Tandem B1 Elements Located in a Mouse Methylation Center Provide a Target for de Novo DNA Methylation*

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A cis-acting methylation center that signals de novo DNA methylation is located upstream of the mouse Aprt gene. In the current study, two approaches were taken to determine if tandem B1 repetitive elements found at the 3′ end of the methylation center contribute to the methylation signal. First, bisulfite genomic sequencing demonstrated that CpG sites within the B1 elements were methylated at relative levels of 43% in embryonal stem cells deficient for the maintenance DNA methyltransferase when compared with wild type embryonal stem cells. Second, the ability of the B1 elements to signal de novo methylation upon stable transfection into mouse embryonal carcinoma cells was examined. This approach demonstrated that the B1 elements were methylated de novo to a high level in the embryonal carcinoma cells and that the B1 elements acted synergistically. The results from these experiments provide strong evidence that the tandem B1 repetitive elements provide a significant fraction of the methylation center signal. By extension, they also support the hypothesis that one role for DNA methylation in mammals is to protect the genome from expression and transposition of parasitic elements.

Several lines of evidence support the hypothesis that a primary function of CpG methylation in mammals is to protect the genome from expression and transposition of parasitic DNA elements (1, 2). Transposons represent ~35% of the human genome, yet they contain the majority of genomic 5-methylcytosine bases. Most transposons are retroposons that have integrated into multiple genomic locations via RNA intermediates. For example, the abundant human Alu elements and the homologous mouse B1 elements apparently arose from the reverse transcription and integration of 7SL RNA (3, 4). DNA methylation has been shown to repress the transcription of Alu sequences both in vitro and in vivo (5, 6). Similarly, DNA methylation apparently represses mouse IAP retroviral element transcription as DNA methyltransferase (Dnmt1)-deficient mouse embryos express dramatically increased IAP transcript levels when compared with wild type embryos (7). Moreover, retroviral long terminal repeats are both methylated and repressed following introduction into embryonal cell types (8).

One prediction of the protective hypothesis for DNA methylation is that retroposons should serve as substrates for de novo DNA methylation (9). We have described previously an 838-base pair methylation center located upstream of the mouse adenosine phosphoribosyltransferase (Aprt) gene (see Fig. 1A) that signals de novo methylation upon transfection into mouse embryonal carcinoma (EC) cells (10). We report here that two tandem B1 elements are located at the 3′ end of the methylation center. These elements are methylated at relatively high levels in embryonic cells with severe Dnmt1 deficiency. Such cells have little or no maintenance methylation activity but retain de novo methylation activity (11). In addition, the B1 elements became methylated de novo when transfected into mouse EC cells. Together, these results suggest that these B1 repetitive elements account for a significant portion of the Aprt methylation center signal.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—The wild type and Dnmt–/– ES cells were grown in leukemia inhibitory factor-supplemented medium as described (11). The EC cells used were P19H22 and DelTG3. P19H22 contains a single allele of the Aprt gene derived from the C3H mouse strain (12, 13). DelTG3 lacks both Aprt alleles (10). Culture and electroporation of the EC cells were performed as described (14).

Bisulfite Sequencing—For P19H22 DNA, bisulfite modification was carried out as described (15) with the following modifications. 2 μg of HpaI-digested genomic DNA (see Fig. 1A) was denatured in 0.3 M NaOH at 75 °C for 5 min. The denatured DNA was mixed with 500 μl of 4.8 M sodium bisulfite and 28 μl of 10 mM hydroquinone, covered with mineral oil, and incubated at 55 °C for 4 h in the dark. DNA was desalted using a QIAquick PCR purification kit (Qiagen) according to the manufacturer’s instructions. The samples were desulfonated in 0.3 M NaOH at 37 °C for 15 min and neutralized by the addition of 5 μl sodium acetate. 1 μl of GenElute-LPA carrier (Sigma) was added, and the samples were precipitated by the addition of 2.5 volumes of 100% ethanol. The precipitated DNA was recovered by centrifugation, and the pellet was rinsed with 75% ethanol and air-dried. The pellet was resuspended in 25 μl of Tris-EDTA and stored at −20 °C before PCR.

For the wild type and Dnmt–/– ES cells, 2 μg of HpaI-digested DNA was modified in a solution of 5.36 mM urea, 3.44 M sodium bisulfite, and 0.5 mM hydroquinone as described (16). The modified samples were desalted, desulfonated, precipitated, and stored as described above for P19H22 DNA.

Bisulfite-modified DNA was subjected to semi-nested PCR using primers specific for the modified DNA. The primers were designed to specifically amplify the modified sense strand. First, 1 μl of modified DNA was subjected to PCR using primers S1 (TTT GAA GGT TTA TGG GAG TTG) and AC (ATC TAA CAC ACA ATC TCC CAT C) (see Fig. 2A). The PCR conditions were as follows: 1 cycle of 95 °C for 9 min; 30 cycles

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§The abbreviations used are: EC, embryonal carcinoma; PCR, polymerase chain reaction.

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of 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s; 1 cycle of 72°C for 3 min. The resultant PCR products were diluted 100-fold, and 1 μl was subjected to 25 cycles of PCR under the same conditions using primers S2 (ATTTTGGATATTGATAATTGGTAAGTGTGTTG) (see Fig. 2A) and AC. Products from this semi-nested PCR were subcloned using a TOPO-TA cloning kit (Invitrogen) according to the manufacturer's instructions and sequenced. In all cases, clones derived from at least two independent PCR reactions were sequenced.

Construct Preparation—Each construct was made by using PCR to amplify the desired fragment (see Fig. 1A) and blunt end-ligating the amplified fragment into the HL construct (see Figs. 1, B and C) (10). Artificial HpaII sites (H') and site-directed mutations were introduced by placing these changes on the primers. The sequence for this region has the accession number U5342.

Southern Blot Procedures—The methods used for Southern blot analysis of DNA preparations from transfectants are described elsewhere (14, 17).

Determination of Methylation Levels for HpaII Sites on Transfected Constructs—Images of Southern blots were acquired with ImageQuant software (Molecular Dynamics), and the intensities of the signal from hybridizations bands were determined with ImageQuant software (Molecular Dynamics). All Southern blot membranes were hybridized with random prime 32P-labeled fragments (Roche Molecular Biochemicals) generated from the N1 probe (see Fig. 1A). The 1.05 (1.2-) and 1.15 (1.3)-kilobase pair hybridization bands (see Fig. 1C) represent methylation of the H1 and H2 sites with or without concomitant methylation of the artificial H* site, respectively. The 0.65 (0.8-) and 0.75 (0.9-) kilobase pair hybridization bands represent methylation of the H1 site, again with or without methylation of the artificial H* site, respectively. Finally, the 0.4- and 0.5-kilobase pair hybridization bands were derived from unmethylated regions (see Fig. 1C). The signal intensity was determined for each hybridization band and adjusted to reflect its relative theoretical degree of hybridization to the N1 probe. The percent methylation for a given HpaII site was determined by dividing the sum of the signal intensities for hybridization bands representing methylation at that site by the sum of the signal intensities from bands representing both methylation and the absence of methylation of that site.

RESULTS

Tandem B1 Elements Were Located at the 3′ End of the Methylation Center—The methylation center (Fig. 1A) is a cis-acting element involved in establishing the methylation pattern upstream of the mouse Aprt promoter (9, 10). Sequence analysis revealed the presence of tandem B1 repetitive elements at the 3′ end of the methylation center (B1–5 and B1–6, Figs. 1A and 2A). These elements are located in a 3′-5′ direction (i.e., tail to head) relative to the 5′ end of the Aprt promoter. The sequences for the B1–5 and B1–6 elements are shown in Fig. 2B. Both B1–5 and B1–6 are similar to the consensus B1 element (18) (92 and 87%, respectively), except that B1–6 lacks the first 13 nucleotides at the 5′ end of the consensus element. The B1–5 and B1–6 elements are 84% homologous with each other.

The B1 Elements Were Methylated in Dnmt1−/− Embryonal Stem Cells—Southern blot analysis demonstrated in previous studies that the HpaII site termed H1 (Fig. 1A) was completely methylated in EC cells and adult tissues (10, 19). To determine if methylation at the H1 site in EC cells is representative of methylation at nearby CpG sites, bisulfite genomic sequencing was used to examine methylation status for all CpG sites within the B1–5 (sites 4–7) and B1–6 elements (sites 2–3) and two flanking CpG sites (sites 1 and 8) (Fig. 2A). As shown in Fig. 2C, all CpG sites were methylated in 15 of 20 alleles analyzed from the P19H22 EC cells, and overall 114 out of a possible 160 CpG sites (93%) were methylated. These results confirm that methylation of the H1 site, as determined by Southern blot analysis, provides a good barometer for methylation of other CpG sites located in the B1 elements or just outside these elements. The H1 site was methylated in 17 of 20 sequenced clones (85%). Therefore, the results also suggest that a low level of unmethylated H1 sites is not detected by Southern analysis or, alternatively, that the bisulfite method slightly overestimates the number of unmethylated H1 sites.

Cells with knockout mutations for the Dnmt1 allele, which encodes the maintenance DNA methyltransferase in mouse cells, have markedly reduced levels of CpG methylation. This is particularly true for cells homozygous for the Dnmt1−/− allele, which has a mutation eliminating catalytic activity (11). However, these cells still retain the capacity for de novo methylation (11) and, therefore, provide a good system in which to test for CpG sites that serve as substrates for de novo methylation. Methylation levels in ES cells wild type for Dnmt1 and homozygous for the Dnmt1−/− allele were determined for seven of the eight CpG sites shown in Fig. 2A. Sequence analysis revealed a CG to TA transition that eliminated CpG site 2 in the ES cell lines, which are 129/Sv-derived. The overall level of methylation for the CpG sites within the wild type ES cells (62%) was lower than that for the EC cells (93%); only 2 out of 11 sequenced clones were methylated at all 7 CpG sites. The CpG sites within and flanking the B1 elements in the Dnmt1−/− ES cells were methylated at unexpectedly high levels, with 19 out of 70 (27%) sites being methylated. This relative level of methylation was 43% that observed for the wild type ES cells. At least 1 CpG site within the B1 elements was methylated in 9 of the 10 clones sequenced, although no specific pattern could be discerned.

De Novo Methylation of the B1 Elements upon Stable Transfection into EC Cells—The observation of a relatively high level of B1 element methylation in the Dnmt1−/− cells suggested that the repetitive element region acts as a signal for de novo DNA methylation. To test this possibility directly, the B1–5 and B1–6 elements were inserted as a dimer (i.e., their normal genomic configuration) into the HL construct (Fig. 1B) to create the HL1–6 dimer construct. The HL construct lacks the meth-
Methylation of the H1 site at its 5’ end was the H1 site for the B1–5 insert and an unmethylated site. CpG sites 1–8 correspond with those shown in panel A of this figure. CpG site 2 is not present in the ES cells. The H1 site at the 5’ end of B1–5 is an artificial site introduced on its 3’ end. The H* sites are artificial HpaII sites for the ability of the B1–5 element to signal DNA methylation (Fig. 5) with average levels for the H1 and H2 sites of 60 and 11%, respectively (Table I). In contrast, methylation of the HL272 construct was observed in only 2 of 8 transfectants with an average level of 6.5% (Table I, Fig. 4). Methylation of the H2 site was not observed. Therefore, the high level of methylation observed for the HL1-dimer transfectants was a direct result of the inserted B1 elements.

To determine the relative contributions of the B1–5 and B1–6 elements to the de novo methylation signal, each element was inserted independently into the HL construct to create HL1–5 and HL1–6, respectively. Again, HpaII sites bracketed the fragments: an artificial 5’ site for B1–5 and two artificial sites for B1–6. The B1–5 element was found to attract de novo methylation (Fig. 5) with average levels for the H1 and H2 sites of 60 and 11%, respectively (Table I). In contrast, methylation of the HL1–6 construct occurred less frequently; only 2 of 6 transfectants displayed any methylation. The average levels of methylation was 16 and 5% for the 3’ H* (i.e. equivalent location of the H1 site on B1–5) and the H2 sites, respectively. Therefore, the B1–5 and B1–6 elements are not functionally equivalent, with a far stronger activity being observed for the B1–5 element.

It has been proposed that clustering of CpG sites contributes to the methylation process (20–22). Although the B1–5 and B1–6 elements have relatively low densities of these sites, a small cluster of CpG sites is present at the 5’ end of the B1–5 element (i.e. the 3’ end of the methylation center). In this region 4 CpG dinucleotides are located within a stretch of 21 base pairs (Fig. 2B). To determine if this cluster is important for the ability of the B1–5 element to signal de novo methylation, all CpG dinucleotides except the one contained within the H1 site were eliminated by site-directed mutagenesis (C → T mutations) to create a construct termed HL1–5SDM. Essential mutations to create a construct termed HL1–5SDM. Essential
the origin of each hybridization band. Fig. 1 shows for a HLB1–5 transfectant. DNA preparations from transfectants were digested with HpaII and PstI and then Southern blot-hybridized with the N1 probe (Fig. 1A). A MspI digest lane is also shown.

**DISCUSSION**

The endogenous targets for de novo DNA methylation in the mammalian genome have not been described (9). In previous work we identified a methylation center upstream of mouse Aprt that can signal de novo methylation when transfected into mouse EC cells and from which methylation can spread (10). We report here that two B1 repetitive elements are located at the 3’ end of the methylation center and have used two approaches to obtain evidence suggesting strongly that this pair can act as a unit to create a strong de novo methylation signal.

The first approach was to examine methylation of CpG sites included within and flanking the B1–5 and B1–6 elements in ES cells homozygous for the Dnmt1−/− allele. This allele contains a knockout mutation in the region of the Dnmt1 gene that encodes the catalytic domain. Therefore these cells lack the enzymatic function responsible for the spreading of methylation and its maintenance, although they still retain the capacity to methylate DNA de novo. Presumably the regions of the genome that remain methylated in these cells are those that attract most strongly de novo methylation. With the bisulfite-sequencing method we demonstrated that CpG sites in the B1 elements under study are methylated at a relative level of 43% in the Dnmt1−/− cells when compared with the same sites in ES cells with wild type Dnmt1 alleles. If the above presumption is correct, these elements are acting as strong signals for de novo methylation. It is noted that the downstream H2 site, which becomes methylated as a function of spreading from the methylation center, is essentially unmethylated in the Dnmt1−/− cells (9).

Methylation of other repetitive elements has also been reported in the Dnmt1−/− ES cells. Using the bisulfite method, Woodcock et al. (23) show that CpG sites within A-repeats are methylated at a consensus relative level of 29% (absolute level of 13%) in the Dnmt1−/− cells when compared with the wild type ES cells. A Southern blot analysis showed methylation of endogenous Moloney murine leukemia retrovirus in the Dnmt1−/− cells, although the absolute and relative levels were not quantified (11). In contrast to these results, a very low level of methylation (<1.4%) was observed for CpG sites within the H19-imprinted region in the Dnmt1−/− allele ES cells. This region is completely methylated on the marked alleles in the wild type ES cells (24).

The second approach we used to examine methylation potential for the B1 elements was to test their ability to become methylated de novo when transfected into mouse EC cells. These cells possess a high capacity for de novo methylation of transfected plasmid constructs that contain the methylation center (10, 14). Although some variability in methylation can be attributed to integration sites, the average methylation levels reflect sequence content of the transfected constructs (25). With this approach we found that the B1 dimer could direct a high level of methylation for the H1 and H2 sites, at average levels near that observed for the corresponding endogenous region (9, 17, 19). A similarly sized contiguous downstream fragment of 272 base pairs that lacked B1 elements failed to elicit a significant methylation response, which confirmed specificity for methylation of the B1 elements and which also confirmed that the H1 site is very close to the 3’ end of the methylation center.

It is interesting to note that the human Alu repetitive element, which is homologous to the mouse B1 element, is found normally as a tandem pair of monomeric units (26). Copies of this element are methylated at high levels in human somatic cells (27, 28). Moreover, spreading of methylation from Alu elements has been suggested as playing a causal role in epigenetic inactivation of some tumor suppressor genes (29, 30). DNA fragments containing B1, B2, or L1 elements have been shown to enhance methylation of a bacterial chloramphenicol acetyltransferase fragment in F9 EC cells (31), although the elements were not tested as isolated units as performed here, and the chloramphenicol acetyltransferase fragment elicited significant methylation by itself. Moreover, the constructs were introduced by the calcium phosphate method, which can often lead to high copy numbers of integrated plasmids. High copy numbers can cause high levels of methylation (32). To prevent this form of nonspecific methylation, we used electroporation to keep copy numbers at low levels (14).

Although the B1–6 element did not become methylated when tested independently, suggesting that loss of the 5’ end decreased its intrinsic methylation signal, an artificial HpaII site placed at its 3’ end (i.e. 5’ end of insert) became methylated at an average minimal level of 59% when combined in tandem with B1–5. Moreover, the CpG sites in B1–6 (two sites in P19 EC cells, one site in the ES cells) were methylated at high levels at their endogenous location in wild type cells (82% in ES cells) and at relatively high levels in the Dnmt1−/− ES cells (27%). This latter observation is taken as evidence that these sites are methylated de novo at their endogenous location despite the relative lack of methylation of B1–6 when tested independently. In total, these data and those obtained with the B1–5 insert suggest that the B1–5 and B1–6 elements act synergistically in their normal tandem orientation to signal DNA methylation. The data obtained with the B1–5 insert further suggest that a single intact B1 element can provide a strong methylation signal. Considering that there are several hundred thousand B1 elements in the mouse genome (26), it is possible that B1 elements account for a significant fraction of mouse genome methylation.
Finally, the results obtained in this study have direct relevance for the hypothesis that a function of DNA methylation in mammalian cells is to protect the genome from expression and transposition of repetitive elements (1, 2). A large number of these elements exist in the mammalian genome, and in general they are highly methylated in somatic cells. As mentioned above, high levels of methylation have been observed for repetitive elements analyzed in Dnmt−/− cells. It has been suggested that methylation of repetitive elements can repress their expression, which is linked to transposition events, in two ways. One is by methylation-mediated transcriptional inactivation, and the second is by increasing the rates of C → T mutations over the course of evolution (2). An example of this type of mutation is the absence of CpG site 2 in B1–6 (Table I) in the 129/Sv strain. Our results provide the first direct demonstration that one class of repetitive elements, i.e. B1 elements derived from 7SL RNA, can signal methylation de novo. Similar findings with other endogenous “parasitic” elements are a logical prediction of the protection hypothesis and are necessary for its further testing.

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