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Methylation of replicating and post-replicated mouse L-cell DNA

(cell permeabilization/CpG methylation/S-adenosyl-L-homocysteine/nearest-neighbor analysis)

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ABSTRACT **We have introduced [α-^{32}P]dGTP into permeabilized cells and measured the degree of methylation at CpG sites by nearest-neighbor analysis. This method reveals a lag of approximately 1 min between DNA synthesis and the modification event. When methylation is inhibited by the addition of S-adenosyl-L-homocysteine in the presence of continued DNA synthesis, the resulting hemimethylated sites are methylated immediately after the release of inhibition. The results suggest that the methylase activity in the cell allows immediate methylation but conditions at the replication fork bring about a short delay in the onset of the modification reaction.**

Animal cell DNA is modified by enzymatic methylation of cytosine residues at CpG-containing sequences (1). The genomic pattern of methylation is inherited from generation to generation by a semiconservative post-replicative mechanism (2, 3). This is accomplished by a maintenance methylase that is specific for the hemimethylated DNA generated during DNA replication (4). Several studies in the past focused on the interrelationship between DNA replication and methylation (5–9). Because of the lack of a convenient experimental system, these efforts yielded conflicting results. One study suggested that in mammalian cells the synthesis and methylation of DNA do not occur simultaneously but may be separated by as much as an hour (10). This conclusion was based on two observations. (*i*) In mouse L cells, DNA methylation continues at a substantial rate for 2 hr after the inhibition of DNA synthesis with hydroxyurea. Furthermore, in cultures of L cells partially synchronized by an 18-hr exposure to aminopterin, the maximum rate of DNA methylation occurred about 1 hr after the maximum rate of DNA synthesis. Similar observations were made by Kappler with a mouse adrenal cell line (5). (*ii*) However, when Kappler used a direct method, a lag of approximately 1 min was observed between the incorporation of deoxycytidine into the DNA and its conversion into 5-methylcytosine (5). The results presented here, from a study in which a technique that allows direct measurement of methylation at CpG sequences during DNA synthesis in an unsynchronized cell culture was used, confirm the lag of about 1 min. Artificially created hemimethylated sites not located at the replication fork are however methylated with no detectable lag. These results suggest that methylatable sites are methylated immediately provided the CpG sites on the newly synthesized DNA are available to the methylase.

MATERIALS AND METHODS

Radioactive [α-^{32}P]dGTP ($\approx$600 Ci/mmol; 1 Ci = 37 GBq) was from New England Nuclear. Tissue culture media and serum were from Biolab Israel (Jerusalem). Proteinase K was purchased from Merck. Micrococcal nuclease, spleen phosphodiesterase, S-adenosyl-L-homocysteine (S-AdoHcy) and dGTP were Sigma products.

Permeabilization and Labeling Procedure. Mouse L cells were grown in 25-cm^2 tissue culture flasks in Dulbecco's modified Eagle's medium to a density of 10^6 cells per flask. Cells were washed with 5 ml of phosphate-buffered saline and subsequently treated with 0.5 ml of permeabilization buffer (75 mM Hepes, pH 7.4/5 mM $MgCl_2$/2 mM dithiothreitol) in the presence of 1 μM [α-^{32}P]dGTP. After 45 sec, this solution was diluted by the addition of 4.5 ml of fresh modified Eagle's medium and the cells were incubated for various time periods. Cells undergoing this permeabilization procedure remain vital and replicate normally. DNA synthesis was terminated by replacing the medium with lysis buffer (10 mM Tris·HCl, pH 8/0.4 M NaCl/1% sodium lauryl sulfate) and the cells were collected with the aid of a rubber policeman.

Preparation of DNA. Lysed cells (1×10^6) were incubated in the presence of proteinase K at 100 μg/ml for 30 min at 37°C. The DNA was purified by phenol and chloroform/isoamyl alcohol extraction. The alcohol-precipitated DNA was dissolved in 50 mM Tris·HCl, pH 8/2 mM EDTA. Labeled DNA was used to analyze the extent of dGTP incorporated (trichloroacetic acid-precipitable radioactivity) as a measure of DNA synthesis and subjected to nearest-neighbor analysis for estimation of the extent of CpG methylation as described below. Bulk DNA of L cells was analyzed for CpG methylation by nick-translation in the presence of Ca^{2+} followed by nearest-neighbor analysis (11).

Analysis of Methylation at CpG Sequences. Labeled DNA ($\approx$1 μg) was digested to deoxynucleoside 3′ monophosphates in 100 mM Tris·HCl, pH 8.5/10 mM $CaCl_2$ using micrococcal nuclease (140 μg/ml) and spleen phosphodiesterase (7 units/ml) for 3 hr at 37°C. The digested DNA was applied to cellulose thin-layer chromatography sheets (Eastman Kodak) and chromatographed in two dimensions as described (11). After autoradiography the radioactive spots were quantitated by scintillation counting. The radioactivity observed in the 5mCyt and cytosine spots was used to calculate the extent of methylation (% CpG methylated).

RESULTS

Previous attempts to characterize the rate of methylation of replicating DNA were inconclusive primarily because indirect methods were used. This prompted us to develop a technique to avoid effects of precursor pool sizes, rates of precursor biosynthesis, and the need for synchronization. Our method was devised to study methylation of newly synthesized CpG sequences in unsynchronous growing cell cultures. This approach allows an estimation of the kinetics of methylation of CpG se-

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Abbreviations: S-AdoHcy, S-adenosyl-L-homocysteine; S-AdoMet, S-adenosyl-L-methionine.

quences in nascent DNA regardless of their distribution in the DNA. The same method has been used to study methylation upstream to the replication fork on hemimethylated DNA stretches created by DNA synthesis in the presence of the methylase inhibitor S-adenosylhomocysteine.

Methylation of Replicating DNA. Mouse L cells were permeabilized briefly in the presence of [α-^{32}P]dGTP, and newly synthesized DNA labeled by the added nucleotide was analyzed by nearest-neighbor analysis for rate of methylation at CpG sequences. Thus, if methylation occurs simultaneously with replication, the newly synthesized CpG residues should be methylated as is the bulk L-cell DNA. The extent of CpG methylation of L-cell DNA has been determined and found to be 65%. When permeabilized L cells were labeled for various short time periods (<2 min), it was found that the newly synthesized labeled DNA was relatively unmethylated, suggesting that DNA methylation must lag behind DNA synthesis at the replication fork (Fig. 1*A*). With long labeling times (10–25 min), the extent of methylation approached the saturation value of about 65%, equal to the degree of methylation of total L-cell DNA (Fig. 1*A* and Table 1). Thus, the CpG-containing sequences that are unmethylated at early labeling times do indeed become methylated following the lag period. The observed kinetics is consistent with a theoretical curve obtained if a 1-min lag is assumed (Fig. 1*A*). A more rigorous representation of the kinetic data indicates that the lag time is 75 sec (Fig. 1*B*). This observed lag represents a maximum value, since it does not take into consideration the time necessary to stop the synthesis reaction and the time elapsed between addition of the nucleotides and their appearance in the DNA. Incorporation into the DNA is probably instantaneous since labeled DNA was observed even after 10 sec of permeabilization in the presence of the radioactive nucleotide.

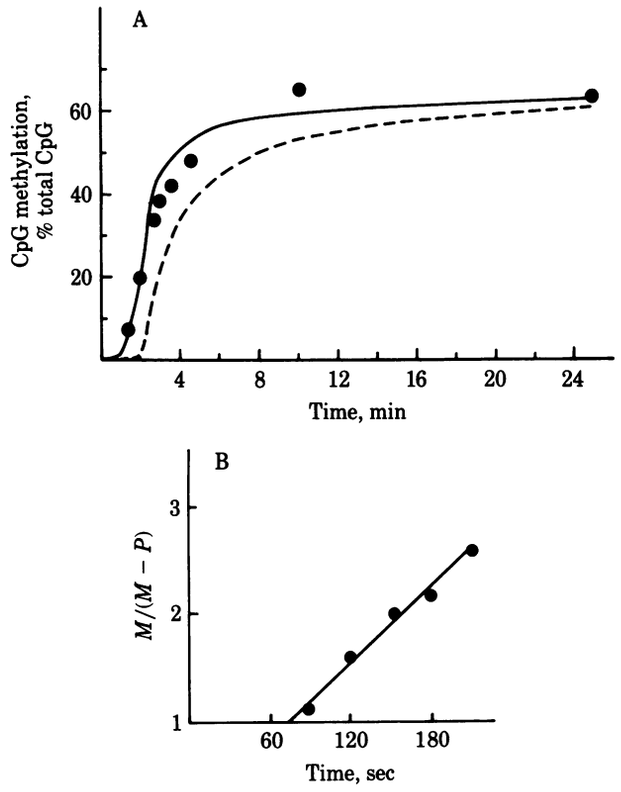

FIG. 1. Kinetics of DNA methylation during replication. (*A*) L cells were permeabilized and labeled, and the DNA was extracted. The extent of CpG methylation was analyzed by the nearest-neighbor method. Theoretical curves for a 1-min lag (——) and a 2-min lag (– – –) were obtained by plotting $M(T - X)/T$ vs. time, where M is maximal methylation (66%), T is the labeling time, and X is the assumed lag time. These labeling times represent the period from the beginning of the permeabilization procedure to cessation of the synthesis reaction, including the 45-sec treatment with Hepes buffer. In general, 5,000 cpm of [α-^{32}P]dGTP were incorporated into DNA per 10^6 cells per min. About 3% of these counts were recovered from the cytosine and 5-methylcytosine chromatographic spots. (*B*) To determine the apparent time lag between DNA synthesis and methylation, the data were plotted as $M/(M - P)$ as a function of time, where P represents the percent of methylation at each time point and M represents the maximum level of DNA methylation (66%). This formulation allows one to obtain the time lag by extrapolation to $P = 0$, since $M/(M - P) = 1$ when CpG methylation (P) is 0.

Table 1. Methylation of post-replicated DNA

Treatment of permeabilized L cells	% CpG methylated
None (25-min labeling)	66
100 μM *S*-AdoHcy (5-min labeling)	10
1 mM *S*-AdoHcy (5-min labeling)	0
1 mM *S*-AdoHcy (5-min labeling) followed by *S*-AdoMet (30 sec)	30
1 mM *S*-AdoHcy (5-min labeling) followed by *S*-AdoMet (1 min)	52
1 mM *S*-AdoHcy (25-min labeling) followed by *S*-AdoMet (30 sec)	30
1 mM *S*-AdoHcy (25-min labeling) followed by *S*-AdoMet (1 min)	50

Mouse L cells were permeabilized and labeled with [α-^{32}P]dGTP and the DNA was purified and analyzed. Treatment with *S*-AdoHcy was by addition to the permeabilization buffer. In experiments in which treatment with *S*-AdoHcy was followed by treatment with *S*-AdoMet, the cells were washed with phosphate-buffered saline and repermeabilized in the presence of 10 μM *S*-AdoMet and 10 μM nonradioactive dGTP, and then the medium was diluted and the DNA from the cells was isolated and analyzed by the nearest-neighbor method.

Methylation of Post-Replicated DNA. The measurements described above are believed to reflect the methylation process that takes place at the replication fork. It was of interest to determine the mode of methylation at other hemimethylated sites found in the DNA. To study this methylation, cells were permeabilized and exposed to [α-^{32}P]dGTP in the presence of *S*-AdoHcy, which is known to inhibit DNA methylation (12). As shown in Table 1, 100 μM *S*-AdoHcy in the medium caused an 85% inhibition of methylation, while addition of 1 mM SAH resulted in complete cessation of methylation activity. Despite the presence of S-AdoHcy hydrolase activity in these animal cells (13), inhibition persists for more than 20 min following the permeabilization procedure. As a result of this treatment (1 mM S-AdoHcy for 5 min), all newly synthesized CpG residues are presumably in a hemimethylated state, having gone through replication without concomitant methylation. When cells after a 5-min inhibition period are repermeabilized in the presence of S-adenosyl-L-methionine (S-AdoMet), methylation of these previously labeled hemimethylated sites proceeds extremely rapidly with no apparent lag. The extent of CpG methylation reaches 30% in 0.5 min and 52% in 1 min. This represents 80% of the maximum obtainable methylation level of 66%. Identical results were obtained following a 25-min inhibition period (Table 1).

DISCUSSION

The present paper describes an approach to the study of the kinetics of methylation of replicating and post-replicated DNA. Brief permeabilization of an asynchronous culture of mouse L cells allows the introduction of labeled dGTP into the cells. The labeled nucleotide, a direct DNA precursor, incorporates in-

stantly (<10 sec) into the DNA, thus providing an appropriate system to study methylation that occurs during replication. A marked difference has been observed between the kinetics of methylation of nascent DNA and methylation of previously synthesized DNA. Although methylation of newly synthesized DNA lags behind the replication process by 75 sec, no corresponding lag is observed in the methylation of DNA that has been synthesized in the presence of S-AdoHcy (a methylase inhibitor). The first type of experiment (Fig. 1) is considered to represent kinetics of methylation at the replication fork. The second type of experiment (analysis of methylation after treatment with S-AdoHcy) (Table 1) represents methylation upstream from the fork and might, therefore, serve as a model for the methylation of repaired DNA.

It has always been a problem to exclude repair DNA synthesis when events involved in DNA replication are studied. The results presented in Fig. 1, however, must generally represent methylation of replicating DNA. This conclusion is based on the following facts: Mouse L cells are devoid of DNA repair activity as compared with mouse primary cultures (14, 15). Furthermore, when repair synthesis is active in a cell, the probability of it occurring at any given site in the genome is very small (<1%) (16), making analysis of methylation following excision/repair difficult. In fact, to prevent the masking over of repair synthesis by replication activity, replication must be suppressed by hydroxyurea (16). In addition, DNA synthesis in permeabilized cells has been shown to represent replication rather than repair (17). Finally, the results of the present study support this conclusion because they indicate that methylation of previously synthesized DNA is characterized by different kinetics than that observed with replicating DNA.

Since the experiments described here were carried out by incorporating dGTP into unsynchronous cell cultures, the methylation observed is S phase methylation. The current data do not answer the question of whether or not methylation of a minor fraction of CpG is postponed. However, methylation of DNA at other stages of the cell cycle has been reported (6, 18). A recent study showed that methylation of certain sequences is completed just before replication in the next S phase (19). In other experiments, it has been shown that some sequences may be methylated when confluent cells are stimulated to divide (16), suggesting the existence of post-replicative methylation.

These observations suggest that some CpG sites capable of methylation may be particularly resistant and therefore methylate slowly. If these sites remain unmethylated before cell division occurs, this will lead to a heritable loss of methylation at some sites. This might well be the mechanism for the process of demethylation that occurs during differentiation. This demethylation has been suggested to explain the phenomenon that many active genes are undermethylated in the tissue of their expression as compared with other tissues and sperm (1).

The results presented here have several implications with regard to understanding of the process of DNA methylation. DNA modification probably occurs fairly rapidly. This observation is consistent with results showing that hemimethylated DNA inserted into L cells by DNA-mediated gene transfer became fully methylated before replication (2). The events immediately following DNA replication are not clearly understood. Both the methylation event and the packaging of the newly synthesized DNA into a nucleosome structure trail close behind the replication fork (20). Our results do not allow one to determine which post-replication event occurs first. The fact that there is efficient methylation of hemimethylated sites that are far removed from the replication fork suggests, but does not prove, that the modification event may take place on a previously formed nucleosomal structure.

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