

Spreading of methylation along DNA

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Mouse DNA methyltransferase is able to catalyse the transfer of a methyl group to certain CG-containing single-stranded oligonucleotides. The presence of a methylcytosine is required for efficient transfer. This methylcytosine may or may not be on the same oligonucleotide as that containing the accepting CG dinucleotide. When the accepting CG dinucleotide forms part of

an unmethylated CG dinucleotide pair, its accepting activity is dramatically reduced. This provides the potential for methylation to spread along the DNA when it is rendered single-stranded at replication. It could also help to maintain fully methylated CG islands and asymmetrically methylated sites.

INTRODUCTION

Mammalian DNA methyltransferase is a maintenance enzyme that regenerates symmetrically methylated CG dinucleotide pairs following replication [1]. As the only enzyme present in most cells, it also appears to be able to methylate *de novo* cytosines not only in CG dinucleotides but also in CNG trinucleotides [2,3] and in unsymmetrical sequences [4], as well as to maintain certain CG dinucleotides in an asymmetrically methylated state [5]. It is also clear that, under certain conditions, methylation can spread along DNA [6,7]. This is rather a lot for one enzyme to do, and we know very little about how these various functions are brought about.

Previous results from our laboratory [8] had shown that a methylcytosine positioned 10 nucleotides away from a CG dinucleotide enhanced the rate of *de novo* methylation at the target cytosine. On closer inspection of the situation by gel electrophoresis, it became clear that some of the samples of oligonucleotide duplexes used contained contaminating single-stranded DNA. When greater care was taken to ensure that all the oligonucleotides were present as duplexes, the only duplex to show significant activity was the one containing a hemimethylated CG dinucleotide. The implication of this observation was that it was the small amount of single-stranded oligonucleotide that had been accepting methyl groups.

This was surprising, as we had previously shown that single-stranded oligonucleotides did not accept methyl groups, although we had not investigated the accepting activities of all of the single-stranded oligonucleotides used. On the other hand, Carotti et al. [9] had concluded that some random sequence polynucleotides could act as methyl group acceptors and, while the present work was in progress, Christman et al. [10] reported similar findings. Moreover, Smith et al. [11] have demonstrated that mispaired cytosines show enhanced accepting ability.

The present paper compares the accepting ability of oligonucleotides in single- and double-stranded form, and shows that only selected single-stranded oligonucleotides accept methyl groups. This accepting ability is blocked when the accepting CG dinucleotide and surrounding nucleotides in the single strand are annealed with their complement to form an unmethylated CG dinucleotide pair. In contrast, methylation of certain single-stranded oligonucleotides is enhanced in the presence of non-accepting single-stranded oligonucleotides of identical primary sequence.

MATERIALS AND METHODS

The oligonucleotides used in this study are listed in Table 1, together with the abbreviations used to describe them. They were made in-house using an Applied Biosystems DNA Synthesizer, model 381A. All oligonucleotides were checked for purity by electrophoresis prior to use.

Assay of DNA methyltransferase was as previously described [8]: 4–40 units (0.7–6.9 μg of protein) of a highly purified enzyme from mouse ascites cells was incubated with oligonucleotide for 1 h using 1 μCi of [^3H]adenosylmethionine (3 μM) as methyl donor. A total of 40 units of enzyme is an excess, in that methyl transfer is proportional to the concentration of DNA substrate (oligonucleotide 22C-10) up to 500 ng/assay. An amount of 100 ng of DNA is sufficient to saturate 4 units of enzyme; 40 units of enzyme methylates about 40% of the target cytosines in 100 ng of oligonucleotide 22C-10 within a 1 h incubation although, probably because of the low affinity of the enzyme for its substrate, the catalytic-centre activity of the enzyme is less than 1.

RESULTS

Single-stranded acceptors

Table 1 shows the accepting activities of 100 ng portions of a series of single-stranded oligonucleotides. It is clear that, while the majority do not accept methyl groups, some show a low level of accepting ability and four, which contain a methylcytosine in *cis* but distant from the target CG dinucleotide, do function as substrates for mouse DNA methyltransferase. Substitution of methylcytosine for C in the CG dinucleotide in oligonucleotide 22C-10 abolishes activity, indicating that it is the CG that is the target for the enzyme. The position of the methylcytosine relative to the target site may be important and is considered below. The short, unmethylated, single-stranded molecules fail routinely to accept methyl groups, although some of the longer ones (e.g. 40Cu, 41Cu and 42Gu) do show limited accepting ability, and the possibility that this might be a result of the formation of fold-back structures is also considered below.

Duplex acceptors

Table 1 also shows that, when these single-stranded molecules are converted into duplexes, accepting activity is generally enhanced. It remains low, however, unless the accepting CG

Abbreviation used: M, methylcytosine (in dinucleotides and above).

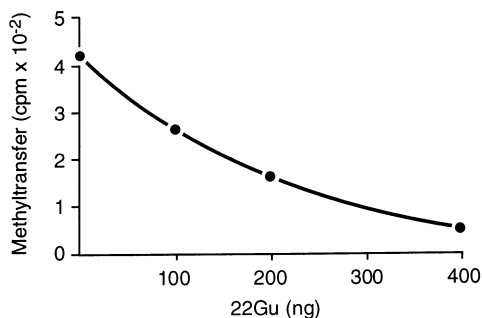
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Table 1 Sequences and acceptor ability of oligonucleotides

The 30-mers are extensions of the 22-mers, but the sequences are unrelated to those of the 40-mers, 41-mers or 42-mers. Unmethylated Cs in CG dinucleotides are underlined. 42Cm has two methylated CGs, as well as two unmethylated CGs and several methylcytosines not in MGs. For measurement of acceptor ability, 100 ng of single-stranded oligonucleotide (about 15 pmol of 22-mer) or duplex DNA was incubated with limiting enzyme and the radioactivity (c.p.m.) in the methylated product assayed. All values are means of at least two determinations (most of four, and some of 12); —, not determined.

| Oligonucleotide | Sequence | Accepting ability (c.p.m.) | |
|-----------------|---|----------------------------|---|
| | | Alone | In duplex form with complementary unmethylated strand |
| 22Cu | ATT CTC <u>CGC</u> CCC ATG GCT GAC T | 5 | 320 |
| 22Cm | M | 0 | 6800 |
| 22C-5 | <u>C</u> M | 0 | 180 |
| 22C-10 | <u>C</u> M | 1420 | 190 |
| 22Gu | unmethylated complement | 0 | — |
| 22Gm | complement with mCG | — | — |
| 30Cu | ATT CAC <u>CGC</u> CCC ATG GCT GAC CAT GGC AGC | — | — |
| 30C-15 | <u>C</u> M | 25 | 130 |
| 30-C-20 | <u>C</u> M | 100 | 190 |
| 30Gu | unmethylated complement | 0 | — |
| 40Cu | CCC CAT CCA CCA CAA <u>CGC</u> TCA GCA <u>ACG</u> CAG CCT AAA AAG G | 320 | 510 |
| 40Cm | <u>C</u> M | 970 | 5760 |
| 40Cma | <u>C</u> MA | 1990 | 7430 |
| 40Gu | unmethylated complement | — | — |
| 41Cu | CCC CAT CCA CCA CAA <u>CGC</u> TCA GCT GGC AGG <u>CAC</u> GGG GCG CA | 120 | 190 |
| 41Cm | M M M M M M | 0 | 5860* |
| 41Gu | unmethylated complement | 0 | — |
| 42Cm' | CCA TTC CAG MAG CTG <u>MCG</u> GTC CMG TGA MGG <u>GCG</u> MTG GAC ACA | 3210 | — |
| 42Gu | unmethylated complement | 160 | — |

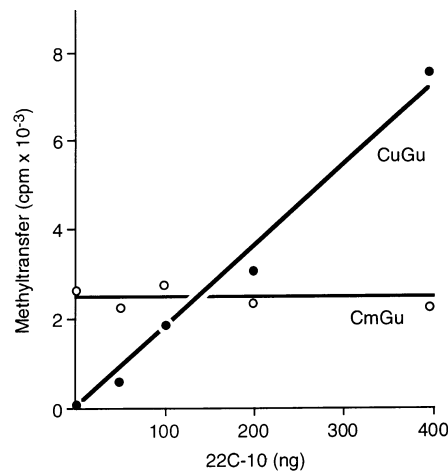
*Incubation of a mixture of 100 ng each of 41Cm and 41Cu gave 1780 c.p.m.

**Figure 1 Quenching of accepting ability on duplex formation**

A 200 ng portion of oligonucleotide 30C-20 was annealed with increasing amounts of 22Gu, which is complementary to the 5' end of 30C-20 including the region containing the target CG dinucleotide. The methyl-accepting ability was then assayed using 10 units of enzyme. The means of duplicate values are plotted.

becomes part of a hemimethylated target site, and this is the basis for the maintenance action of these enzymes.

The exception to the enhanced activity is with oligonucleotides such as 22C-10, where the unusual accepting activity of the single strand is lost on duplex formation. Figure 1 confirms that this is true even when the extra methylcytosine is in a single-stranded tail of an otherwise duplex molecule, indicating that, for the enhanced activity, the accepting CG dinucleotide must not be locked into an unmethylated CG dinucleotide pair.

**Figure 2 Competition between single-stranded DNA and unmethylated or hemimethylated duplex DNA**

A 200 ng portion of the indicated 22-mer duplex was mixed with increasing amounts of 22C-10, and methyl transfer activity was measured using 10 units of enzyme. The means of duplicate assays are plotted.

Binding to 22C-10 competes with binding to duplex DNA

Oligonucleotide 22C-10 can still act as an acceptor in the presence of unmethylated (Figure 2) or fully methylated (results not

Table 2 Effect of addition of oligonucleotide 22C-10 on the methylation of duplex DNA

Incubations contained 5 units of methyltransferase and 400 ng of the indicated duplex, either alone or in the presence of 100 ng of oligonucleotide 22C-10. The values represent radioactivity (c.p.m.) incorporated into the DNA. The values for the inhibition of methylation assume that methylation of duplex DNA is not affected by the single-stranded oligonucleotide.

| Duplex (400 ng) | Methylation (c.p.m.) | | Inhibition of methylation of 22C-10 (%) |
|-----------------|----------------------|--------------------|---|
| | Alone | + 100 ng of 22C-10 | |
| None | — | 2200 | 0 |
| 22Cu/22Gu | 15 | 460 | 80 |
| 22Cm/22Gu | 1975 | 2290 | 86 |
| 22Cm/22Gm | 0 | 1570 | 29 |

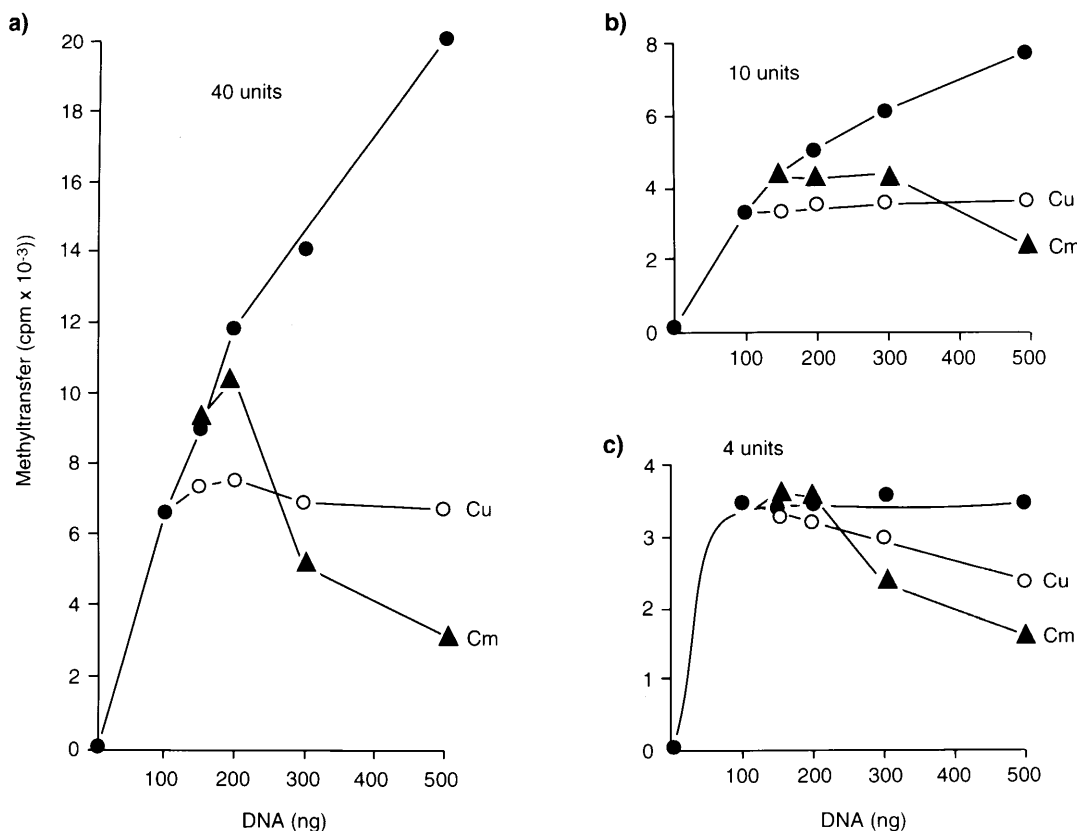
shown) duplex, indicating that 10 units of enzyme is sufficient to bind to 200 ng of duplex and up to 400 ng of 22C-10. However, under these conditions a hemimethylated duplex is able to sequester most or all of the enzyme, and the single strand now fails to accept methyl groups. By reducing the amount of enzyme we were able to devise conditions in which all duplexes were in competition with the single-stranded oligonucleotide 22C-10 (Table 2). This provided a method for comparing the affinity of binding of the enzyme to various duplex oligonucleotides. It is clear that the highest affinity is shown for the hemimethylated

duplex, with the unmethylated duplex not far behind, whereas the symmetrically methylated duplex shows a much lower affinity for the enzyme. This result is similar to those observed using gel retardation methods [12], although in those experiments the unmethylated duplex showed an affinity more similar to that of the symmetrically methylated duplex.

Non-complementary oligonucleotides can affect accepting activity

The following experiments were carried out using 100 ng of oligonucleotide 22C-10 in the presence of increasing amounts of competitor oligonucleotide. With 4 units of enzyme, the reaction goes at maximum velocity with this amount of 22C-10 (Figure 3c), but when an excess of enzyme (40 units/assay) is used the amount of methyl transfer is almost directly proportional to the amount of 22C-10 up to 500 ng/assay (Figure 3a). In the presence of 100 ng of 22C-10, the addition of up to a 4-fold excess of 22Cu has little effect on methyl transfer, except when enzyme is limiting (Figure 3c). However, a 4-fold excess of 22Cm always inhibits methyl transfer (Figures 3a–3c). This inhibition, which is independent of the amount of enzyme used, suggests that, in this case, inhibition is brought about by the interaction of the two DNA molecules (see below) rather than by a direct effect of 22Cm on the enzyme.

Particularly when the enzyme is in excess, smaller amounts of 22Cm stimulate methyl transfer (Figure 3a). The reason for the stimulation is not known, but it may reflect the separate binding

**Figure 3** Stimulation of methylation by identical, non-acceptor, oligonucleotides

Increasing amounts of 22C-10 (●), or 100 ng of 22C-10 with increasing amounts of 22Cu (○) or 22Cm (▲), were incubated in the presence of (a) 40 units, (b) 10 units or (c) 4 units of methyltransferase. The means of duplicate assays are plotted.

Table 3 Short duplex regions that can be formed by fold-back or as staggered duplexes by the 22-mers

Structures II and IV have no target CG; structure I has a mismatched CG:CT dinucleotide pair; structure III has a mismatched, hemimethylated, CG:MT dinucleotide pair. Cu and Cm can form staggered structures with C-10 that have little or no activity (I and IV). If these structures are formed they will interfere with the formation of the C-10 fold-back structure and therefore inhibit methyl transfer. This is not seen with low amounts of Cu and Cm. Formation of structure III by adding Cu does not increase the amount of MT, and so will have no effect on activity. See the text for further details.

| Duplex | Structure | Activity | Fold-back | Staggered (top strand first) |
|--------|---------------|----------|-----------|---|
| I | TCCGCC | Low | 22Cu | 22Cu 22Cu |
| | AGTCGG | | | 22Cu 22Cm 22C-10 22Cm 22C-10 22Cu |
| II | TCMGCC | None | 22Cm | 22Cm 22Cm |
| | AGTCGG | | | 22Cm 22Cu |
| III | TCCGCC | High | 22C-10 | 22Cu 22C-10 |
| | AGTMGG | | | 22C-10 22C-10 |
| IV | TCMGCC | None | — | 22Cm 22C-10 |
| | AGTMGG | | | |

to the enzyme of the MG of 22Cm (where M is methylcytosine) and the CG of 22C-10 (see below).

When the enzyme is limiting, incorporation into 100 ng of 22C-10 is inhibited by 22Cu (Figure 3c) and also by the unrelated oligonucleotides 42Gu, 41Cu and 41Gu (60–80% inhibition is seen using 100 ng of these oligonucleotides; results not shown). Such findings probably reflect the sequestration of limiting enzyme by an unproductive DNA-enzyme complex.

Formation of fold-back structures or staggered duplexes

To what extent are the above observations a result of the (transient) formation of hemimethylated duplexes? This could occur by fold-back of a single strand, a reaction that will be favoured if the CG dinucleotides are separated by a minimum

Oligonucleotides 22Cu and 22Cm can form staggered structures with 22C-10 that have little or no activity (structures I and IV in Table 3). If these form they will interfere with the formation of structure III by 22C-10 and therefore inhibit methyl transfer. This could explain the inhibitory effects of high levels of 22Cm on the methylation of 22C-10 even in the presence of excess enzyme. This is not seen with low amounts of 22Cu and 22Cm, probably reflecting the smaller chance of intermolecular reactions at low DNA concentrations. Formation of structure III by adding 22Cu to 22C-10 does not increase the overall amount of structure III, and so will have no effect on activity.

Another possible target that might form between 22Cu and 22Cm is a transient duplex that generates only a CG/MG dinucleotide pair and does not involve the complementary cassette. As 22Cu and 22Cm are both inactive either alone or in combination, it appears that preferential formation of the more stable structure I or structure II prevents separate interaction of the methylcytosine with the enzyme. Such interaction appears possible with 40Cma (see below) and could explain the stimulation of incorporation seen with equal amounts of 22C-10 and 22Cm when excess enzyme is used (Figure 3a).

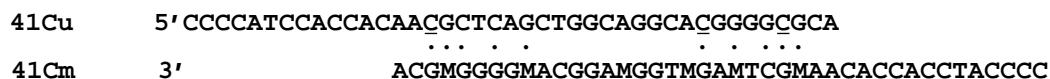
For 22C-5 the CG and the M cannot be combined to form a fold-back structure or a staggered duplex, as the M is not present in a complementary cassette. However, 22C-5 could form structure I as a fold-back or as a staggered duplex that should behave exactly as does 22Cu. Similar arguments apply to 30C-15. All three of these oligonucleotides have minimal accepting ability (Table 1).

Oligonucleotide 30C-20 can also form 4 bp of structure I; it can also fold back, with a 14 base loop, or generate staggered molecules with the structure:



The fact that the activity of 30C-20 is not as high as that of 22C-10 probably reflects the relative stabilities of structures I and V.

41Cm (which lacks any target CG dinucleotides) strongly stimulates methylation of 41Cu. These oligonucleotides can be arranged to give two hemimethylated CG trinucleotide pairs, but other than this there is very little complementarity:

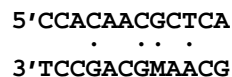


distance. The other way in which single strands can form duplex regions is by two strands annealing with each other to form a short duplex with single-stranded tails. In this case there would be no minimum distance required between the CG dinucleotide and the methylcytosine, as they would be on different strands.

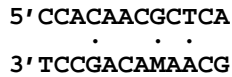
The 22-mers have only the one CG dinucleotide but, as Smith et al. have pointed out [11], accepting activity is greater if the target C is unpaired. The 22-mers could fold back (with a four- or six-base loop), or form staggered duplexes to give the structures shown in Table 3. These structures are possible because the oligonucleotides have a sequence (cassette) complementary to that surrounding the CG dinucleotide. Changing the base sequence of 22C-10 to abolish the complementary cassette while retaining the M 10 bases from the target CG causes loss of accepting ability (result not shown).

Oligo 42Cm' shows considerable accepting activity (Table 1), and can form a partial duplex involving a methylcytosine and a CG dinucleotide by folding back on itself in several ways, although none of these involve more than three contiguous base pairs.

40Cm, and more particularly 40Cma, show high activity as single strands (Table 1), strongly supporting the interpretation that a mismatched, hemimethylated, CG duplex structure might be involved [11]. However, the only structure that can be formed by 40Cm as a staggered duplex or as a fold-back with a 4-base loop is:



As a 4-base loop is unlikely to add to stability, even this structure can probably form only as a staggered duplex. For 40Cma the structure is:



Such structures would be predicted to be totally unstable in solution, but may be stabilized on the enzyme surface following the separate interaction of the two single strands with the enzyme. However, no stimulation of methylation is seen in mixtures of 22Cu and 22Cm, where a CG:MG dinucleotide pair can be generated in the same way as suggested for 40Cm. Such oligonucleotides may not be available in solution as a result of the formation of the more stable pentanucleotide pairs (seen in structures I and II, Table 3) that are able to saturate limiting amounts of enzyme. However, in the presence of excess enzyme, stimulation is seen with some mixtures of 22C-10 and 22Cm (Figure 3a), which are also capable of forming the inactive structures I, II and IV.

DISCUSSION

In 1986, Carotti et al. [9] reported findings with single-stranded polynucleotides containing a random base sequence and concluded that the human methyltransferase probably contains two DNA binding sites, each of which binds a recognition site on single-stranded DNA. On interaction with duplex DNA, the two strands are separated, with the target CG binding to the active site and the 'allosteric' site binding a methylcytosine that is normally paired with the target CG. It is known that the base opposite the target cytosine is unimportant [11], and it is also known that a region distinct from the active site is responsible for maintenance methylation in eukaryotic methyltransferases [13,14]. Carotti et al. [9] considered it unlikely that significant regions of fold-back DNA could be formed by their polynucleotides, but Christman et al. [10], in a paper published while the present work was in progress, claim that very short complementary regions may form duplexes transiently on the enzyme surface and that these regulate the methylation occurring at the replication fork.

Essentially, these three studies come to similar conclusions, i.e. that methylcytosines situated on the same single-stranded DNA molecule are able to stimulate methylation of nearby CG dinucleotides. However, our results give further support to the contention that the methylcytosine does not activate only when in *cis*, but is also able to activate methylation when it is present on a totally separate DNA molecule. In this case the two single-stranded DNA molecules presumably make contact with the same enzyme molecule, with the methylcytosine binding to the allosteric or regulatory site while the target CG dinucleotide binds to the active site. From a consideration of oligonucleotide 40Cma, one can conclude that the presence of a C:G or M:G pair adjacent to the target C is sufficient to activate methyl transfer. Although not essential, longer regions of complementarity may increase the amount of methyl transfer occurring by increasing the chance of transient duplex formation prior to, or following, interaction with the enzyme. However, where complementarity generates a stable unmethylated region of duplex DNA, this no longer provides a suitable substrate for the enzyme. This could be considered to be another regulatory mechanism whereby formation of some short duplexes may

sequester target cytosines so that they are no longer free to interact with the enzyme.

If short duplex regions (such as structures I-V; see the Results section and Table 3) do form in solution, the oligonucleotides might be expected to form a network of interacting molecules, with the 5' and 3' sequence blocks interacting with different oligonucleotides. Mixed solutions of oligonucleotides 22C-10 and 22Cu would be linked through structures I and III, and solutions of 22C-10 and 22Cm would contain all four possible structures. No evidence for stable interactions has been found, in that the oligonucleotides migrate as monomers on gel electrophoresis, confirming that, if anything, fold-back structures are more likely to form in solution.

We can imagine several situations in which methylation of single strands might apply *in vivo*. At the replication fork (and to a lesser extent during transcription) the DNA is rendered single-stranded. This is true particularly for the lagging strand, but it applies to both parental strands following helicase action. Were the methyltransferase to have access to the DNA at this time, then clusters of CG dinucleotides, of which one or more were initially methylated, might all become methylated by a looping-back process. This would have the effect of maintaining a high level of methylation, particularly where target sites are clustered, as in CG islands. However, if one CG failed to get methylated in one generation, this would be of little consequence, as the defect would be reversed in succeeding generations. This would reduce the chance of island demethylation with the consequent activation of unneeded genes. This mechanism would also lead to the rapid spreading of methylation over regions of repeated DNA such as the CCG repeat present at the fragile X site [15].

The second situation also arises at the replication fork, where an Okazaki piece may transiently align itself with the identical parental sequence on the other branch of the fork and, thereby, inherit its methylation pattern. This would be particularly applicable to palindromic sequences and could explain how patterns of hemimethylation could be maintained, as has been observed in several situations [5]. Another way in which patterns of hemimethylation might be conserved is by generating a duplex patch on the unmethylated strand by annealing it to a short unmethylated oligonucleotide at the time of maximum exposure to DNA methyltransferase.

A third scenario could result in the transfer of a pattern of methylation from one copy of a repeated sequence to all other copies, and may play a part in repeat induced methylation observed in plants and lower eukaryotes [16,17], or even in the RNA-induced methylation seen on viroid infection of plants [18].

Rather than trying to find applications for our results with these models, we may be able to learn more about how the methyltransferase functions. Given a hemimethylated duplex, the enzyme is able to flip out the unmethylated cytosine [19]. How does the mammalian enzyme recognize which is the unmethylated cytosine, and why does it not flip out the methylcytosine? How does it recognize a hemimethylated site? Either the enzyme works at random, which seems unlikely, or it is able to recognize a methylCG and flip out the complementary cytosine. Indeed, Smith et al. [11] have shown that the G opposite the target cytosine is unnecessary for enzymic action. Unlike the prokaryotic enzymes, the mammalian enzymes must be able to recognize the methylcytosine in a hemimethylated CG pair and react with it. Our results would suggest that this is possible even if this methylcytosine is on a different DNA molecule or on an adjacent region of the same strand. We would suggest that this might be the normal situation and that the mammalian enzyme has two sites: one for a methylcytosine (not necessarily in a CG dinucleotide) and one for the target cytosine

that is normally in a CG dinucleotide. The first step for the enzyme is to find a methylcytosine and bind firmly to this stretch of DNA; this presumably does not involve a flip-out mechanism, the methyl group being accessible in the major groove or in single-stranded DNA. The second step is to find the neighbouring CG dinucleotide and methylate the cytosine. The ideal situation is when the nearest CG is complementary to the methylated cytosine that has already been bound (i.e. the site is hemimethylated); under these conditions the enzyme is able to flip out the target cytosine into the active site. If the only CG is further along the same strand then physical constraints will dictate its suitability as an acceptor. If this CG is present in duplex DNA it will be inaccessible to the enzyme (i.e. spreading of methylation cannot occur along duplex DNA and is probably restricted to S-phase, when the DNA is rendered single-stranded). When dealing with relatively high concentrations of short oligonucleotides (as is the *in vitro* situation), there is clearly no constraint that the methylated cytosine and the target CG are on the same oligonucleotide, although we feel that this may apply *in vivo* only to repeated DNA sequences.

When enzyme is limiting, duplex hemimethylated DNA is able to sequester the enzyme so that none (or less) remains to methylate a single-stranded acceptor such as 22C-10. The longer the region of duplex DNA around the target cytosine, the greater the chance of both sites on the enzyme being occupied by the two complementary strands. This would explain how the enzyme binds most avidly to hemimethylated duplex and less avidly to fully methylated or unmethylated DNA. Similar conclusions have been arrived at using gel retardation studies and from kinetic studies [12]. These results show that, although the enzyme can interact with a methylcytosine in single-stranded DNA, it preferentially interacts with methylcytosine in duplex DNA.

Such a complex would be only transient unless an appropriate target cytosine was close at hand.

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