DNA Methylation at Mammalian Replication Origins*

(Received for publication, December 7, 1998, and in revised form, May 12, 1999)

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In *Escherichia coli*, DNA methylation regulates both origin usage and the time required to reassemble pre-replication complexes at replication origins. In mammals, at least three replication origins are associated with a high density cluster of methylated CpG dinucleotides, and others whose methylation status has not yet been characterized have the potential to exhibit a similar DNA methylation pattern. One of these origins is found within the −2-kilobase pair region upstream of the human c-myc gene that contains 86 CpGs. Application of the bisulfite method for detecting 5-methylcytosine at specific DNA sequences revealed that this region was not methylated in either total genomic DNA or newly synthesized DNA. Therefore, DNA methylation is not a universal component of mammalian replication origins. To determine whether or not DNA methylation plays a role in regulating the activity of origins that are methylated, the rate of remethylation and the effect of hypomethylation were determined at origin methylated, the rate of remethylation and the effect of hypomethylation were determined at origin

In early embryos undergoing rapid cell cleavage (e.g. frogs, flies, sea urchin, fish), initiation of DNA replication appears neither to require specific DNA sequences nor to occur at specific DNA sites. However, as development progresses past the blastula stage, initiation of DNA replication begins to occur at specific sites (1, 2). Thus, it is not surprising that initiation sites for DNA replication in cultured mammalian cells occur at specific genomic loci (3). For example, a 200-kb region at the human β-globin gene (4, 5) and a 500-kb region at the mouse IgH gene (6) are both replicated from a single initiation locus. Moreover, what previously had been viewed as random initiation events distributed throughout “initiation zones” in *Schizosaccharomyces pombe* and hamster cells more likely reflects the presence of several strongly preferred initiation sites (7, 8). Nevertheless, the precise size and composition of these initiation loci, as well as the parameters that define them remain the subject of intense investigation.

The fact that specific sites for initiation of DNA replication can be developmentally acquired makes it clear that initiation sites in the metazoans are determined at least in part by epigenetic parameters. These parameters include nuclear structure, chromatin structure, and events that occur during G1 phase of the cell division cycle (reviewed in Ref. 3). In addition, replication origins are determined by specific DNA sequences, although the precise nature of these sequences is not yet known. Initiation occurs at the same well defined sites within a large genetic locus that is present as a single copy per haploid genome as it does in cells that have amplified the locus into hundreds of copies (9–12). Therefore, each copy must contain specific cis-acting sequences that determine where replication can begin. Moreover, replication origins exhibit initiation activity when they are translocated to other chromosomal sites (13–15), and origin activity can be eliminated by deletion of specific sequences either within the initiation site or at some distance away (4, 5, 13, 16). The distal sequences may act as enhancers to alter chromatin structure, while the proximal sequences may act as assembly sites for pre-replication complexes. Several reports of autonomously replicating sequence elements that function in mammalian cells and cell extracts have been documented in detail and shown to correspond to initiation sites for DNA replication in mammalian chromosomes (17–20). This does not exclude the possibility that, under appropriate conditions, other sequences may promote autonomous plasmid DNA replication in mammals (21) as they do in *Xenopus* eggs (22) and in yeast (23).

One mechanism by which chromatin structure or nuclear organization might affect origin activity is through DNA methylation at replication origins. In *Escherichia coli*, DNA methylation plays a direct role in regulating the efficiency of origin usage and the timing of origin activation (see “Discussion”). In mammals, two replication origins in hamster cells (24) and one

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‡ Recipient of a postdoctoral fellowship from the Deutsche Forschungsgemeinschaft.

** Supported by Public Health Service Grant GM53819.

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1 The abbreviations used are: kb, kilobase pair(s); bp, base pair(s); ori-, origin; OBR, origin of bidirectional DNA replication; “CpG, methylated CpG dinucleotide; **C, 5-methylcytosine; CHO, Chinese hamster ovary; PCR, polymerase chain reaction; DHFR, dihydrofolate reductase; Br-DNA, bromo-DNA.

2 T. Kobayashi, T. Rein, J. Bogan, and M. L. DePamphilis, unpublished data. Malott, M., and Leffak, M. (1999) Mol. Cell. Biol., in press.
in human cells (32) are associated with an unusually dense cluster of methylated CpG dinucleotides (CpGs) while other origins contain sufficient CpGs to generate a similar pattern of DNA methylation (24). In one case (24), these CpGs were shown to be methylated in active origins as well as in the total cell population. Earlier reports that mammalian replication origins were associated with an unusual “densely methylated island” consisting of ~100 to ~500 base pairs in which all cytosines were methylated, regardless of their dinucleotide composition (25, 26), apparently resulted from incomplete DNA cleavage by methylation-sensitive restriction endonucleases and incomplete reaction of DNA with bisulfite followed by selective amplification of unreacted DNA segments (24, 27).

DNA methylation at mammalian DNA replication origins might affect initiation of DNA replication in at least two ways. First, DNA methylation might delay reinitiation at origins in mammalian cells, as it does in E. coli. For example, while newly replicated DNA appears to be rapidly remethylated throughout most of the genome (28, 29), remethylation is delayed in as yet undefined parts of the genome for up to 6 h (30). These genomic regions may represent replication origins. Since the tumor suppressor p21WAF1/CIP1 can disrupt the interaction between PCNA and DNA methyltransferase (31), p21 could potentially play a dual role at replication origins by regulating activation of prereplication complexes through control of Cdk2-cyclin A and E protein kinase activity and by delaying remethylation of newly synthesized DNA. Following the E. coli scenario, this would allow hemimethylated origin DNA to be sequestered by proteins that are sensitive to its methylation status, thus delaying reassembly of a prereplication complex.

Alternatively, DNA methylation might promote specificity in the selection of initiation sites, because DNA methylation can alter DNA secondary structure (38, 39) and 5-methylcytosine in DNA can bind specific proteins (40), one of which (MeCP2) also binds to nuclear matrix (41). These properties could alter chromatin structure to make some sites more or less accessible, or bind to nuclear matrix (41). These properties could alter chromatin structure to make some sites more or less accessible, or bind to DNA methyltransferase (31), p21 could potentially play a dual role at replication origins by regulating activation of prereplication complexes through control of Cdk2-cyclin A and E protein kinase activity and by delaying remethylation of newly synthesized DNA. Following the E. coli scenario, this would allow hemimethylated origin DNA to be sequestered by proteins that are sensitive to its methylation status, thus delaying reassembly of a prereplication complex.

These considerations raised two important questions. First, is DNA methylation a feature common to all mammalian replication origins? Second, does DNA methylation play a role in regulating the rate at which prereplication complexes are reassembled, because origins are rapidly remethylated. Nevertheless, hamster cells containing hypomethylated DNA no longer exhibited site-specific initiation of DNA replication in the ori-β locus, revealing that DNA methylation does affect the initiation process at some origins. This could occur either directly by facilitating the binding or activity of replication proteins or indirectly by altering the concentrations of critical replication proteins.

**MATERIALS AND METHODS**

**Mapping Methylated Cytosines in Specific DNA Sequences**

The bisulfite method was used to detect methylated cytosines at specific DNA sequences as described previously (24). For analysis of nascent DNA, 1 μg of 4x174 DNA was added as carrier prior to bisulfite conversion.

**Isolation Of Newly Synthesized DNA from Early Replication Bubbles**

**Nascent Strand Length**—To isolate nascent DNA of different lengths, CHO K1 cells were cultured and synchronized at their G1/S transition by a double aphidicolin block as described previously (8). The fraction of cells at their G1/S boundary was determined by fluorescence-activated cell sorting analysis to be ~90%. DNA was labeled, extracted, fractionated by sucrose gradient centrifugation, and purified as described previously (8), except that sucrose gradient fractions were collected that contained DNA from 400 to 4000 residues in length.

**Replication Time**—To isolate nascent DNA at different times after cells were released into S phase, cells synchronized at their G1/S transition were washed twice with prewarmed complete medium and then cultured for 14 min in complete medium before pulse-labeling nascent DNA for 1–2 min at 37 °C by the addition of 1 μM 3H-labeled deoxycytidine and 100 μM 5-bromodeoxyuridine (BrdUrd). Cells then were washed twice with prewarmed Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum and cultured in fresh prewarmed medium for 1 min to 6 h. DNA was isolated and purified as described previously (8).

**c-myc Origin DNA**—HeLa cells were synchronized at their G1/S transition by serum starvation and aphidicolin arrest as described for CHO K1 cells (8). Cells were released into S phase for 10 min by washing twice with prewarmed medium before labeling nascent DNA for 5 min with BrdUrd. Cells were washed and incubated in fresh medium for another 4 h. Chromosomal DNA was extracted and digested with restriction endonucleases (8). Nascent Br-DNA then was affinity-purified and reacted with bisulfite. Alternatively, nascent DNA was isolated from synchronized HeLa cells and fractionated according to size by gel electrophoresis after sedimentation through two sequential cesium chloride gradients as described previously (20).

**RESULTS**

**Some Replication Origins Are Not Methylated**—An origin of bidirectional DNA replication (OBR) has been identified upstream of the human c-myc gene (Fig. 1) using several independent criteria. A replication initiation site was initially located within a 3.5-kb region 5’ to the c-myc gene by extension in vitro of nascent DNA strands that had been initiated in vivo (51). Subsequent analysis of nascent DNA strand lengths from unsynchronized HeLa cells located the OBR within a ~2-kb locus centered ~1.5 kb upstream of exon 1 (47). This method also mapped an origin at the same position upstream of the chicken c-myc gene (46). Analysis of the distribution of short nascent
DNA strands between the two DNA templates in synchronized HeLa cells revealed several strand switches within this region, consistent with the presence of one or more replication initiation sites (48). A 2.4-kb segment from this region exhibits autonomous, semiconservative replicating sequence activity in both transfected cells and cell extracts, and replication begins within this 2.4-kb locus (17, 20). Although most replication bubbles were centered ~1.4 kb upstream of promoter P1 (17), this region may contain more than one autonomously replicating sequence element (19, 52). The 2.4-kb c-myc initiation locus contains 110 CpG dinucleotides, or 1 CpG per 22 bp.

To determine whether or not methylated CpG dinucleotides (mCpGs) are a feature of most, if not all, mammalian replication origins, the methylation status of this region was determined using the bisulfite method. Of the currently available methods for mapping 5-methylcytosine (5mC) at specific genomic locations, the bisulfite method provides the greatest sensitivity and is able to identify the methylation status of every cytosine within a large sequence (53). Attention was focused on the 2-kb region from map position 200 to 2200 that contains the two changes in nascent strand polarity, the OBR detected by nascent Br-DNA was affinity-purified and analyzed for 5-methylcytosines. Again, all cytosines were found to be unmethylated.

Because the PCR primers (Table I) were devoid of CpG dinucleotides, bisulfite should convert all of the cytosines at these genomic sites into uracils. Therefore, these PCR primers, which were designed to be complementary to converted DNA, did not select for either methylated or unmethylated CpG sites in the c-myc origin locus. Plasmid DNA carrying the 2.4-kb HindIII fragment that contains the c-myc replication origin was used to display the sequence when it is completely unmethylated. The fact that one cytosine (position 344) in this control sequence lies within a bacterial DNA cytosine methylase (dcm) recognition sequence confirmed that the bisulfite procedure would have detected methylated cytosines if they had been present.

Since this bisulfite protocol does not detect DNA methylation in less than 5–10% of the population (53), it was possible that only a subpopulation of the cells were methylated at the c-myc origin and that initiation of DNA replication at the c-myc origin occurred only in this subpopulation. Therefore, the methylation status of the c-myc origin was analyzed in newly synthesized DNA. If active replication origins are methylated, then DNA methyltransferase will remethylate the nascent DNA strand at each hemimethylated CpG dinucleotide.

HeLa cells were synchronized at their G1/S-transition and released into S phase for 10 min, and then nascent DNA was labeled for 5 min with BrdUrd. Cells were washed and incubated in fresh medium for another 4 h to allow sufficient time for remethylation to occur. Chromosomal DNA was extracted and cleaved with restriction enzymes to eliminate the possibility that subsequent purification of nascent Br-DNA retrieved c-myc sequences whose origin may not have fired but were connected to an active, BrdUrd-labeled origin located some distance away. Nascent Br-DNA was affinity-purified and analyzed for mCpGs using the bisulfite method as described previously (27). An example of these data is shown in Fig. 2, and the total results are summarized in Fig. 1. Nascent DNA from the c-myc replication origin, like total DNA, was devoid of mC.

Theoretically, a signal from methylated DNA at active origins might have been obscured in these experiments if replication forks from neighboring origins had traveled through this region in some cells. This could produce nascent c-myc DNA that was unmethylated. Therefore, nascent DNA in randomly proliferating HeLa cells was labeled with BrdUrd, and Br-DNA 4–8 kb in length was isolated and then analyzed for 5-methylcytosines. Again, all cytosines were found to be unmethylated.
At Methylated Replication Origins, Remethylation Rapidly Follows Replication.—To determine whether or not nascent DNA at replication origins containing \(^{5}\text{mCGs}\) is rapidly remethylated, the rate of remethylation at ori-\(\beta\) was measured both as a function of time after release into S phase and as a function of nascent DNA strand length. Ori-\(\beta\) is a primary initiation site for bidirectional DNA replication that has been identified in Chinese hamster ovary (CHO) cells by six different origin mapping protocols in 12 different studies (summarized in Ref. 8). Ori-\(\beta\) has been localized to a 2-kb region (8) centered at map position 2400 (Fig. 3) was determined as a function of time after release into S phase and as a function of DNA methylation for human DNA (Fig. 3; Refs. 24 and 27).

To measure the rate of remethylation at ori-\(\beta\), CHO cells were synchronized at their G 1/S transition by arresting them with aphidicolin, a specific inhibitor of replicative DNA polymerases \(\alpha\) and \(\delta\). They were then released into S phase down-steam of the DHFR gene in hamster cells that has been identified as a primary (high frequency) initiation site by six different origin mapping protocols (summarized in Ref. 8). Closed lollipops denote methylated cytosines at CpG dinucleotides that were determined using four independent methods, including the bisulfite method (24, 27). Nine of these \(^{5}\text{mCGs}\) are clustered within a 356-bp region (shaded box) adjacent to the OBR that was defined by the transition between discontinuous and continuous DNA synthesis (55). The CpG cluster contains an A:T element containing 60 adenines on one strand and 60 thymines on the other with only four single base pair interruptions within the dense CpG cluster. A micrococcal nuclease-hypersensitive site (MNase HS site) that is specific for cells at their G 1/S phase border and for DNA associated with nuclear matrix maps to this A:T element (100). Map positions are based on the sequence under GenBank\textsuperscript{TM} accession number X94372.

These results showed that nascent ori-\(\beta\) DNA was completely remethylated within 1–3 min of DNA synthesis. A strong signal from methylated DNA (indicated by cytosine residues in the C lane at the positions of CpG dinucleotides) was evident within 1 min of pulse labeling, whereas the signal from

DNA Methylation at Mammalian Replication Origins

| Number | Sequence 5’ to 3’ | Map position |
|--------|-------------------|--------------|
| 1      | TTT TTT GGA GAT AAA AGG | 201-222     |
| 2      | CTA TAC ATT TAA CTC CAA | 465-445     |
| 3      | TTT TAG CTA AAT TAA TAG | 445-465     |
| 4      | TTA TCT CCT CCA CCA AAT CCA | 732-711     |
| 5      | AAG TTG CTT TGG GAG GGT TGG | 709-730     |
| 6      | ATT TTA ACT ACC TTA CTA AAA | 994-973     |
| 7      | TAA TGT TGG GAA GGG GGT TAA | 971-992     |
| 8      | AAA TCT CTA AAA AAA ACA AAT | 1250-1299   |
| 9      | GCT TTA TAA GGT TTA TTA TTA AAT | 1214-1233   |
| 10     | AAA TCC CCA ATA TCT CTA CCA | 1409-1388   |
| 11     | GAA TAA TAA GGA GGT GGT TCG | 222-242     |
| 12     | CTT TCC TCT CCC TCC ACC AG | 700-681     |
| 13     | CTA AAT ACT AAT AAA AAT ACA C | 940-919     |
| 14     | AGG GAA TAA TTA TTA TTT ATT AAT | 793-813     |
| 15     | GCT AAA TTT TAA TTA GCT TAA | 987-1008    |

FIG. 2. Identification of the methylated status of CpG dinucleotides in the human c-myc gene replication origin. Plasmid, genomic, and nascent DNA from HeLa cells was treated with bisulfite and amplified with PCR using primers 1 and 4 (Table I). PCR products were sequenced using primer 2. The closed lollipops identifies the methylated dcm site in plasmid DNA. The open lollipops designate unmethylated CpG dinucleotides. T, G, C, and A above the sequencing lanes indicate the nucleotide identified. Nucleotides 201–429 are shown as an example.

FIG. 3. Hamster DHFR gene ori-\(\beta\). ori-\(\beta\) is a 2-kb locus downstream of the DHFR gene in hamster cells that has been identified as a primary (high frequency) initiation site by six different origin mapping protocols (summarized in Ref. 8). The open lollipops designate unmethylated cytosines in CpG dinucleotides. T, G, C, and A above the sequencing lanes indicate the nucleotide identified. Nucleotides 201–429 are shown as an example.
nascent DNA strands 400 or 500 nucleotides long; mCpGs were run in parallel. DNA methylation was not detected in analytical gel electrophoresis in which DNA length standards length of nascent DNA in various fractions was determined by centration through a neutral sucrose gradient. The average BrdUrd. After 15 min, chromosomal DNA was purified, heat-transition and then released into culture medium containing DNA methylation at nucleotide positions 2372–2443 using the bisulfite method. Sense strand DNA was selectively amplified with primers 2s and 3s and sequenced with primer 4s (24). Top panel, the positions of cytosines within CpG dinucleotides are identified by lollipops. Open lollipops identify unmethylated CpGs, and closed lollipops identify methylated CpGs. A, C, G, and T indicate the nucleotide identified in each lane. Reference DNA was taken from randomly proliferating CHO K1 cells. Bottom panel, the fraction of methylation at each of the five CpGs shown in the top panel was estimated using the program NIH Image after scanning autoradiographs, averaging the five CpGs, and plotting them as a function of time after the BrdUrd pulse.

unmethylated DNA (indicated by thymidine residues in the T lane at the positions of CpG dinucleotides) was weak and disappeared completely at later times during the chase period (Fig. 4). This conclusion was confirmed and extended by analysis of DNA methylation as a function of the length of the nascent DNA strand. CHO cells were again synchronized at their G1/S transition and then released into culture medium containing BrdUrd. After 15 min, chromosomal DNA was purified, heat-denatured, and then fractionated according to length by sedimentation through a neutral sucrose gradient. The average length of nascent DNA in various fractions was determined by analytical gel electrophoresis in which DNA length standards were run in parallel. DNA methylation was not detected in nascent DNA strands 400 or 500 nucleotides long; mCpGs were first detected in nascent DNA fragments of 700 nucleotides, and DNA methylation was completed in nascent DNA fragments with an average length of 4000 nucleotides (Fig. 5). These results showed that DNA methylation did not occur concomitantly with DNA synthesis but was closely linked to DNA synthesis, because nascent ori-β DNA was completely remethylated by the time 4000 nucleotides had been synthesized.

**DNA Hypomethylation Results in Loss of Initiation Site Specificity**—To determine whether or not DNA methylation plays any role in the initiation of DNA replication in mammalian cells, CHO cell lines were obtained from D. Woodcock that had been selected for their ability to survive treatment with 5-aza-2′-deoxycytidine, an inhibitor of DNA methyltransferase (56). Total genomic DNA methylation in these cell lines was reduced 25–30%. In one of these cell lines (CHO C14), the relative intensities of each of the 15 mCpGs within a ~2-kb region containing the ori-β OBR and the high density cluster of CpG dinucleotides (Fig. 3) varied significantly (Fig. 6). The fraction of mC at each CpG dinucleotide revealed that, on average, CHO C14 cells were only 49% as methylated in the ori-β locus and 47% as methylated in the CpG cluster as were CHO K1 cells. The same CpG dinucleotides were completely methylated in CHO K1 (Figs. 4 and 5 and Ref. 24) and CHO Scc30 cells, a clonal isolate of CHO K1 that was parent to CHO C14 (data not shown).

To determine whether or not DNA hypomethylation affected initiation of DNA replication, CHO C14 cells were analyzed for initiation at origins β and β′ using the same “nascent strand abundance assay” that we used to identify these origins in CHO K1 cells (8). Since nascent DNA strands on the order of 1 kb long originate predominantly from newly formed replication bubbles, the number of strands containing a particular sequence will be proportional to the frequency of initiation events at that sequence. This assay is the most sensitive, quantifiable assay currently available for detecting replication origins in mammalian cells.

Cells were synchronized at their G1/S boundary and then released into S phase in the presence of BrdUrd to label nascent DNA. DNA was purified, denatured, and then fractionated by sucrose gradient centrifugation to isolate chains 800 ± 200 nucleotides long. Nascent Br-DNA was purified from this pool by affinity chromatography and analyzed by competitive PCR to determine the relative concentrations of 15 different genomic sites. Two sites (H and I) were located in a region of the DHFR gene where initiation events have not been detected by any origin mapping protocol, including two-dimensional gel electro-
Competitive PCR measures the number of copies of a specific sequence in a DNA sample by employing an internal competitor DNA to correct for variation in the efficiency of amplification by different PCR primer sets and between different PCR reactions using the same primer set. The target DNA and competitor DNA share the same primer recognition sites, but reactions using the same primer set. The target DNA and competitor DNA to correct for variation in the efficiency of amplification by different PCR primer sets and between different PCR reactions using the same primer set.

The two slower migrating bands are heteroduplexes between target and competitor DNA that formed during the final cycle of denaturation and renaturation. Therefore, the amount of target and competitor DNA in these heteroduplexes was included in determining the actual amount of target DNA present so that the ratio of [total C]/[total T] was linear as a function of competitor DNA added to the polymerase chain reaction (8). The number of copies of each sequence was determined both in the nascent Br-DNA fraction and in a sample of 0.8 kb of DNA isolated from nonproliferating cells, and the ratio of nascent Br-DNA to nonreplicating DNA at each PCR primer set was determined in order to eliminate any inaccuracy in our competitor DNA concentrations. These ratios were then normalized to the average ratio observed for probes H and I. Thus, regardless of the actual number of copies detected in each experiment, the relative number of copies at each sequence will reveal preferential sites of initiation.

The results revealed that hypomethylated CHO C14 cells, in contrast to fully methylated CHO K1 or CHO Scc30 cells, did not initiate DNA replication preferentially at either ori-β or -β′ (Fig. 7B). Samples of Br-DNA isolated from either C14 cells (example in Fig. 7A), K1 cells (example in Fig. 4 of Ref. 8), or Scc30 cells (data not shown) contained ~200 copies of sequences defined by primer set 1 (DHFR gene). However, samples of Br-DNA from K1 and Scc30 cells contained 8–17 times more sequences at primer set 6 (ori-β peak) than samples of

![Fig. 6. Ori-β methylation status in a hypomethylated hamster cell line. The methylation status of ori-β in CHO C14 cells was determined using the bisulfite method as described previously (24). The gel shows an example of bisulfite sequence analysis for nucleotides 2375–2487 using primers 2a and 3a for amplification and primer 4a for sequencing. Lollipops designate positions of cytosines in CpG dinucleotides, and the extent of their shading reflects the fraction of C in that position. The fraction of methylation at each CpG plotted next to the sequence position was determined by measuring the ratio of cytosine to thymine in the data generated by the bisulfite method for mapping 5-methylcytosine.

![Fig. 7. Absence of site-specific initiation of DNA replication in a hypomethylated hamster cell line. A. Competitive PCR was used to quantify the relative amounts of specific sequences in CHO C14 cells, as described previously (8). A fixed amount of newly synthesized Br-DNA was amplified in the presence of increasing amounts of competitor DNA. DNA products were resolved by gel electrophoresis and stained with ethidium bromide. Primer sets I (DHFR gene), 6 (ori-β peak), 9, and 13 are shown as examples. Total target DNA (T) = (T:T + T:C + C:T)/2 and the total competitor DNA (C) = (C:C + T:C + T:T)/2. T:C and C:T are homoduplexes (gel bands marked H). T:C and C:T are heteroduplexes. Background staining in gels was subtracted from DNA bands before calculations were made. B, the relative abundance of 15 different sequences (see Fig. 3 in Ref. 8) was determined on newly synthesized BrLed-labeled DNA from CHO C14 cells synchronized at their G1/S boundary, and on nonreplicating DNA from serum-starved CHO C14 cells. The ratios of nascent Br-DNA/nonreplicating DNA were calculated in each experiment, and the amount of each probe was normalized to the average of probes H and I (DHFR gene). Since initiation events have never been detected near probes H and I by any origin mapping method (including two-dimensional gel electrophoresis), numbers of 1 or less (shaded area) indicate the absence of initiation events. Mean values for two independent experiments with CHO C14 cells (●) and for five independent experiments with CHO K1 cells (○), same as in Ref. 8) were plotted on their nucleotide "map position" (GenBankTM accession no. X94372). The region shown is from 12 to 27 kb downstream of the 3′-end of the DHFR gene. Error bars indicate S.E. Primer sets H, I, 6, and 12 were used to confirm in one experiment that CHO Scc30 cells behaved like CHO K1 cells (data not shown).]
Br-DNA from C14 cells. Therefore, initiation events at ori-β and -β′ in CHO C14 cells were reduced to background.

**DISCUSSION**

A role for DNA methylation in the regulation of DNA replication in mammalian cells was suggested two decades ago based on the *E. coli* paradigm (57). The *E. coli* origin of chromosome replication, ori-C, is a 245-bp region containing 11 GATC sites (58) that are methylated at the N-6 position of adenine by deoxyadenosine methyltransferase (59, 60). Control of replication timing within each cell is critically dependent on the level of deoxyadenosine methyltransferase (61). The activity of both ori-C and the closely related ori-R in bacteriophage P1 is regulated by methylation on two levels: sequestration of hemimethylated origin DNA through SeqA (62) and severely reduced origin activity in unmethylated DNA (63). When ori-C is replicated, the GATC sequences within ori-C become hemimethylated (64, 65) and sequestered at the cell membrane (64) through association with SeqA protein (62, 66–68). SeqA inhibits both the formation and activity of prepriming complexes (62, 66–69). Methylation at ori-C also influences the local DNA structure and stability (62, 66–71) and its interaction with replication proteins (72). Hemimethylated ori-C is refractory to further initiation events (73–75) due to the inability of DnaA protein to access ori-C (76), thus preventing premature reinitiation when free DnaA levels are still high (64, 65, 76). OriC persists in this hemimethylated/sequestered state for ~8 min, or about 10 times longer than for most other sites on the *E. coli* chromosome (64, 65).

Thus, although deoxyadenosine methyltransferase is not required for *E. coli* cell proliferation, the methylation status at replication origins in *E. coli* regulates both origin efficiency and the rate at which replication origins can be reused. In fact, GATC sites and the dam methylation system are conserved among enteric bacteria, suggesting that this form of regulation is widespread (77). Nevertheless, it is not universal, since it is absent from *Bacillus subtilis* and *Pseudomonas*. An analogous situation may exist in eukaryotes.

**DNA Methylation at Mammalian Replication Origins**

One would not expect DNA methylation to be universally required for initiation of DNA replication in eukaryotes, because mammals appear to use the same proteins for assembly of prereplication complexes as do yeast and flies, and these eukaryotes are virtually devoid of methylated bases (60). Nevertheless, two replication origins have been identified in hamsters (ori-β, ori-RPS14; Ref. 24) and one in humans (ori-dnm1C1; Ref. 32) that are associated with a high density cluster of CpG dinucleotides. Other replication origins, such as the human c-myc origin, are located within CpG islands and therefore contain sufficient CpG dinucleotides to form analogous high density clusters of CpGs (24). CpG islands are generally assumed to be completely unmethylated, but prior to the present study, only a few CpG islands have been examined using the bisulfite method, and these studies were limited to no more than ~0.3 kb of sequence (78–83).

Results presented here demonstrate that the ~2-kb region upstream of the human c-myc gene that contains a replication origin is devoid of 5-methylcytosines (Fig. 1). This was true both for total cellular DNA and for newly synthesized DNA. Our analysis, which included all 86 CpG dinucleotides between positions 200 and 2150, is consistent with the limited data available from previous studies on the mammalian c-myc gene locus. Previous studies using methylation-sensitive restriction endonucleases did not detect CpGs until ~5 kb downstream of this region (84–86), suggesting that this CpG island is exceptional, because it is completely demethylated over a much larger range than previously reported for CpG islands (usually about 1 kb (87)). A small subset of CpG dinucleotides downstream of the promoter (84, 86, 88, 89) as well as within the promoter region in mouse and human cells (85, 90) have been examined by methylation-sensitive restriction endonucleases and found to be unmethylated. Six CpG dinucleotides within the origin region (positions 1036–1156) were found to be unmethylated using the hydrazine method (85), but this method would not detect methylation in less than 25% of the molecules (53, 85). Another study using the bisulfite method also concluded that the human c-myc origin was unmethylated (28), but this study examined only 10% of the CpGs (positions 764–1095), and these were located in the region of lowest CpG density (Fig. 1). Thus, they were not representative of the high density cluster of CpGs described for methylated origins (24, 32). Moreover, this study detected only eight CpGs in a region that contains nine. Finally, one of the PCR primers (“MYC OUT”) used in this study contained a CpA dinucleotide that is complementary to bisulfite-treated DNA only if the genomic CpG is not methylated. As previously shown (24, 25, 27), this can lead to selective amplification of DNA molecules that are not methylated, giving a false impression that most of the cells contain unmethylated DNA at this locus. Nevertheless, taken together with the results reported here and with results showing that the human lamin B2 replication origin is not methylated (28), it is clear that DNA methylation is not required for initiation of DNA replication at all initiation sites in mammalian cells. Like transcription factor binding sites (18, 91), clusters of CpGs may be components of some origins but not of others.

It has been suggested that most, if not all, unmethylated CpG islands contain replication origins (50). However, the hamster DHFR gene has a CpG island of ~1 kb at its 5’-end (92), but at least seven different origin mapping methods have failed to detect DNA replication initiation events in the DHFR gene or its promoter region (18). The observation that some CpG islands serve as replication origins may simply reflect the fact that some transcription factors can facilitate origin activity, while others do not (18, 91).

**Potential Roles for DNA Methylation in the Initiation of Mammalian DNA Replication**

**Regulating Reinitiation**—One potential role for DNA methylation at mammalian replication origins could be to delay the reassembly of prereplication complexes, analogous to the role of DNA methylation at *E. coli* origins. In *E. coli*, the time required to replicate the genome is 40 min (93). Therefore, remethylation of ori-C is delayed until ~20% of the chromosome has replicated (8 min/40 min) or until each replication fork has traveled ~470 kb. In CHO K1 cells, the time to replicate the genome is ~8 h.³ Therefore, remethylation of the dense CpG cluster at ori-β was delayed until ~0.6% of the genome was replicated (3 min/480 min) or until each replication fork had traveled 2–4 kb. Thus, the delay in remethylation at ori-β was no greater than the delay reported for replication forks distributed throughout the mammalian genome (29) and was at least 10 times faster than observed at replication origins in *E. coli*. However, remethylation at replication origins in hamster cells did not occur concomitantly with DNA replication, as previously suggested for monkey and human cells (28), but was delayed until >500 nucleotides had been synthesized (Fig. 5). It has been reported that amplification of a mixture of unmethylated and methylated, bisulfite-converted alleles can be biased toward one or the other methylation status (94). If such a bias existed at ori-β, it would affect significantly

³ T. Rein, unpublished data.
only values between 10 and 90% methylation (94) and therefore would not affect this conclusion. The rapid remethylation observed at hamster ori-β was consistent with the rapid remethylation observed with a collection of nonspecified origin sequences isolated from monkey and human cells (28). Thus, DNA methylation cannot play a significant role in regulating reassembly of prereplication complexes in mammalian cells as it does in E. coli.

Facilitating Binding or Activity of Initiation Proteins at Specific Sites—Relative to CHO K1 and CHO ScC30 cells, CHO C14 cells were only 49% as methylated at the ori-β locus and 47% as methylated in the Cpg cluster. Remarkably, this level of hypomethylation eliminated preferential initiation of DNA replication at ori-β and ori-β', revealing that DNA methylation is either directly or indirectly involved in determining where replication begins.

Direct involvement of DNA methylation at replication origins would occur if DNA methylation facilitated either the association of proteins at specific sites or the function of proteins bound to replication origins. These roles are analogous to those proposed for transcription factors at replication origins (18, 91, 95). For example, Cpg dinucleotides bind specific proteins such as MeCP2, and these proteins can alter chromatin structure through their association with other proteins (96, 97) and can bind to nuclear matrix (41). Furthermore, the Cpg cluster at ori-β contains a 60-bp A:T element (adenines on one strand, thymines on the other, Fig. 3). Similar A:T elements serve as essential components of replication origins in the fission yeast, S. pombe (98), and can stimulate transcription from yeast promoters, apparently via nucleosome phasing (99).

Flanking the A:T element with mCpG dinucleotides may accentuate its function. In fact, the A:T element at ori-β becomes hypersensitive to micrococcal nuclease (101, 102). Forks are hypersensitive to micrococcal nuclease (101, 102).

REFERENCES

1. Sasaki, T., Sawado, T. Yamaguchi, M., and Shinomiya, T. (1999) Mol. Cell. Biol. 19, 547–555
2. Hyrien, O., Marie, C., and Méchal, M. (1995) Science 270, 994–997
3. DePamphilis, M. L. (1999) BioEssays 21, 5–16
4. Aladjem, M. I., Groudine, M., Brody, L. L. (1993) EMBO J. 13, 2701–2707
5. Kelly, R. E., DePamphilis, M. L., Braeger, B. W., and Wahl, G. M. (1995) Mol. Cell. Biol. 15, 4136–4148
6. Viro-Pearman, T., Linn, R. H., and Chambon, A. C. (1993) Mol. Cell. Biol. 13, 5901–5942
7. Aladjem, M. I., Rodewald, L. W., Kolman, J. L., and Wahl, G. M. (1998) Science 281, 1005–1009
8. Gabai, S., Klar, A., Meuth, M., and Chard, H. (1989) Cell 57, 909–920
9. Orr-Weaver, T. L. (1991) BioEssays 13, 97–105
10. Kajita, R. F., Li, X., Mesner, L. D., Dijkwel, P. A., Lin, H. B., and Hamlin, J. L. (1998) Mol. Cell. Biol. 2, 797–806
11. Bergerth, S., Trivedi, A., Daniel, D. C., Johnson, E. M., and Leffak, M. (1995) J. Mol. Biol. 245, 92–109
12. DePamphilis, M. L. (1996) DNA Replication in Eukaryotic Cells (DePamphilis, M. L., ed) pp. 45–86, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
13. McWhinnie, C., Waltz, S. E., and Leffak, M. (1995) DNA Cell Biol. 14, 365–379
14. Trivedi, A., Waltz, S. E., Kamath, S., and Leffak, M. (1998) DNA Cell Biol. 17, 885–896
15. Krysan, P. J., Smith, J. C., and Gullick, M. P. (1995) Mol. Cell. Biol. 15, 2688–2696
16. Blow, J. J., and Chong, J. P. J. (1996) DNA Replication in Eukaryotic Cells (DePamphilis, M. L., ed) pp. 971–982, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
17. Kipling, D., and Kearsley, S. E. (1990) Mol. Cell. Biol. 10, 265–272
18. Rein, T., Zorbis, H., and DePamphilis, M. L. (1997) Mol. Cell. Biol. 17, 416–426
19. Asaf, E. S., and Ouf, D. J. (1994) Mol. Cell. Biol. 14, 5636–5644
20. Tashcheva, E. S., and Ouf, D. J. (1995) Somat. Cell Mol. Genet. 19, 369–383
21. Rein, T., Natalie, D. A., Gartner, U., Niggemann, M., DePamphilis, M. L., and Zorbis, H. (1997) J. Biol. Chem. 272, 10201–10208
22. Araujo, F. D., Knox, J. D., Szyf, M., Price, G. B., and Zannis-Hadjopoulos, M. (1998) Mol. Cell. Biol. 18, 3475–3482
23. Gruenbaum, Y., Szyf, M., Cedar, H., and Razin, A. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 4919–4922
24. Woodcock, D. M., Simmons, D. L., Crowther, P. J., Cooper, I. A., Trairorn, K. J., and Morley, A. A. (1988) Exp. Cell Res. 176, 102–112
25. Huang, L. S.-H., Iani, H.-I., Koh, T.-W., Xiu, G., and Li, B. F. (1997) Science 277, 1996–2000
26. Araujo, F. D., Knox, J. D., Pfaff, D. J., Price, G. B., and Zannis-Hadjopoulos, M. (1998) Mol. Cell. Biol. 18, 3475–3482
27. Gruenbaum, Y., Szyf, M., Cedar, H., and Razin, A. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 4919–4922
28. Woodcock, D. M., Simmons, D. L., Crowther, P. J., Cooper, I. A., Trairorn, K. J., and Morley, A. A. (1988) Exp. Cell Res. 176, 102–112
29. Huang, L. S.-H., Iani, H.-I., Koh, T.-W., Xiu, G., and Li, B. F. (1997) Science 277, 1996–2000
30. Weitzel, J. M., Buhrmeister, H., and Stra¨tling, W. H. (1997) DNA Cell Biol. 16, 414–421
31. Weitzel, J. M., Buhrmeister, H., and Stra¨tling, W. H. (1997) DNA Cell Biol. 16, 414–421
32. Adams, R. L. P. (1996) Biochem. J. 305, 309–320
33. Szyf, M., Zouzov, V., and Tanigawa, G. (1991) J. Biol. Chem. 266, 10027–10030
34. Vogel, M. C., Pascopoulos, T., Müller-Hermelink, H. K., Drabkovsky, D., and Pfeifer, G. P. (1988) FEBS Lett. 236, 9–13
35. Wu, S., and Gilbert, D. (1996) Science 271, 1270–1272
36. Phu, V., and Gilbert, D. (1996) Science 271, 1270–1272
37. Vassilev, L., and Johnson, E. M. (1990) J. Biol. Chem. 265, 5650–5655
38. Waltz, S. E., Trivedi, A. A., and Leffak, M. (1996) Nucleic Acids Res. 24, 1887–1894
DNA Methylation at Mammalian Replication Origins

49. Simon, I., and Cedar, H. (1996) in DNA Replication In Eukaryotic Cells (M. L. DePamphilis, ed) pp. 387–408, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

50. Delgado, S., Gomez, M., Bird, A., and Antequera, F. (1998) EMBO J. 17, 2426–2435.

51. Leffak, M., and James, C. D. (1989) Mol. Cell. Biol. 9, 586–593.

52. Iguchi-Arima, S. M., Okazaki, T., Itani, T., Ogata, M., Satoh, Y., and Ariga, H. (1988) EMBO J. 7, 3135–3142.

53. Rein, T., DePamphilis, M. L., and Zorbas, H. (1998) Nucleic Acids Res. 26, 2255–2264.

54. Clark, S. J., Harrison, J., Paul, C. L., and Frommer, M. (1994) Nucleic Acids Res. 22, 2990–2997.

55. Burhans, W. C., Vasileiev, L. T., Caddle, M. S., Heintz, N. H., and DePamphilis, M. L. (1998) Mol. Gen. Genet. 259, 8033–8036.

56. Taylor, J. (1978) in DNA Synthesis: Present and Future (Molineux, I. and Kohiyama, M., eds) pp. 149–159, Plenum Press, New York.

57. Oka, A., Sugimoto, K., Takamani, M., and Hirota, Y. (1990) Mol. Gen. Genet. 178, 9–20.

58. Geier, G. E., and Modrich, P. (1979) J. Biol. Chem. 254, 1408–1413.

59. Antequera, F., Tamame, M., Villanueva, J. R., and Santos, T. (1984) J. Biol. Chem. 259, 981–989.

60. Boye, E., and Lobner-Olesen, A. (1990) EMBO J. 9, 4083–4089.

61. Abeles, A., Brendler, T., and Austin, S. (1993) Cell 74, 162, 230–240.

62. Brendler, T., Abeles, A., and Austin, S. (1995) EMBO J. 14, 3135–3142.

63. Abeles, A., Brendler, T., and Austin, S. (1993) Cell 74, 162, 230–240.

64. Ogden, G. B., Pratt, M. J., and Schaechter, M. (1988) Cell 54, 127–135.

65. Campbell, J. L., and Kleckner, N. (1990) Cell 62, 967–979.

66. Lu, M., Campbell, J. L., Boye, E., and Kleckner, N. (1994) Cell 77, 413–426.

67. Slater, S., Wold, S., Lu, M., Boye, E., Skarstad, K., and Kleckner, N. (1995) EMBO J. 14, 2426–2435.

68. von Freiesleben, U., Rasmussen, K. V., and Schaechter, M. (1994) EMBO J. 13, 3672–3678.

69. Van der Vliet, P. C. (1996) Cell 84, 275, 1245–1252.

70. Mancino, R., Ohtsuki, H., Nakagawa, T., and Maeda, Y. (1988) Mol. Gen. Genet. 213, 619–624.

71. Van der Vliet, P. C. (1996) Cell 84, 275, 1245–1252.

72. Mancino, R., Ohtsuki, H., Nakagawa, T., and Maeda, Y. (1988) Mol. Gen. Genet. 213, 619–624.

73. Mancino, R., Ohtsuki, H., Nakagawa, T., and Maeda, Y. (1988) Mol. Gen. Genet. 213, 619–624.

74. Mancino, R., Ohtsuki, H., Nakagawa, T., and Maeda, Y. (1988) Mol. Gen. Genet. 213, 619–624.