

A DNA-histone linear complex coiled to generate a fiber is thought to be a basic element of the chromosome of all higher organisms (10). The specificity of trypsin for the basic amino acid side chains that appear to be involved in bonds to DNA and its effect on the structure of chromosomes and of nucleohistone fibers (10) suggest that unmasking of DNA methylation sites might be implicated in the activation. However, it should be noted that Chatterjee and Walker (11), using about the same concentration of trypsin which has been used in the present work, have found partial degradation of histones only after 3 hours incubation of nucleohistones with the enzymes. Thus, the simple interpretation of unblocking of DNA methylation sites by loss of histones from the chromosome should not be taken for granted. A more subtle mechanism involving the action of a basic protein, which would be a component of a complex DNA methylation apparatus, might be a better explanation of our results.

The lack of any action on DNA methylation by DNA-ase added to the incubation mixtures suggests that no effect of activation of DNA is involved as that which occurs for DNA when it is used as substrate for DNA polymerase (12).

Several other mechanisms can explain the activation of DNA methylation as, for instance, activation of DNA-cytosine methylase(s), production of an activator from an inactive preactivator protein, inactivation of an inhibitor protein. But whatever the mechanism a protein(s) rich in arginine and lysine appear(s) to be involved in the activation of DNA methylation.

The time dependence of DNA methylation in the first 10 min is linear in the absence of trypsin (1) while in the presence of trypsin the rate increases at least in the first 5 min. Thus the time kinetics in the presence of trypsin suggests either time increasing activation of the enzyme methylation complex or increase in the first minutes of incubation of unblocking of DNA methylation sites.

We will defer to a later publication results and discussion on the type of DNA sites that are methylated in the experiments described here, namely, if the methylated DNA sites are physiological sites which should be methylated also *in vivo* either on completion of the chromosome replication cycle or later upon development as predicted by the synchron model (1-5). It is also possible, however, that the sites are abnormal, namely, sites which would never be methylated *in vivo* (non-physiological sites), unmasked by the conditions of the *in vitro* experiments.

We are investigating whether the trypsin activation of DNA methylation exists also in animal tissues where no cell multiplication occurs.

Distribution of the label between DNA-5-methylcytosine and DNA-thymine. As reported under results and in a previous publication (1), the only DNA bases which are labeled in the experiments with isolated nuclei from developing sea urchin embryos incubated in the presence of isotopic methyl SAM are 5-methylcytosine and thymine. However, the distribution of the label between the two bases varies from experiment to experiment. The distribution might depend on the species of the sea urchin and on the stage of the embryo used as well as on the method of the preparation of the nuclei. In the instances in which more than 10% of the label is found in thymine (Table III) it seems unlikely that the labeled thymine originates because of some technical problem in the isolation and purification procedure of the base. However, at the present stage of the work no mechanism of the origin of the labeled thymine can be inferred with certainty.

It should be remarked that the addition of trypsin appears especially to increase the methylation of DNA cytosine. On the basis of the hypothesis of the DNA-modification complexes, namely, the DNA-cytosine methylase apparatus and the DNA-5-methylcytosine aminohydrolase apparatus (1) it is likely that the methods which permit to detect one complex might not be those optimal for the detection of the other. Recent work in our laboratory suggests that changes in the preparation of nuclei and in the conditions of incubation increase the amount of the label in thymine.

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