Identification of Initiation Sites for DNA Replication in the Human\textsuperscript{*} \textit{dnmt1} (DNA-methyltransferase) Locus

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Vertebrates have developed multiple mechanisms to coordinate the replication of epigenetic and genetic information. \textit{Dnmt1} encodes the maintenance enzyme DNA-methyltransferase, which is responsible for propagating the DNA methylation pattern and the epigenetic information that it encodes during replication. Direct sequence analysis and bisulfite mapping of the 5′ region of DNA-methyltransferase 1 (\textit{dnmt1}) have indicated the presence of many sequence elements associated with previously characterized origins of DNA replication. This study tests the hypothesis that the \textit{dnmt1} region containing these elements is an origin of replication in human cells. First, we demonstrate that a vector containing this \textit{dnmt1} sequence is able to support autonomous replication when transfected into HeLa cells. Second, using a gel retardation assay, we show that it contains a site for binding of origin-rich sequences binding activity, a recently purified replication protein. Finally, using competitive polymerase chain reaction, we show that replication initiates in this region in \textit{vivo}. Based on these lines of evidence, we propose that initiation sites for DNA replication are located between the first intron and exon 7 of the human \textit{dnmt1} locus.

Mammalian DNA replication initiates from multiple sites throughout S phase (1). These sites are determined both by cis-acting DNA sequences, known as replicators, and by trans-acting elements, defined by initiator proteins that bind to the replicator (1, 2). Because of the complexity of the mammalian genome, initiation of replication has been studied in greater detail in prokaryotes and lower eukaryotes. However, a number of techniques, including methods for the isolation of newly synthesized DNA in combination with competitive PCR techniques, have led to the identification of new mammalian replication initiation regions as well as characterization of additional initiation sites at established origins (3–8). The best example of such a region is the extensively studied initiation region of the human \textit{dnmt1} locus.

Metazoan chromosomal origins of replication comprise specific sequence motifs. Replication initiation elements can be moved to new chromosomal sites and still initiate replication (9–11), whereas deletions of specific sequences in these elements abolish their ability to initiate replication (11, 12). The ability of specific mammalian DNA sequences to support autonomous replication of plasmids into which they have been inserted has been used in the past as an assay for the presence of an origin of replication in that sequence (13–20). In several instances, the ability of certain specific sequences to support autonomous replication of plasmids has been directly validated by mapping of the same sequences as chromosomal initiation sites for replication in living cells (16).

Whereas only a few origins of replication have been identified in mammals, certain sequence motifs have been found to be common in most of these origins (21). These motifs include A/T-rich regions, ATTAT and ATTTA nuclear matrix attachment motifs, and yeast ARS consensus sequence elements (WTTATTTT), identified in autonomously replicating sequences in \textit{Saccharomyces cerevisiae} (20, 22). Furthermore, an origin binding activity (OBA)\textsuperscript{*} was recently purified from HeLa cells (23) through its ability to bind to the 186-bp minimal replication origin of \textit{ors} 8 (13) and a 36-bp sequence (A3/4) that is found in a number of mammalian replication origins. OBA sediments at approximately 150 kDa in a glycerol gradient, and it cofractionates with DNA polymerases α and δ, topoisomerase II, and replication protein A (23).

In addition to genetic elements, epigenetic components such as DNA modification by methylation and chromatin structure, have been proposed to be characteristic determinants of origins of replication (6, 24, 25). We have previously shown that origins of replication like regulatory regions of genes are differentially methylated (26). Furthermore, it was recently shown that nonmethylated CpG islands are enriched in early replicating nascent DNA (6). However, because of the limited number of mammalian origins of replication that have been characterized thus far, it is still too early to draw general conclusions regarding the critical genetic and epigenetic determinants of origin function and its differential regulation. Additional origins have to be characterized to allow for understanding of the general rules governing origin function in mammals.

DNA methylation is a post-replicative covalent modification of DNA that is catalyzed by the DNA-methyltransferase enzyme (\textit{DNMT1}) (27–29). In vertebrates, 60–80% of cytosines in CpG sequences are methylated; the nonmethylated CpGs are the critical genetic and epigenetic determinants of origin function and its differential regulation. Additional origins have to be characterized to allow for understanding of the general rules governing origin function in mammals.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank\textsuperscript{TM} / EBI Data Bank with accession number(s) AF119248. § To whom correspondence should be addressed: Dept. of Pharmacology and Therapeutics, McGill University, 3655 Drummond St., Montreal, Quebec H3G 1Y6, Canada. Tel.: 514-388-7107; Fax: 514-388-6690; E-mail: mazy@pharma.mcgill.ca.

\textsuperscript{1} The abbreviations used are: \textit{dhfr}, dihydrofolate reductase; kb, kilobase(s); bp, base pair(s); \textit{dnmt1}-DNA-methyltransferase 1; ARS, autonomous replicating sequence; OBA, origin-rich sequences binding activity; PCR, polymerase chain reaction; DNMT1, DNA-methyltransferase 1.
methyltransferase (5′-AGAGACATCTTGGAGAATGTG) (p1, sense, starting at bp 240), 5′-AGTGAGCCGGTATGTGATCA (p2, antisense, starting at bp 643), 5′-GATTGCTAGGGGAGTGTATAGTTG (primer used to design competitor at c1); c2 region, 5′-AGACGTAGTTACATCCCG (p3, sense, ~6.3 kb downstream of p2), 5′-GCTTCTTACGGTTCTTCG (p4, antisense, ~7 kb downstream of p2), 5′-TATATTCAGAGCATCTGAG (primer used to design competitor at c3; c3 region, 5′-TCAGGGTCTACGCCACAT (p5, sense, starting at bp 2906), 5′-TCCCAAGCTTACACGTAGT (antisense, starting at bp 3300), 5′-ATTACACTACGGCAAATGCTGCTCAGT (primer used to design competitor at c5); c4 region, 5′-ACTACGATAGCGCGG (p7, sense, starting at bp 1718), 5′-TCATGCATTCACTGCTACG (p8, antisense, starting at bp 1768), 5′-GCTACACGAGCAGATGTA (p9, antisense, starting at bp 2155), 5′-GACTCTGCATGACATGAC (primer used to design competitor at c4); c5 region, 5′-CTTTCCAGTTCAGCAAGG (p10, antisense, ~3.5 kb upstream of p1), 5′-CAAGGACAATTCGACTGTTTGCATGTTT (primer used to design competitor at c5). Approximate distances between some of the dnm1 primers were calculated from Southern blot analysis (28).

The following primers were used for the c-myc locus (GenBank accession number J00120): MO region, 5′-TCCGGTTGTAATACAAA (p11, sense, starting at bp 761), 5′-TCTTCAAGGGTCTTCCCT (p12, antisense, starting at bp 1134), 5′-AAATCGAAGATCTTTCGAGGCAC (primer used to design competitor at MO); MF region, 5′-GCTTCGAGATGAGCTGAGT (p13, sense, starting at bp 7845), 5′-ATGGACTGGGTGATGCTCAG (p14, antisense, starting at bp 7675), 5′-GTTCATTCAATGCTGCTGC (p15, antisense, starting at bp 6701); 5′-TTCCTGGGAAGAGTGGAGTGGAGGCA (primer used to design competitor at MF). The PCR reactions for c1 and c2 regions were performed as follows: 3 min at 94 °C, then 30 cycles of 1 min at 94 °C, 1 min at 55 °C, 1 min at 72 °C, followed by 5 min at 72 °C. The PCR reactions for the remaining regions were performed as follows: 3 min at 94 °C, then 30 cycles of 1 min at 94 °C, 1 min at 60 °C, 1 min at 72 °C, followed by 5 min at 72 °C. Isolation of nascent DNA, c-myc PCR primer design, and competitor construction were done as described previously (5, 26, 39, 40).

Bisulfite Mapping Analysis—The following primers were used for the dnm1 site (bp 1 was assigned to the first 5′ bp of the c1 construct): 5′-TATATGTTTGTAGGGTTGAT (Mor1; sense, starting at bp 6); 5′-GATCTGTTATATATATTTTATTG (antisense, starting at bp 6); 5′-GCTCCCATGCTGCTGCT (p14, antisense, starting at bp 643), 5′-TCTTCCCTGGAAGAGTGGAGTGGAC (primer used to design competitor at MF). The PCR reactions for c1 and c2 regions were performed as follows: 3 min at 94 °C, then 30 cycles of 1 min at 94 °C, 1 min at 60 °C, 1 min at 72 °C, followed by 5 min at 72 °C. Isolation of nascent DNA, c-myc PCR primer design, and competitor construction were done as described previously (5, 26, 39, 40).

Sequence Elements Associated with Characterized Origins of DNA Replication Are Present in the First Intron of the dnm1 Locus—Sequence analysis of a 2.38-kb construct (c1 construct) (Fig. 1, A and B)—containing intron sequence immediately upstream of exon 2 revealed several sequence elements that are associated with characterized origins of replication (21). These elements include a 536-bp stretch that is 77% AT-rich, at least 19 ATTA and ATTTA nucleotide matrix attachment motifs (21), a perfect match of the 11-bp ARS consensus sequence (TTTIRATATTW) of the yeast S. cerevisiae (22), a methylated CpG island (25, 26), and a region homologous to the binding sites of OBA, a human origin binding protein (22).

The c1 construct Is Able to Support Autonomous Replication—To ascertain whether the dnm1 sequence indeed contained an origin of replication, we first tested the ability of the c1 construct to support autonomous replication using a density shift assay following bromodeoxyuridine incorporation into nascent DNA, as described previously (13, 17, 19). The results are
**Fig. 2A** demonstrate a density shift of the c1 construct corresponding to incorporation of bromodeoxyuridine into one (heavy-light) or both of the nascent DNA strands (heavy-heavy), indicating one and two or more rounds of replication, respectively. The SK plasmid vector, on the other hand, did not exhibit a similar shift, and all plasmid DNA was recovered in the unreplicated (light-light) form of input DNA (Fig. 2B). The linearity of each gradient was verified by measuring the refractive index of every other fraction.

**The dnmt1 A3/4 Homologous Region Is Able to Bind OBA**—Sequence analysis revealed a 30-bp stretch that is 86% homologous to the 36 bp A3/4 sequence, identified as part of the binding site for OBA.2 An oligonucleotide of this 30-bp dnmt1 sequence was synthesized, and its binding activity to OBA was assayed by gel retardation in the presence of excess nonspecific competitor poly(dI-dC). The results (Fig. 3) show that the dnmt1 oligonucleotide is able to bind to and form a complex with OBA (lane 3) of the same size as the complex formed when the 36 bp A3/4 oligonucleotide is used (lane 6). This binding is specific, because the dnmt1 oligonucleotide was able to compete for the OBA binding (lanes 4 and 8) as effectively as the 36-bp A3/4 oligonucleotide when it was used as competitor (lanes 5 and 7).

**Initiation Sites for DNA Replication Are Present within the** dnmt1 **Locus**—To quantitatively analyze the replication origin activity of the c1 construct and map the initiation site(s) in it, a series of competitive PCR experiments were performed to measure and compare nascent DNA abundance of five different regions within the dnmt1 locus (Fig. 4). The top gel (Fig. 4A) of each of the regions (c1-c5, MO, and MF) corresponds to the competitive PCR amplification of HeLa genomic DNA used to standardize the differences among primers and competitors with respect to their amplification efficiencies. The linearity of each of the competitive PCR analysis was verified by plotting the ratio of competitor DNA concentration over target DNA.

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2 M. Ruiz, D. Matheos, G. B. Price, and M. Zannis-Hadjopoulos, submitted for publication.
concentration (y axis) versus the concentration of competitor DNA (x axis) (Fig. 4B). Small nascent DNA ranging in size between 800 bp to 1.3 kb was isolated by the nascent strand extrusion method (5, 26, 40) followed by sucrose gradient sedimentation and further purification on agarose gel, as described previously (5). The bottom gel (Fig. 4AII) of each of the regions (c1-c5, MO, and MF) corresponds to the competitive PCR amplification of HeLa nascent DNA, and the bar graphs of the c-myc locus (Fig. 4C) and the dnmt1 locus (Fig. 4D) represent the nascent DNA abundance obtained by the respective competitive PCR assays. Two regions, MO and MF, within the well characterized initiation region associated with the c-myc gene (39) were used as control for the quality of the nascent DNA preparation (Fig. 4C). The results (Fig. 4C) show that a previously characterized c-myc origin of replication (MO) is present in the nascent DNA preparation at \(6 \times 10^3\) copies of nascent DNA, whereas no signal is observed for a region located 7 kb downstream from the origin of replication (MF), indicating that the nascent DNA used did not contain degraded parental DNA (5, 26). The same preparation of nascent DNA was used to determine the relative abundance of five regions within the dnmt1 locus. The results (Fig. 4D) indicate that two regions of DNA located between the first intron and the seventh exon of dnmt1 (c1 and c3) are highly abundant in the nascent DNA fraction, containing approximately \(3 \times 10^4\) copies of nascent DNA each. In contrast, the relative abundance of regions of dnmt1 residing approximately 3 kb upstream of the c1 region (c5) or 5 kb downstream of the c3 region (c2) are far less abundant, containing \(6 \times 10^3\) copies of nascent DNA (c5) and no nascent DNA (c2), respectively. The data are consistent with the hypothesis that at least two major initiation sites for DNA replication are localized between the first intron and exon 7 of the dnmt1 gene.

A Methylated CpG Cluster Is Associated with the Initiation Sites for DNA Replication—A number of origins of replication, among them ori \(\beta\) at the dhfr locus, have been previously shown to be associated with a methylated CpG cluster (25), whereas other origins of replication have been shown to be unmethylated in the associated CpG sequences (6, 26), suggesting differential methylation of replication origins. To determine whether dnmt1 is associated with either a methylated or unmethylated CpG cluster, we performed a bisulfite methylation mapping analysis of a CpG cluster located within the c1 construct region (Fig. 5A). Twelve clones were selected, and the eight CpGs residing in this region were analyzed in each clone...
Fig. 4. Competitive PCR mapping of an in vivo origin of replication residing in the *dnmt1* locus. A series of competitive PCR experiments were performed to measure and compare the abundance of 5 different regions within the *dnmt1* locus (c1-c5) in nascent DNA. To normalize the differences in primer and competitor amplification efficiencies, HeLa genomic DNA was used as template for the competitive PCR assay (*A*, top gel for each indicated regions). The linearity of each of the competitive PCR analysis was verified by plotting the ratio of competitor DNA concentration over target DNA concentration (y axis) versus the concentration of competitor DNA (x axis) (Fig. 4B). Small nascent DNA ranging in size between 800 bp to 1.3 kb was used as template for the competitive PCR assay (*AII*, bottom gel for each of the indicated regions). The competitor molecule number used in each competitive PCR is displayed on top of every gel. As a control for the purity of the nascent DNA preparation, we determined the abundance of the *c-myc* origin of replication (MO) and a sequence residing 7 kb downstream (MF) (*C*). The position of each of the tested regions is illustrated in the schematic diagrams of both *dnmt1* and the *c-myc* locus below the respective bar graphs. The bar graph results (*D*) represent nascent DNA abundance for each indicated *dnmt1* region. ATG, exonal location of proposed initiation codons; NLS, nuclear localization signal.
with respect to their methylation status. Six of them were methylated in all clones tested; 1 CpG was unmethylated, and interestingly, 1 CpG was methylated in 6 of the 12 clones tested (Fig. 5, A and B). A sample result (Fig. 5B) of the bisulfite analysis indicates the methylated CpGs and their respective positions. The results show that the dnmt1 initiation region is associated with a methylated CpG cluster, as was previously demonstrated for the dhfr ori B (25).

DISCUSSION

This paper describes the identification of initiation sites for DNA replication located between the first intron and exon 7 of the dnmt1 locus. This finding is supported by several lines of evidence. First, a plasmid containing the c1 region of dnmt1 is able to replicate autonomously. Second, this region contains a binding recognition sequence and binds to OBA, a protein that binds to origins of replication. Third, this region of dnmt1 contains several sequence elements characteristic of known origins of DNA replication, such as, an A/T-rich region, matrix attachment motifs, a methylated CpG cluster, and a perfect match to the yeast ARS consensus sequence (21). Finally, this region is abundant in nascent DNA, as are other characterized origins of replication, as determined by competitive PCR. Two primary replication initiation sites were identified, one localized in the c1 region and another localized in the c3 region; these regions are approximately 2 kb apart from each other (Fig. 4D). This finding is similar to recently described observations in the Chinese hamster ovary dhfr origin of replication, where initiation was shown to occur primarily from two sites, ori β and ori β′ (7).

The data presented in this paper further support the previous observations that regions of initiation of DNA replication share common sequence features (21). A 536-bp, 77% A/T-rich element is present in the c1 region of the dnmt1 locus, suggesting a possible function as a low melting region or as a DNA-unwinding element (21). This region also contains a perfect match for the yeast ARS consensus sequence, although previous reports have indicated that these sequences are not essential for mammalian origin function (18, 21). Nineteen ATTA and ATTTA matrix attachment motifs are present in the 2.4-kb c1 construct; these motifs constitute the core elements recognized by the homeobox domain from species as divergent as flies and humans and are frequently present in matrix attachment sites of several eukaryotic genes in addition to numerous eukaryotic and viral origins of DNA replication (41).

A methylated CpG cluster is associated with the dnmt1 replication initiation region. DNA methylation is now recognized as a fundamental mechanism of epigenetic regulation of genomic processes such as transcription, recombination, imprinting, development, carcinogenesis, and replication timing (36). Some origins of replication bear a cluster of heavily methylated CpG sites (25), whereas other origins, such as c-myc, are associated with a nonmethylated CpG cluster (26). Taking these findings into account, it seems that origins of replication exhibit differential methylation patterns similar to those observed in promoter elements of several genes (36). It remains unclear what role methylation plays in replication. It has been recently suggested that early activated origins are associated with nonmethylated CpG islands (6). The identification of origins that are representative of either methylation profile will allow the testing of the hypothesis that methylation plays a role in differential activation of origins of replication.

The c1 construct containing all the structural elements mentioned above is able to support episomal autonomous replication when transfected into HeLa cells. Results from utilization of in vivo assays of autonomous replication have been controversial, because some studies have demonstrated that only large fragments (>10 kb) could support autonomous replication (15), whereas several other studies showed that small specific sequences could successfully support autonomous replication (13–19). In addition, plasmids carrying ori β of the dhfr locus replicated autonomously both in vivo and in vitro regardless of their size, whereas plasmids of equivalent size inserts, but with random sequence, did not (16). Furthermore, the capacity of several specific sequences to support autonomous replication of plasmids correlates directly with their chromosomal mapping sites (16).

Regarding the significance of the co-localization of initiation of replication with the regulatory region of the dnmt1 gene, an
attractive possibility would be that the physical association of the dnmt1 regulatory region with initiation sites for DNA replication plays a role in coordinating the replication of the genetic information with that of the epigenetic information. Future experiments should resolve this question.

In summary, several lines of evidence, both structural and functional, demonstrate the presence of at least two major initiation sites for DNA replication residing in the region contained between the first intron and exon 7 of the dnmt1 locus.

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