CORRELATION BETWEEN SYNTHESIS AND METHYLATION OF DNA IN HELA CELLS+

Domenico Geraci, Tamilla Eremenko, Roberta Cocchiara, Aldo Granieri, Eduardo Scarano and Pietro Volpe

International Institute of Genetics and Biophysics, Naples, and Chair of Molecular Biology, University of Naples, Italy

Received January 28, 1974

SUMMARY - For the whole cell cycle the methylation of DNA was studied in synchronized HeLa cells and in nuclei isolated from them. In the intact cells the methylation of DNA cytosine runs parallel to DNA synthesis. The pattern of DNA cytosine methylation by the isolated nuclei is almost identical to that obtained with the whole cells. Since the isolated nuclei do not synthesize DNA, it is shown that DNA methylation continues for at least 30 min after DNA synthesis is over. No DNA minor thymine is found in the isolated nuclei.

In the cells of higher organisms the only minor methyl base found in DNA is 5-methylcytosine (1-3). In eukaryotes as in bacteria the methyl group of DNA 5-methylcytosine originates from the methyl group of SAM[1].

The mechanism of DNA methylation in vivo is, however, very poorly understood. Kappler (3) has recently suggested that in animal cell DNA methylation follows DNA synthesis by about 2 min. Other authors (5-8) have suggested that methylation may continue for some hours after synthesis of DNA is completed.

Isolated nuclei, which in the absence of deoxynucleoside triphosphates do not synthesize DNA (9), are able to methylate their own DNA (10-12). They appear to be, thus, a useful tool for studying the mechanism of DNA methylation.

In this paper experiments are reported on the correlation between DNA synthesis and DNA methylation in synchronized HeLa cells and in nuclei isolated from them at hourly intervals during the cell cycle.

+ These findings were presented at the 9th International Congress of Biochemistry, July 1973, Stockholm, Sweden. Abstract book, 3m64.

1 Abbreviations: ^{14}C-MET, [^{14}C-methyl] L-methionine; SAM, S-adenosyl-L-methionine; ^{3}H-SAM, [^{3}H-methyl] S-adenosyl-L-methionine; SDS, Sodium dodecyl sulfate.

Address: International Institute of Genetics and Biophysics, Via Marconi 10, 80125 Naples, Italy.

MATERIALS AND METHODS

The materials used were obtained as indicated: ^{14}C-MET (1 m Ci/mg) and ^{3}H-SAM (4.55 Ci/m-mole) from New England Nuclear Corp.; Tris and EDTA from Merck A.G., Darmstadt; SDS from K&K Laboratories; POP and POPOP from Packard Instrument Corp.; pronase B grade pure from Calbiochem. Pronase was heated at 80°C pH 5 for 10 min before use.

1. Cell synchronization. HeLa cells were subcultured in Joklik-modified minimum essential medium. Calf serum was added to a final concentration of 10% to the suspension media. The cultures were maintained in spinners at 37°C ± 0.2 and aerated by a constant flow of 5% CO_2 in air (13). Cells were synchronized with a double thymidine block (14, 15). They were sedimented in a Sorvall GSA rotor at 1,000 rev/min for 10 min and resuspended in 4 l of medium containing 2 mM thymidine to initiate synchronization at the density of 0.5 x 10^6 cells/ml. After 24 hr the cells were sedimented, resuspended in fresh medium without thymidine and left to grow under these conditions for 8 hr. At this time a second thymidine block was given after which the cells, when replaced into normal medium without thymidine, entered the phase S.

2. Preparation of nuclei. These were isolated with minor modification of the method proposed by Penman (16). At hourly intervals throughout the mitotic cycle, aliquots of synchronized cell suspension containing 100 x 10^6 cells were centrifuged in a Sorvall GSA rotor at 1,000 rev/min. The harvested cells were washed in a large volume of fresh minimum essential medium, resuspended in 5 ml of buffer (0.01 M NaCl; 0.0015 M $MgCl_2 \cdot 6H_2O$; 0.01 M Tris-HCl pH 7.4), kept in ice for 5 min and then homogenized in a teflon potter for 2 min. Nuclei were precipitated at 1,500 g for 90 sec and washed in 0.1 M Tris-HCl pH 7.4.

3. Labeling in vivo and in vitro. In the in vivo experiments, 100 x 10^6 cells were suspended in 25 ml of medium lacking methionine and incubated with 0.225 mCi of ^{14}C-MET for 1 hr. The methionine lacking medium was supplemented with 10% calf serum previously depleted of free amino acids on Sephadex G-50 (17). The labeling was performed at 37°C under stirring and a constant flow of 5% CO_2 in air. In the in vitro experiments, the purified nuclei from 100 x 10^6 cells were incubated for 30 min at 37°C in 300 µl of 0.003 M Tris-HCl pH 7.4, containing 25 µCi of ^{3}H-SAM.

4. DNA extraction and base separation. Both in the in vivo and in vitro sets of experiments, the purified nuclei were treated with three volumes of 1% SDS-0.004 M EDTA. They were incubated with 1 mg/ml pronase overnight at 37°C. The mixture was then brought to 0.5 N NaOH and left for 16 hr at 37°C. After the addition of 2 mg carrier calf thymus DNA, $HClO_4$ was added to a final concentration of 5%. DNA was then centrifuged at 10,000 g and washed three times with cold 0.5% $HClO_4$. The hydrolysis to bases was performed with 60% $HClO_4$ (15 µl/mg DNA) at 100°C for 1 hr. The hydrolyzate was brought to a volume of 0.3 ml by lyophilization and spotted on 3 MM Whatman paper (5 A.U. 260 nm/cm). The four bases were separated in i-propanol-HCl-H_2O (65:16.7:18.3). Each base was then purified in three sequential unidimensional chromatograms using i-propanol-H_2ONH_4OH (85:15:1.3) as solvent. The guanine spot was eluted with 0.1 N HCl, the others with water. 5-methylcytosine was not separated from cytosine because no label enters the pyrimidine ring. The eluted material was dissolved in Bray's solvent and its radioactivity measured in a Packard scintillation radiospectrometer.

RESULTS AND DISCUSSION

The utilization of the methyl group of methyl-labeled L-methionine for DNA synthesis in HeLa cells cultured as indicated here is similar to that in developing sea urchin embryos (1, 3). Beside the methylation of DNA cytosines by SAM, the label from the methyl group of L-methionine by oxidation to intermediates of the one carbon unit enters the methyl group of thymine and the purine ring of adenine and guanine. No label from the methyl group of L-methionine enters the pyrimidine ring. By measuring the rate of incorporation of ^{14}C from the methyl group of L-methionine into DNA adenine, guanine and thymine it is possible to evaluate the rate of DNA synthesis. The rate of DNA methylation is determined by the incorporation of ^{14}C from the methyl group of L-methionine into DNA 5-methylcytosine. The determination of the rates of DNA synthesis and DNA methylation depends on the assumption that the internal pools of L-methionine and DNA precursors do not appreciably change during the cell cycle under the conditions of the present experiments. Therefore, in HeLa cells DNA synthesis and DNA methylation can be studied by using only ^{14}C-MET.

Samples of synchronized HeLa cells were withdrawn at hourly intervals during the cell cycle, starting from the time of entering the S-phase and

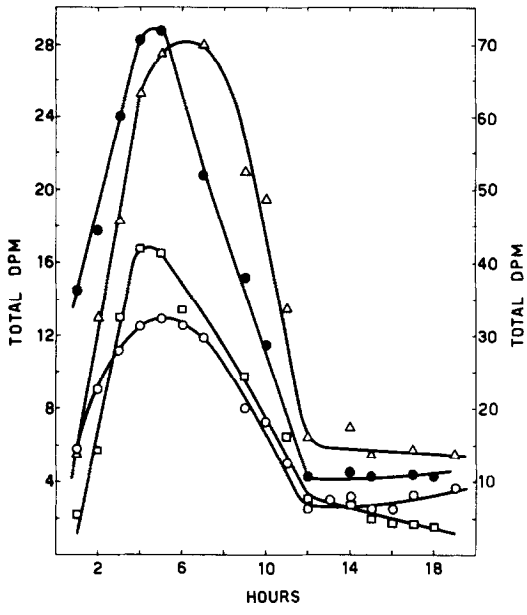

Fig. 1 - DNA synthesis and DNA methylation in vivo. The set of experiments follows at hourly intervals the whole HeLa cell mitotic cycle, starting from removal of thymidine from the culture medium. Left ordinate: (●-●), adenine; (o-o), 5-methylcytosine. Right ordinate: (Δ-Δ), thymine; (□-□), guanine.

---

were incubated with ^{14}C-MET. Fig. 1 shows the labeling of the isolated DNA bases. The label in adenine, guanine and thymine is proportional to DNA synthesis and the label in 5-methylcytosine is proportional to DNA methylation. This demonstrates that DNA methylation parallels DNA synthesis. The slight displacement of the peak of thymine labeling with respect to the peaks of labeling of adenine, guanine and 5-methylcytosine might be explained by a time decrease of the pool of DNA thymine precursors because the cells were synchronized by the deoxythymidine double block method.

The isolated nuclei incubated in vitro in the presence of ^{3}H-SAM methylate their own DNA and the only product of the DNA methylation is 5-methylcytosine (Fig. 2). Moreover, the shape of the curve is identical to that of 5-methylcytosine of Fig. 1. Namely, the in vitro rate of DNA methylation with isolated nuclei is proportional at each point of the cell cycle to the in vivo rate of DNA methylation. Because in the isolated nuclei no DNA synthesis occurs, the

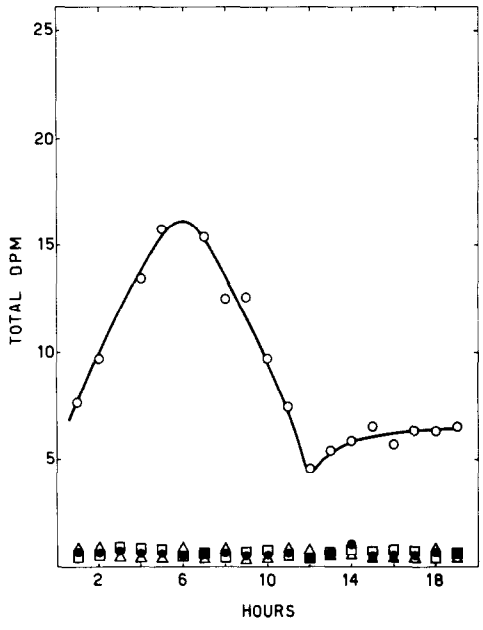

Fig. 2 - DNA methylation in vitro. The set of experiments follows the whole HeLa cell mitotic cycle, starting from removal of thymidine from the culture medium. Nuclei were isolated and treated at hourly intervals, as in Methods. (●-●), adenine; (Δ-Δ), thymine; (□-□), guanine; (o-o), 5-methylcytosine.

data suggest that DNA methylation continues for at least 30 min after DNA synthesis is over.

The data suggest that DNA methylation is proportional to the new DNA synthesized and not to changes of DNA methylase activity. The results obtained with the isolated nuclei demonstrate, on the other hand, that DNA methylation continues for at least half an hour when DNA synthesis is not occurring.

It is interesting that in nuclei, isolated from the nondifferentiating stabilized HeLa cells, and incubated in the presence of ^{3}H-SAM, no label is found in DNA thymine, while in nuclei isolated from developing sea urchin embryos and incubated as above there is preliminary evidence of the occurrence of a DNA minor thymine (18, 19).

REFERENCES

1. Scarano, E., Iaccarino, M., Grippo, P. and Winckelmans, D., J. Mol. Biol. 14, 603 (1965).
2. Brown, G.-M. and Attardi, G., Biochem. Biophys. Res. Commun. 20, 298 (1965)

3. Grippo, P., Iaccarino, M., Parisi, E. and Scarano, E., J. Mol. Biol. 36, 195 (1968).
4. Kappler, J. A., J. Cell Physiol. 75, 21 (1970).
5. Burdon, R. H. and Adams, R.L.P., Biochim. Biophys. Acta 174, 322 (1969).
6. Adams, R.L.P., Biochim. Biophys. Acta 254, 205 (1971).
7. Adams, R.L.P., Nature New Biology 244, 27 (1973).
8. Evans, H.H., Evans, T.E. and Littman S., J. Mol. Biol. 74, 563 (1973).
9. Lynch, W.E., Brown, R.F., Umeda, T., Langreth, S.G. and Lieberman, I., J. Biol. Chem. 245, 3911 (1970).
10. Morris, N.R. and Pih, K.D., Cancer Res. 31, 433 (1971).
11. Burdon, R.H., Biochim. Biophys. Acta 232, 359 (1971).
12. Tosi, L., Granieri, A. and Scarano, E., Exptl. Cell Res. 72, 257 (1972).
13. Volpe, P. and Eremenko, T., Eur. J. Biochem. 32, 227 (1973).
14. Puck, T.T., Cold Spring Harb. Symp. Quant. Biol. 29, 167 (1964).
15. Volpe, P. and Eremenko, T., Methods in Cell Biology, Ed. by D.M.Prescott, Academic Press, N.Y., vol. VI, Chapter 4, p.113-126 (1973).
16. Penman, S., J. Mol. Biol. 17, 117 (1966).
17. Piez, K.A., Oyama, V.I., Levintow, L. and Eagle, H., Nature 183, 59 (1959).
18. Grippo, P., Parisi, E., Carestia, C., Scarano, E., Biochemistry 9, 2605 (1970).
19. Tosi, L. and Scarano, E., Biochem. Biophys. Res. Commun. 55, 470 (1973).