Solid phase synthesis and restriction endonuclease cleavage of oligodeoxynucleotides containing 5-(hydroxymethyl)-cytosine

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ABSTRACT
Emerging data suggest an important role for cytosine methylation in tumorigenesis. Simultaneously, recent studies indicate a significant contribution of endogenous oxidative DNA damage to the development of human disease. Oxidation of the 5-methyl group of 5-methylcytosine (5mC) residues in DNA results in the formation of 5-(hydroxymethyl)cytosine (hmC). The biological consequences of hmC residues in vertebrate DNA are as yet unknown; however, conversion of the hydrophobic methyl group to the hydrophilic hydroxymethyl group may substantially alter the interaction of sequence-specific binding proteins with DNA. Central to both biophysical and biochemical studies on the potential consequences of specific DNA damage products such as hmC are efficient methods for the synthesis of oligodeoxynucleotides containing such modified bases at selected positions. In this paper, we describe a method for the placement of hmC residues in oligodeoxynucleotides using established phosphoramidite chemistry. In addition, we have examined the influence of specific hmC residues on enzymatic cleavage of oligodeoxynucleotides by the methylation-sensitive restriction endonucleases Mspl and HpaII.

INTRODUCTION
5-(Hydroxymethyl)cytosine (hmC, Fig. 1) is a modified pyrimidine found in Escherichia coli phage T4 DNA (1). In vertebrates (2), the only naturally occurring modified base is 5-methylcytosine (5mC). However, hmC can be formed in vertebrate DNA by in situ oxidation of the methyl group of 5mC. In solution, it has been demonstrated that 5mC can be converted to hmC by reaction with hydroxyl radicals (3), gamma (4) and UV irradiation (5). In calf thymus DNA, hmC residues have been identified by post-labelling methods (6), and a specific glycosylase activity which excises hmC residues has been identified in extracts of calf thymus (7).

The role of 5mC in higher organisms is as yet unknown; however, it is believed that DNA methylation may play a key role in gene control and cellular differentiation (8). More recently, attention has focused on alterations in the distribution of 5mC residues and tumorigenesis (9,10). The most studied reaction of 5mC is deamination to thymine. It is estimated that approximately eight 5mC residues deaminate to thymine per human cell per day (9).

The rate of oxidation of the methyl group of 5mC has not yet been measured, but may be estimated based upon comparison with the rate of oxidation of the thymine methyl group. Oxidation of thymine residues in DNA to the corresponding 5-(hydroxymethyl)uracil (hmU) residues is considered to be an important source of endogenous oxidative DNA damage (11,12) and it has been estimated that ~620 thymine residues are oxidized to hmU per cell per day (13). In solution, 5mC is slightly more reactive toward hydroxyl radical attack than is thymine (14). In human cells, the ratio of thymine to 5mC residues is ~30 (2), and we would therefore predict that ~20 5mC residues would be oxidized to hmC per cell per day. As the estimated rates of 5mC deamination and oxidation are of the same order of magnitude, it is likely that formation of hmC residues in vertebrate DNA is a biologically important reaction.

In order to perform biochemical and biophysical studies on the consequences of hmC formation in DNA, it is necessary to have a method for the synthesis of defined-sequence oligodeoxynucleotides containing hmC residues at selected sites. Whereas placement of 5mC residues at selected sites requires the same protection strategy as unmodified cytosine, introduction of the

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5-hydroxymethyl group of hmpC presents a significant synthetic challenge as this group must be selectively protected and chemically distinguished from the 5'-hydroxyl group. In this paper, we present a synthetic method for the placement of hmpC residues at selected sites using standard phosphoramidite chemistry. The base composition of oligodeoxynucleotides synthesized with this method is confirmed by both enzymatic hydrolysis and high performance liquid chromatography (HPLC) analysis as well as acid hydrolysis and gas chromatography/mass spectrometry (GC/MS) analysis. We also examine the influence of 5°C and hmpC residues on the enzymatic cleavage of synthetic oligonucleotides containing these modified bases with the methylation-sensitive restriction nucleases MspI and HpaII.

MATERIALS AND METHODS

Materials

5-Hydroxymethyl-2'-deoxuryridine (hmpdU) was prepared by the method of Shiao et al. (15). 2-Cyanoethanol alcohol, trifluoroacetic acid, acetic anhydride, pyridine, 1,2,4-triazole, triethylamine, 1,4-dioxane, trimethylsilyl chloride, benzoyl chloride, sodium sulfate, DMAP (4-dimethylaminopyridine), 4,4'-dimethoxytrityl chloride, 2-cyanoethyl-N,N-diisopropylphosphoramidite, methane, dichloromethane, tolune and silica gel were obtained from Aldrich. Silica gel H was obtained from Fluka and DNA grade G25 resin was obtained from Pharmacia. Ammonium hydroxide (29.8%) was obtained from Mallinckrodt. Normal grade G25 resin was obtained from the United States Biochemical Corporation and 32P-ATP was obtained from ICN.

Results

The product was then isolated by silica gel chromatography. Obtained, 3.05 g (76% yield); 1H NMR (300 MHz, d6 DMSO) δ, 7.85 (s, H6), 7.44 (s, NH), 6.68 (s, NH) 6.15 (t, H1', J=6.0 Hz), 5.22 (d, 3'OH), 5.02 (t, 5'OH), 4.23 (m, H3', 5'-CH2O-), 3.78 (m, H4'), 3.58 (m, H5', H5''', -CH2CH2CN), 2.78 (m, -CH2CH2CN, J=6.3 Hz), 2.12 (m, H2',H2''). (Found: C, 50.06; H, 5.66; N, 13.35; O, 31.27; Calc. for C13H17N2O6C; C, 50.16; H, 5.51; N, 13.49; O, 30.84%)

5-(2-cyanoethoxy)methyl-2'-deoxycytidine (3). 5-(2-Cyanoethyl-oxymethyl)-2'-deoxuryridine (2) was converted to 5-(2-cyanoethoxy)methyl-2'-deoxycytidine (3)' by the method of Divakar and Reese (16). 5-(2-Cyanoethoxy)methyl-2'-deoxuryridine (400 mg, 1.3 mmol) was dried by coevaporation of dry pyridine. Pyridine (50 ml) was then added, followed by dropwise addition of acetic anhydride (1.23 ml, 13 mmol). The solution was stirred at room temperature for 3 h. Anhydrous methanol (5 ml) was added and the mixture was stirred for an additional 5 min. Solvents were removed under reduced pressure and the product was dried to a foam by coevaporation of toluene. The acetylated derivative was used directly for the next conversion.

A separate mixture containing 1,2,4-triazole (0.81 g, 11.7 mmol) and acetonitrile (20 ml) was stirred at 0°C, and treated by dropwise addition of phosphorus oxychloride (242 µl, 2.6 mmol), followed by triethylamine (1.6 ml, 11.2 mmol). The dry acetylated nucleoside was dissolved in a minimal volume of acetonitrile and added to the triazole mixture. Stirring was continued for 1 h. Solvents were removed under reduced pressure and the residue was dissolved in dichloromethane and washed with brine and distilled water. The organic phase was dried with sodium sulfate and filtered. Solvents were removed under reduced pressure and the product was dissolved in 1,4-dioxane (10 ml) and treated with 29% aqueous ammonia (2 ml). The mixture was stirred at room temperature for 2 h. Solvents were removed under reduced pressure and the product was isolated by silica gel chromatography. Obtained, 305 mg (76% yield); 1H NMR (300 MHz, d6 DMSO) δ, 7.85 (s, H6), 7.44 (s, NH), 6.68 (s, NH) 6.15 (t, H1', J=6.0 Hz), 5.22 (d, 3'OH), 5.02 (t, 5'OH), 4.23 (m, H3', 5'-CH2O-), 3.78 (m, H4'), 3.58 (m, H5', H5''', -CH2CH2CN), 2.78 (m, -CH2CH2CN, J=6.3 Hz), 2.03 (m, H2', H2'').

N°-benzoyl-5-(2-cyanoethoxy)-methyl-5'-O-(4'-4'-dimethoxytrityl)-2'-deoxycytidine-3'-O(2-cyanoethyl)-N,N-diisopropylphosphoramidite (4). 5-(2-Cyanoethoxy)methyl-2'-deoxycytidine (3) was converted to N°-benzoyl-5-(2-cyanoethoxy)methyl-2'-deoxycytidine by the method of Ti et al. (17). 5-(2-cyanoethoxy)methyl-2'-deoxycytidine (1 g, 3.23 mmol) was dried by coevaporation of dry pyridine. Pyridine (16 ml) was added, followed by trimethylsilyl chloride (2.06 ml, 16.2 mmol). After 15 min at room temperature, the stirred solution was treated with benzoyl chloride (1.9 ml, 16.5 mmol). The solution was continuously stirred for an additional 3 h. The mixture was then cooled to 0°C and water (3 ml) was added. After 5 min, 29% aqueous ammonia (4 ml) was added and the mixture was stirred at room temperature for 15 min. Solvents were removed under reduced pressure and the residue was dissolved in dichloromethane and washed with saturated aqueous sodium bicarbonate and brine. The organic phase was dried with sodium sulfate and filtered. Solvents were removed under reduced pressure and the product was isolated by silica gel chromatography. Obtained, 0.95 g (71% yield); 1H NMR (300 MHz, d6 DMSO) δ, 8.20–8.25, 7.52–7.65 (m, aromatic), 7.90 (s, H6), 6.18 (t, H1', J=6.0 Hz), 5.32 (d, 3'OH), 5.12 (t, 5'OH), 4.47 (m, H3'), 4.29 (m, 5'-CH2O-), 3.87 (m, H4'), 3.55–3.70 (m, H5', H5''', -CH2CH2CN), 2.79 (t, -CH2CH2CN), 2.25 (m, H2', H2'').

N°-benzoyl-5-(2-cyanoethoxy)methyl-2'-deoxycytidine was converted to the protected 5'-dimethoxytrityl-3'-phosphoramidite by standard methods (18). Dimethoxytrityl was added to the 5'.
5-(Hydroxymethyl)-2′-deoxyctydine (5). In order to generate an authentic marker of hm dC, 5-(2-cyanoethoxy)methyl-2′-deoxyctydine (3) was treated with aqueous ammonia at 60°C overnight. Quantitative conversion to hm dC was observed by TLC. 1H NMR (300 MHz, d6 DMSO) δ, 7.75 (s, H6), 7.34 (s, NH), 6.59 (s, NH) 6.17 (t, H1′, J=6.0 Hz), 5.20 (d, 3′OH), 5.00 (m, 5′OH, CH2OH), 4.18 (m, H3′, 5′-CH2OH), 3.77 (m, H4′) 3.56 (m, H5′, H5′′), 1.99 (m, H2′, H2′′).

### Oligonucleotide synthesis and purification

The hm C-phosphoramidite (4) was dissolved in dry acetonitrile and placed in one of the additional ports of the DNA synthesizer. The standard 1.3 μmol synthesis cycle with retention of the 5′ terminal dimethoxytrityl group was used without modification. Following synthesis, the solid support was suspended in concentrated aqueous ammonia in a sealed vial and heated at 65°C for 60 h. The trityl-containing oligonucleotide was purified by HPLC on a Hamilton PRP semi-preparative column using 0.1 M triethyl ammonium acetate and an acetonitrile gradient. The purified trityl containing oligonucleotide was detritylated in 80% acetic acid for 10 min, 11.0 x 10^3, 265 nm; dA, 13.8 min, 12.0 x 10^3, 259 nm. All bases were identified by their characteristic retention times and mass spectra. Observed retention times (min) and most abundant ion (m/z) for the silylated derivatives are thymine, 9.4 min, m/z 255; cytosine, 10.9 min, m/z 254; hmC 11.3 min, m/z 254; hmC 13.7 min, m/z 357; adenine 14.9 min, m/z 264; and guanine, 17.6 min, m/z 352. Solutions of known concentration of the free bases were used to generate standard curves relating base concentration to integrated chromatogram peak areas at appropriate mass ion currents. Based upon these standard curves, we were able to verify that all bases were present in synthetic oligonucleotides in correct ratios.

### Restriction enzyme cleavage of the oligonucleotide

Portions of the oligonucleotides were 32P-end labelled and purified by G25-spin columns. Duplexes were prepared by combining equimolar portions of the upper and lower oligonucleotide strands in restriction nuclease buffer, heating for 5 min at 90°C followed by slow cooling. Restriction enzyme (20 U) was added and cleavage reactions were allowed to proceed for 15 to 30 min at 37°C. Oligonucleotide duplexes were precipitated with ethanol, resuspended in the gel loading buffer and analyzed by gel electrophoresis on a 20% polyacrylamide denaturing gel. Gels containing labelled oligonucleotides were visualized with a PhosphorImager.

### Analysis of base composition

The purified oligonucleotides were enzymatically digested using nuclease P1 and bacterial alkaline phosphatase (19). The liberated deoxynucleosides were separated by HPLC using a reverse phase column and a gradient of 0.05 M sodium phosphate, pH 4.0 with increasing methanol (0 to 20%). Detection was provided by a photodiode array detector. The deoxynucleosides were chromatographically separable and identifiable based upon their characteristic UV spectra. Retention times, molar absorptivity (pH 4.0, 265 nm, M^-1 cm^-1) and absorbance maxima (nm) for the deoxynucleosides are hm dC, 2.8 min, 11.0 x 10^3, 274 nm; dG, 7.6 min, 12.3 x 10^3, 252 nm; dT, 8.7 min, 9.5 x 10^3, 265 nm; dA, 11.8 min, 13.0 x 10^3, 259 nm. All deoxynucleosides were present in the correct ratios based upon integration of the HPLC chromatogram at 265 nm.

The oligo was also acid hydrolyzed to liberate the constituent free bases. The bases were silylated and analyzed by GC/MS as previously described (20). Bases were identified by their characteristic retention times and mass spectra. Observed retention times (min) and most abundant ion (m/z) for the silylated derivatives are thymine, 9.4 min, m/z 255; cytosine, 10.9 min, m/z 254; hmC 11.3 min, m/z 254; hmC 13.7 min, m/z 357; adenine 14.9 min, m/z 264; and guanine, 17.6 min, m/z 352. Solutions of known concentration of the free bases were used to generate standard curves relating base concentration to integrated chromatogram peak areas at appropriate mass ion currents. Based upon these standard curves, we were able to verify that all bases were present in synthetic oligonucleotides in correct ratios.

### RESULTS

Previously, we presented a strategy for the selective protection of the 5-hydroxymethyl group of hm dU and placement of hm U residues in synthetic oligonucleotides (21). It was envisaged that hmU residues could be incorporated conveniently by formation of the 4-triazole derivative of the hmU-phosphoramidite as described for other 4-substituted pyrimidines (22). In our hands, however, conversion of the hmU-phosphoramidite to the corresponding triazole derivative did not go to completion, perhaps due to a steric problem introduced by the acetylated hydroxymethyl group in the adjacent 5-position. Attempts to separate the triazole
Figure 2. Synthetic scheme for the preparation of the \(^{13}C\) phosphoramidite. (a) cyanoethyl alcohol/trifluoroacetic acid; (b) acetic anhydride/pyridine; (c) triazole/phosphoryl chloride/triethylamine/acetonitrile; (d) dioxane/aqueous ammonia; (e) trimethylsilyl chloride/pyridine; (f) benzoyl chloride/pyridine/ammonia; (g) 4,4'-dimethoxytrityl chloride/DMAP/pyridine; (h) cyanoethyl-N,N-diisopropylchlorophosphoramidite/diisopropylamine/acetonitrile; (i) concentrated aqueous ammonia/60°C.

phosphoramidite by silica gel chromatography resulted in substantial generation of starting material. As a second strategy, we attempted to selectively acetylate the 5-hydroxymethyl group of 5-hydroxymethyl-2'-deoxycytidine (hm dC) as described previously for hm dU (21); however, we observed quantitative cleavage of the glycosidic bond.

As an alternative strategy, we exploited the known reactivity of the 5-hydroxymethyl group of hm dU to condense with alcohols as well as carboxylic acids (21,23,24). hm dU was reacted with cyanoethanol in the presence of a catalytic amount of trifluoroacetic acid to form the 5-cyanoethyl ether in high yield. The cyanoethyl derivative of hm dU quantitatively regenerates hm dU by \(\beta\)-elimination when treated overnight with aqueous ammonia at 60°C. The cyanoethyl group is routinely used as a phosphate protecting group for oligonucleotide synthesis (18) and Christopherson and Broom (25) have used the cyanoethyl group for base protection in the synthesis of 2’-deoxy-6-thioguanosine-containing oligonucleotides.

The 5-protected hm dU derivative (Fig. 2, 2) formed by reaction of hm dU with cyanoethanol was then converted to the corresponding deoxyctydine derivative by the method reported by Divakar and Reese (16). Fortunately, the 4-triazole group is displaced by ammonia at room temperature whereas the cyanoethyl protecting group remains intact. The 5-cyanoethyl protected hm dC derivative (Fig. 2, 3) was then benzylated, tritylated and phosphitylated using established methods (17,18). The hm dC-phosphoramidite (4) prepared by this method was incorporated into synthetic oligonucleotides with efficiency indistinguishable from normal bases. Because hm dC and dC are difficult to separate by HPLC, we first prepared a 12-base test sequence containing hm dC, dT, dG and dA: 5’ (T G \(^{13}C\) A) 3.

Figure 3. HPLC chromatogram of the deoxynucleosides obtained following enzymatic digestion of an oligonucleotide containing dT, dG, dA and hm dC. The small peak at 9.6 min is protected hm dC.

The hm dC-containing oligonucleotide was deblocked by treatment in aqueous ammonia at 60°C for 60 h and purified by HPLC. Although 12 h is sufficient to remove the cyanoethyl protecting group from the monomer, prolonged treatment is required to remove the protecting group from the protected residue in the oligonucleotide. A portion of the hm dC-containing oligonucleotide was enzymatically digested with nuclease P1 and bacterial alkaline phosphatase. The liberated deoxynucleosides were then analyzed by HPLC using a reverse phase column.

The chromatogram shown in Figure 3 shows four predominant peaks corresponding to the three normal deoxynucleosides and hm dC as well as a minor peak eluting at 9.6 min. The hm dC peak was identified by co-chromatography with an authentic standard. The UV spectrum of the hm dC peak, obtained with the diode array detector (Fig. 4, top), shows absorbance maxima and minima of 273 nm and 251 nm respectively, consistent with reported values (26).

The hm dC obtained by HPLC chromatography was dried, silylated and analyzed by GC/MS. The mass spectrum of the silylated \(^{13}C\) peak at 13.7 min is shown in Figure 4 (lower). The most abundant ion is the parent ion at m/z 357. Additional ions resulting from fragmentation of the 5-substituent are identified in Figure 4 (lower).

The minor peak in the HPLC chromatogram (Fig. 3) eluting at 9.6 min is the cyanoethyl-protected hm dC derivative (Fig. 2, 3). Based upon integration of the chromatogram peaks, deprotection of the \(^{13}C\) residue was ~96% complete. No peak corresponding to \(^{13}C\) dU, which could result from hydrolytic deamination of hm dC, was observed. Treatment of the oligonucleotide with sodium methoxide overnight at room temperature eliminated residual traces of the cyanoethyl-protected hm dC.

Having established the synthetic method for placement of hm dC residues, we prepared a series of longer oligonucleotides containing cytosine, 5°C or hm dC at selected sites (Table 1). The sequence of the oligonucleotide contains the recognition site for
the MspI/HpaII restriction endonucleases. Following deprotection and purification, the composition of the oligonucleotides was examined by GC/MS following acid hydrolysis in 60% formic acid. Data was collected in the selected ion mode using the most abundant ions of each of the bases in the oligonucleotides. The results are shown in Figure 5.

An oligonucleotide corresponding to the upper strand (Materials and Methods), containing only A, T, G and C generated four chromatographic peaks identified by their characteristic retention times and mass spectra (Fig. 5, upper). An oligonucleotide of the same sequence except for the replacement of the inner cytosine of the MspI/HpaII cut site with 5mC (Table 1, Duplex 2) was prepared and analyzed in an identical fashion. The silylated 5mC peak is observed at 11.3 min (Fig. 5, center). An additional oligonucleotide was then generated in which the same inner cytosine residue was replaced by hmC. The hmC peak is observed at 13.7 min (Fig. 5, lower). As reported previously for the analysis of hmU in oligonucleotides (11,12), we also observe that acid hydrolysis results in considerable destruction of hmC as indicated by the reduced intensity of the hmC peak in Figure 5 (lower). The size of the observed hmC chromatographic peak is consistent with the expected amount of hmC in the oligonucleotide based upon results obtained following acid hydrolysis of standards. The selected ion chromatogram corresponding to the most abundant ion of the silylated hmC derivative (m/z 357) is shown in the inset of Figure 5 (lower).

The oligonucleotides were combined with cytosine-containing complementary sequences to generate a series of duplexes with asymmetrically modified cytosine residues at either the inner or outer cytosine residue of the C/CGG recognition sequence. Complementary strands were also synthesized with 5mC to generate symmetrically modified recognition sites (Table 1). Oligonucleotide duplexes, 32P-labelled in both strands, were incubated with either MspI or HpaII for 15 and 30 min respectively. The resulting oligonucleotide fragments were then separated by electrophoresis on a 20% polyacrylamide denaturing gel and analyzed by a PhosphorImager. The results obtained are shown in Figure 6.

Previously it had been reported that hmC-containing bacteriophage DNA is not cleaved by either MspI or HpaII (27). In the bacteriophage DNA, however, both cytosine residues of the recognition site, in both strands are replaced by hmC. Only by chemical synthesis is it possible to selectively modify one of the cytosine residues. We observe that the duplex containing hmC at

Figure 4. Characterization of hmC from the synthetic oligodeoxynucleotide. (Top) UV spectrum of the hmC peak obtained with the diode array detector. (Bottom) Mass spectrum of the silylated hmC peak derived from the HPLC chromatogram shown in Figure 3.

Figure 5. Sum of the selected ion chromatograms (parent ions) of oligodeoxynucleotide hydroxylates containing (top) all normal bases, (center) an oligonucleotide in which one cytosine has been replaced by 5mC, and (lower) an oligonucleotide in which one cytosine has been replaced by hmC. The base sequence corresponds to the upper strand (Materials and Methods).

Figure 6. Cleavage of synthetic oligonucleotides by restriction endonucleases: (Top) HpaII, (Bottom) MspI. Lane markers 1 through 9 correspond to the duplexes shown in Table 1.
the inner position (Table 1, Duplex 3; Fig. 6, lane 3) is cleaved by MspI, however, if the $^{5}$mC residue is placed at the outer cytosine position (Table 1, Duplex 5; Fig. 6, lane 5), the modified strand is protected from MspI cleavage whereas the unmodified strand is cleaved. The same pattern is observed when the $^{5}$mC residue is replaced by $^{5}$tC. The only difference observed between $^{5}$mC and $^{5}$tC is that $^{5}$tC at the outer position (Table 1, Duplex 4; Fig. 6, lane 4) completely protects the modified strand from MspI cleavage whereas the $^{5}$mC-containing strand (lane 5) is cleaved to a small extent. Placement of either $^{5}$tC or $^{5}$mC at either position completely protects the modified strand from cleavage by HpaII and the cleavage efficiency of the unmodified complementary strand is reduced. Previously, it has been reported that the placement of $^{5}$tC within the MspI/HpaII recognition site selectively protects only the modified strand of the duplex from cleavage (28). The similarity in results obtained with $^{5}$tC and $^{5}$mC-containing oligonucleotides with these restriction endonucleases provides additional support of the validity of the method reported here for placement of $^{5}$mC residues in oligodeoxynucleotides.

**DISCUSSION**

We report here a method for the synthesis of oligodeoxynucleotides containing $^{5}$mC residues at selected sites. This strategy is based upon the selective reactivity of the 3-hydroxymethyl group of $^{5}$mC with alcohols under acidic conditions. The cyanoethyl ether protecting group generated is stable toward ammonia at room temperature, allowing conversion from the protected uracil derivative to the protected cytosine derivative. At elevated temperatures in aqueous ammonia, the cyanoethyl ether is cleaved by $\beta$-elimination. Prolonged ammonolysis is required to remove the protecting group from the oligonucleotide which would prevent simultaneous use of the protected $^{5}$mC derivative reported here with alkaline-labile modified bases.

The base composition of the oligonucleotides prepared by this method has been verified by both enzymatic digestion and HPLC analysis as well as acid hydrolysis and GC/MS. The validity of this synthetic method is further supported by studies with methylation-sensitive restriction nucleases in which it is demonstrated that the placement of $^{5}$mC and $^{5}$tC similarly protect oligodeoxynucleotides from cleavage.

The mechanism by which cytosine methylation influences gene control is as yet unknown, however, it is suspected that such an effect may be modulated through sequence specific DNA–protein interactions sensitive to the presence of the methyl group. Oxidation of $^{5}$tC to $^{5}$mC could impact DNA–protein interactions because replacement of the hydrophobic methyl group by the hydrophilic hydroxymethyl group may prevent or alter the binding of proteins having hydrophobic binding clefts for the $^{5}$tC methyl group. Recently, we demonstrated that replacement of T by $^{5}$mC residues increases the dissociation rate of restriction nucleases from the cleaved substrates (29).

A mechanism by which $^{5}$tC oxidation could have a heritable effect upon methylation patterns is by blocking methyl-directed methylation following DNA replication. It is known that hemimethylated DNA, generated during DNA replication, is a substrate for cytosine methyltransferase and that the cytosine 5-methyl group in the parental strand directs the enzymatic methylation of the complementary progeny strand. Smith and coworkers have previously shown that the ability of cytosine 5-substituents to direct methylation of the complementary strand is a function of the size of the substituent (30). A sharp maximum was seen at the size of the methyl group. Oxidation from methyl to hydroxymethyl not only increases the size of the substituent significantly, but the hydrophilic hydroxyl group is likely well hydrated and may not be accommodated within a binding cleft designed to hold a hydrophobic methyl group. If unrepaird, oxidation of the 5-methyl group could then prevent maintenance methylation. The influence of $^{5}$mC on the methylation is as yet unknown, however, such studies are currently in progress.

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