In the cell, Dnmt1 is the major enzyme in maintenance of the pattern of DNA methylation after DNA replication. Evidence suggests that the protein is located at the replication fork, where it could directly modify nascent DNA immediately after replication. To elucidate the potential mechanism of this process, we investigate the processivity of DNA methylation and accuracy of copying an existing pattern of methylation in this study using purified Dnmt1 and hemimethylated substrate DNA. We demonstrate that Dnmt1 methylates a hemimethylated 958-mer substrate in a highly processive reaction. Fully methylated and unmethylated CG sites do not inhibit processive methylation of the DNA. Extending previous work, we show that unmethylated sites embedded in a hemimethylated context are modified at an approximately 24-fold reduced rate, which demonstrates that the enzyme accurately copies existing patterns of methylation. Completely unmodified DNA is methylated even more slowly due to an allosteric activation of Dnmt1 by methylcytosine-containing DNA. Interestingly, Dnmt1 is not able to methylate hemimethylated CG sites on different strands of the DNA in a processive manner, indicating that Dnmt1 keeps its orientation with respect to the DNA while methylating the CG sites on one strand of the DNA.

In vertebrates, DNA is modified at cytosine residues to form 5-methylcytosines. DNA methylation occurs predominantly in the context of CG dinucleotides, which are methylated to 60–80%. The modified CGs are not randomly distributed but form a tissue- and cell-specific pattern (1, 2). Methylation is involved in epigenetic regulation of gene expression, in the context of X chromosome inactivation, genomic imprinting, and development (3). On the other hand, methylcytosine is a hot spot of mutation, and aberrant methylation is involved in aging and cancer (4).

5-Methylcytosine is introduced into the DNA after replication by DNA methyltransferases. Until now, three families of DNA methyltransferases have been identified in mammals: Dnmt1, Dnmt2, and Dnmt3 (5). All cytosine-C5-methyltrans-
tases contain a domain of ~500 amino acid residues, which is characterized by the presence of 10 conserved amino acid motifs, shared between prokaryotic and eukaryotic cytosine-C5-methyltransferases (6, 7). In addition to the catalytic parts (5, 7), Dnmt2 is the smallest enzyme among the eukaryotic methyltransferases. It consists only of the catalytic part, which is similar to prokaryotic cytosine-C5-methyltransferases. It was shown only recently to be an active methyltransferase (8).

The Dnmt3 family comprises three different proteins, Dnmt3a, Dnmt3b, and Dnmt3L. Dnmt3a and Dnmt3b have a regulatory N-terminal domain, which is not essential for catalysis (9, 10). Both enzymes do not distinguish between unmethylated and hemimethylated substrates and are supposed to mediate de novo methylation together with Dnmt1 (11–13). Dnmt3a is distributive on unmethylated substrates, whereas Dnmt3b is a processive enzyme (9). The third member of the Dnmt3 family, Dnmt3L, does not have a functional catalytic domain and is catalytically inactive (14).

Dnmt1 prefers hemimethylated DNA over unmethylated DNA up to 40-fold in vitro (15). In vivo, it is supposed to be responsible for copying the existing methylation pattern of the DNA and is therefore called maintenance methyltransferase (5). Dnmt1 is a very large protein of 184 kDa. It has an extended N-terminal regulatory domain that contains a nuclear localization sequence (16), a replication foci targeting domain (17), and a cysteine-rich Zn2+ binding domain (18, 19). Another domain shows homology with Polybromo-1 protein from chicken (16), which plays a role in the transport of Dnmt1 to the replication foci as well (20). Many proteins have been reported to interact with the N-terminal part of Dnmt1 by yeast two-hybrid and/or interaction assays (21–27). It seems that the N terminus forms a platform for the binding of proteins involved in chromatin condensation and gene regulation. One example is the proliferating cell nuclear antigen, a protein also known as a processivity factor for the DNA polymerases ε/δ (27, 28).

Previous evidence for a processive methylation of DNA by Dnmt1 dates back more than 20 years, when Bestor and Ingram (29) investigated the mechanism of a new DNA methyltransferase activity, later identified to be Dnmt1. In their experiments, they showed a higher methylation rate of Dnmt1 on longer DNA molecules, a finding that suggests that Dnmt1 is a processive enzyme. However, this result could also be explained by a preferential binding of the enzyme to longer substrates, not necessarily involving repetitive methylation on the same substrate molecule. In addition, due to technical reasons, unmethylated DNA, which is not the preferred substrate of the enzyme and does not reflect the physiological situation, was used in these experiments. Since a processive DNA methyl-
unmet: forward: 5′-GCATGCACCTGCTATCCAGATGGCAG-3′
back: 3′-CGATGCATGCCGCGCTTAACTGCTAG-5′

hemi: forward: 5′-GCATGCACCTGCTATCCAGATGGCAG-3′
back: 3′-CGATGCATGCCGCGCTTAACTGCTAG-5′

hemi 2: forward: 5′-GCATGCACCTGCTATCCAGATGGCAG-3′
back: 3′-CGATGCATGCCGCGCTTAACTGCTAG-5′

hemi 3: forward: 5′-GCATGCACCTGCTATCCAGATGGCAG-3′
back: 3′-CGATGCATGCCGCGCTTAACTGCTAG-5′

hemi-turn: forward: 5′-GCATGCACCTGCTATCCAGATGGCAG-3′
back: 3′-CGATGCATGCCGCGCTTAACTGCTAG-5′

Scheme 1. Sequences of the oligodeoxynucleotides used for the methylation and restriction analysis of the 80-mer substrates.

Scheme 2. Sequences of the oligodeoxynucleotides used as PCR primers to amplify the 958-mer substrates.

5′-P1: 5′-GGAAGCGGGACGATCATCGCCTCGCGG-3′
P2: 5′-GGAACGGCGAGGACGATCATCGCCTCCGG-3′

5′-P-P1: 5′-cCGGGGGCGCTCATCGCCTCGCGG-3′
P2: 5′-cGGGCGGGCGCTCATCGCCTCCGG-3′

Dnmt1 Methylates DNA Processively

MATERIALS AND METHODS

Oligodeoxynucleotides—Oligodeoxynucleotides were purchased from Interactiva (Ulm, Germany) in HPLC-1 and PAGE-purified form. The concentration of oligodeoxynucleotide stocks were calculated from the A260 nm using molar extinction coefficients provided by the supplier. Double-stranded oligodeoxynucleotide substrates were formed by annealing identical amounts of complementary single-stranded oligodeoxynucleotides. The 80-mer oligodeoxynucleotide contained three repeated sequences, comprising 20 nucleotides, flanked by 10 variable nucleotides at each site to prevent nonspecific annealing. The target site for methylation is situated in the middle of the 20-mer repeated sequence. The oligodeoxynucleotides displayed different methylation status. In the case of the hemi3 oligonucleotide substrate, 2 nucleotides were removed to remove the restriction site. In the case of the hemiturn oligonucleotide substrate, the entire central 20-mer repeated sequence was inverted. In Scheme 1, the recognition sequence for Dnmt1 is indicated with bold letters, and the GATC recognition sequence of the Sau3AI restriction enzyme is underlined.

The substrate plasmid pAT153 (NCBI accession number: L08853) was used as template for the generation of a longer hemimethylated substrate. It was mutagenized by the addition of a Sau3AI cleavage site at position 1129 and thereby create a substrate with four GATG sites but no additional GATC site. The mutation was introduced by a PCR-based mutagenesis protocol after Jeltsch and Lano (30). For this mutagenesis, the first step is to create a megaprimer, with two primers, one of them containing the mutation. The GATC recognition sequence of the Sau3AI restriction enzyme is underlined.

Oligodeoxynucleotides used as primers for the generation of a longer hemimethylated substrate were purchased from Interactiva in HPLC-purified form.

The upper or lower strand of the substrate was phosphorylated with T4 polynucleotide kinase and [γ-32P]ATP. The oligodeoxynucleotides were annealed by adding equal amounts of the complementary strands, heating 10 min to 90 °C, and slowly cooling to ambient temperature. After precipitating the DNA with ethanol, the exact amount of DNA was measured by OD260 nm using an extinction coefficient of ε260 nm = 20 cm1 liter/g. These substrates contain repetitive sequences that may potentially lead to a variety of secondary structures and formation of stem loop structures. We have confirmed successful annealing by native polyacrylamide gel electrophoresis (Supplemental Fig. 2) and suitable molecular mass markers of known concentration.

Methylation and Restriction Analysis of the 80-mer Oligodeoxynucleotide Substrates—The upper or lower strand of the substrate was phosphorylated with T4 polynucleotide kinase and [γ-32P]ATP. The oligodeoxynucleotides were annealed by adding equal amounts of the complementary strands, heating 10 min to 90 °C, and slowly cooling to ambient temperature. After precipitating the DNA with ethanol, the exact amount of DNA was measured by OD260 nm using an extinction coefficient of ε260 nm = 20 cm1 liter/g. These substrates contain repetitive sequences that may potentially lead to a variety of secondary structures and formation of stem loop structures. We have confirmed successful annealing by native polyacrylamide gel electrophoresis (Supplemental Fig. 2) and suitable molecular mass markers of known concentration.

Methylation reactions were carried out at 37 °C in methylation buffer (20 mM HEPES, 1 mM EDTA, pH 7.5) containing 1 mM S-adenosyl-l-methionine (Sigma) and 100 μg/ml bovine serum albumin. The substrate was used at a concentration of 3.8 nm DNA. The reaction was started by the addition of enzyme to a final concentration of 6.5 nM in all experiments, except for the experiment with unmethylated oligodeoxynucleotide, in which the Dnmt1 concentration was increased 10-fold (65 nM).

The methylation reaction was started by adding ethanol, and the precipitated and purified DNA was digested with 4 units of Sau3AI. For the Sau3AI digest, additional unmethylated 80-mer oligodeoxynucleotide was added to a final concentration of 50 nM to improve the efficiency of the cleavage reaction (31). Cleavage products were separated on a 10% (w/v) polyacrylamide gel containing 7 M urea and analyzed using an Instant Imager (Canberra Packard). All the
experiments were repeated at least four times. Despite prolonged incubation times and the addition of stimulatory oligonucleotides, the digestion of the 80-mer substrate with Sau3AI was never found to be complete, which had to be considered in the quantification of the exact amount of methylation by Dnmt1. Due to incomplete cleavage, a certain amount of unmethylated DNA was not cleaved, suggesting that it was methylated. For this reason, the relative amount of incompletely cleaved DNA in relation to the amount that was completely cleaved was calculated from control reactions for each gel and each experiment (typically in the range of 2–5%). This fraction was used for correction of the Dnmt1-treated samples. Incompletely digested DNA contributes to the amount of intermediate detectable in the analysis, although it does not originate from incompletely methylated substrate. Therefore, the contribution of incomplete digestion to the apparent amount of intermediates was corrected by calculating the amount of incomplete digestion for each lane, by multiplication of the amount of cleaved DNA with the fraction of incomplete digestion in the respective experiment. For example, assume that 3% incomplete digestion was detected in the control lane, and in the kinetic analysis, one lane shows 5% intermediates and 70% unmethylated substrate. Under these circumstances, incomplete digestion contributes by 2.1% (= 70% × 0.03) to the amount of intermediate detected. Therefore, the real amount of incompletely methylated DNA is 2.9% (= 5% – 2.1%).

**Methylation Analysis of a longer Hemimethylated Substrate**—We also studied the processivity of Dnmt1 using a hemimethylated substrate with a length of 957 bp, which contains four GATCG sites. To generate the substrate, we used a protocol modified after Thomas et al. (32). The 957-mer oligonucleotide was obtained by PCR with the primers 5′-P1 and P2, using the mutated plasmid pAT153 as template (PCR protocol: 1 × 95 °C for 3 min; 30 × 95 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min; 1 × 72 °C for 5 min). This PCR product was methylated at all CG sites in both DNA strands by the CG-specific methytransferase M.SssI (kindly provided by Dr. M. Roth, Institut für Biochemie, Justus-Liebig Universität Giessen, Giessen, Germany); the completeness of the methylation was confirmed by digestion of the DNA with R.HpaII (MBI Fermentas). The methylated PCR product was digested by Λ-exonuclease (New England Biolabs) in the buffer provided by the company for 60 min at 37 °C, 1 unit was used for 1 μg of PCR product. Λ-Exonuclease selectively digests one strand of double-stranded DNA from a phosphorylated 5′-end. Since the forward primer in the PCR reaction was phosphorylated, it leads to the selective removal of the upper strand of the PCR product. Digestion with Λ-exonuclease was complete as judged from a native polyacrylamide gel, in which no double-stranded PCR product was detectable after digestion. Subsequently, the methylated single strand was used as template for a primer extension reaction. In this 50-μl reaction, the template, primer 5′-P1, and dNTPs were incubated with Taq polymerase (Stratagene) in the buffer provided by the company supplemented with 0.37 MBq [α-32P]ATP (Hartmann Analytic). The temperature profile of the extension reaction was: 95 °C for 3 min, 65 °C for 30 s, 72 °C for 5 min, 90 °C for 1 min, 62 °C for 30 s, 72 °C for 5 min, 88 °C for 2 min, 60 °C for 30 s, 72 °C for 15 min. The hemimethylated substrate was purified with a spin column (Macherey-Nagel), and the amount of DNA was determined using an extinction coefficient of ε260 nm = 20 cm mg⁻¹. The final hemimethylated substrate was incubated with HpaII and Sau3AI restriction enzyme. HpaII did not cleave the DNA, confirming the completeness of the hemimethylation since hemimethylated CCGG sites are not cut, whereas Sau3AI was cleaving the substrate (Supplemental Fig. 3).

Methylation reactions with Dnmt1 were carried out at 37 °C in methylation buffer (20 mM HEPES, 1 mM EDTA, pH 7.5) containing 1 mM S-adenosyl-L-methionin (Sigma) and 100 μM/mI bovine serum albumin. The substrate was used at a concentration of 2 μM DNA; the reaction was started by the addition of the enzyme to a final concentration of 4 nM. The methylation reaction was stopped at different time points by the addition of 5 volumes of 100% ethanol. After ethanol precipitation, the DNA was digested by Sau3AI as described previously. Cleavage products were separated on a 6% (w/v) native polyacrylamide gel, and the radioactivity was analyzed using an Instant Imager (Canberra Packard).

**RESULTS**

The aim of our studies was to investigate the processivity and accuracy of the DNA methylation reaction by Dnmt1. For this purpose, we have analyzed the processivity of Dnmt1 on substrates differing in the length, the number of CG target sites, and the methylation state of these sites. We employed a restriction protection assay in which the DNA after methylation is incubated with a methylation-sensitive restriction enzyme such that methylated sites are not cut. With this method, the rate of methylation of each individual site can be analyzed, and one can follow the appearance and disappearance of intermediates of the methylation reaction precisely. Since Dnmt1 prefers hemimethylated substrates and one of its functions is to act as the maintenance methytransferase after DNA replication, we have employed hemimethylated substrates modified in one DNA strand and studied the methylation of the second DNA strand. To this end, one needs to distinguish between hemimethylated and fully methylated CG sites. However, there is no restriction enzyme available that recognizes a CG site and at the same time distinguishes between the hemimethylated and fully methylated state of the substrate. Therefore, we have designed substrates containing GATCG sites in which the recognition sequence of Sau3AI (GATC) is overlapping with the CG recognition sequence of Dnmt1. Sau3AI cleaves the DNA when the GATC site is not methylated. If the CG of the lower strand is methylated (GATCG/CmGATC), the methyl group is not part of the recognition site of the restriction enzyme, and the site is cleaved although the CG site is hemimethylated. After Dnmt1 has modified the hemimethylated CG site, a fully methylated CG site is generated. The methyl group introduced by Dnmt1 is in the recognition site of Sau3AI (GATCG/CmGATC), and DNA cleavage is inhibited (Fig. 1) because Sau3AI does not cut hemimethylated GATC sites (33).

**Methylation of a 958-bp Substrate**—Processivity is dependent on the length of the substrate, and in vitro, Dnmt1 is confronted with a very long stretch of hemimethylated DNA after DNA replication. To simulate this situation in vitro, a long substrate, in which all CG sequences are hemimethylated, is required. Such substrates can be produced following a published protocol (32). In a first step, a 958-mer PCR product containing four GATCG sites was generated using an upper primer phosphorylated on its 5′-end. Then, this PCR product was methylated with M.SssI. By Λ-exonuclease digestion, which specifically hydrolyzes one strand of double-stranded DNA starting from a phosphorylated 5′-end, a methylated single strand was obtained. In the third step, the single strand was used as template for a primer extension reaction using radioactive [α-32P]ATP to produce a double-stranded DNA, containing four hemimethylated GATCG sites (Fig. 2A). The completeness of the hemimethylation was confirmed by complete protection of the substrate against HpaII digestion (Supplemental Fig. 3). Since the substrate is internally labeled, all Sau3AI cleavage fragments are detectable on the gel, even
after incomplete methylation of the DNA by Dnmt1. The processivity of the methylation reaction can be deduced from the pattern of unmethylated DNA, fully methylated DNA, and methylation intermediates. A distributive enzyme will leave its substrate after each turnover. Thus, for statistical reasons, the enzyme will bind to unmethylated substrates in the early phases of the reaction, and DNA molecules carrying one, two, or three methyl groups will accumulate before significant amounts of completely methylated final product appear. In contrast, a processive enzyme will stay on each substrate until it is completely methylated such that the final product appears even at the earliest time points of the reaction. A simulation of a distributive and a processive methylation reaction of the 958-mer oligonucleotide is shown in Fig. 2C for comparison. We have performed these experiments under conditions of excessive or equimolar enzyme. However, due to an unfavorable binding constant and low absolute concentrations, this does not implicate more than one enzyme being bound to each substrate on average. Therefore, our results can be interpreted in a theoretical framework assuming that on each substrate molecule, just one enzyme is active.

The time course of methylation of the 958-mer oligonucleotide by Dnmt1 shows that there are only a few intermediates detectable (total amount <5%, Fig. 2, B and D). The high degree of processivity of the reaction is illustrated by comparison of the reaction profile shown in Fig. 2D with simulated profiles, calculated under the assumption of a fully distributive and fully processive reaction (Fig. 2C). Our data demonstrate that 95% of all substrates are methylated at all four sites in a processive reaction. The probability to continue DNA methylation after each catalytic event therefore can be estimated to be 98.8% (0.988^4 = 0.95). We conclude that Dnmt1 methylates hemimethylated DNA, comprising 958 bp in a highly processive manner.

Methylation of Synthetic 80-mer Oligonucleotides—After having shown that Dnmt1 modifies DNA in a processive reaction, we attempted to investigate the influence of the methylation state of adjacent CG sites on the processivity as well as the accuracy of the enzyme in copying an existing methylation pattern. To this end, synthetic oligonucleotide substrates were employed to investigate partially unmethylated and fully methylated substrates as well. These experiments were carried out using 80-mer oligonucleotide substrates containing three 20-mer repeats; 10 nucleotides specific for each end were added to ensure accurate annealing. The GATCG recognition site of Dnmt1 is embedded in the repeats, and the repeated structure was chosen to ensure that the methylation of Dnmt1 is not influenced by different flanking nucleotides, and all three sites are modified at the same intrinsic rate. The oligodeoxynucleotide substrates were radioactively labeled at one 5′-end. Therefore, only fragments containing the original 5′-end were detectable on the gel. After Sau3AI cleavage of an unmethylated substrate, a 19-mer oligonucleotide product is generated, whereas the fully methylated 80-mer oligonucleotide is not cleaved at all. If the methylation is not complete, intermediate products could appear, with lengths of 39 or 59 nucleotides, depending on whether one or two restriction sites are protected. Additional intermediates are possible but not detectable.

Fig. 2. Methylation of a hemimethylated 957-bp PCR substrate. A, a schematic drawing of the substrate. The CG sequence is drawn as a square, and the red squares indicate the methylated cytosines. The position of Sau3AI cleavage is indicated by arrows. The substrate is internally labeled, B, an example of the Dnmt1 methylation. The incubation time is shown in minutes, and the minus sign indicates the control digest without Dnmt1 methylation. Sau3AI digest is indicated. The fully cleaved substrate fragments are indicated in red, and the fully protected product is indicated in blue. The two intermediates that accumulate to a detectable level correspond to molecules methylated at two sites, either at the first two CG sequences (length of the intermediate, 443 bp) or at the central two recognition sequences (length of the intermediate, 635 bp) and are indicated in green. C, a simulation of the methylation reaction assuming a completely distributive or processive reaction. In the graph, the amounts of cleavage fragments corresponding to unmethylated DNA (red), methylation intermediates (green), and fully methylated DNA (blue) are indicated. D, a quantitative analysis of the gel shown in panel B. The total radioactivity in the gel between the 317-bp substrate band and the 957-bp product band was considered as an intermediate and was used in the calculation.
Dnmt1 Methylates DNA Processively

Accuray of DNA Methylation by Dnmt1—One biological function of Dnmt1 is to copy an existing methylation pattern after DNA replication, which requires Dnmt1 to methylate hemimethylated CG sites but not to methylate unmodified sites. Here, we wanted to address experimentally the question of the accuracy of Dnmt1 in copying methylation patterns. To this end, we have used a substrate containing an unmethylated CG site flanked by two hemimethylated sites and investigated whether Dnmt1 methylates the unmethylated CG site (Fig. 5A). The oligonucleotide with an unmethylated CG sequence showed a high accumulation of the 39-mer intermediate during the time course of the methylation reaction, indicating that the first hemimethylated CG site was methylated, but the central site was left unmodified (Fig. 5B). The 59-mer intermediate that would appear if the substrate is only modified at the first hemimethylated CG and the central unmethylated CG was not detectable at any time point during the reaction progress curve. This indicates that the unmethylated CG sequence is modified much more slowly than both hemimethylated ones. The fully methylated product appeared very slowly; after 60 min, only approximately 50% of product was formed, indicating a slow methylation also at the central site. Then, the apparent rate of methylation at the unmodified site is 0.5/hr (50% methylated after 60 min). In contrast, 50% of the 19-mer cleavage product has disappeared after ~2.5 min, indicating that the hemimethylated site is modified with a rate of 12/hr (50% methylated after 2.5 min). Therefore, the hemimethylated site is modified ~24 times faster than the unmethylated one.

Processivity of Methylation Of Substrates Containing Two Hemimethylated Sites Separated by an Unmethylated Site—In the experiment described above, two hemimethylated sites were separated by one unmethylated CG. Since the methylation of the hemimethylated CG sequences occurred much faster than that of the unmethylated site, with this substrate, one cannot decide whether Dnmt1 is moving over the unmethylated CG site to methylate the substrate in a processive manner. To decide whether Dnmt1 is methylating hemimethylated CG sequences separated by an unmethylated CG sequence in a processive manner, we used the substrate that carries the unmethylated CG site but no Sau3AI site between the two hemimethylated sequences. This reduces the number of Sau3AI recognition sites to two, and on the gel, only one intermediate will be visible (Fig. 6B). The time course of methylation demonstrates that up to 10% of the intermediate is formed. Since there is only one intermediate possible, this leads to the con-
clusion that Dnmt1 methylates in 90% of all substrates in a processive manner, although an unmethylated CG is in between. This degree of processivity is comparable with that observed with the fully hemimethylated 80-mer oligonucleotide, indicating that the unmethylated site separating the hemimethylated ones does not interfere with the processive methylation of the site of the unmethylated CG.

**Fig. 4. Methylation of a hemimethylated oligonucleotide.** A, a schematic drawing of the substrate. The CG sequence is drawn as a square, and the red squares indicate the methylated cytosines. The position of Sau3AI cleavage is indicated by arrows. 32P with a circle indicates the radioactively labeled strand. B, an example of the Dnmt1 methylation. The incubation time with 6.5 nM Dnmt1 is shown in minutes, and the minus sign indicates the control digest without Dnmt1 methylation. All samples are Sau3AI-digested. The detectable cleavage products are drawn beside the gel in a schematic manner (red, the 19-bp fragment, representing the fully cleaved substrate; orange, the 39-bp methylation intermediate; green, the 59-bp methylation intermediate; blue, the 80-bp fully protected product). C, average data of four independent experiments, with the radioactive label in the upper or lower strand. The color code is used as explained in panel B.

**Fig. 5. Methylation of an oligonucleotide with an unmethylated CG sequence flanked by two hemimethylated CG sequences.** A, a schematic drawing of the substrate. The CG sequence is drawn as a square, and the red squares indicate the methylated cytosines. The position of Sau3AI cleavage is indicated by arrows. 32P with a circle indicates the radioactively labeled strand. B, an example of the Dnmt1 methylation. The incubation time with 6.5 nM Dnmt1 is shown in minutes, and the minus sign indicates the control digest without Dnmt1 methylation. All samples are Sau3AI-digested. The detectable cleavage products are drawn beside the gel in a schematic manner (red, the 19-bp fragment, representing the fully cleaved substrate; orange, the 39-bp methylation intermediate; green, the 59-bp methylation intermediate; blue, the 80-bp fully protected product). C, average data of four independent experiments, with the radioactive label in the upper or lower strand. The color code is used as explained in panel B.
methylation of the DNA. Conjunction of both experiments with the substrates containing two hemimethylated sites separated by one unmethylated one leads to the conclusion that on the one hand, Dnmt1 is methylating the unmethylated CG site very slowly, and on the other hand, it methylates the following hemimethylated CG site fast. This shows that Dnmt1 modifies the DNA specifically and processively at hemimethylated CG sequences and ignores unmethylated CG sites in between.

Processivity of Methylation of Substrates Containing Two Hemimethylated Sites Separated by a Fully Methylated Site—Finally, we wanted to investigate whether fully methylated CG sequences stimulate Dnmt1 and/or whether such fully methylated sequences might disturb the processivity of the enzyme. For this purpose, we used a substrate that carries a fully methylated CG site flanked by two hemimethylated CG sequences (Fig. 7A). The fully methylated CG sequence in the center is not cleaved by Sau3AI; therefore, only one methylation intermediate is possible. The time course shows that Dnmt1 methylates the two hemimethylated sequences separated by a fully methylated CG site processively (Fig. 7C). From the data, we conclude that Dnmt1 methylates 85% of the substrates in a processive manner, indicating that the additional methylation does not disturb Dnmt1 but also does not increase the processivity of methylation or the rate of the reaction.

Methylation Reaction of Hemimethylated Substrates Methylated in Different Strands of the DNA—As a control, we wanted to address the question whether the observed processivity by Dnmt1 could be due to a rapid reassociation of Dnmt1 to the same DNA strand after dissociation. To this end, we investigated the methylation of a substrate in which the central repeated sequence was inverted, still providing a hemimethylated CG site but carrying the methylation on the other DNA strand (Fig. 8A). With this substrate, we could detect whether, during processive methylation of one DNA substrate, Dnmt1 keeps the same orientation in respect to the target strand. In this case, Dnmt1 should not be able to methylate the inverted site on the lower strand during processive methylation of the target sites on the upper strand. If Dnmt1 leaves the DNA after each turnover, it could rebind in two orientations to the DNA, placing the unmethylated cytosine of either strand in the active site of the DNA and methylating the target sites on both strands in an apparently processive manner.

Our comparison between the methylation of a hemimethylated substrate and the inverted hemimethylated substrate shows that the 39-mer intermediate is accumulating only during the methylation reaction of the inverted substrate (Fig. 8B). This demonstrates that the first hemimethylated site is methylated but that the inverted one is not, and the intermediate having the first two sites methylated is not detectable at all. Therefore, during the reaction, the methylation of the inverted target site (on the lower strand) is not coupled to the methylation of the hemimethylated sites (on the upper strand). This result demonstrates that during processive methylation of the DNA, Dnmt1 does not change its orientation toward the two strands of the DNA, only one of which can be methylated in each orientation of the enzyme. It is not in agreement with a dissociation/rapid reassociation mechanism because in this model, the reassociation should occur in a random orientation.

It should be noticed that after binding to the DNA in an orientation that supports methylation of the lower strand, Dnmt1 will rapidly convert the inverted substrate into a substrate carrying a fully methylated site in between two hemimethylated ones. This is not detectable in our experimental setup. However, as shown previously, this substrate is methylated in a processive reaction at both hemimethylated sites. If the chance of binding is equal to both the DNA strands, one would expect that half of the inverted substrates should be methylated in a reaction that appears fully processive. 

A processive methylation of both strands of the DNA also would be compatible with a dimeric mode of action of Dnmt1.
Dnmt1 Methylates DNA Processively

**FIG. 7.** Methylation of an oligonucleotide containing a fully methylated CG sequence flanked by two hemimethylated CG sequences. A, a schematic drawing of the substrate. The CG sequence is drawn as a square, and the red squares indicate the methylated cytosines. The position of Sau3AI cleavage is indicated by arrows. $^{32}$P with a circle indicates the radioactively labeled strand. B, an example of the Dnmt1 methylation. The incubation time with 6.5 nM Dnmt1 is shown in minutes, and the minus sign indicates the control digest without Dnmt1 methylation. All samples are Sau3AI-digested. The detectable cleavage products are drawn beside the gel in a schematic manner (red, the 19-bp fragment, representing the fully cleaved substrate; green, the 58-bp methylation intermediate; blue, the 80-bp fully protected product). C, an average data of four independent experiments, with the radioactive label in the upper or lower strand. The color code is used as explained in panel B.

**DISCUSSION**

During every S-phase, DNA replication converts ~40 million methylated CG sequences into the hemimethylated state. These hemimethylated CG sequences have to be methylated fast and precisely without methylation of unmethylated CG sites to maintain the original methylation pattern of the DNA. This is done by the maintenance DNA methyltransferase Dnmt1. The catalytic mechanism of Dnmt1 and DNA methyltransferases in general comprises several steps: first, the enzyme binds to the DNA nonspecifically, and then, it searches the substrate until it reaches its specific recognition sequence. Here, the methyltransferase binds specifically and undergoes a conformational change that activates the enzyme, and finally, the site becomes modified. After methyl group transfer, the methyltransferase either stays on the modified DNA, searching for another target site, or leaves the substrate. A processive methyltransferase has a high propensity to stay on the DNA after each turnover and search for the next recognition sequence by linear diffusion. Therefore, many target sites are modified on the same substrate molecule in one DNA strand. A distributive methyltransferase, in contrast, would leave the DNA after each methyl group transfer, leading to the generation of many DNA molecules that carry just one or a few methyl groups in both DNA strands. In the family of prokaryotic DNA methyltransferases, there are examples for both types of enzymes (9, 30, 35–38). To distinguish experimentally between these modes of action, one has to detect the formation and disappearance of methylation intermediates, i.e. DNA molecules that are partially methylated. If the methyltransferase works in a processive manner, then during the time course of the reaction, the original substrate and fully methylated product exist at the same time in the absence of intermediates. The processivity of Dnmt1 is a highly relevant issue in vivo because evidence exists that the enzyme could be attached to the replication fork, which would require a highly processive mode of action (17, 20, 27, 39–41).

So far, one publication addresses the processivity of Dnmt1. Bestor and Ingram (1983) have demonstrated that Dnmt1 methylates longer substrates faster than shorter ones and have concluded that Dnmt1 is a processive enzyme (29). However, there are alternative interpretations of their results, such as preferential binding of the enzyme to longer substrates, which could also lead to higher methylation rates of the longer substrates. In addition, the degree of processivity could not be detected, and the authors used unmethylated linear DNA, which is not the preferred substrate for Dnmt1. We have now developed a novel assay system to investigate the processivity of DNA methylation by Dnmt1. We show that it methylates hemimethylated substrates as long as 1000 bp in a highly processive manner, with a propensity of 98.8% to perform the next turnover on the same substrate after each methylation event. This means that on average, about 60 methyl groups would be introduced on a substrate of sufficient length. If one assumes that a CG is encountered every 60th bp (as it is in human DNA), a DNA section comprising ~3600 bp would be methylated by Dnmt1 in a processive manner. However, processivity is influenced by the conditions. Factors that increase the nonspecific binding of the enzyme to the DNA, such as longer DNA substrates or lower salt concentrations, usually increase the processivity. The effect of the DNA length on processivity is reflected in our data by the difference in processivity of the methylation reaction of the 958- and the 80-mer oligonucleotide. It is difficult to compare our results with the in vivo situation in which the salt concentrations are higher but
the length of the DNA molecules is also higher. Moreover, in vivo molecular crowding will tend to increase the processivity. In any case, our results demonstrate that Dnmt1 can methylate DNA in a processive reaction.

The apparent processivity observed in our study could be explained by an alternative model as well, instead of indicating that the enzyme moves along one DNA molecule. One could hypothesize that the enzyme is inherently distributive but that it rapidly reassociates to the same DNA molecule after each turnover. Such preferential reassociation to the same substrate molecule could be stimulated by the low substrate concentrations in our experiments. To analyze whether such a dissociation/reassociation model is valid in this case, we investigated whether Dnmt1 methylates hemimethylated CG sites on both strands of the DNA in a processive reaction. In this context, it is important to consider that Dnmt1 can bind to the DNA in two orientations such that either the upper strand or the lower strand of the DNA can be modified. Since in the dissociation/reassociation model, reassociation should occur in random orientation, one would expect processive methylation of both strands of the DNA. However, our experiments show that Dnmt1 cannot methylate CG sites on different strands of the DNA processively. This result, together with the processivity shown in the previous experiments, clearly demonstrates that Dnmt1 keeps its orientation with respect to the DNA while methylating one strand of the DNA in a processive reaction. This finding can only be rationalized if the enzyme permanently keeps in touch with the DNA during the whole reaction. It implicates a continuous movement along the DNA and is not in agreement with a dissociation/reassociation mechanism.

In addition, we show in this study that the activity of Dnmt1 toward unmethylated CG sites is at least 1000-fold reduced when compared with hemimethylated DNA. In the context of DNA carrying a defined methylation pattern, a 24-fold preference for hemimethylated target sites was observed. In fact, this preference could be even larger because we could only detect methylation of unmodified sites at time points when hemimethylated target sites are no longer available. This is an important result demonstrating that Dnmt1 is able to copy existing patterns of DNA methylation with high accuracy.

It has been demonstrated previously that a fully methylated DNA can stimulate Dnmt1 allosterically to methylate unmethylated CG sequences (11, 15). A similar stimulation was observed here because we show that unmethylated sites next to hemimethylated sites are modified faster than on fully unmethylated DNA. This is obvious because the central CG site in substrate hemi2 is modified with a 24-fold reduced rate, whereas completely unmethylated substrates are modified at a 1000-fold reduced rate. This result suggests that Dnmt1 has two different methylation modes: on hemimethylated DNA, the enzyme works quickly, processively, and accurately; on unmethylated DNA, it operates in a slow and distributive manner that can be stimulated by allosteric activation of the enzyme by methylated sites nearby. This process could be involved in the maintenance of methylation at highly modified DNA sequences. After incomplete postreplicative methylation in these regions, the allosteric stimulation of Dnmt1 by methylated DNA nearby would increase the rate of methylation of the unmodified site in later stages. It makes DNA methylation behave in an all-or-none fashion, meaning that only unmethylated DNA remains unmethylated, whereas moderately modified DNA tends to become fully methylated. This all-or-none

![Methylation of hemimethylated substrates methylated in the same or different strands of the DNA.](http://www.jbc.org/)

**Panel A:** A schematic drawing of the two substrates. The CG sequence is drawn as a square, and the red squares indicate the methylated cytosines. The position of Sau3AI cleavage is indicated by arrows. ^32P with a circle indicates the radioactively labeled strand. The direction of the repeated sequence is written below for clarity. **Panel B:** An example of the Dnmt1 methylation. The incubation time with 6.5 nM Dnmt1 is shown in minutes, and the minus sign indicates the control digest without Dnmt1 methylation. All samples are Sau3AI-digested. The detectable cleavage products are drawn beside the gel in a schematic manner (red, the 19-bp fragment, representing the fully cleaved substrate; orange, the 39-bp methylation intermediate; green, the 59-bp methylation intermediate; blue, the 80-bp fully protected product). **Panel C:** Average data of four independent experiments of the two substrates in comparison. The color code is used as explained in panel B.
behavior can increase the efficiency of DNA methylation to act as a binary device, switching gene expression on or off.

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Dnmt1 methylates DNA processively.
