Vol. 95, No. 2, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 31, 1980 Pages 630-637

DNA METHYLATION OF LIVER AND HTC CELLS DURING CORTICOSTEROID INDUCTION

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Received May 16, 1980

SUMMARY: The status of DNA methylation, as measured by m^5C[2] content of nuclear DNA, was examined during corticosteroid mediated TAT induction in rat liver and in dividing and nondividing HTC cells. The m^5C content, determined by HPLC, was not significantly altered in HTC cells during TAT induction whether the cells were in the logarithmic or stationary growth phase. In the liver of adrenalectomized rats where the range of corticosteroid effects is greater than in HTC cells, a small but significant decline in genomic levels of m^5C was detected between 1 to 8 hr post-induction. The alterations in DNA methylation did not fluctuate during induction by more than 8% in the liver or 7.5% in HTC cells. These results demonstrate that no gross change or elevation in m^5C content is detected in two, different, hormonally responsive hepatocellular systems during gene activation.

Eukaryotic DNA is modified by methylation of a small proportion of cytosine residues (0.1 - 8.0%) forming the methylated base m^5C (1). A number of studies have reported alterations in genomic levels of DNA methylation in various tissues (2,3), between germ and somatic cells (4), and during development (5,6) and aging (7). However, several of these reports that indicated differences between tissues and during development have been controverted by others who demonstrated constant levels of methylation (8,9). Although the function of DNA methylation is still obscure in higher eukaryotes, recent findings in several laboratories have correlated gene activity with undermethylation (10-12). These studies have been limited to the analysis of changes in methylation of CCGG sequences detected by cleavage with the isoschizomer pair of restriction enzymes HpaII/MspI (13-15). However, although methylation occurs primarily in the dinucleotide CpG (16,17), evidence, including that from direct sequencing of satellite repeats (18,19), DNA with other restriction enzymes (8), indicate that other methylated sequences may be

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[2]Abbreviations used are: m^5C, 5-methylcytosine; HPLC, high pressure liquid chromatography; HTC, hepatoma tissue culture; TAT, tyrosine aminotransferase

0006-291X/80/140630-08$01.00/0

functionally significant. Hence, the study of genomic level of methylation which encompasses all sequences is required for a more exact correlation with DNA function, until methods become available to study the status of individual sequences.

Recently, Vanyushin and colleagues (7,20) have reported that corticosteroids caused a rapid and marked elevation in the methylation of DNA which suggested a more direct or specific role of DNA methylation. Under corticosteroid stimulation, the liver begins active RNA synthesis (21) including ribosomal RNA (22) that is reflected in a change in availability of chromatin-DNA template to transcription (23). Several proteins, notably TAT, alanine aminotransferase, glutamine synthetase, and tryptophan oxygenase (24,25), are induced in the liver. Rat HTC cells retain the ability to synthesize TAT and for this reason have been used as a model system to study corticosteroid action (26,27).

To attempt a more exact correlation between the induction of a prototypic enzyme, TAT, with the level of DNA methylation, we analyzed the nuclear DNA of HTC cells and the liver of adrenalectomized rats under corticosteroid stimulation. To determine the actual content of m^5C we determined the absolute levels of nucleotides by HPLC.

MATERIALS AND METHODS

Animals and Cells

Male Sprague Dawley rats, weighing 125 - 150 g, underwent adrenalectomy. These animals were fed a normal diet ad libitum with 0.9% saline instead of water for a period of 8 days prior to corticosteroid exposure. Hydrocortisone suspension (Sigma, 10 mg/ml in sterile saline) was administered intraperitoneally at a dose of 200 mg/kg body weight. Animals were injected at 9:00 a.m. in order to avoid diurnal variations in responsiveness (28).

HTC cells (provided by Dr. R. Stellwagen) were maintained in spinner culture using tricine buffered Swim's S77 media (Gibco) containing 10% fetal calf serum (Colorado Serum) and supplemented with additional glucose, glutamine, and bicarbonate according to Thompson et al. (26). Cells were maintained in logarithmic growth by splitting and refeeding the cultures daily, or held in stationary phase by allowing cell density to reach 1 - 1.2 x 10^6 cells/ml and then refeeding at that cell density for 1 - 2 days. Viability, determined by trypan blue exclusion, was greater than 90% in those cultures used for experiments. HTC cells were induced with 10^{-6} M hydrocortisone diluted into the media from fresh 0.01 M stock prepared in absolute ethanol (29).

TAT Assay

TAT activity was measured according to the procedure of Diamondstone (30) as modified by Stellwagen (31). HTC cells (2 - 5 x 10^6) were washed in 0.15 M KCl, then lysed by 3 cycles of rapid freezing and thawing in TAT assay buffer (0.05 M KPO_4, pH 7.6, 0.5 mM α-ketogluterate, 0.2 mM pyridoxal phosphate. The lysate was clarified by centrifugation at 15,000 x g for 15 min, and the supernatant recovered to assay total protein and TAT activity.

For liver TAT, a slice of approximately 0.5 g was homogenized in TAT assay buffer in a loose Dounce homogenizer, sonicated briefly for 15 sec, and centrifuged as described above. Protein concentration was determined by the dye method of Bradford (32) that employs BioRad reagent and bovine serum albumin as a standard.

DNA Purification and Base Analysis

Rat liver nuclei were isolated from livers as described previously (33). HTC nuclei were rapidly isolated by vigorously vortexing the cells in 5 ml of 0.3 M sucrose, 10 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.4), 0.2% Nonidet P-40 (Shell), and sedimenting at 1,100 x g for 5 min at 0 - 2 °C. This step was repeated twice but the NP-40 was omitted in the final step.

DNA was purified from these nuclei by proteinase K digestion, phenol-Sevag deproteinization, RNase A and T1 treatment, and 0.5 M alkali digestion for 30 min at 60 °C as described previously (33). DNA was hydrolyzed to bases with concentrated HF for 1.5 hr at 80 °C, and analyzed by HPLC on a Partisil 10-SCX column (0.6 x 25 cm, Whatman) eluted isocratically with 0.07 ammonium formate (pH 3.2) at room temperature in a Glenco HPLC system. Bases were identified relative to the elution of authentic compounds, and their quantity determined by measurement of the base peak area at 280 nm and divided by the base extinction coefficient at 280 nm at pH 3.2. The m^5C content of each sample was determined in triplicate. For each time point, the mean of three samples and their standard deviation (σ) was reported. Statistical significance of the results was examined by unpaired t-test and an analysis of variance (F-test).

RESULTS

The levels of DNA methylation in a population of cells is partially determined by the proportion of cells undergoing DNA synthesis, since nascent DNA is relatively undermethylated (33,34). This is not a major concern in the adult hepatic tissues since in these, DNA synthesis is almost undetectable (35) and, in addition, corticosteroids are known not to exert an effect on cell proliferation in the liver (36). However, for a valid comparision of DNA levels of methylation in HTC cells during corticosteroid induction, it was necessary to maintain these cells in a stationary state where hormonal responsiveness is still

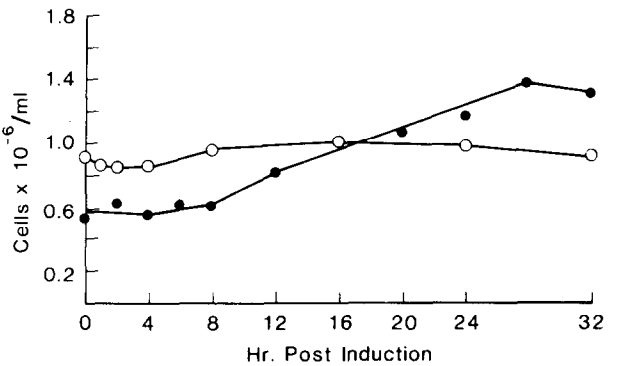

Fig. 1. Cell growth kinetics during hydrocortisone induction. For growing HTC cells, a half liter culture was split and refed at cell density of 0.5 x 10^6 /ml. After 2 hr, hydrocortisone was introduced at 10^{-6} M and cell counts monitored (●—●). For stationary HTC cells, a half liter was grown to cell density of 1.2 x 10^6/ml and kept at that density for two days. Cells were induced with 10^{-6} M hydrocortisone and cell counts monitored (○—○).

preserved. Figure 1 shows the cell density as a function of time after corticosteroid induction in the stationary and logarithmically dividing HTC cells that were used in these experiments.

The induction of TAT in both liver and HTC cells is shown in Figure 2. The livers of adrenalectomized rats showed a very rapid induction of TAT that achieved a maximum level corresponding to a 21-fold increase over basal level of 0.62 munits/mg protein after 8 hr. Induction in the liver of an animal administered hydrocortisone intraperitoneally was transient and a rapid decline to basal levels was observed 24 hr after induction. In the HTC cells, the induction was slower, taking approximately 16 hr to reach a maximum level, and a slower decline was exhibited. In logarithmically growing cells, the induction proceeds to a level comparable to the liver; a 24-fold increase over basal level of 0.2 munit/mg protein is observed. In a stationary cell culture induced by similar dose of hydrocortisone, TAT levels are increased 11-fold from a basal level of 0.26 munits/mg protein. Although a subsequent decline in TAT levels is observed in HTC cells, this phase is prolonged in comparison to that observed in the liver of an adrenalectomized animal.

The relative level of nuclear DNA methylation as a function of time during TAT induction is shown in Figure 3. In the normal adult liver (Panel A), the m^5C content is 3.42 ± 0.17% m^5C/total cytosines. There is a statistically significant decrease in m^5C content, lasting approximately 8 hr, beginning 1 hr after hormonal stimulation. This rapid, small yet significant decline in m^5C was observed in three different studies, only one of which is reported here. Thereafter, levels were not significantly different from uninduced control liver. The range in values for the m^5C content in the control liver and induced liver fluctuated between 3.46 ± 0.21% to 3.18 ± 0.17%. This corresponds to less than an 8% change in overall methylation of the genome.

In Panel B, the m^5C content of HTC nuclear DNA during induction is shown for HTC cells in logarithmic growth. The basal level of DNA methylation during proliferation (3.64 ± 0.24% m^5C is lower than in nondividing cells (3.77 ± 0.19% m^5C Panel C). This finding is in accord with the predicted contribution of HTC cells in S phase which contain relatively undermethylated newly replicated DNA (33,34). As shown from the overlapping stippled areas in Panels B and C, the level of DNA methylation is the HTC DNA after

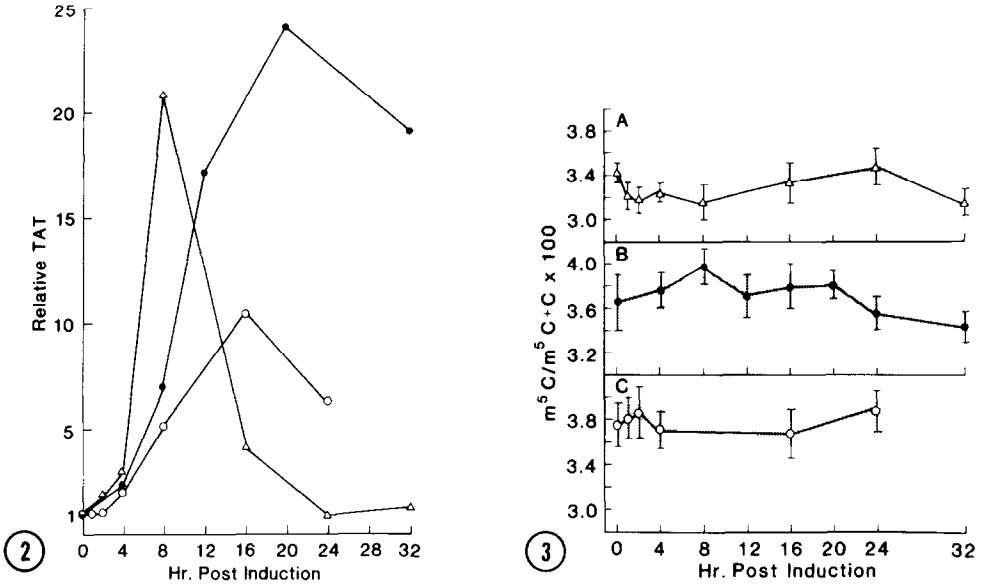

Fig. 2. Kinetics of induction after hydrocortisone treatment. Hepatic levels of TAT were measured in livers of individual, adrenalectomized animals given 200 mg/kg hydrocortisone (Δ—Δ). Basal values for TAT ranged between 0.05 and 0.62 munits/mg protein. Aliquots of HTC cells were removed after 10^{-6} M hydrocortisone induction and TAT measured. TAT levels in logarithmically growing HTC (●—●) of Fig. 1. TAT level in nondividing cells in stationary phase (o—o) of Fig. 1. Basal levels for TAT were 0.2 and 0.25 munits/mg protein in logarithmically growing and stationary phase HTC cells, respectively.

Fig. 3. m^5C content of DNA after hydrocortisone induction. Panel A, m^5C content of individual rat liver nuclear DNA after hydrocortisone induction (Δ—Δ). m^5C content is mean of three liver nuclear DNA preparations. Error bar indicates standard deviation of the mean. Stippled area is range of uninduced m^5C content. Panel B, m^5C content of HTC nuclear DNA after induction in logarithmically growing culture (●—●). Panel C, m^5C content of HTC nuclear DNA after induction in stationary cell culture (o—o). m^5C content of HTC cells is mean of three separate aliquots treated independently. TAT levels for each time point are shown in Fig. 2.

corticosteroid induction was not statistically different from the basal uninduced control levels. The m^5C content in the logarithmic cultures ranged from between 3.95 ± 0.25% and 3.43 ± 0.16%; for stationary cultures it ranged between 3.86 ± 0.17% and 3.66 ± 0.22%. This amounted to a fluctuation of less than 7.5% and 5.5%, respectively, in the genomic levels of DNA methylation during the induction of HTC cells. This apparent constancy in m^5C content is even more evident in the culture of predominantly undividing HTC cells (Panel C).

DISCUSSION

Results from numerous investigations on the genomic levels and distribution of m^5C have been adduced to support the concept of specific differences in DNA methyla-

tion of tissues and during differentiation (2-6) and an hypothesized connection between methylation and gene activity (2,20,37). To ascribe a functional role for these large changes in genomic levels of DNA methylation, Scarano et al. (37), Holliday and Pugh (38), Riggs (39), and Sager and Kitchin (40) have put forth theoretical treatments as to mechanistic activation and/or regulation of genes by DNA methylation.

Some of these proposals appeared to be supported by the work of Vanyushin and colleagues (7,20) who measured the level of m^5C in hepatic DNA during corticosteroid stimulation and reported large changes in m^5C content. After 4 hr, a 25% increase was observed which peaked after 8 hr with a 71% increase. No change was observed in the spleen while brain increased by 25%. However, our results, obtained by HPLC analysis of m^5C content of liver nuclear DNA following comparable stimulation with hydrocortisone, demonstrate clearly that the overall level of DNA methylation fluctuates less than 8%. In stimulated HTC cells, no significant alteration was demonstrated despite a 20-fold stimulation in TAT synthesis. Furthermore, in the liver, the alteration was towards a decrease in DNA methylation. Pollock et al. (9) have recently reported that no statistically significant fluctuation in genomic levels of DNA methylation took place during sea urchin and mullet development despite earlier studies which had indicated large changes when analyzed by radioactive precursor incorporation (6) or by spectrophotometric measurement of bases separated by paper chromatography (2,3,5,7,20). Furthermore, the variation between tissue in mouse and rabbit have been recently determined by HPLC to be less than 10% (8), while earlier workers reported far greater differences (2,3,20).

In those studies where relatively constant levels of methylation were determined, HPLC methodologies were employed for base separation and quantitation. The HPLC methods are highly accurate, sensitive and reproducible compared to paper chromatographic and radiolabeled precursor incorporation methods where interference or one carbon pool metabolic problems, respectively, can hinder the analysis (9,41).

Our findings of relatively constant genomic levels of DNA methylation do not exclude the possibility of changes in methylation occurring during a switchover in genetic program. Small but significant changes in the genomic levels of m^5C towards undermethylation of teratocarcinoma cells induced to differentiated *in vitro* have been demon-

strated by HPLC (42). Using restriction enzymes to probe for methylation of CCGG and other CG methylatable sequences, Singer et al. (8) have confirmed similar changes during teratocarcinoma differentiation.

Our results indicate that fluctuations in DNA methylation occur during corticosteroid induction but that they are rather small when compared to overall levels of methylation in the genome. Such fluctuations may be limited because the alterations to which they are related are limited to that small fraction of total genes responsive to corticosteroid stimulation, or alternatively, involve more global over-and-undermethylation of genomic sites that "average out." The latter appears to be unlikely in view of evidence provided by Mandel and Chambon (11) and Kuo et al. (12) that certain sites are variably methylated under conditions affecting gene regulation while others are constantly methylated. The decline in m^5C content in the DNA from normal, mitotically quiescent liver is perplexing since no enzyme system for direct base demethylation has yet been discovered (43). However, such loss of m^5C via the formation of minor thymine as dectected in the developing sea urchin (37,44) but not in HeLa cell DNA (45) by a DNA-m^5C deaminase, might account for these findings.

ACKNOWLEDGMENTS

This investigation was supported by Grants CA20657 and CA21927 awarded by the National Cancer Institute, Department of Health, Education and Welfare. The authors would also like to acknowledge the excellent technical assistance of Sherry Hauft, Carol Keiffer, and Ted Barna and Julie Collins and Catherine Johns for their manuscript preparation.

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