An unusual “densely methylated island” (DMI), in which all cytosine residues are methylated on both strands for 127–516 base pairs, has been reported at mammalian origins of DNA replication. This report had far-reaching implications in understanding of DNA methylation and DNA replication. For example, since this DMI appeared in about 90% of proliferating cells, but not in stationary cells, it may regulate origin activation. In an effort to confirm and extend these observations, theDMI at the well characterized ori-β locus 17 kilobases downstream of the dhfr gene in chromosomes of Chinese hamster ovary cells was checked for methylated cytosines in genomic DNA. The methylation status of this region was examined in randomly proliferating and stationary cells and in cell populations enriched in the G1, S, or G2 + M phases of their cell division cycle. DNA was subjected to 1) cleavage by methylation-sensitive restriction endonuclease, 2) hydrazine modification of cytosines followed by piperidine cleavage, and 3) permutate modification of 5-methylcytosines (mC) followed by piperidine cleavage. The permutate reaction is a novel method for direct detection of mC residues that complements the more commonly used hydrazine method. These methods were capable of detecting mC in 2% of the cells. At the region of the proposed DMI, only one mC at a CpG site was detected. However, the ori-β DMI was not detected in any of these cell populations using any of these methods.

DNA methylation at CpG dinucleotides in mammalian cells has been implicated as an important component of such pivotal processes as transcription (1), imprinting (2), carcinogenesis (3), development (4), and replication timing (5). CpG methylation is carried out by a methyltransferase that is associated with replication foci in the nucleus (7). In general, this enzyme methylates specifically hemimethylated CpG dinucleotides in genomic DNA. The methylation status of this region was examined in randomly proliferating and stationary cells and in cell populations enriched in the G1, S, or G2 + M phases of their cell division cycle. DNA was subjected to 1) cleavage by methylation-sensitive restriction endonuclease, 2) hydrazine modification of cytosines followed by piperidine cleavage, and 3) permutate modification of 5-methylcytosines (mC) followed by piperidine cleavage. The permutate reaction is a novel method for direct detection of mC residues that complements the more commonly used hydrazine method. These methods were capable of detecting mC in 2% of the cells. At the region of the proposed DMI, only one mC at a CpG site was detected. However, the ori-β DMI was not detected in any of these cell populations using any of these methods.

CpG dinucleotides can occur but at a low rate (8). In addition, this enzyme occasionally methylates cytosines in sequences other than CpG (9, 10). However, given the specificity of the mammalian methyltransferase, this non-target methylation should be of little significance in vivo because the pattern of non-CpG methylation will not be maintained during the subsequent round of DNA replication.

Recently, an unusual form of DNA methylation has been implicated as an important component of mammalian origins of DNA replication. A densely methylated island (DMI) has been reported at three different mammalian origins of DNA replication (11, 12). These DMIs were found only in proliferating cell populations and consisted of a 127-, 258-, or 516-bp stretch of DNA in which all cytosines were methylated, regardless of their sequence context (12). The discovery of DMIs has several important implications. First, this unprecedented methylation pattern suggested that a second (or modified) methylation enzyme exists in mammalian cells. Interestingly, limited proteolysis of the mammalian DNA methyltransferase stimulates its ability to methyle unmethylated CpG dinucleotides de novo (13, 14). Second, the rapid loss of DMIs when cells stop proliferating implies an active demethylation process. In fact, repair enzymes might be involved in active demethylation (15, 16). Third, the characteristics of DMIs strongly suggest a role in initiation of DNA replication. For example, since the 16 known mammalian origins (17) lack a common extensive sequence homology, a DMI may mark initiation sites for DNA replication. Alternatively, DMIS might provide a means to recognize origins that have already replicated. Remethylation at the 11 dam methylation sites at Escherichia coli’s ori-C is delayed about 10 min, during which time the hemimethylated ori-C is bound to the outer bacterial membrane in a form that cannot reinitiate replication (18, 19). The DMI might play an analogous role in mammalian cells where nuclear structure is required for site-specific initiation of DNA replication (20, 21). In fact, site specificity is established during the middle of G1 phase in each cell cycle (22), about the time when DNA methyltransferase activity appears and then increases during S phase (23–25). Therefore, it was imperative to confirm the existence of a DMI at a well characterized mammalian replication origin and to determine whether or not a DMI appears at a specific time during the cell division cycle.

The DMIS described above were identified using the bisulfite method to detect 5-methylcytosines (mC). Bisulfite catalyzes the hydrolytic deamination of cytosines, but not mC, to uracils...
Absence of DMI at Replication Origins

(26–28). However, this method suffers from the fact that conversion of cytosines to uracils requires that DNA be in a single strand state (29, 30). Thus, failure to properly denature the genomic DNA or partial renaturation during the bisulfite treatment can lead to persistence of cytosines that will be interpreted erroneously as the presence of \textsuperscript{mC} (31). Therefore, we investigated cytosine methylation by three alternative and independent methods. The first method used methylation-sensitive restriction endonucleases to identify specific sites that are methylated and, therefore, resistant to cleavage. Cleavage was monitored by direct visualization of the DNA products using Southern blotting and hybridization. Alternatively, uncleaved genomic DNA was quantitated by PCR amplification. Southern blotting and hybridization imposes the fewest DNA manipulations and provides a direct estimate of the fraction of cells containing a DMI, whereas PCR monitoring allows the detection of very small DMI fractions. However, digestion by restriction endonucleases detects only a subset of potential methylation sites. The second method is able to detect \textsuperscript{mC} at any nucleotide position by modifying DNA with hydrazine and then cleaving the modified cytosines with piperidine. Although hydrazine, like bisulfite, reacts with cytosines, but not with \textsuperscript{mC} (32), hydrazine modification is independent of the strandedness of the DNA. However, because \textsuperscript{mC} is identified by the absence of a band in the cytosine sequence of genomic DNA that is present in the cytosine sequence of cloned DNA (i.e. “negative display” of \textsuperscript{mC}), background cleavage events or closely spaced bands may lead to ambiguity. This problem was overcome by a third method. Permanganate at slightly acidic pH can modify thymines and \textsuperscript{mC} but not cytosines (33). Permanganate reactivity requires that the DNA be single stranded, but in contrast to bisulfite, the strandedness of the DNA can be monitored by the concomitant reaction of thymines, which also takes place only in single-stranded DNA. To our knowledge, the permanganate/piperidine method for detection of \textsuperscript{mC} has never before been applied to complex genomes. Therefore, conditions for the permanganate reaction were optimized for this purpose, and the method is presented here as a valuable tool for investigation of DNA methylation in complex genomes.

Using the methods described above, cytosine methylation was investigated at the DMI locus at \textit{ori}-\textbeta, an origin of bidirectional replication located 17 kb downstream of the \textit{dfhr} gene in Chinese hamster ovary (CHO) cells (17). Methylation was examined in randomly proliferating and stationary CHO K1 cells containing two copies of the \textit{dfhr} gene region per diploid genome and in CHO C400 cells containing ~10\textsuperscript{3} tandem copies of this region. CHO K1 cells were also isolated in the G1, S or G2 + M phases of their proliferation cycle. In no case was the DMI detected, although the sensitivity of the methods allowed detection of \textsuperscript{mC} in 2\% of the total DNA.

EXPERIMENTAL PROCEDURES

Cell Culture and Cell Harvesting—CHO were propagated after 1:20 dilution of a confluent starting culture in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 0.4 mM l-glutamine and 5 (CHO K1) or 10\% (CHO C400) fetal calf serum, at 37 °C in 5\% CO\textsubscript{2}. Proliferating cells were harvested when they reached ~50% confluency (about day 3). To obtain stationary cells (G\textsubscript{0}), cells at about 30% confluency were washed with PBS and cultured for another 5 days in DMEM + 0.4 mM l-glutamine + 0.5% fetal calf serum (12). Further culturing of cells at low serum, to increase the portion of G\textsubscript{0}, resulted in slow cell death without change of the FACS profile (data not shown). For harvesting, cells were washed with PBS and trypsinized. Trypsin digestion was stopped by adding DMEM + 10\% fetal calf serum. The cells were collected by centrifugation at 1,000 \times g for 10 min at 4 °C and resuspended in PBS on ice. The profile of the cell cycle was checked before and after harvesting by FACS analysis (see below). Cells were used for elution or immediately for DNA isolation.

Elutriation—Centrifugal elutriation was carried out on 1.2 \times 10\textsuperscript{8} proliferating growing CHO K1 cells in 5 ml of PBS using a Beckman rotor (JE-5.0) and centrifuge (model J-6/ME) at 2,500 rpm. The flow rate was gradually increased until cells reached the upper region in the chamber. 1-2 fractions were washed free of adhering cells (34). The cells were kept on ice, and an aliquot (~1 \times 10\textsuperscript{6} cells) of each fraction was used for FACS analysis. DNA was prepared from the remaining cells.

FACS Analysis—Prior to harvesting, adherent cells in one 75 cm\textsuperscript{2} flask were rinsed once with DMEM lacking serum and stained as described (34) with some modifications. Briefly, 5 ml of sterile cold staining solution (4 mM sodium citrate, pH 7.0, 5 µg/ml propidium iodide, 0.5% Triton X-100, and 10 µg/ml freshly added RNase A) was added to the cells and then placed at 4 °C for 45 min in the dark. Residual adhering cells were dislodged by pipetting. After harvesting or elutriation, the cells were rinsed in PBS, centrifuged at 1,000 \times g for 5 min at 4 °C, resuspended in the above staining solution, and held at 4 °C for 45 min in the dark. The cell suspensions (~1 \times 10\textsuperscript{5} cells/ml) were analyzed by flow cytomtery (Epics XL/MCL 4 Color Coulter). Between 5,000 and 20,000 fluorescent events were counted. The FACS profile of cells treated by this method was stable for at least 2 weeks.

Genomic DNA Extraction, Enzymatic Digestions, and Quantitative Southern—Cells at (~5 \times 10\textsuperscript{6} per ml in PBS) were mixed with an equal volume of lysis buffer (1% sarcosyl, 75 mM Tris-Ch, pH 8.0, 25 mM EDTA, 100 µg/ml proteinase K) was added to the suspension. Cells were lysed by incubation at 50 °C overnight. The solution was extracted twice with TE-equilibrated phenol and once with chloroform:isoamyl alcohol (24:1). RNase A was added to 100 µg/ml, incubated for 3 h at 37 °C, and dialyzed extensively against TE buffer for 2 days. Genomic DNA was concentrated under vacuum to 0.1 of the previous volume and dialyzed once more against 0.1 x TE buffer. After this step, DNA had a final concentration of 0.1 µg/µl.

Cleavage of genomic DNA with \textit{Alu}I and \textit{Mbo}II was performed according to the instructions of the manufacturer. An unrelated ~100-bp DNA fragment containing an \textit{Alu}I site, radioactively labeled at one 5’-end, was added to the \textit{Alu}I digests of genomic DNA. Completeness of \textit{Alu}I cleavage was monitored by autoradiography after an aliquot of the \textit{Alu}I digestion was subjected to gel electrophoresis. Only the genomic DNA from reactions in which the labeled fragment was completely cleaved was used for Southern blotting (below). A second control for completion of both enzymatic digestions was performed by monitoring the average length of DNA after gel electrophoresis and ethidium bromide staining. In order not to obscure the subsequent Southern blot analysis, the radioactive phosphate was removed from the end-labeled fragment by treatment with calf intestine phosphatase. Digested genomic DNA was phenol-extracted, ethanol-precipitated, and dissolved in TE at 1 µg/µl.

For Southern blot analysis, equal amounts of \textit{Alu}I- or \textit{Mbo}II-labeled genomic DNA of logarithmically growing or stationary CHO cells were run in parallel with radioactively end-labeled DNA molecular weight markers on a 2.5\% BIOZYME “small DNA” agarose gel in TBE buffer. Ten \% DNA and 1 µg/ml yeast RNA at 50 °C overnight were used. After electrophoresis, the DNA was denatured by soaking the gel in 0.2 M NaOH, 5 mM EDTA for 40 min. The gel was neutralized with 0.5 M sodium phosphate buffer, pH 6.5, and equilibrated in 10 mM sodium phosphate buffer, pH 6.5. The DNA was electrophoretically transferred onto a nylon Hybond-N\+ membrane (Amersham Corp.) in a Bio-Rad Mini-PROTEAN II blotting chamber in 10 mM sodium phosphate buffer, pH 6.5, at 250 mA and 30 V overnight. Complete transfer was verified by comparing the autoradiographs of the labeled DNA marker in the gel and on the membrane prior to hybridization. The transferred DNA was fixed by placing the membrane on Whatman paper soaked in 0.4 M NaOH for 20 min and baking it for 2 h at 80 °C. This treatment ensures complete retention of DNA on the membrane during hybridization, as verified by autoradiography of the labeled DNA marker prior to and after hybridization. Genomic DNA was hybridized with end-labeled primers 6C and 6E in Church buffer (32) supplemented with 250 µg/ml yeast RNA at 50 °C overnight and washed as described (32). Hybridization signals were visualized by autoradiography.

PCR Detection of Cleavage Pattern of Methylation-sensitive Restriction Enzymes at Genomic DNA from Synchronized Cells—Proliferating and elutriated CHO cells were grown as described above. Cells grown to early-, mid-, or late-G\textsubscript{1} were harvested at 40, 120, or 195 min, respectively, after re-plating cells obtained by mitotic selection (21). Progression into S phase, which occurred at approximately 4 h after replating, was monitored by incorporation of [\textsuperscript{3}H]thymidine into parallel cultures (21). At the indicated time, cells were washed free of medium using cold (4 °C) PBS, released by trypsinization, and counted.
Absence of DMI at Replication Origins

**TABLE I**

| Primers used in LM-PCR at the dhfr ori-β |
|----------------------------------------|
| Sequence position numbers refer to GenBank™ accession number X94372. |

| General primers | A | B |
|-----------------|---|---|
|                 | = | 5'-GGG GTG ACC CGG GAG ATC TGA ATT C |
|                 | = | 5'-GAG TTA ACG TC |

| Sequence specific primers |
|---------------------------|
| **Set 1**                |
| C (2661–2678)            | = | 5'-CTT TCT TCC CTT TCT CTC AGA TGA GTC C |
| D (2675–2703)            | = | 5'-TTC TCA GTG AGT CCA CTT GCT TTA AAA TC |
| E (2688–2718)            | = | 5'-CAC TTT CCT AAT CAG GTC TTA AAG AGC C |

| **Set 2**                |
| C (2928–2902)            | = | 5'-TAT TGG ATT AGA GGC CAT TCG TGG AGC |
| D (2914–2888)            | = | 5'-CCA TCT GTG GAG CTG TCG TGT TTT CTC |
| E (2898–2870)            | = | 5'-TGT GTT TTC TCA AAA ACC TCG TTT CAT GC |

| **Set 3**                |
| C (3211–3238)            | = | 5'-AGG TTT AAT CCA TTG TCA TTG TTG CAG G |
| D (3227–3252)            | = | 5'-CAT GAT TGC AGG AAG TAT GGT GCC CC |
| E (3240–3265)            | = | 5'-AGT ATG GTG GCC CAC AGG CAG ACA TG |

| **Set 4**                |
| C (3163–3193)            | = | 5'-GAA ACT CTT ATA AAG GAA AGC AAT TAT TGG G |
| D (3181–3209)            | = | 5'-AGC AAT TAT TGG CTC CTT ACA GGT TTA ATC C |
| E (3194–3221)            | = | 5'-TCC AGC TTA CAG TCA GGT TTA AAG CAC GC |

| **Set 5**                |
| C (2539 to 2878)         | = | 5'-TTT CTA CTG CCG TAT TAT AAG ACA AAT GTC |
| D (2587 to 2882)         | = | 5'-AAG ACA AAT GTC AGC ATG AAG GCA GAG |
| E (2571 to 2900)         | = | 5'-CAT GAA GCC AGG TTT TTC TCA AAA CAC AGC |

| **Set 6**                |
| C (3014 to 3040)         | = | 5'-GTT TCG TGT AGC TTA GGT TAG AGG TAG |
| D (3023 to 3049)         | = | 5'-GTC ACA TTA GGT TAG ACT CTG AAC |
| E (3036 to 3061)         | = | 5'-AGT GCA TCT AGC ATG AAG CAG CTG GGG |

| **Set 7**                |
| C (3433–3404)            | = | 5'-GAC TAT TTA CTA AGT CTC ATC AGC AAT AGC |
| D (3421–3395)            | = | 5'-AGT CTC ATC AGA AAT AGC AAG CAG CTG GGG |
| E (3404–3377)            | = | 5'-CAA GCT GGT GGA TTA GAT ACC TCT TAG TAC C |

| **Set 8**                |
| C (3061–3036)            | = | 5'-CTT GGG AGG CCA GTG CAG AAT CCA |
| D (3049–3023)            | = | 5'-GTT CAG ATG CTC AAT TGC TGA TGT CAC |
| E (3040–3014)            | = | 5'-CTA CTT AAC TGA TGT CAT ACC ACA GCA ACC |

DnaA site was radioactively labeled at one 5'-end by standard methods. CpG sites of an aliquot were methylated by the bacterial SssI methylase (New England Biolabs). Methylated and unmethylated fragments were cleaved with MspI (New England Biolabs) or HpaII (Boehringer Mannheim) according to the manufacturer’s instructions. Hydrazine/piperidine treatment of methylated and unmethylated fragments was performed as described (35). KMnO₄/piperidine treatment was as described (33) with the modification that the pH of the sodium acetate buffer was lowered to 4.1. The products of all cleavage reactions were analyzed on a sequencing gel.

Analyses of DNA Methylation in a Synthetic PCR DNA Fragment by Chemical Probes—A 790-bp fragment of the DNA region around the proposed DMI was synthesized by PCR on a cloned DNA template by using primer 1D (Table I) and a second primer 5'-ATA ATA AAA AAA CTA GTT TGT ATG CAT TTT ATG G-3' (positions 3475–3442) and either dCTP or methyl-dCTP at a final nucleoside triphosphate concentration of 200 μM. The PCR product was purified from a low melting point agarose gel by isochromphoresis (36). Hydrazine/piperidine or KMnO₄/piperidine treatment of fully methylated and fully unmethylated fragments was performed as described above. Guanine cleavage was as described (35). Cleavage products were visualized by LM-PCR.

Analyses of DNA Methylation in Genomic DNA by Chemical Probes—Conditions for base-specific modification of genomic DNA by hydrazine in vitro, followed by piperidine-catalyzed cleavage, have been described previously (32, 37). Genomic DNA was cut with PstI to reduce its viscosity before treatment with hydrazine/piperidine. Ligation-mediated polymerase chain reaction (LM-PCR) was carried out as described previously (38) and modified (39, 40). Conditions for base-specific modification of genomic DNA by permanganate in vitro followed by piperidine-catalyzed cleavage have been described previously (33) and were modified as follows. High molecular weight DNA was cut with PstI to reduce its viscosity, phenol-extracted, and ethanol-precipitated. A 50-μl sample containing 50 μg of genomic DNA in TE buffer was heated to 90 °C for 2 min and immediately placed on ice. 200 μl of 30 mM NaOAc, pH 4.1, was added at room temperature (22 °C), before adding 50 μl of freshly prepared 6 mM KMnO₄. The sample was incubated for 3 min at room temperature and terminated by adding 20 μl of β-mercaptoethanol. DNA was precipitated in 0.3 M NaOAc, pH 5.0, and 2 volumes of
ethanol, dissolved in TE buffer, and precipitated again. The sample was dissolved in 100 µl of 10% piperidine, incubated for 30 min at 90 °C, and then quenched at 0 °C. Piperidine was removed by vacuum centrifugation.

**RESULTS**

The Putative ori-β DMI Was Not Detected by a Methylation-sensitive Restriction Endonuclease, Southern Blot Detection—A densely methylated island (DMI) was reported to overlap with the minimal origin of bidirectional replication (OBR) ori-β located about 17 kb downstream of the dihydrofolate reductase (dhfr) gene in hamster CHO K1 cells (Ref. 12; see Fig. 1). This DMI exhibited four critical features: (a) all cytosines of both strands in a region of 516 bp were methylated, irrespective of dinucleotide composition; (b) both borders were well defined; (c) at least 86% of CHO K1 cells of an asynchronous, proliferating culture displayed this unusual methylation pattern; (d) serum-deprived, stationary cells lost their DMI, but regained it after resuming growth.

In an effort to confirm these results, the methylation state of the putative ori-β DMI was investigated using a method that required the fewest manipulations of genomic DNA. DNA was digested with a restriction endonuclease, AluI, that recognizes and cleaves AGCT, a non-CpG site, but is inhibited when the cytosine in either strand is methylated (41). DNA products were then analyzed by Southern blotting and hybridization. AluI has three recognition sites within the DMI region (Fig. 2C). A methylation-insensitive restriction endonuclease, MboII, was used to determine the general accessibility of DNA to restriction enzymes (41). MboII has two recognition sites with the sequence GAAGA/TCTTC in the DMI (Fig. 2C). Inhibition of AluI cleavage due to methylation of the recognition sequence and insensitivity of MboII was confirmed by digestion of a PCR fragment spanning the DMI region, which was synthesized in the presence of either dCTP or 5′dCTP (“Materials and Methods”; data not shown). DNA from CHO K1 cells containing a single copy of the dhfr locus per haploid genome and from CHO C400 cells, which have amplified this locus about 80–85% of the total population.

Complete digestion of genomic DNA with AluI was monitored by the concomitant cleavage of a short, radioactively labeled fragment containing a unique AluI site, which was included in the cleavage reaction. Genomic DNA on the membrane was hybridized with two radioactive primers located at adjacent sites at the center of the DMI. As shown in Fig. 2B, lanes 4 and 7, cleavage of genomic DNA of both logarithmically growing CHO K1 and CHO C400 cells yields a fragment of 221 bp (arrows), which is expected when AluI is not inhibited by methylation (compare Fig. 2C). The same fragment with the same intensity was visible when DNA from stationary cells was analyzed (Fig. 2B, compare lanes 3 and 4 and lanes 7 and 8). All four possible AluI fragments (Fig. 2C) appeared when the membrane was hybridized with a synthetic PCR fragment spanning the entire DMI region (data not shown). In no case did we obtain a fragment of 740 bp, which should appear if all three AluI sites within the DMI were resistant to cleavage due to methylation (see Figs. 2B and 3C, broken lines). Cleavage of all DNAs with MboII yielded the expected fragment of 411 bp, indicating unimpaired accessibility of the DNA at this region (Fig. 2B, lanes 1, 2, 9 and 10, arrows; Fig. 2C). These results reveal that at least some non-CpG sites of the proposed DMI region of most proliferating or stationary CHO cells must be unmethylated.

The Putative ori-β DMI Was Not Detected by Four Methylation-sensitive Restriction Endonucleases, PCR Detection—The fraction of genomic DNA that resists cleavage to AluI or other methylation-sensitive restriction endonucleases can be quantitated by PCR using flanking primers, since only those molecules that resist cleavage allow synthesis of a full-length PCR product. Using this assay Tasheva and Roufa (12) concluded...
that ~86% of proliferating CHO cells contained a DMI at ori-β and ori-RPS14 (12). To determine whether or not a DMI could be detected at ori-β using the same strategy employed by Tasheva and Roufa (12), the methylation-sensitive restriction endonucleases (41) AluI (AGCT), AccI (GTMKAC), Alw26I (GGATC), or MslI (CAYN_RTG) were incubated individually with DNA from proliferating CHO cells isolated in our laboratory or with DNA samples provided by Tasheva and Roufa. All four of these enzymes cut within the putative DMI at ori-β, and AluI and MslI cut within the putative DMI at ori-RPS14, another locus shown to contain an OBR (43). DNA products were amplified using PCR under conditions designed to give a linear response between the amount of template included in the reaction and the amount of product obtained (data not shown). Following digestion with these enzymes, no amplification product was detected at ori-β using the same strategy employed by Tasheva and Roufa (12), the methylation-sensitive restriction endonucleases (41) AluI (AGCT), AccI (GTMKAC), Alw26I (GGATC), or MslI (CAYN_RTG) were incubated individually with DNA from proliferating CHO cells isolated in our laboratory or with DNA samples provided by Tasheva and Roufa. All four of these enzymes cut within the putative DMI at ori-β, and AluI and MslI cut within the putative DMI at ori-RPS14, another locus shown to contain an OBR (43). DNA products were amplified using PCR under conditions designed to give a linear response between the amount of template included in the reaction and the amount of product obtained (data not shown). Following digestion with these enzymes, no amplification product was detected at ori-β (Fig. 3, lanes 1–4). On the other hand, when genomic DNA was digested with XbaI, a restriction endonuclease that cuts outside the putative DMI, the putative DMI region was amplified (Fig. 3, lane 5). Equivalent results were obtained with genomic DNA from quiescent CHO cells and from CHO cells synchronized in their early, mid, or late G1 phases (data not shown). Control digestions using a PCR DNA fragment that had been generated in the presence of 5-methyl-dCTP demonstrated that the enzymes used would not cut methylated DNA even when an excess of incubation time and enzyme is used (see "Materials and Methods"; data not shown). To provide a quantitative assay for the detection of the highest portion of the cells possessing a DMI, the PCR conditions were adjusted to detect residual uncleaved molecules. It was estimated that a DMI, as defined by Tasheva and Roufa (12), cannot exist in more than 2% of the cells. Taken together, these results confirmed the absence of the putative DMI at ori-β. Similar results were obtained at ori-RPS14.

Hydrazine and Permanganate Are Complementary Probes for Methylcytosines—The methylation state of the ori-β DMI was further investigated at nucleotide resolution using two chemical probes, hydrazine and permanganate, that discriminate between cytosine and methylcytosine. Hydrazine reacts only with cytosine (32). To ensure specificity of this method, a 5′-end-labeled DNA fragment (unrelated to ori-β) was methylated at all CpG dinucleotides using bacterial SssI methylase. Complete methylation was confirmed by digestion of an aliquot of

![Image](http://www.jbc.org/)
13–17 that are discussed later) was slightly more retarded relative to unmethylated DNA (Fig. 4, lanes 7 and 10 and also lanes 12 and 15–18, see below). This most probably resulted from the increased mass due to the methyl groups.

Permanganate modifies thymines and methylcytosines in single-stranded DNA but not cytosines (33). Modified residues can be cleaved by treatment with piperidine. The specificity of this method was also confirmed using the same DNA fragments as described above for hydrazine. To specifically enhance the methylcytosine reaction, several parameters were optimized, the most important of which was reducing the pH from 4.8 to 4.1. Under these conditions, all methylcytosines were cleaved to the same extent as thymines (Fig. 4, compare lanes 11 or 13 with lane 12). In addition, a single guanine at position G28 was also cleaved. This was not surprising, because some depurination (creating piperidine-sensitive cleavage sites) was expected due to the slightly acidic pH (33). Most importantly, all cytosines remained unreactive. This method, therefore, complements the hydrazine method and has the same degree of specificity, albeit in a reciprocal manner.

It was also necessary to determine the sensitivity limit of chemical probing of the DNA, i.e. what percentage of methylated cytosines at a particular site can be still detected in a mixed, inhomogeneous DNA molecule population also containing unmethylated cytosines at the same site. This was achieved by mixing increasing amounts of unmethylated to decreasing amounts of methylated fragment, both of which had been probed with hydrazine, keeping total DNA amount constant (Fig. 4, lanes 14–18). Analysis of these mixtures revealed that at least 25% of methylcytosine is required to give a slight

**Fig. 5.** Analysis of cytosine methylation at the dhfr ori-β region using hydrazine or permanganate. A, synthetic PCR fragment. LM-PCR analysis of a fully unmethylated (lanes 1–3) or fully methylated (lane 4) synthetic PCR fragment, treated with dimethyl sulfate/piperidine (lane 1) or hydrazine/piperidine (lanes 2–4), in the presence (lane 2) or absence (lanes 3 and 4) of 1.5 M NaCl. Arrows indicate methylcytosine positions. See text for explanations. B–C, genomic DNA. A region is shown which was obtained with primer set 2 (B) or primer set 6 (C) on an unmethylated synthetic PCR fragment (SF, lanes 1 and 4) treated with hydrazine/piperidine in the presence of 1.5 M NaCl (HZ) or on genomic DNA (gen.; lanes 2, 3, 5, and 6) treated with hydrazine/piperidine (HZ, lanes 2 and 3) or with potassium permanganate/piperidine (KM; lanes 5 and 6). DNA was obtained from logarithmically growing (log) or stationary (stat) cells, as indicated. The methylated CpG residue at 2849 is indicated by an arrow. Brackets indicate the borders of the putative DMI region. The gradual decrease in intensity of all bands toward the upper region of lanes 2 and 3 in B is due to a fortuitous stronger hydrazine reaction with the genomic as compared with the synthetic DNA (lane 1).
visible decrease in band intensity compared with a homogene-
ous population with only cytosine at that site (Fig. 4, lane 17
versus 18). In these analyses, greater retardation of T and C
bands of methyl-DNA than those of methyl-free DNA in the
same lane for the reason explained above is clearly evident.

**Hydrazine and Permanganate Detect a Methylcytosine at ori-β, but Did Not Detect a Densely Methylated Island—**

To determine whether the DMI was present in only a small
portion of the DNA from an asynchronous proliferating popu-
lation of cells, DNA was isolated from CHO K1 cells in different
phases of their cell division cycle. Proliferating CHO K1 cells,
cleared with restriction enzymes to reduce its viscosity, and then
treated with either hydrazine or permanganate as described above. Two examples of these
analyses are provided. One region should contain the border of the DMI at position 2806 (Fig. 5B; Fig. 1). The other region
should contain an internal portion of the DMI (Fig. 5C). The
products of a hydrazine reaction designed to display cytosines
only were obtained using an unmethylated PCR fragment as a
substrate (Fig. 5B, lane 1). The products from a hydrazine
reaction designed to display both cytosines and thymines using
DNA from stationary and proliferating cells are shown in Fig.
5B, lanes 2 and 3, respectively. Reduced cytosine reactivity
compared with the reactivity of nearby thymine residues indi-
cates that a cytosine is methylated. For example, the intensity
of the cytosine band at position 2849 was much less than that
of a nearby thymine (position 2852) but was equivalent to the
background in these lanes (Fig. 5B, lanes 2 and 3). This
indicates that this cytosine is methylated in most, and probably all,
genomic DNA molecules. This cytosine is a constituent of a
CpG site and served as an internal control for the specificity
and sensitivity of the hydrazine reaction. In contrast, all other
cytosines of the displayed genomic region (Fig. 5B, lanes 2
and 3) were as reactive toward hydrazine as nearby thymines in
the same lanes. Therefore, given the sensitivity of this method (Fig.
4), at least 75% of the genomic DNA must be unmethylated.

These conclusions were confirmed using the permanganate
method on single-stranded DNA of the same genomic region.
The cytosine at position 2849 was methylated, because it re-
acted with permanganate in DNA from stationary or prolifer-
ating cells (Fig. 5B, compare lanes 5 and 6). No other cytosine residue
gave a signal above background in this analysis (Fig. 5B, com-
pare lanes 5 and 6 with lane 4), suggesting that no other
cytosine in this region was methylated. Since all thymines in
this region reacted with permanganate, the non-reactivity of
cytosines did not result from the presence of double-stranded
DNA. Beside thymines, guanines also gave slightly weaker
 cleavage signals. These signals may have resulted from limited
permanganate oxidation or from acidic depurination of genomic
DNA (33). This was more apparent here than in pilot experi-
ments (Fig. 4), probably due to enhanced DNA fragmentation
cau sed by the initial heating at 95 °C in LM-PCR.

Analysis of an internal DMI region that was expected to be
fully methylated revealed that all cytosines in at least 75% of the
DNA from stationary or proliferating CHO K1 cells were
unmethylated (Fig. 5C). No cytosine residue was resistant to
modification by hydrazine (Fig. 5C, compare lanes 2 and 3 with
lane 1), and all cytosine residues were insensitive to permang-
anate (Fig. 5C, compare lanes 5 and 6 with lane 4). A total of
about 90% of the entire putative DMI region and both DMI
boundaries were analyzed in proliferating CHO K1 cells, and parts
of the DMI were analyzed in stationary CHO K1 and CHO
C400 cells (Fig. 5 and data not shown). Taken together, the
data obtained from hydrazine and permanganate methods for
14C detection revealed only one 14C residue that is a constituent
of the single CpG within the putative ori-β DMI. Given the
sensitivity of these methods (Fig. 4), the remaining cytosines on
either strand of the DMI region were unmethylated in at least
75% of the DNA from either proliferating or stationary cells.

To determine whether the DMI was present in only a small
portion of the DNA from an asynchronous proliferating popu-
lation of cells, DNA was isolated from CHO K1 cells in different
phases of their cell division cycle. Proliferating CHO K1 cells
were fractionated by centrifugal elutriation and analyzed by
FACS (Fig. 6). The results revealed a distribution between G1,
S, and (G2 + M) phases of about 1.6:1.4:1, respectively. The
enrichment factor for the G1 (fraction 1), S (fraction 4), and G2

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**Figure 6.** FACS analysis of elutriated fractions of a proliferating
CHO K1 cell population. Abscissa, fluorescence of propidium iodide-
stained nuclei in arbitrary units. Ordinate, count events. Enriched
fractions are emphasized on the right of the figure. See text.
Absence of DMI at Replication Origins

+ M phases (fraction 6) was 2.5–4-fold. Analysis of the DNA from these fractions by either the hydrazine or permanganate methods failed to reveal the presence of a DMI (data not shown). Since the detection limit for \(^{35} \text{C}\) was 25% (Fig. 3), and assuming that the DMI would be present in only one of these phases, at least 90–94% of the DNA molecules in the total population lacked a DMI at ori-β.

**DISCUSSION**

The report of an unusual form of DNA methylation closely associated with origins of bidirectional replication exclusively in the chromosomes of actively proliferating mammalian cells constituted an important discovery of what appeared to be a major component of mammalian replication origins (11, 12). The experiments described here had two objectives. The first was to confirm the existence of a DMI at the well characterized dhfr ori-β locus in proliferating CHO cells, and the second was to determine whether or not this DMI appeared at a specific time during the cell division cycle.

In the original report describing densely methylated islands (DMI), two methods were used to demonstrate the presence of a DMI at the dhfr ori-β locus in 86% of proliferating CHO cells (12), 1) conversion of cytosines to uracils by bisulfite treatment of DNA, and 2) sensitivity of genomic DNA to cleavage by AluI. In an effort to confirm this report, three independent, stringently controlled techniques were applied to investigate the ori-β DMI, 1) sensitivity of genomic DNA to cleavage by different methylation-sensitive restriction enzymes, 2) sensitivity of cytosines to cleavage by hydrazine/piperidine treatment, and 3) sensitivity of methylcytosines to cleavage by permanganate/piperidine treatment. Control experiments demonstrated that the methods for detecting \(^{35} \text{C}\) in genomic DNA at the nucleotide level would have detected a DMI if present in as little as 25% of the total cell population. In addition to comparing proliferating and nonproliferating CHO and CHO C400 cells, CHO cells were also fractionated by centrifugal elutriation to enrich for populations of G1, S, and G2 + M phase cells. Analysis of these individual cell populations meant that a DMI would have been detected in as few as 6–10% of the cells examined. The PCR-based detection of DNA molecules resistant to cleavage by methylation-sensitive restriction endonucleases would have detected a DMI if present in as little as 2% of the cells.

Both the hydrazine and permanganate methods identified the single, stable \(^{35} \text{CpG}\) within the putative ori-β DMI. This \(^{35} \text{CpG}\) was present in >90% of the proliferating CHO K1 (two copies of this locus) and CHO C400 (~1000 copies of this locus) cells, and the same \(^{35} \text{CpG}\) also was present in stationary cells. Therefore, these methods had no difficulty in detecting methylated cytosines in genomic DNA. However, none of the three methods used detected a DMI. How might the discrepancy between these results and those reported previously (12) be explained?

The bisulfite method is subject to an artifact that could easily account for the appearance of DMIs. Bisulfite converts cytosines to uracils only when the DNA is single stranded. If the DNA sample is not completely denatured, sequences that failed to react with bisulfite will contain stretches of unmethylated cytosines that will falsely indicate cytosine methylation (29, 30, see also Ref. 31). If PCR primers are used to amplify the unmethylated regions in the belief that they contain methylated cytosines, then even a small contamination of unconverted DNA may yield a PCR product that contains only unconverted cytosines. One way to distinguish between cytosines that did not react with bisulfite because they were methylated from cytosines that did not react because they were present in double-stranded DNA is to apply independent methods for detection of methylated cytosines that are not subject to the same artifact.

One such method used by Tasheva and Roufa (11, 12) to determine the fraction of genomic DNA that contained a DMI was to measure the fraction of DNA that could be cleaved by digestion with AluI, a restriction endonuclease that cannot cut when its recognition site is methylated. However, the inability of AluI to cut genomic DNA can result either from methylation of its DNA recognition site or simply from incomplete digestion. In fact, while plasmid DNA or PCR-generated DNA was easily cut by AluI in our hands, we initially encountered considerable difficulty cutting genomic DNA to completion. Incompletely digested genomic DNA yielded a full-length product after PCR. However, if this same genomic DNA was re-digested with AluI, no PCR product was obtained (data not shown). Thus, any method using AluI, or any other methylation-sensitive enzyme as a tool for methylation analysis, would be subject to possible incomplete reactivity. Therefore, a stringent control for the enzyme activity should be included. Such a control was included in our experiments by monitoring the degree of cleavage of an unrelated fragment in the same incubation reaction where cleavage of genomic DNA took place. This control was not provided in previous studies of DMIs (12) and therefore leaves open the possibility that AluI simply failed to cut genomic DNA efficiently in these experiments. Additionally, DNA from stationary cells was not investigated to show that in the absence of the putative DMI, AluI would cut DNA to completion.

To determine the methylation status at every cytosine within the putative DMI, we used hydrazine to modify cytosines, which allows cleavage at these positions by piperidine. Hydrazine is the classical method for sequencing DNA and for identifying methylated cytosines (\(^{35} \text{C}\)) in genomic DNA (32, 35). Since hydrazine does not modify \(^{35} \text{C}\), piperidine will not cleave at these positions, and the corresponding cytosine band will be absent in a sequencing gel. The advantage of this method over bisulfite is that its reactivity and specificity does not depend on the strandedness of the DNA. The hydrazine method detected only one \(^{35} \text{C}\) within the putative ori-β DMI, and this was at a CpG dinucleotide, the classical site for \(^{35} \text{C}\). Moreover, it was present in stationary cells as well as proliferating cells.

To confirm this conclusion, we developed the use of permanganate to complement the hydrazine method. At low pH, permanganate modifies \(^{35} \text{C}\) as well as T and allows cleavage at these positions by piperidine. Since permanganate at low pH does not modify \(^{35} \text{C}\), piperidine will not cleave at these positions, and the corresponding cytosine band will be absent in a sequencing gel. The advantage of this method over bisulfite is that its reactivity and specificity does not depend on the strandedness of the DNA. The hydrazine method detected only one \(^{35} \text{C}\) within the putative ori-β DMI, and this was at a CpG dinucleotide, the classical site for \(^{35} \text{C}\). Moreover, it was present in stationary cells as well as proliferating cells.

Stringent application of the bisulfite method that was employed by Tasheva and Roufa (11, 12) confirms the absence of a DMI at ori-β and also at the RPS14 origin (31). Thus, replication origins are not associated with the unusual methylation pattern known as a DMI. Unusual, dense non-CpG DNA methylation is most likely an artifact of the bisulfite method (31). Since other important findings of unusual DNA methylation were based on this method (e.g. Ref. 44), we suggest that any
such findings be reevaluated either by independent methods as presented here or by a modified bisulfite procedure (31).

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