Volume 13 Number 8 1985 Nucleic Acids Research

The characteristics of DNA methylation in an *in vitro* DNA synthesizing system from mouse fibroblasts

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Received 5 December 1984; Revised and Accepted 28 March 1985

ABSTRACT

An in vitro DNA synthesizing system from mouse fibroblasts has been used to study DNA methylation. DNA methylation occurs in two phases, one at the replication fork and the other farther behind it. Although 4% of the dCMP residues in mouse cell DNA are mdCMP, only 1.7% of the total [α^{32}P]dCMP in newly replicated DNA is methylated in vitro. No methylation of Okazaki fragments was detected. Nearest neighbor analysis of the newly replicated DNA revealed that, although 40% of the CpG dinucleotides were methylated, significant amounts of cytosine methylation were also found in CpC, CpT, and CpA dinucleotides.

INTRODUCTION

DNA methylation, a postreplicative modification of DNA, has been implicated in many important biological functions in both prokaryotes and eukaryotes (for review see 1). In prokaryotes, DNA methylation has been demonstrated to direct restriction-modification and has been implicated in DNA mismatch repair, while in mammalian cells, it has been linked to the control of gene expression. Although prokaryotes contain both 6-methyladenine and 5-methylcytosine, mammalian cells have only the 5-methylcytosine modification. The partially purified mammalian DNA methyltransferases do not show the sequence specificity that has been observed with the prokaryotic DNA methylases. The mammalian enzymes have been reported to methylate predominantly the sequence $_5$CpG$_3$ and to a lesser extent other cytosine dinucleotides (2, 3, 4).

In vivo studies of mammalian DNA methylation and replication have resulted in many apparent contradictions in the temporal relationship of DNA methylation to DNA synthesis, in the methylation of Okazaki fragments, and in the effect of DNA-damaging agents on methylation (for review see 5). In addition, the properties of the semipurified DNA methylases have not been consistent with in vivo observations. An approach to circumvent these difficulties would be the use of a well-characterized in vitro DNA synthesizing system to analyze the direct methylation of DNA using the methyl donor S-adenosylmethionine

(AdoMet). In this report the mechanism of DNA methylation is studied using an in vitro system that has been used successfully in the past to elucidate the mechanism of mammalian DNA synthesis (6), the repair of uracil-containing DNA (7) and the purification of enzymes involved in the initiation of DNA replication (8). Our results show that 1) there are at least two classes of DNA methylation, one linked to replication and one lagging significantly behind it; 2) Okazaki fragments are not methylated; and 3) significant cytosine methylation occurs in dinucleotides other than CpG.

MATERIALS AND METHODS

Chemicals and Enzymes

Nonidet P-40, sodium phosphoenol pyruvate and AdoMet (purified as described previously, 9) were obtained from Sigma. All of the ribonucleotides and deoxyribonucleotides were from P.L. Biochemical. RNAse A, pancreatic DNAse I, spleen phospodiesterase, snake venom phosphodiesterase, micrococcal nuclease, and proteinase K were from Boehringer Mannheim. T1 ribonuclease was from Bethesda Research Laboratories. All radioactively labeled compounds were purchased from Amersham. Deoxyazacytidine was a gift from L. Kedda of the N.I.H.

Cells

Mouse fibroblast cell line B6 was grown in monolayer in Dulbecco's modified eagle medium supplemented with 8% fetal calf serum and 100 units/ml each of penicillin and streptomycin. Cells were passaged using standard tissue culture techniques and harvested during log phase for experiments.

Preparation of Whole-Cell Lysate

Cells were grown in 490-cm^2 roller bottles (Corning) and were harvested by washing with Dulbecco's phosphate buffered saline without Ca^{2+} or Mg^{2+} (PBS) and treating briefly with 0.05% trypsin at room temperature. The cells were resuspended in 10 ml of complete medium at 4 °C and centrifuged at 600xg for 10 min at 4°C. The cells were then washed twice with Ca^{2+}-and Mg^{2+}-free phosphate buffered saline at 4 °C, and the whole cell lysate was prepared as described previously (6, 7).

In Vitro DNA Synthesis

In vitro DNA synthesis was performed as described previously (6, 7), except that all incubations contained 8 μM AdoMet. The specific activities of the radioactively labeled precursors and the concentrations of bromodeoxyuridine triphosphate (BrdUTP), cytosine arabinoside triphosphate (ara-CTP), and dideoxythymidine trisphosphate (ddTTP) are indicated in the figure legends. All incubations were performed at 37 °C for the times indicated. DNA synthesis

was monitored by the incorporation of radioactively labeled dNTP's. The total radioactive incorporation was measured as described previously (7).

In Vitro DNA Methylation

Two different methods were used to monitor DNA methylation. In both cases the in vitro reaction mixtures were identical to those described for DNA synthesis. However, in the first method, the total DNA methylation occurring in this in vitro system was determined by following the transfer of ^{3}H-methyl from [^{3}H-methyl]AdoMet to DNA using an acid precipitation procedure. The procedure was the same as that described for DNA synthesis except that the precipitate was collected by centrifugation for 10 min in an Eppendorf centrifuge and washed twice with 2 N HCl and three times with 95% ethanol. The pellet was dried and resuspended in 200 µl of 100 mM Tris-HCl (pH 8.0), 1% sarkosyl, and 2 mM EDTA. Proteinase K (125 µg) was added, and the sample was incubated at 55°C for 2 h until the pellet completely dissolved. The DNA was again precipitated with 2 N HCl and collected on GF/C filters as described above. HPLC analysis of enzymatically digested DNA treated in this manner demonstrated that all of the ^{3}H radioactivity was in mdCMP (5-methyldeoxycytidine-5'-monophosphate).

In the second method, the in vitro DNA synthesis reaction mixture contained [α^{32}P]dCTP, and the extent of DNA methylation into newly synthesized DNA was determined by measuring the conversion of incorporated [^{32}P]dCMP to [^{32}P]mdCMP chromatographically. The in vitro reactions were stopped by first precipitating with 2 N HCl as described above. After proteinase K treatment, the sample was phenol extracted two times and ethanol precipitated. The resulting pellets were washed twice with 95% ethanol, dried and resuspended in 10 mM Tris-HCl (pH 7.6), and 2 mM Na_3EDTA. RNAse A and T1 ribonuclease (100 µg/ml and 50 units/ml, respectively) were added to each sample and incubated for 2 h at 37 °C. The samples were treated again with proteinase K, phenol extracted, and ethanol precipitated. The resulting DNA was resuspended in 100 µl of Tris-HCl (pH 7.6) and 2 mM Na_3EDTA and dialyzed against the same buffer. For monitoring the conversion of incorporated [^{32}P]dCMP to [^{32}P]mdCMP, the DNA was routinely digested to 5'-dNMP's in a 50-µl reaction mixture containing up to 30 µg of DNA, 10 mM $MgCl_2$, 2 mM $CaCl_2$, 20 mM Tris-HCl (pH 8.0), and 5 µg of pancreatic DNAse I at 37 °C for 1 h; 1 µg of snake venom phosphodiesterase was added and digestion continued for an additional hour. The samples were evaporated to dryness, resuspended in 20 µl of 0.5 M NaH_2PO_4 (pH 5.0), and analyzed by HPLC as described below.

Sedimentation Analysis of DNA

In vitro DNA synthesis and methylation reactions (300 µl) were stopped by

the addition of 700 µl of a solution containing 4.23 M guanidinium thiocyanate, 25 mM Na_3EDTA, 0.5% sarkosyl, and 100 mM β-mercaptoethanol (the pH of which was adjusted to 7.6 by the addition of 1 M Trizma Base (Sigma) to approximately 10 mM). After repeated passages through a 21-gauge needle to shear the DNA, the solution was layered onto a discontinuous CsCl gradient containing 1.0 ml of 1.0 M CsCl in the guanidinium thiocyanate solution used above and 1.5 ml each of 4.5 M CsCl and 5.7 M CsCl in 20 mM Tris-HCl (pH 7.6) and 2 mM Na_3EDTA. The samples were centrifuged for 20 h in the SW 50.1 rotor (Beckman) at 35,000 rpm at 20 °C. The gradient was fractionated by piercing the bottom of the tube and collecting 10-drop fractions. The DNA peak was located by acid precipitation of a 5-µl aliquot of each fraction. The DNA was then pooled, dialyzed extensively against 10 mM Tris-HCl (pH 7.6) and 2 mM Na_3EDTA, and then treated with proteinase K, phenol extracted and ethanol precipitated as described above. The resulting DNA was dissolved in 200 µl of 10 mM Tris-HCl (pH 7.6) and 2 mM Na_3EDTA.

Neutral isopycnic CsCl gradients were performed in 20 mM Tris-HCl (pH 7.6), 3 mM Na_3EDTA, and 0.2% sarkosyl in 56% (w/w) CsCl. Alkaline isopycnic CsCl gradients were the same as above but contained 0.3 M NaOH and 58.6% (w/w) CsCl. Centrifugation was performed in the VTi 65 rotor (Beckman) at 40,000 rpm for 48 h at 20 °C. Fractions (10 drops) were collected and analyzed as described above.

For the analysis of the size distribution of the DNA, NaOH was added to the DNA solution to a final concentration of 0.3 M and alkaline sucrose velocity sedimentation analysis was performed as described previously (7) but in the SW 50.1 rotor at 45,000 rpm at 20 °C for 11 h.

Nearest Neighbor Analysis

For nearest neighbor analysis, *in vitro* reactions (300 µl) containing 50 µl of whole-cell lysate and either [α^{32}P]dATP, -dCTP, -dGTP, or -dTTP at 8.2 Ci/mmole were stopped by precipitation with 2 N HCl, and the DNA was purified by the second method described above for measuring the extent of methylation in newly replicated DNA. The DNA (29 µg) was digested to 3'dNMP's in a 50-µl reaction mixture containing 10 mM $CaCl_2$, 20 mM Tris-HCl (pH 8.0), and 1 µg of micrococcal nuclease at 37 °C for 1 h. The pH was then adjusted to pH 5.0 by the addition of 5 µl of 1 M sodium acetate (pH 5.0) and 4 µl of 200 mM Na_2EDTA, and the mixture was digested for an additional hour with 2 µg of spleen phosphodiesterase. The samples were evaporated to dryness, resuspended in 20 µl of 0.5 M NaH_2PO_4 (pH 4.2), and analyzed by HPLC as described below.

HPLC Analysis

HPLC analyses were performed using a 15-cm C-18 column (R.E. Gourley, Inc.) in either 0.5 M NaH_2PO_4 (pH 5.0) for separation of 5'-dNMP's (10) or 0.5 M NaH_2PO_4 (pH 4.2) for separation of 3'-dNMP's. The retention times (min) for the latter were: C (2.6), mC (5.0), G (6.8), T (8.3), A (14.6). The flow rates were at 1 ml/min and the absorbence at 280 nm was monitored wth a Spectromonitor III from LDC. Radioactivity was monitored by collecting 12-drop fractions directly into scintillation vials using an LKB 2112 Redirac fraction collector and adding 1 ml of H_2O and 10 ml of Aquassure (New England Nuclear Corp).

RESULTS

The In Vitro DNA Synthesis System Can Be Used For The Study of DNA Methylation

The lack of a well characterized genetic system for mammalian cells has led to the development of subcellular systems to study complex biological functions. To study the mechanism of DNA methylation, we used an *in vitro* system that involves the mild detergent lysis of the cells, which leaves the nuclei intact (6). The whole cell lysate supports DNA synthesis which can be monitored by the incorporation of labeled dNTP's. It is also possible to measure DNA methylation either by following the transfer of the methyl group from [^{3}H-methyl] AdoMet to DNA or by monitoring the conversion of incorporated [^{32}P]dCMP to [^{32}P] mdCMP chromatographically. The results of such an analysis are shown in Fig. 1. DNA synthesis is linear for 30 min while DNA methylation increases non-linearly for the same period of time.

Since some *in vivo* studies have shown that there is a lag in DNA methylation following synthesis (11, 12, 13), the effects of various DNA polymerase inhibitors on both DNA methylation and replication were examined *in vitro*. As expected, α-polymerase was found to be the enzyme responsible for DNA replication, since DNA synthesis was inhibited 90% by the α-polymerase inhibitor ara-CTP and was only slightly affected by the β-polymerase inhibitor ddTTP. After a brief lag, ara-CTP inhibited DNA methylation by 50%, whereas ddTTP showed no detectable inhibition of *in vitro* DNA methylation (Fig. 1B). The addition of the nonhydrolyzable ATP analog β,γ -imido-ATP or the exclusion of the nucleoside triphosphates (either of which totally inhibited DNA synthesis) only partially inhibited DNA methylation. These results suggest that, *in vitro*, some DNA methylation may be lagging DNA replication.

To test whether the level of *in vitro* DNA methylation seen in the presence of ara-CTP was nonspecific methylation that was due to the preparation of the whole-cell lysate, cells were treated *in vivo* prior to harvest either with cy-

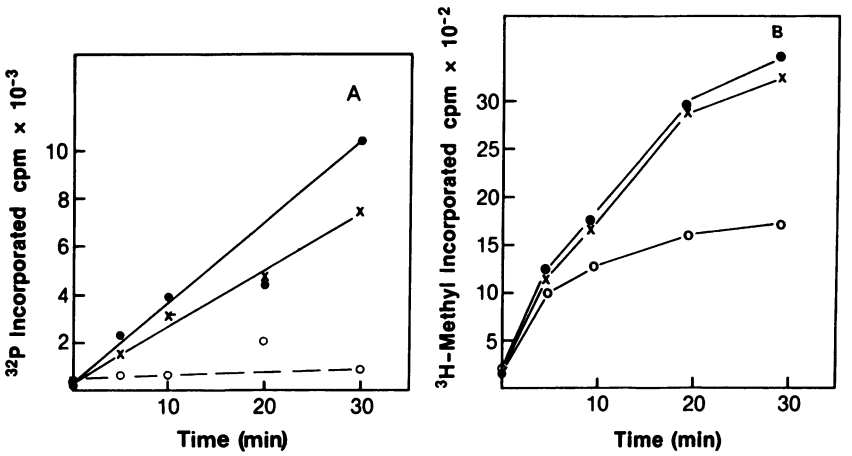

Figure 1. Effect of DNA polymerase inhibitors on in vitro DNA synthesis or methylation. Reaction mixtures (150 µl) containing 25 µl of the whole-cell lysate were incubated under the standard conditions at either (A) 40 µM [α^{32}P]dTTP (0.2 Ci/mmole) and 8 µM AdoMet or (B) 40 µM dTTP and 8 µM [^{3}H-methyl] AdoMet (24 Ci/mmole) (●-●). Both ddTTP (x-x) and ara-CTP (o-o) were added to 300 µM. At the times indicated 25 µl portions were removed and incorporation was measured as described in the Materials and Methods.

tosine arabinoside (ara-C) or with deoxyazacytidine, an inhibitor of DNA methylation (14). The whole cell lysate was prepared from the treated cells by the standard procedure and assayed for both DNA synthesis and methylation. The results are shown in Fig. 2. Both in vitro DNA synthesis and methylation were completely inhibited by pretreatment of cells in vivo with ara-C. These results indicate that de novo methylation is not occurring in vitro suggesting that the in vitro ara-CTP-independent DNA methylation is not an artifact of preparation of the whole-cell lysate but that DNA methylation may be lagging significantly behind the replication fork. The araC inhibition of DNA synthesis in vivo presumably allowed the lagging DNA methylation to catch up with the replication fork. Pretreatment in vivo with deoxyazacytidine only partially inhibited DNA synthesis but completely blocked DNA methylation in vitro. These results are consistent with in vivo studies (14,15,16) in which azacytidine has been shown to completely block DNA methylation.

Since the heritable pattern of DNA methylation could, in theory, be controlled by DNA demethylation, the stability of the methylated DNA was examined in a pulse-chase experiment. Whole cell lysate was incubated with [^{3}H-methyl] AdoMet for 10 min and then a 10-fold excess of unlabeled AdoMet was added and

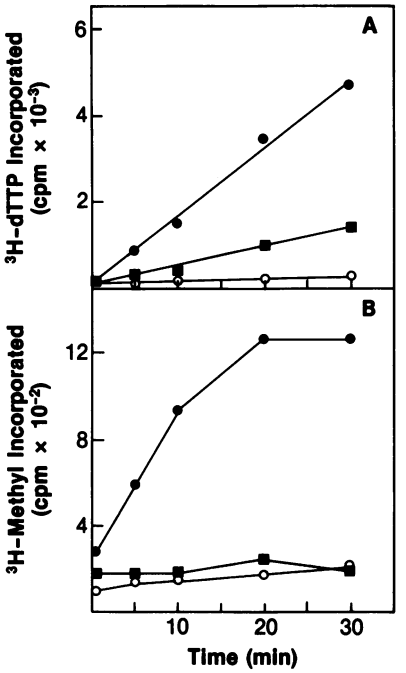

Figure 2. Effect of the in vivo addition of araC or deoxyazacytidine on in vitro DNA synthesis and methylation. Cells were treated either with ara-C (1 mM) at 37 °C in complete medium for 1 h prior to harvest (o-o) or with deoxyazacytidine (10 μM) for 48 h prior to harvest (■-■). Control samples were left untreated (●-●). Cells were harvested and the whole-cell lysate was prepared as described in the Materials and Methods. Reaction mixtures were identical to those described in Fig. 1 for DNA synthesis (A) or DNA methylation (B). Incorporation was measured as described in the legend to Figure 1.

the sample was incubated for an additional 40 min. The addition of unlabeled AdoMet was omitted in the control sample. As shown in Fig. 3, the in vitro methylated DNA showed no loss of methylation during the times examined, demonstrating that DNA "demethylation" does not occur to any significant level in this system.

DNA Synthesized In Vitro is Undermethylated

The above results indicated that in vitro DNA methylation occurred in both-newly replicated and previously synthesized DNA. The extent of methylation in newly replicated DNA can be determined in two ways, either by measuring the conversion of incorporated [^{32}P]dCMP to [^{32}P]mdCMP or by following the transfer of ^{3}H-methyl from [^{3}H-methyl]AdoMet to DNA synthesized in the presence of the density label BrdUTP. Thus, DNA was labeled in vitro in the presence of BrdUTP with either [^{3}H-methyl]AdoMet or [α^{32}P]dCTP. The DNA was purified by the guanidinium thiocyanate procedure and rebanded in alkaline CsCl gradients to determine the amount of newly replicated DNA that was methylated. As expected due to the semi-conservative nature of DNA synthesis, most of the newly replicated, ^{32}P-labeled DNA banded at a heavy density (Fig. 4, region I). However, more than 50% of the ^{3}H-methylated DNA banded at a light density (region III)

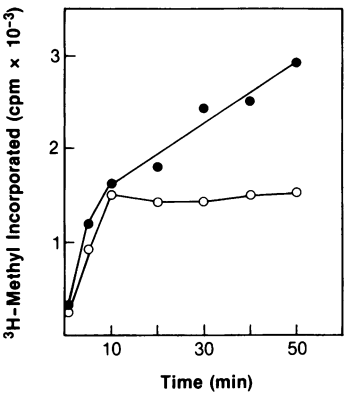

Figure 3. Stability of ^{3}H-methyl incorporated into DNA. Two reaction mixtures (300 µl) containing 50 µl of whole-cell lysate were incubated under the standard conditions at 40 µM dTTP and 8 µM [^{3}H-methyl]AdoMet (24 Ci/mmole). At 10 min, 25 µl of buffer A was added to one sample (●-●), while to the other 25 µl of AdoMet (670 mM) was added to a final concentration of 80 µM (o-o); the samples were then incubated further. Incorporation was measured as described in the legend to Figure 1.

corresponding to previously synthesized DNA. These results agree with our finding that DNA methylation is only partially inhibited by ara-CTP in vitro (Fig. 1), and support our conclusion that DNA methylation is occurring in two phases: one at the replication fork (sensitive to ara-CTP) and one farther behind it (resistant to ara-CTP).

The ^{3}H-methyl label in both the newly replicated and the previously synthesized DNA's (Fig. 4, regions I and III, respectively) was analyzed by HPLC and shown to be present exclusively as mdCMP. Based on the specific activities of both

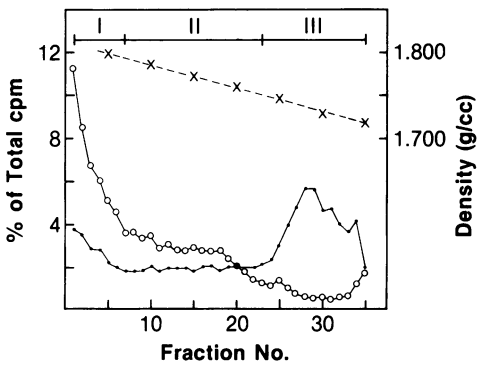

Figure 4. The in vitro methylation of newly synthesized DNA. Reaction mixtures (600 µl) containing 100 µl of whole-cell lysate were incubated for 20 min under the standard conditions at either 20 µM [α^{32}P]dCTP (0.8 Ci/mmole) and 8 µM AdoMet or 20 µM dCTP and 8 µM [^{3}H-methyl]AdoMet (42 Ci/mmole). In each reaction mix brdUTP (200 µM) was substituted for dTTP. The DNA was isolated by the guanidinium thiocyanate procedure, and an aliquot of each was rebanded on two separate alkaline CsCl gradients as described in the Materials and Methods. (o-o) [α^{32}P]dCTP-labeled DNA (54,900 cpm); (●-●)[^{3}H-methyl]AdoMet-labeled DNA (10,500 cpm); (x-x) density.

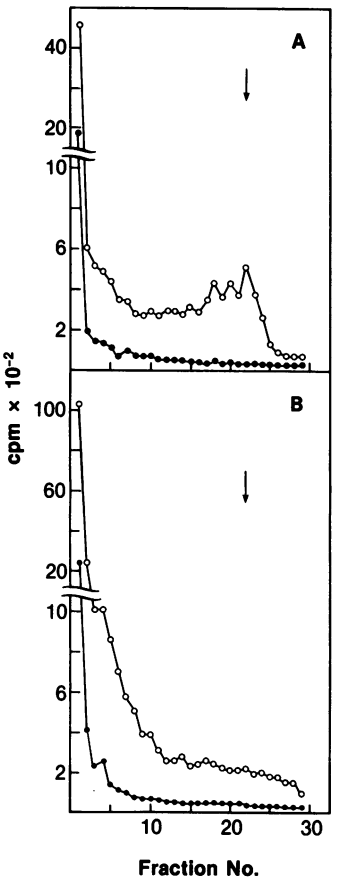

Figure 5. Undermethylation of Okazaki fragments. Reaction mixtures (300 µl) containing 50 µl of whole cell lysate were incubated under the standard assay conditions containing both 20 µM [α^{32}P]dCTP (17 Ci/mmole) and 10 µM [^{3}H-methyl]-AdoMet (81 Ci/mmole). At 1 min the reaction was either stopped (A) or dCTP and S-adenosyl-homocysteine were added to final concentrations of 2 mM and 500 µM respectively and incubation was continued for an additional 20 min (B). The samples were prepared for alkaline sedimentation velocity centrifugation as described in the Materials and Methods. The arrow indicates the position of 4S DNA. Sedimentation is from right to left. o-o ^{32}P; ●-● ^{3}H.

the [^{3}H-methyl]AdoMet and the [α^{32}P]dCTP, one can estimate that 1% of the dCMP in the newly replicated DNA is methylated. However, direct measurement of the conversion of incorporated [^{32}P]dCMP to [^{32}P] mdCMP showed that 1.7% of the dCMP in newly replicated DNA was converted to mdCMP. This discrepancy is likely due to the intracellular pool of AdoMet which can be calculated to be approximately 30 mM. Integration of the A_{280} profile showed that bulk DNA contains 4% mdCMP. These results show that *in vitro* replicated DNA is undermethylated and also imply that there may be a significant lag in methylation following replication.

Under Methylation of Okazaki Fragments

DNA synthesis in mammalian cells proceeds by a semi-discontinuous mechanism in which DNA synthesized on the lagging strand occurs via small molecular weight intermediates (4 S) which are precursors to high-molecular-weight DNA (6, 17).

As shown in Fig. 5A, these intermediates (Okazaki fragments) could be detected in our whole cell lysate system during a 1 min pulse with [α^{32}P]dCTP. When the reaction was continued for an additional 20 min in the presence of 100-fold excess of unlabeled dCTP, essentially all of the labeled DNA becomes high molecular weight (Fig. 5B). Similar results for DNA synthesis both *in vivo* and *in vitro* have been reported for human lymphoblasts (6, 18).

Because the previous results showed that the newly replicated DNA was undermethylated by approximately 50% *in vitro*, we investigated the extent of methylation of Okazaki fragments. Due to the inhibition of DNA synthesis by high concentrations of AdoMet (50% inhibition at 300 μM, data not shown), S-adenosylhomocysteine, a competitive inhibitor of AdoMet (19), was added in a 50-fold excess after 1 min and the reaction was continued for 20 min. During the 1 min pulse with [^{3}H-methyl]AdoMet virtually all of the radioactivity appeared in high molecular weight DNA (Fig. 5A). No methylation of Okazaki fragments was detected *in vitro* since no elongation of the ^{3}H-labelled DNA into higher molecular weight DNA was observed. Based on the specific activity of each radioisotope, methylation could have been detected at a level of 0.6% or nearly one-seventh the level of mdCMP found in B6 cellular DNA. These results demonstrate that Okazaki fragments were undermethylated *in vitro* and suggest that the bulk of DNA methylation occurred after Okazaki fragments were ligated.

Density Distribution of Methylated DNA

Mouse cells contain an AT-rich satellite DNA that has been reported to be overmethylated (20). To determine whether a disproportionate amount of DNA methylation occurred in such a subpopulation of DNA, we purified the DNA's synthesized *in vitro* in the presence of either [^{3}H]dTTP or [^{3}H-methyl]Adomet by the guanidinium thiocyanate procedure and then rebanded the DNA's without the proteinase K treatment on CsCl gradients. As shown in Fig. 6, the *in vitro* methylated DNA had the same density distribution as the newly replicated DNA, suggesting that there was no gross disparity between the densities of the DNA being synthesized and that being methylated.

The ^{3}H-labeled material banding at the lighter density in the [^{3}H-methyl]AdoMet-labelled sample was the result of protein methylation. This was demonstrated both by its sensitivity to proteolytic digestion and by sodium dodecylsulfate-polyacrylamide gel electrophoretic analysis. Visualization of protein bands by staining with Coomassie Blue showed that most of the proteins are histones, and visualization by flourography showed that more than 90% of the protein methylation occurred in histones H3 and H4 (data not shown), the only histones methylated *in vivo*. Taken together, the above results indicate that this *in vitro*

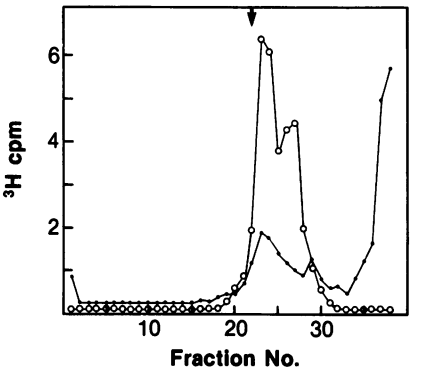

Figure 6. Neutral CsCl gradient analysis of DNA methylated in vitro. Reaction mixtures (600 µl) containing 100 µl of whole-cell lysate were incubated for 20 min under the standard conditions at either 20 µM ^{3}H-dTTP (7 Ci/mmole) and 8 µM AdoMet or 20 µM dTTP and 8 µM [^{3}H-methyl]AdoMet (19 Ci/mmole). The DNA was isolated by the guanidinium thiocyanate procedure, and an aliquot of each was rebanded on neutral CsCl gradients as described in the Materials and Methods. The arrow indicates the position of ^{32}P-labeled pBR322 added as a density marker. (O-O) [^{3}H]dTTP-labeled DNA (cpm x 10^{-3}); (●-●) [^{3}H-methyl]AdoMet-labeled DNA (cpm x 10^{-2}).

system mimics in vivo protein methylation as well as DNA methylation.

Newly Replicated DNA is Methylated in Sequences Other than CpG

In mammalian cells, cytosine methylation has been reported to occur mainly in the sequence CpG, although other deoxycytidine dinucleotides are also methylated (2, 21, 22, 23). Since some DNA methylation occurred on newly replicated DNA (Fig. 4), we used nearest neighbor frequency analysis of this DNA to determine whether in vitro DNA methylation also occurs in the sequence CpG. Thus, whole-cell lysates were incubated in the presence of either [α^{32}P] -dATP, -dCTP, -dGTP, or -dTTP for 30 min and analyzed as described in the Materials and Methods. The results of such an experiment are shown in Table I. The nearest neighbor frequencies were similar to those reported previously (24) for BHK21 cells, and, as expected, the total CpG dinucleotide was underrepresented, indicating that the replicated DNA was representative of the total cellular DNA and was not a subset with an abnormal distribution of bases. In the newly replicated DNA, 40% of the total CpG dinucleotides were methylated, which agrees with other in vivo experiments showing that not all CpG sequences are methylated (25). We were also able to detect significant methylation of the sequences CpA, CpC, and CpT. Although a direct comparison between the various CpX dinucleotides was not possible, we were able to determine that one-sixth of the total methylation is in the dinucleotide CpC. This was accom-

Table 1. Nearest neighbor frequencies of DNA synthesized in vitro.

	5'[α^{32}P]dXTP			
3'dNMP	C	G	T	A
C	25.8	3.3	25.9	26.4
G	18.6	22.9	17.5	21.1
T	30.9	38.5	32.6	21.8
A	24.3	33.3	23.4	30.1
mC	0.3	2.0	0.5	0.5

The amount of label transferred to the 3'dNMP was analyzed as described in the Materials and Methods. Nearest neighbor frequencies are the percentage of total label incorporated into newly synthesized DNA in the sequence NpX.

plished by determining the level of cytosine methylation in the [α^{32}P]dCTP labeled DNA to be 1.7%, a level also found previously in the experiment using BrdUTP as a density label (these results indicate that incorporated BrdUMP has no effect on the level of DNA methylation and agree with previous results obtained *in vivo* (26)). Since nearest neighbor analysis showed that 0.3% of the total [α^{32}P]dCMP incorporated was in the sequence mCpC, then 0.3%/1.7% or one-sixth of the cytosine methylation was in this dinucleotide. Similar analysis of the other samples was not possible. However, after pancreatic DNAse I digestion of DNA methylated *in vitro* with [^{3}H-methyl] AdoMet and HPLC separation of the resulting dinucleotide diphosphates, greater than 90% of the ^{3}H-methyl label was found in the dinucleotide pCpG (data not shown). Nonetheless, the nearest neighbor frequency analysis does indicate that a low but significant amount of cytosine methylation on newly replicated DNA occurs in sequences other than CpG.

DISCUSSION

Through the use of a subcellular system, we have dissected the mechanism of DNA methylation in a manner similar to that performed previously for DNA replication and repair (6, 7). This *in vitro* system appears very similar to that developed for human lymphoblasts (6) because DNA synthesis occurs discontinuously by the synthesis of Okazaki fragments, which are subsequently ligated into higher molecular weight DNA. Replication is also inhibited 90% by the addition of ara-

CTP, an inhibitor of α-polymerase. In addition, the DNA product has a density distribution and nearest neighbor frequency similar to those reported previously.

This *in vitro* system has been shown to reflect authentic *in vivo* DNA replication (18), and in the present study has also been demonstrated to be a valid system for the study of DNA methylation. Using this system, we have verified *in vivo* observations concerning DNA methylation and also provided quantitation that was unavailable from the *in vivo* experiments. We have also shown that *in vitro* DNA methylation occurs while DNA is being replicated; however, the newly replicated DNA contains only 50% of the mdCMP found in total DNA. This result suggests that although 50% of the DNA methylation occurs near the replication fork, the remaining 50% lags significantly behind replication. Similar results have been reported for *in vivo* experiments with mouse fibroblasts (13), whereas other studies (27) have shown that *in vivo* CpG methylation lags replication by only 75 sec. However, in these latter experiments neither the total DNA methylation occurring during replication nor the methylation of other cytosine-containing dinucleotides was measured. Methylation of preexisting DNA was also not determined.

In addition, we have shown that although newly replicated DNA is partially methylated *in vitro*, there is no detectable methylation of Okazaki fragments. Similar results with Okazaki fragments have also been reported *in vivo* (28, 29). In one case, the incorporation of [^{14}C]deoxycytidine (424 cpm) was too low to detect a 4% level of DNA methylation. In the other case, 4% methylation would have resulted in 100 cpm above background in 5-methylcytosine and none was detected. The methylation of Okazaki fragments *in vivo* (30) has been reported. However, it is not certain that this DNA represented authentic Okazaki fragments since the labeling times were relatively long and there was insignificant elongation into high-molecular-weight DNA.

Significant methylation of CpC and CpT dinucleotides has been reported previously (22). However, reasonable estimates of the relative percentage of methylation in each CpX dinucleotide is impossible to extract from these published data. We report here that although 40% of the CpG dinucleotides are methylated and, although CpG represents the major methylation, a significant proportion of the total methylation in the newly replicated DNA is in the dinucleotide CpC. Detectable levels of cytosine methylation are also found in CpA and CpT.

In *Escherichia coli*, the undermethylation of adenine is a cornerstone of the model for DNA mismatch repair, where the DNA repair enzymes are directed to the incorrect base by the undermethylation of the daughter strand. Methylation of Okazaki fragments does occur *in vivo* (31), but they are undermethylated by

35% with respect to adenine methylation. This fact coupled with the inability of purified bacterial methylases to methylate homologous DNA suggests that very little adenine undermethylation may be sufficient to direct mismatch repair. We find undermethylation of Okazaki fragments and significant undermethylation of newly replicated DNA in in vitro experiments with mammalian cells. These results point to the possible existence of methyl directed mismatch repair similar to that proposed for bacterial systems. These facts point to the existence of at least two forms of DNA maintenance methylation, one linked to replication and one lagging behind. Similar conclusions have been reached from in vivo experiments (32). The undermethylation could be a signal for DNA mismatch repair (for which recent evidence has been obtained in mammalian cells (33)) and/or for chromosome folding and packaging. For instance, once the DNA is replicated, all methylation sites become, by definition, hemi-methylated. The initial round of DNA methylation occurs at approximately half of these sites, leaving the remaining sites in their hemi-methylated state. The DNA is then checked for mistakes, the remaining sites are methylated, and the DNA is assembled into its appropriate chromosomal stucture. Such a mechanism could explain the incomplete methylation of newly replicated DNA.

Thus, this in vitro system, which has in the past served as a model for the study of replication and repair, has allowed us to ask pertinent questions regarding methylation and replication. We have been able to quantitate the amount of DNA methylation in newly replicated DNA and to demonstrate that significant levels of DNA cytosine methylation is occurring in dinucleotides other than CpG. Future experiments with this system should provide useful insight into the role that methylation plays in the mechanism of DNA replication, repair, and chromosome structure. Finally, in order to duplicate the in vivo properties of the DNA methyltransferase, it may not be sufficient to purify the catalytic activities, but it might be necessary to reconstitute protein-DNA complexes that have some of the properties of chromatin. The development of this in vitro system represents an important step in this direction.

ACKNOWLEDGEMENTS

The authors wish to thank Ms. D. Plentovich for her expert technical assistance and Drs. S. Austin and M. Gellert for helpful suggestions during the course of this work.

Research sponsored by the National Cancer Institute, DHHS, under contract No. NO1-CO-23909 with Litton Bionetics, Inc. The contents of this publication

do not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

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