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SIZE OF THE DIRECTING MOIETY AT CARBON 5 OF CYTOSINE AND THE ACTIVITY OF HUMAN DNA(CYTOSINE-5)METHYLTRANSFERASE

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M13 DNAs in which carbon 5 of each deoxycytidine residue in one strand is replaced with a bulky group are very good substrates for human DNA(cytosine-5)methyltransferase. Rate enhancements of up to 35 fold are obtained depending on the size of the moiety at C-5. The enzyme appears optimally suited to sense a methyl group in one strand at this position. Alkaline density gradient analyses of the distribution of methyl groups applied to 5-BrdCyd or 5-IdCyd substituted DNA reveal that these groups serve to direct the enzyme to methylate the unsubstitued strand.

Experimental evidence for the existence of a mammalian DNA (cytosine-5)methyltransferase (E.C. 2.1.1.37) activity with a strong preference for hemimethylated d(pCG) sites was first provided by Gruenbaum et al. (1). Enzymes with this specificity may play an essential role in the somatic inheritance of cytosine methylation patterns (2,3). Most cytosine methylation occurs at the d(pCG) dimer in eukaryotes (4), and a small subset of these sites appear to have the capacity to prevent gene expression (5-7). In light of the demonstration (8,9) of the faithful somatic inheritance of modification at d(pCG) dimers, methylation of certain of these sites may have the capacity to clonally silence genes.

Highly purified mammalian DNA methyltransferase preparations retain a strong preference for hemimethylated d(pCG) dimers (10,11). Here we report that the capacity of the preparation from human placenta to methylate DNA depends on the size of the moiety at C-5 of cytosine directing the methylation.

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MATERIALS AND METHODS: [^{3}H]AdoMet was from Research Products International with a specific activity of 22 Ci/mmole. Deoxynucleotide triphosphates were from P-L Biochemicals. Dithiothreitol, and 2-mercaptoethanol, were from Sigma Chemical Co. Hpa II restriction endonuclease was from New England Biolabs. DNA Polymerase I (Klenow fragment) and Msp I restriction endonuclease were from Boehringer Mannheim.

5-FdCyd was prepared by amination of 5-FdUrd (Roche Laboratories) using the method of Sowers et al.(in preparation). 5-FdCyd was converted to the triphosphate essentially as described in (12). The product was purified using DEAE-cellulose. The identity of the purified 5-FdCTP was confirmed by negative ion mass spectrometry (mass/charge $C_9H_{14}O_{13}P_3F^-$: observed 483.98; calculated 483.97).

For the synthesis of uniformly substituted heteroduplex DNAs, 5-MedCTP, 5-BrdCTP, 5-IdCTP,and 5-FdCTP were used in place of dCTP during primer extension as described in reference (13). DNA methyltransferase (Fraction VI enzyme) was prepared as previously described (11) except that DEAE-cellulose was used to remove nucleic acid from hydroxyapatite concentrated material prior to Dodecyl-Sepharose chromatography.

A unit of activity is that amount of enzyme that will catalyze the incorporation of 1 pmole of methyl groups into trichloroacetic acid insoluble material in one hour in a reaction mixture containing 50 mM Tris-Cl, pH 7.8; 50mM NaCl; 10% vol/vol glycerol; 150 ug/ml heat denatured M. lysodeikticus DNA; 1.4 uM [^{3}H]AdoMet; 10mM EDTA; 10 ug/ml RNAse; and 2mM dithiothreitol at 37^o (11). Fraction IV had a specific activity of 12,000 units/mg protein (U/mg).

DNA methyltransferase was present at a final concentration of about 250 U/ml unless otherwise stated. The reaction rate with M13 DNA was found to be linear for about 8 minutes. Initial velocities were estimated from a single time point taken 5 minutes after initiating the reaction with enzyme. For alkaline density gradients, BrdCyd-containing DNA (12 ug) was labelled with [^{3}H]methyl groups by incubation with 370 U/ml methyltransferase in a 300 ul reaction volume for 12 min. After ethanol precipitation, the DNA was denatured and separated by alkaline density gradient centrifugation (14). Fractions (300 ul) were collected from the bottom of the tube. DNA concentrations were estimated from the absorbance at 260 nm. Tritium incorporation was measured in trichloracetic acid precipitable material as described for the enzyme assay (11).

RESULTS: The synthetic DNAs showed relative mobilities near that of the open circular form of the biologically produced double stranded M13 DNAs (see Fig. 1). The synthetic products were digested with Hpa II, Msp I, and Hinf I. Typical results are given in Fig. 1. Msp I which is sensitive to methylation of cytosine at the outside position in its d(pCCGG) recognition site was able to cleave dCyd, and 5-FdCyd containing DNAs, but was unable to cleave 5-BrdCyd, 5-IdCyd or 5-MedCyd containing DNAs. Identical results were obtained with Hpa II which recognizes the same sequence but is sensitive to methylation of the internal cytosine in the sequence. Hinf I was able to cleave the synthetic products, although the DNAs containing 5-substi-

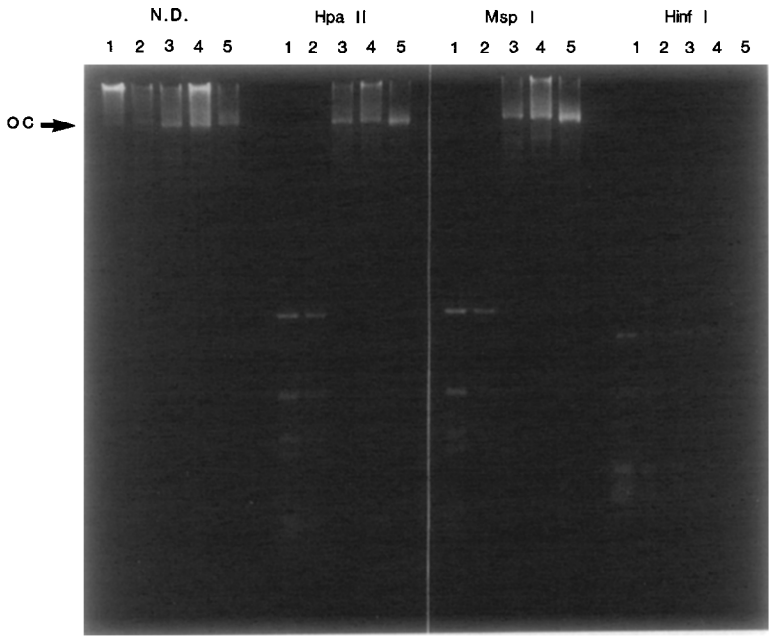
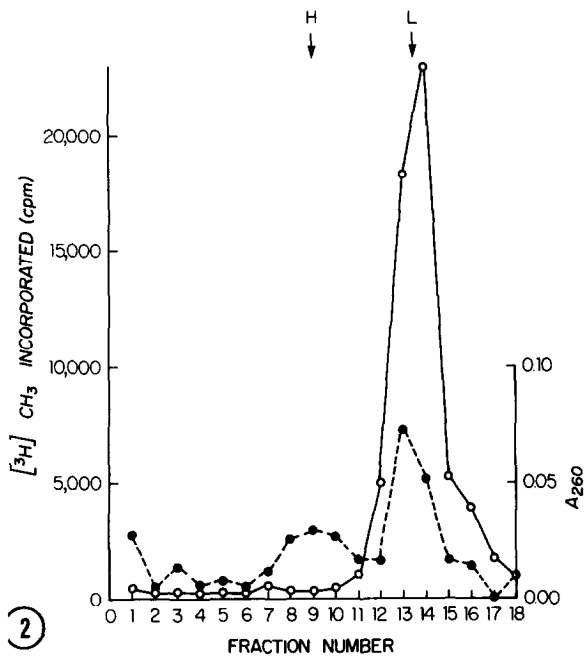


Fig. 1. Restriction Analysis of M13 DNAs
Synthetic M13 DNAs were subjected to digestion with Hpa II (27 U/ug), Msp I (27 U/ug), and Hinf I (45 U/ug for all but 5-IdCyd containing DNAs where 60 U/ug was used). DNAs (0.5 ug) which were not digested (N.D.) were separated by electrophoresis through 1.5% agarose gels along with the digested DNAs. OC: position of open circular form of the M13 duplex. For each series, Lane 1: dCyd substituted DNA; Lane 2: 5-FdCyd substituted DNA; Lane 3: 5-BrdCyd-substituted DNA; Lane 4: 5-MedCyd-substituted DNA; Lane 5: 5-IdCyd-substituted DNA.

tuted cytosines were poorer substrates than DNA containing unmodified cytosine.

Saturation curves for 5-FdCyd and dCyd substituted DNAs were hyperbolic. Those for the 5-BrCyd, 5-MedCyd, and 5-IdCyd substituted substrates showed a marked substrate inhibition above about 3 ug/ml (not shown). Purification of the synthetic DNAs by gel filtration to remove small molecules, or phenol: chloroform extraction to remove proteins did not alter the ability of the substrate DNAs to inhibit the enzyme.

Density gradient analysis of 5-BrdCyd substituted M13 heteroduplex DNA that had been methylated *in vitro* by DNA methyltransferase (Fig. 2) revealed that the enzyme applied methyl groups almost exclusively to the light strand. The ratio of [^{3}H]methyl observed in the plus strand to that in the minus strand was about 380:1. Similar results were obtained with 5-IdCyd substituted DNA (not shown).



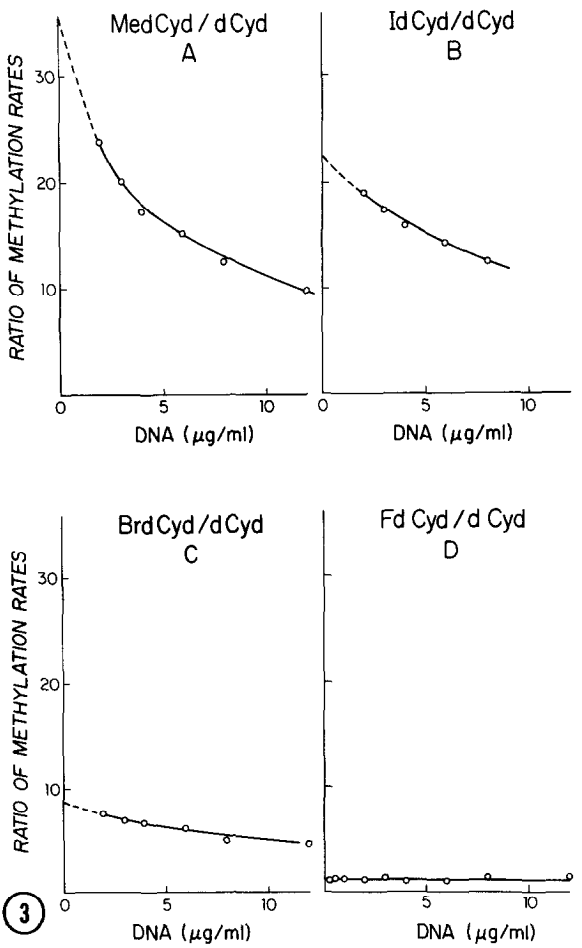


Fig. 2. Alkaline Gradient Analysis of in vitro Methylated DNA Approximately 6 ug (75,000cpm) 5-BrdCyd-containing heteroduplex DNA was applied to the gradient after it had been tritium-labelled by the methyltransferase. O---O ^{3}H, ●---● A_{260}. L:position of light DNA. H:position of heavy DNA.

Fig. 3. The Effect of DNA Concentration on Enzyme Selectivity The ratio of the activities is plotted as a function of DNA concentration. A smooth extrapolation to zero DNA concentration yields an estimate of the maximal selectivity ratio.

One measure of enzyme selectivity in comparing two substrates is the ratio of the reaction rates (1,10,11). A comparison of this type is made in the experiments depicted in Fig. 3. Since this ratio is a smooth function of DNA concentration, the data permit an extrapolation to zero DNA where substrate inhibition is expected to be minimal for each type of molecule. A plot of the ratios obtained by extrapolation as a function of the van der Waals radius for the substituent moieties (15) is given in Fig. 4. This measure of selectivity reaches a sharp maximum at the radius of the methyl group.

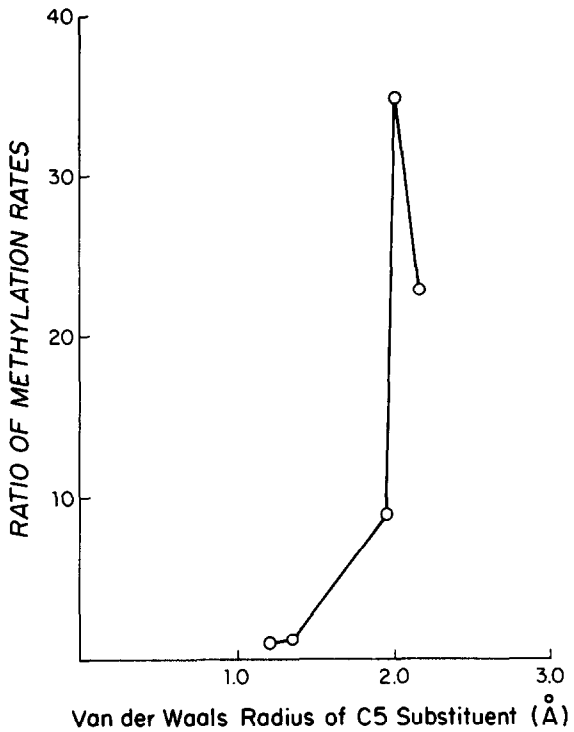


Fig. 4. The Effect of the Size of the Moiety at C-5. Maximal methyltransferase selectivity (see Fig. 3) is plotted as against the van der Waals radius of the cytosine substituent.

DISCUSSION: The restriction anaylsis of the substrate DNAs (Fig.1) shows that they are composed largely of duplex DNA because an appropriate cleavage pattern can be produced by each of the enzymes. Phi X 174 DNA in which 5-MedCyd is present at every dCyd site on one strand is not cleaved by Hpa II or Msp I but is cleaved by Hinf I (16). Our results show that this is also true for 5-BrdCyd and 5-IdCyd substituted M13 DNA. 5-FdCyd substituted DNAs are cleaved by all three enzymes. Since a fluorine atom is only slightly larger than a hydrogen atom, the methylation sensitive restriction enzymes are unaffected by it. However, cleavage is blocked by the larger bromine, methyl and iodine moieties. HinfI did not effectively cleave 5-IdCyd containing DNA even though it is not influenced by a methyl group in its cleavage site. This may be similar to the inhibitory effects seen with certain other restriction enzymes when halogenated bases are present in their recognition sequences (see ref. 17 for example).

5-BrdCyd, 5-MedCyd, and 5-IdCyd in one strand served as a strong stimulus for methylation of DNA by the enzyme prepara-

tion. Alkaline density gradient centrifugation showed that the enzyme applied methyl groups almost exclusively to the light strand in 5-BrdCyd and 5-IdCyd substituted DNAs. The rate enhancement demonstrates that this is not merely due to the lack of methylatable sites on the heavy strand. These results show that 5-BrdCyd and 5-IdCyd in one strand can direct the enzyme to methylate DNA in the opposite strand. In addition the result argues that the enzyme is specific for C-5 of cytosine (1), since no significant methylation occurs on the strand in which the C-5 of each cytosine is blocked by iodine or bromine.

The magnitude of the stimulus provided by the group at C-5 is dependent on its size. The plot of the selectivity ratio determined as shown in Fig. 3 shows a sharp maximum at the size of the methyl group. DNAs substituted with a bulky group in the major groove on each cytosine may have to adopt an altered overall structure that could influence enzyme-DNA interactions. Thus general structural differences in the DNAs could account for some of the effect we see. However, the enzyme clearly has a means by which it senses the size of the group on C-5, and this mechanism is optimized to a methyl group.

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