METHYLATION OF DNA IN L CELLS ON REPLICATION

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1. Introduction

The data available at present on the methylation of newly-synthesized DNA in animal cells are still scanty and contradictory. On the one hand, it is believed that in animals there is a time gap between replication and methylation of DNA [1,2], and that newly-synthesized DNA is not methylated at all [2]. Other authors have reported, however, that methylation is inherent in the formation of highly polymeric DNA molecules [3,4].

Here, an attempt has been made to study DNA methylation during replication in a culture of transformed mouse fibroblast cells (L cells). Synthesis and methylation of newly-synthesized DNA fragments were studied after resolution of the latter in an alkaline sucrose gradient. The results presented below show that the formation of the 5-methylcytosine residues in the newly-synthesized DNA of L cells proceeds at the same time as the synthesis of Okasaki fragments. Further, the synthesis of precursors, longer than the Okasaki fragments, of highly polymeric DNA involves additional methylation of some cytosine residues.

2. Experimental

2.1. *Cultivation of cells*

2-*O*-Methylcholantrene-transformed C3H murine embryo fibroblasts were used. The cells were grown in glass flasks in a culture medium containing 45% 199 medium in Hank's solution, 45% medium with 0.5% lactalbumin hydrolysate in Hank's solution and 10% cattle serum. The cells were grown in continuous culture and passaged every 4–5 days. The replication and methylation of the newly-synthesized DNA were studied at high and low cell densities on the surface of the flask (1–2 × 10^5 and 4–5 × 10^5 cells/cm^2, respectively) 12 h after stimulation of the cells by replacement of the culture medium.

2.2. *Incubation of the cells with [^{3}H]thymidine, [^{14}C]uridine and [*methyl-^{3}H*]methionine*

The cells were incubated in 5 ml Hank's solution with 20 μCi [^{3}H]thymidine (15 mCi/mmol, USSR) for 5–10 min or with 200 μCi [U-^{14}C]uridine (450 mCi/mmol, Amersham) for 30 min or in 5 ml Hank's solution containing 20 μg/ml thymidine, 20 μg/ml adenine, 1.5 mg/ml sodium formate and 0.5 μCi L-[*methyl*-^{3}H]methionine (9.5 μCi/mmol, Amersham) for 10–20 min. Incubation was interrupted by replacing the medium with cold Hank's solution containing 0.01 M sodium azide. The cells were removed from the surface of the flask with the help of 0.2% trypsin solution, suspended in medium 199 and sedimented by 5 min centrifugation at 1000 × *g*. In the chase experiments, after incubation the cells were kept in a medium containing 15 μg unlabelled thymidine or 60 μg unlabelled methionine in 5 ml Hank's solution, respectively, and then treated as above.

2.3. *Analysis of radioactivity incorporation into newly-synthesized DNA fragments*

The cell sediment was treated with pronase (100 μg/ml, Serva) for 45 min at 37°C in a solution containing 0.15 M NaCl, 0.015 M sodium citrate and 0.25% SDS. Incubation was interrupted by addition of SDS up to 1% and 3 N NaOH to 0.3 N. The cell lysate was layered on a linear 5–20% sucrose gradient containing 0.1 N NaOH and 0.2% SDS and centrifuged in a Spinco L-2 centrifuge (Beckman, rotors SW-39, SW-27) or K-32 centrifuge (USSR, rotor C-45) at 20°C. Ribosomal 28 S, 18 S and 5 S RNA from Krebs II ascite carcinoma cells in a 5–20% linear sucrose gradient containing 0.1 M NaCl and 2% SDS were

used as reference points. The gradients were fractionated dropwise from the bottoms of the tubes and calf thymus DNA was added to the fractions as a carrier. The radioactivity of the incorporated [^{3}H]thymidine was measured in the acid-insoluble material.

To study the methylation of the newly-synthesized DNA fragments, the acid-insoluble material was additionally washed 3 times with 2 ml cold 2% $HClO_4$ and twice with 2 ml cold ethanol containing 2% $HClO_4$. The acid-insoluble material of the fractions was hydrolysed in 0.2 ml 5% $HClO_4$ at 90°C and the radioactivity determined in a dioxane scintillation liquid in Mark II or Mark III scintillation counters (Nuclear Chicago) with a counting efficiency of 20–25%, the error being $\leqslant$10%.

For determination of the degree of methylation of cytosine residues in the newly-synthesized DNA in the presence of [^{14}C]uridine DNA fragments, the acid-insoluble material of the alkaline sucrose gradient fractions was hydrolyzed in 57% $HClO_4$ and the bases were resolved by two-dimensional ascending thin-layer chromatography (TLC) on cellulose (Filtrak, GDR) in the presence of authentic 5-methylcytosine (Serva) as a marker [5]. The zones that corresponded to cytosine and 5-methylcytosine were placed in counting vials and eluted with water for 4–5 h at 37°C. The degree of methylation of cytosine residues in DNA was determined as the ratio of the radioactivity of $m^5C \times 100/(C + m^5C)$.

2.4. *Determination of degree of methylation in total DNA*

Fibroblasts were incubated with 10 μCi/ml [U-^{14}C]-uridine for 24 h, then DNA was isolated from the cells as in [6] and hydrolysed into bases, the bases were resolved by TLC [5], and the radioactivity of cytosine and 5-methylcytosine was measured as described previously.

3. Results and discussion

3.1. *DNA synthesis in cultures of different cell density*

In our experiments, after a short-term (5–10 min) incubation of the cells with [^{3}H]thymidine, radioactive DNA occurs, after centrifugation in an alkaline sucrose gradient, in individual peaks of 5,13,18,23, 28,33 S and higher (fig.1,2). This agrees with the idea of discontinuous synthesis of DNA in animal cells. One can see that in mouse fibroblasts DNA synthesis

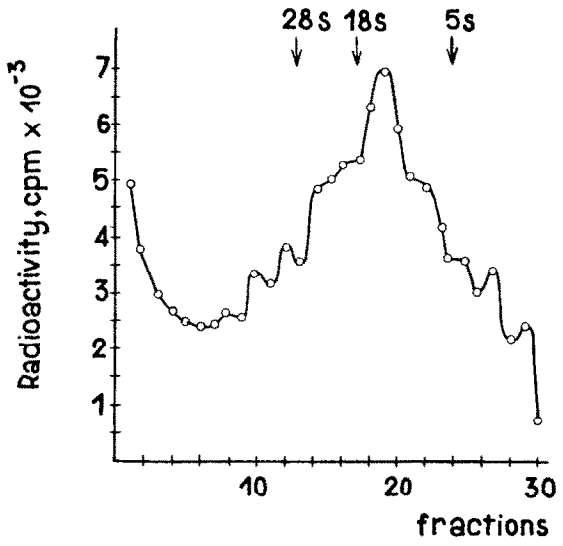

Fig.1. Incorporation of [^{3}H]thymidine into DNA at low cell concentration in a culture of mouse fibroblasts. The cells (0.8×10^5/cm^2) were incubated for 5 min with [^{3}H]thymidine, 0.3 ml cell lysate was layered on 4.6 ml of 5–20% alkaline sucrose gradient and centrifuged for 4 h at 38 000 rev./min and 20°C (rotor C-45).

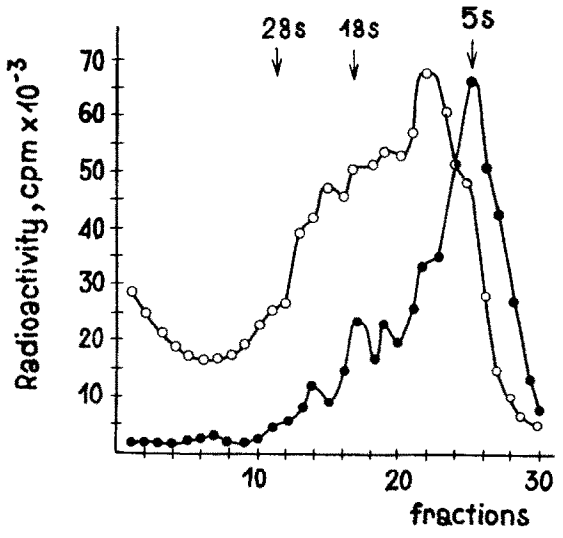

Fig.2. Incorporation of [^{3}H]thymidine into DNA at high cell concentration (4×10^5/cm^2) in a culture of mouse fibroblasts. (–•–) Incubation of cells with [^{3}H]thymidine for 10 min; (–○–) same, followed by incubation for 3 h at 37°C in a medium containing unlabelled thymidine; Cell lysate (0.3 ml) was layered on 4.6 ml alkaline sucrose gradient and centrifuged for 4.5 h at 36 000 rev./min at 20°C (rotor SW-39).

involves formation of Okasaki fragments (~5 S) which are subsequently linked together.

When the cell density is not high ($1-2 \times 10^5/cm^2$), the radioactivity quickly goes to the fragments of >5 S. At the same time, at a high concentration of the cells ($4-5 \times 10^5/cm^2$), even after 10 min incubation with [^{3}H]thymidine, the newly-synthesized DNA is mostly represented by low-molecular-weight fragments of 5 S. A noticeable shift of radioactivity towards the zones of 13,18,23 S and higher is observed only 3 h after the cells are transferred into a medium containing non-labelled thymidine (fig.2).

Thus, the rate of ligation of the Okasaki fragments depends on the density of the cells in the culture and sharply decreases at high cell concentrations.

3.2. *Methylation of DNA fragments on replication*

In separate experiments, we have established by hydrolysis of DNA into bases and their resolution by TLC, followed by radioactivity assay, that 80–100% of the radioactivity incorporated from [*methyl*-^{3}H]-methionine into DNA fragments is found in 5-methylcytosine.

After 20 min incubation of a culture with low cell density in the presence of [*methyl*-^{3}H]methionine, the radioactivity is found in DNA fragments of 5,13, 28–33,45 and 55 S (fig.3). On the other hand, at high cell concentrations, like on incubation with [^{3}H]thymidine, the label is again localized in the Okasaki fragments (fig.4). Under these growth conditions, the shift of the radioactivity to the larger fragments was observed 3 h after the medium with radioactive methionine was replaced by that containing unlabelled methionine (fig.4).

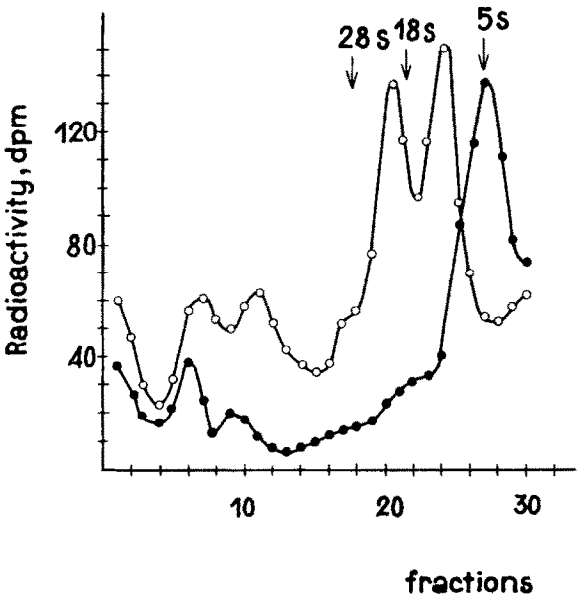

Fig.4. Incorporation of radioactivity from [*methyl*-^{3}H]-methionine into DNA at a high cell concentration (4×10^5/cm^2) in a culture. (–●–) Incubation of cells with [*methyl*-^{3}H]methionine for 10 min; (–○–) same, followed by 3 h incubation in a medium containing unlabelled methionine. Cell lysate (2 ml) was layered on 30 ml 5–20% alkaline sucrose gradient and centrifuged for 16 h at 15 000 rev./min 20°C (rotor SW-27).

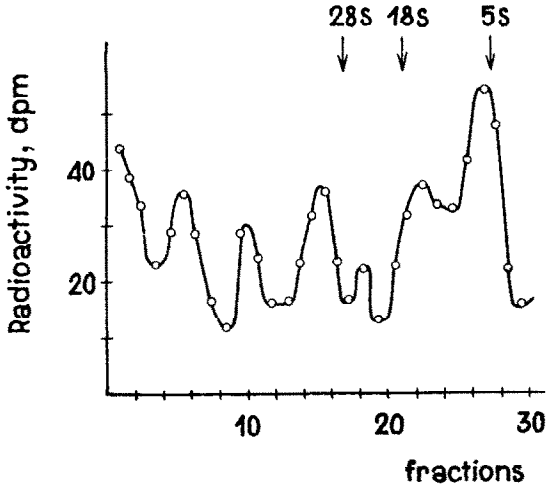

Fig.3. Incorporation of radioactivity from [*methyl*-^{3}H]-methionine into DNA at low cell concentration in a culture. Cells (1.4×10^5/cm^2) were incubated for 20 min with [*methyl*-^{3}H]methionine; 2 ml cell lysates were layered on 30 ml 5–20% alkaline sucrose gradient and centrifuged at 20°C for 16 h at 15 000 rev./min (rotor SW-27).

The patterns of DNA radioactivity in alkaline sucrose gradients after the incubation of the cells with [^{3}H]thymidine or with [*methyl*-^{3}H]methionine are very similar, i.e., the positions of the major part of the radioactivity peaks on the corresponding profiles are the same. In addition, in some cases we observed incorporation of radioactivity from [^{3}H]-thymidine and [*methyl*-^{3}H]methionine into DNA fragments of <5 S (fig.1 and unpublished results).

Consequently, in transformed mouse fibroblasts, not only are Okasaki fragments methylated, but also shorter, newly-synthesized fragments of <100 nucleotide residues.

3.3. *The level of methylation of cytosine residues in DNA fragments of different lengths and in total DNA of L cells*

The cells incubated for 24 h with [^{14}C]uridine at different growth-phases of the culture ($1-5 \times 10^5$ and 4×10^5 cells/cm^2) have the same level of total DNA methylation (table 1).

As seen from table 1, the $m^5C \times 100/(C + m^5C)$ ratio is not equal for DNA fragments of $\leqslant 5$ S and >5 S (respectively, 2.7–2.8 and 4.1–4.2) and does not depend on the concentration of the cells on the surface of the flask. This also means that the formation of 5-methylcytosine residues in short fragments of DNA occurs immediately after their synthesis, and their accumulation as a result of inhibition of ligation (at high cell density) does not entail overmethylation. However, on ligation of Okasaki fragments and formation of polynucleotides of >5 S, a certain increase in the 5-methylcytosine content is observed in the DNA fragments.

Thus, in animal cells (mouse fibroblasts) there exists DNA methylation during replication. This DNA methylation involves at least two steps, i.e., at the beginning the newly-synthesized short fragments (Okasaki fragments and shorter ones) are methylated and then an additional DNA methylation occurs at the level of the newly-formed ligated (>5 S) DNA molecules.

The methylation of low molecular weight fragments of newly-synthesized DNA (Okasaki and fragments <5 S) detected, as well as the absence of overmethylation of the Okasaki fragments on retardation of their ligation in cultures of high cell concentrations, show that during replication DNA methylation in fibroblasts starts immediately on the synthesis of the DNA fragments that are the least accessible to methylase(s). The DNA-methylase(s) that is (are) operative during the replication should apparently be looked upon as being one of the functioning components of the active replication complex. It is not excluded that additional formation of 5-methylcytosine residues at the level of DNA fragments >5 S may involve some other enzyme, different in its properties from the DNA-methylase that modifies cytosine residues in the Okasaki fragments.

Table 1
The level of methylation of cytosine residues in newly-synthesized fragments and total DNA in cultures of different cell densities

No. cells/cm^2	$m^5C \times 100/(C + m^5C)$ $(\bar{x} \pm G)$		
	DNA fragments $\leqslant 5$ S	>5 S	Total DNA
2×10^5	2.7 ± 0.30	4.2 ± 0.10	4.4 ± 0.10
4×10^5	2.8 ± 0.10	4.1 ± 0.05	4.3 ± 0.20

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