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Intracellular distribution of DNA methyltransferase during the cell cycle

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The intracellular distribution of DNA methyltransferase has been analyzed in synchronously proliferating human cells. The localization of DNA methyltransferase was determined immunocytochemically using monoclonal antibodies directed against this enzyme. DNA methyltransferase was found to accumulate predominantly in nuclei with weak cytoplasmic staining. The DNA methyltransferase antigen was absent in early G_1 phase, appeared in late G_1 prior to the onset of DNA synthesis and persisted throughout S and G_2 phases of the cell cycle. Mitotic cells showed a particularly strong staining intensity. These results show that DNA methyltransferase levels fluctuate during the cell cycle. This has possible implications on the stability of the DNA methylation pattern.

DNA methyltransferase; DNA methylation; Immunocytochemistry; Cell cycle

1. INTRODUCTION

The function of DNA methylation in mammals, although still a subject of debate, is in many cases related to regulation of gene expression (reviewed in [1,2]). The clonal stability of DNA methylation patterns is guaranteed by a DNA methylating enzyme, which transfers methyl groups to hemimethylated 5′-CpG-3′ dinucleotides shortly after DNA replication. This enzyme, DNA cytosine-5-methyltransferase (DNA methyltransferase, EC 2.1.1.37) strongly prefers hemimethylated over unmethylated DNA substrates and its activity resides in a single polypeptide of 190 kDa [3]. However, the enzyme possesses a considerable de novo DNA methyltransferase activity in vitro, which transfers methyl groups to previously unmethylated sites [4–7]. This de novo activity is assumed to be suppressed in the cell in order to avoid changes of the DNA methylation pattern except in certain stages of differentiation and development, when the methylation pattern is specifically altered [8]. In non-proliferating cells, where apart from DNA repair processes the DNA methylating enzyme is obviously dispensable, the level of DNA methyltransferase was shown to be indeed extremely low [3,9,10]. DNA methyltransferase activity increases dramatically, when cells are exposed to a proliferation stimulus [9,10]. The availability and distribution of DNA methyltransferase within the cell may be an important factor for the control of methyl group transfer reactions with important implications on the stability of DNA methylation patterns.

Here, we have analyzed the intracellular distribution of DNA methyltransferase in synchronously proliferating human carcinoma cells by immunocytochemical techniques.

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2. MATERIALS AND METHODS

2.1. *Cell culture*

HeLa cells (ATCC, CCL2, obtained from Flow Laboratories) and HEp-2 cells (ATCC, CCL23, obtained from Flow

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Laboratories) were grown at 37°C as monolayers in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and antibiotics (penicillin, 100 units/ml; streptomycin, 100 μg/ml).

2.2. *Cell synchronisation*

Cells were synchronized by selection of accumulated mitotic cells [11]. The cell monolayers were incubated for 12 h in the presence of colcemid (0.005 μg/ml medium). Mitotic cells were detached from the plastic surface by shaking the dishes and collected by centrifugation. The cells were resuspended in culture medium and plated onto microscopic slides at a concentration of 1×10^5 cells/ml. Between 80 and 90% of the plated cells were identified as mitotic cells. The cells were grown for one cell cycle (22 h) and were then processed for immunocytochemistry and thymidine incorporation studies.

2.3. *Cell cycle monitoring*

At 3 h intervals, slides were tested for thymidine incorporation as follows: the cells were incubated with 1 μCi [^{3}H]thymidine (Amersham Buchler, spec. act. 93 Ci/mmol) in 1 ml medium for 1 h at 37°C. After removal by trypsinisation, cells were washed in phosphate buffered saline (PBS) and disrupted in 0.5 N NaOH. Radioactivity in perchloric acid-insoluble material was determined by liquid scintillation counting.

2.4. *Immunocytochemistry*

After the indicated time periods (3 h intervals), the microscopic slides were washed in PBS. Cells were fixed in absolute methanol for 10 min at room temperature. Non-specific binding was blocked by preincubating the slides in PBS containing 1% horse serum. The slides were then incubated for 1 h at room temperature with purified monoclonal anti-DNA methyltransferase antibody M1F6D7/5C10 [12] at a concentration of 10 μg/ml (unless otherwise indicated) in PBS. Immune complexes were visualized with biotinylated horse anti-mouse IgG followed by peroxidase reaction with diaminobenzidine as substrate (Vectastain® , Vector Laboratories). The slides were dehydrated in graded ethanol and mounted in Eukitt® . As a control for unspecific binding the monoclonal anti-DNA methyltransferase antibody was omitted or substituted by normal mouse serum. None of the controls yielded a positive staining reaction.

3. RESULTS

Cultures of human carcinoma cell lines HeLa and HEp-2 were synchronized by the mitotic detachment technique. The cells were allowed to grow for one cell cycle and were analyzed for the presence of DNA methyltransferase antigen in the second cell cycle after synchronisation. Progression of synchronized cells through the cell cycle was monitored by thymidine pulse labelling at indicated time periods (fig.1). In HeLa cells, DNA synthesis started to increase significantly after about 8–9 h and in HEp-2 cells 10–12 h after the beginning of the second cell cycle. Maximal rates of DNA synthesis were measured after 15 h in both cell types.

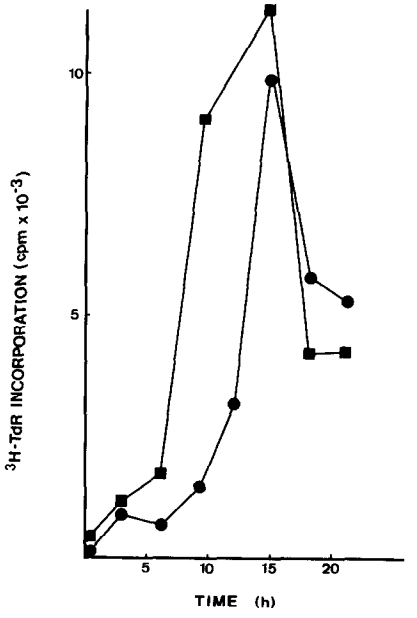

Fig.1. Monitoring of the cell cycle by thymidine pulse labelling. HeLa cells (■) and HEp-2 cells (●) were pulse labelled in the second synchronized cell cycle with [^{3}H]thymidine as described in section 2.

The cells were processed for immunocytochemistry at 3 h intervals. HeLa cells are shown in fig.2, HEp-2 cells in fig.3. No immunocytochemical signal was obtained in postmitotic early G_1-phase cells (figs 2A and 3A). Cells in late G_1 phase showed a significant staining of nuclei and a weak staining of cytoplasmic regions (figs 2B and 3B). At the time point of the maximal rate of DNA synthesis, the nuclei showed an intense granular staining pattern (figs 2C and 3C). This pattern remained almost unchanged throughout later S phase and G_2 phase of the cell cycle (fig.3D). In figs 2D and 3D some mitotic cells are visible. They show a particularly strong accumulation of the DNA methyltransferase antigen (fig.3D). When more diluted antibody solutions are used, the condensed chromosomes are predominantly stained in mitotic cells (fig.2D). Very similar cell cycle-dependent distribution patterns of the DNA methyltransferase antigen were obtained in HeLa and HEp-2 cells (figs 2 and 3) and in a human melanoma cell line (not shown).

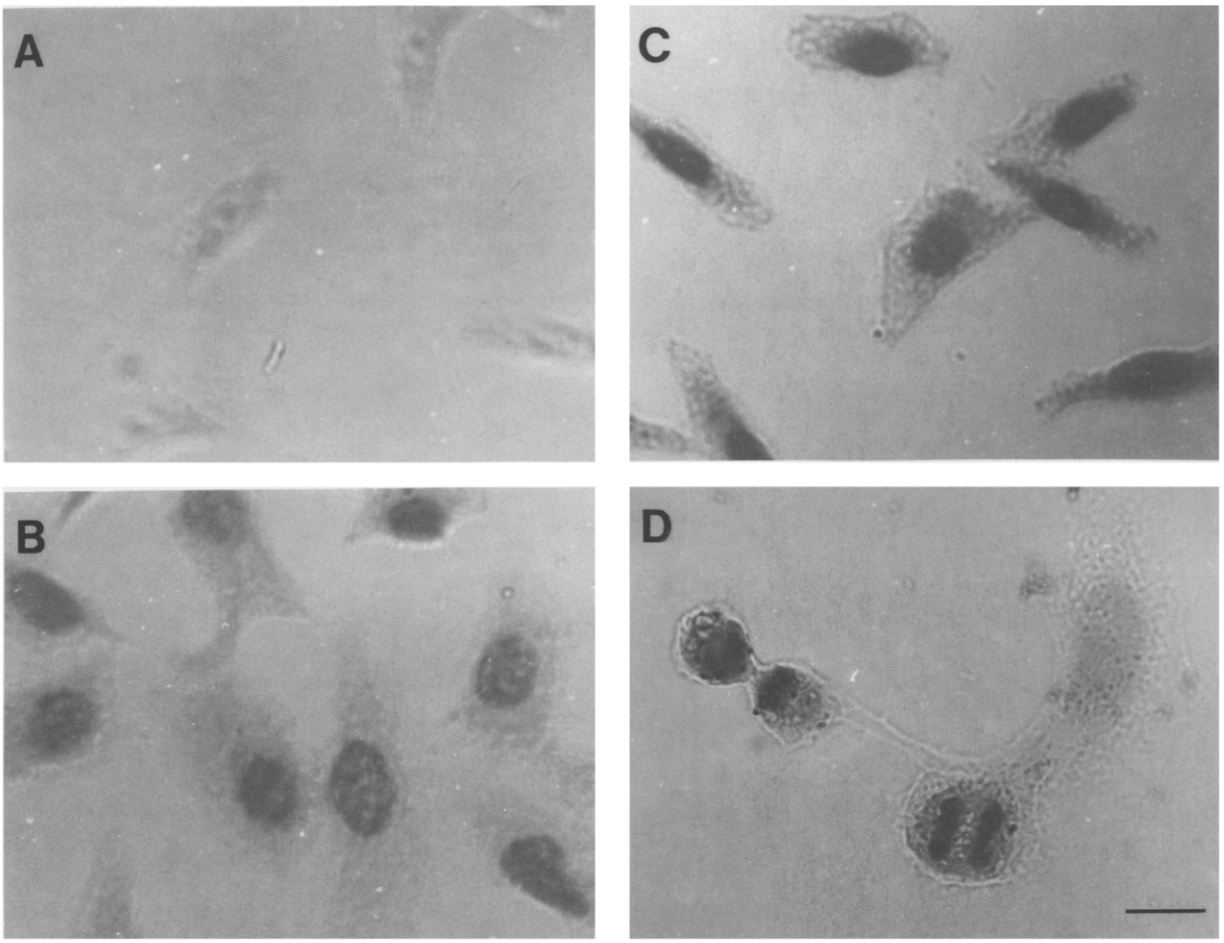

Fig.2. Cell cycle-dependent distribution of DNA methyltransferase in HeLa cells. (A) Cells in early G_1 phase (3 h); (B) late G_1 phase (6 h); (C) S phase (15 h); (D) mitotic cells (24 h). The concentration of monoclonal antibodies in (D) was 1 μg/ml. The cell in the right upper part belongs to either the G_2 phase or very early G_1 phase. Bar, 20 μm.

4. DISCUSSION

DNA methyltransferase is nonrandomly distributed throughout the different phases of the cell cycle. The localization of the DNA methyltransferase antigen is predominantly nuclear, which is consistent with biochemical investigations using different cellular compartments for extraction and activity determination [12]. Our immunocytochemical data show that DNA methyltransferase is absent in early G_1 phase, appears in late G_1, and accumulates and persists throughout S and G_2 phases of the cell cycle. The presence of DNA methyltransferase in the G_2 phase may explain the phenomenon of delayed DNA methylation occurring several hours after replication [13]. Mitotic cells are characteristic for a strong immunochemical staining. This may be due to a release of the DNA methyltransferase antigen from nuclear structures which are inaccessible for the anti-DNA methyltransferase antibody in interphase cells. Indeed, it has been shown that DNA methyltransferase is associated with nuclear matrix like structures [12–14], although the amounts of enzyme present within these structures are difficult to quantify. The absence of the DNA methyltransferase antigen in early G_1 phase implies that the enzyme disappears shortly after completion of cell division either by

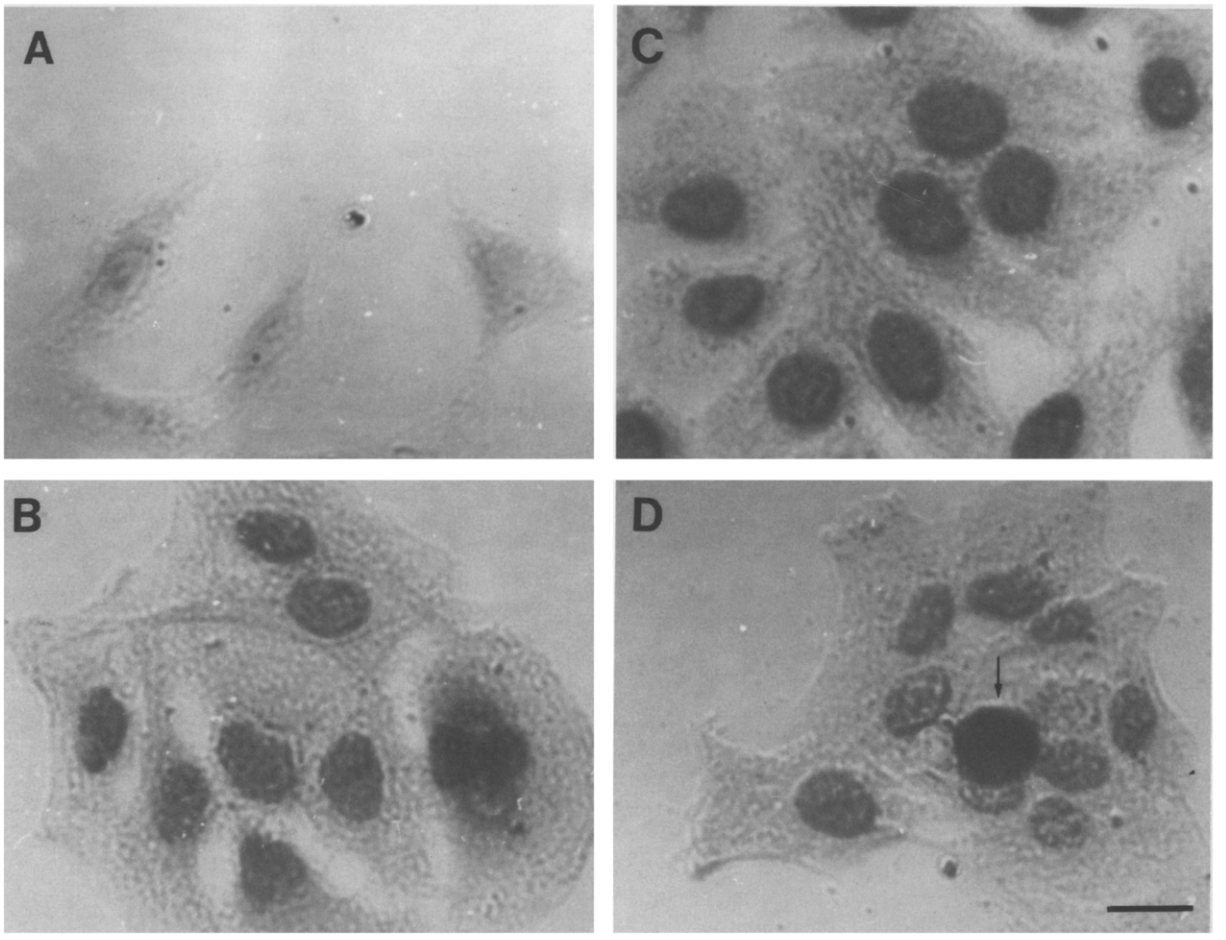

Fig.3. Cell cycle-dependent distribution of DNA methyltransferase in HEp-2 cells. (A) Cells in early G_1 phase (3 h); (B) late G_1 phase (6 h); (C) S phase (15 h); (D) late S and G_2 phase (21 h). The arrow indicates a mitotic cell. Bar, 20 μm.

redistribution into antibody-inaccessible structures or by a rapid decomposition.

In previous studies, DNA methyltransferase levels have been analyzed in peripheral lymphocytes before and after mitogen stimulation. Activity determination [10] and immunocytochemical studies [9] have shown that DNA methyltransferase is absent in unstimulated peripheral lymphocytes but appears just prior to the onset of DNA synthesis. DNA methyltransferase levels were found to be coupled to proliferation also in hepatectomized rat livers [10]. Analysis of DNA methyltransferase activities throughout the HeLa cell cycle presented evidence for two activity peaks, one in G_1 phase, and a second in S phase [15]. Our system determining antigen concentrations did not show a biphasic distribution. Delfini et al. [15] have used double thymidine block for cell synchronisation. Their results also indicate the absence of DNA methyltransferase in early G_1 phase. The fluctuation of DNA methyltransferase levels throughout the cell cycle resembles the cell cycle-dependent appearance of a number of other enzymes and proteins involved in DNA synthesis. Similar to DNA methyltransferase, the levels of DNA topoisomerase II are very low in early G_1-phase cells [16]. The amount of DNA polymerase α increases rapidly during S phase, but unlike DNA methyltransferase the polymerase is redistributed to the daughter cells after mitosis and

persists there through the whole G_1 phase [17]. The DNA polymerase σ interacting replication factor cyclin is detectable only in S-phase cells [18].

The fluctuation of DNA methyltransferase during the cell cycle may be of considerable regulatory importance. In early G_1-phase cells and also in non-replicating cells (G_0 cells) DNA methyltransferase is obviously dispensable except for methylation of DNA repair patches (although it is not known, whether one and the same enzyme is responsible for methylation in replication and repair processes). An important question pertinent to our understanding of the stability of DNA methylation patterns is: How is the de novo activity of DNA methyltransferase suppressed in the cell? It is clear that one way to achieve this suppression and to exclude inappropriate changes of the DNA methylation pattern is to avoid the presence of the enzyme at possible de novo methylation sites. This functional aspect may underlie the fluctuation of DNA methyltransferase levels during the cell cycle.

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