Subunit and Functional Size of Human Placental DNA Methyltransferase Involved in de Novo and Maintenance Methylation*

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The subunit molecular size of human DNA methyltransferase isolated from nuclear extracts of placenta was determined on the electroblotted polypeptides after sodium dodecyl sulfate-polyacrylamide gel electrophoresis and compared with the functional size by high performance size exclusion chromatography on Superose 12 and γ radiation inactivation analysis. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis results indicated a subunit mass of 120 ± 10 kDa, while the functional size data indicates that the enzyme operates both in de novo and maintenance modes as a dimer of molecular mass 220 ± 15 kDa with no evidence of monomers in solution of ionic strength between 0.1 and 0.8 M NaCl. The 220-kDa activity carried out the transmethylation of both hemi- and unmethylated DNA substrates. There was no evidence for separate functional catalytic sites on each monomer subunit acting independently when engaged in methylation of hemimethylated or single-stranded DNA from the invariance of radiation inactivation target size with these substrates. The radiation inactivation target size was 230 ± 15 kDa.

DNA (cytosine-5)-methyltransferase (EC 2.1.1.37) has been purified from human tissues, with disparate results (1-4). Zucker et al. (3) have purified a single 126-kDa polypeptide from human placenta which carried out DNA methylase activity (138,000-fold purification). Pfeifer et al. (4) have employed monoclonal antibodies against DNA methyltransferase from P815 mouse mastocyte cells that cross-reacted with the human enzyme to immunoaffinity purify the placental DNA methyltransferase (10,343-fold purification). This dimer in solution showed no indication of monomerization at very high or low ionic strength; however, under the former condition evidence of a 440 ± 20-kDa species was observed. The 220-kDa species was most active in carrying out the methylation of dl-dC-containing polynucleotides followed by hemimethylated and unmethylated substrates (last) in agreement with the results of Pedrali-Ney and Weissbach (5).

EXPERIMENTAL PROCEDURES

Enzymes and Chemicals—S-adenosyl-L-[methyl-3H]methionine was purchased from ICN (8 Ci/mmol); DNA polymerase I large fragment (Klenow enzyme) was from Bethesda Research Laboratories, and 5-methyl-2'-deoxycytidine 5'-triphosphate, poly(dI-dC), and poly(dC-dG), were from Pharmacia P-L. Biochemicals. Other biochemicals and enzymes including β-galactosidase, catalase, calf intestine alkaline phosphatase, horse liver alcohol dehydrogenase, acetocholinesterase, fraction 5 bovine serum albumin, and high-molecular-weight protein standards were from Sigma. Micrococcus luteus DNA was isolated from freeze-dried cells (Behring Diagnostics) by the procedure of Marmur (6). M13 mp9 phage DNA was prepared according to Messing and Vierra (7). Hemimethylated M13 substrates were prepared as described in Ref. 8.

DNA Methyltransferase Preparation and Assay—Human placental nuclei were prepared by the procedure of Bloble and Potter (9), using solutions with 0.1% Nonidet P-40 (Bethesda Research Laboratories) and 1 mM phenylmethylsulfonyl fluoride. Nuclei were extracted with buffer A (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol, 5% glycerol) using 0.8 M KC1 added to a final concentration of 0.42 M determined conductimetrically. The nuclear extract was centrifuged for 1 h at 160,000 × g to remove residual chromatin and processed by batch absorption and elution from phosphocellulose (PC1, Whatman), followed by concentration on hydroxyapatite (Bio-Rad HP), as described for rat liver DNA methyltransferase in Ref. 10. This removed all contaminating nucleic acid and rendered the preparation completely dependent on exogenous DNA. The standard assay measures the incorporation of 3H-methyl from [3H]AdoMet (specific activity, 17,600 dpm/pmol) into acid-precipitable DNA where 1 unit of activity is equivalent to the transfer of 1 pmol of CH3/h. The concentration of substrates was 2 μg with 2.5 μM [3H]AdoMet (specific activity, 17,600 dpm/pmol) in a volume of 140 μl in buffer A. The reactions were carried out at 37°C for various times as noted, stopped with 0.63 M NaOH by heating at 60°C for 15 min, and processed as described in Ref. 10. Reaction kinetics were linear for all substrates tested except hemimethylated DNAs which tended to plateau between 30 min and 1 h (8).

Radiation Inactivation—A Theratron 80 Cobalt 60 machine (Atomic Energy Commission of Canada, Ltd.) was modified for radiation inactivation studies. A special plexiglass block was machined

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1 The abbreviations used are: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; AdoMet, S-adenosylmethionine; HPSEC, high performance size exclusion chromatography.

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to fit into the 24" beam sector field at a distance of 5 cm from the surface of the source (5,763 Ci ²⁶²Hg). The block was designed to hold four 0.5-ml Eppendorf tubes symmetrically located to receive an equidose of radiation. Forced ambient air was circulated to maintain temperature in the range of 20-30 °C during the radiation period, which ranged up to 25 min. Crude DNA methyltransferase extracts (0.5-1 mg) were dialyzed against buffer A minus glycerol containing 0.1 M phenylmethylsulfonyl fluoride and were lyophilized to minimum volumes, generally twice, so that a very small residue was visible as a pellet in the bottom of the tube. This was necessary to control tube assayed by the method of Craven et al. (12); calf intestine alkaline phosphatase (D₃₇: 4.3 megarads, 140 kDa), using 2.4 milliliters assayed by the method of Morton (19); and acetylcholinesterase (D₃₇: 0.51 megarads, 105 kDa), 25 µg assayed by the method of Wermuth and Brodbbeck (14). The target size was extrapolated from linear regression by least squares of the data to give D₃₇ values and then calculated from the relation Mₑ = 6.4 × 10⁶/D₃₇ (11). These data were further checked by the ratio of inactivation slopes method of Lo et al. (15) that is independent of absolute beam calibration that can vary from run to run. These two methods gave comparable results for γ inactivation target size of DNA methyltransferase. In all the target size experiments the DNA methyltransferase samples contained the above enzymes as internal standards. Two samples/time point were radiated, providing replicates. After radiation, the samples were recovered twice with 400 µl of Tris-HCl, pH 7.4, gently rocked for 1 h at 4 °C whereupon 40-µl aliquots were used to assay each of the separate activities in duplicate giving quadruplicate values.

**Polyacrylamide Gel Electrophoresis Analysis of Active Enzyme—** SDS-PAGE on 7.5% acrylamide (8 × 10-0.05 cm) MiniLab gels (Bio-Rad) was performed using Laemmli’s procedure (18), modified with the inclusion of 1 mM cysteine in the running buffers. Crude lyophilized DNA methyltransferase (50 µg, -9 poly(dC-dG) units) was solubilized in 0.5 M glycerol, 0.5% SDS, 5 mM dithiothreitol, 1 mM EDTA and held for 1 h at 37 °C before electrophoresis at 100 V for approximately 1 h. The gel lanes were sectioned and the lane containing high molecular weight standards (Sigma) was silver stained (19), while the lane containing enzymatic activity were electrophoretically run on a sheet of Schleicher & Schuell (BA 80 0.15 µm) nitrocellulose. Electrobolting was performed in a Trans-phor cell (Bio-Rad) using the following buffer: 192 mM glycine, 25 mM Tris-HCl (pH 8.2), 1 mM cysteine, 1 mM EDTA, 0.1% SDS, 5 mM dithiothreitol, and 20% methanol for 2 h at 0.2 A. The nitrocellulose membrane was rinsed twice in excess buffer A plus 0.1% Nonidet P-40 at 4 °C for 4-6 h to effectuate a detergent exchange of the SDS and renature the proteins. The nitrocellulose strips corresponding to each lane were subsequently sectioned into 5-mm transverse pieces for assays, which were carried out with 2.5 µM [³²P]AdoMet for 8 h at 37 °C, using 1 µg of poly(dI-dC) as a substrate (estimated recovery, 0.38%). The samples containing membrane-immobilized enzyme and soluble reaction mix were subsequently treated as described previously as the enzyme in solution (10).

**RESULTS**

Several methods for the detection of DNA methyltransferase activity following SDS-PAGE were evaluated in order to make a determination of the molecular mass of DNA methyltransferase that could exist either as an active monomeric protein or complex protein having catalytic subunits. Huber & al. (20) have shown that Hpail methyltransferase and eukaryotic DNA methyltransferase from calf thymus and rat neuronal cells could be renatured in situ in DNA substrate containing SDS-PAGE gels. The activity could be subsequently detected by radiography of the in situ methylated substrate upon incubation of the gel with [³²P]AdoMet. Since this method gave very high background after long exposure times that were not interpretable, we sought other methods of renaturing the enzyme following separation by SDS-PAGE. High efficiency electrobolt transfer of proteins in SDS buffers to nitrocellulose membranes that necessitated recovery of enzymatic activity in the immobilized state was evaluated. When calf intestine alkaline phosphatase, an enzyme that is moderately stimulated in the presence of SDS, was electrobolted and the activity assayed on individual 5-mm slices of the membrane for hydrolysis of p-nitrophenyl phosphate, a single peak of activity at approximately 140 kDa was detected (Fig. 1A). The silver stain gel of the calf intestine alkaline phosphatase preparation gave a predominant band at 140 kDa over a smear of background staining. This procedure was applied to the DNA methyltransferase preparation from placenta nuclei, but it was necessary to incorporate several modifications in order to obtain a satisfactory methylation signal with the most efficient substrate poly(dI-dC). These included the incorporation of phenylmethylsulfonyl fluoride, a protease inhibitor in all buffers during preparation of the extract; denaturation for 1 h at 37 °C rather than boiling for 3 min; and inclusion of cysteine as a free radical scavenger during electrophoresis and electrobolting. Finally, extensive detergent exchange with 0.1% Nonidet P-40 promoted the renaturation of the immobilized enzyme on the membrane.

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**FIG. 1.** SDS-polyacrylamide gel electrophoresis of human placental DNA methyltransferase. After electrophoresis the gel was electrobolted to nitrocellulose membrane, renatured by detergent exchange, and assayed as described under “Experimental Procedures.” Panel A, activity profile for electrobolted and immobilized calf intestine alkaline phosphatase (140 kDa). Panel B, activity profile for electrobolted and immobilized human placenta DNA methyltransferase assayed with poly(dI-dC). Panel C, photograph of silver-stained high molecular mass standards: myosin (205 kDa, Rₚ 0.07); β-galactosidase (116 kDa, Rₚ 0.27); phosphorilase b (95.5 kDa, Rₚ 0.32); bovine serum albumin (65 kDa, Rₚ 0.41); and ovalbumin (45 kDa, Rₚ 0.47); carbonic anhydrase (30 kDa, Rₚ 0.76).
With these modifications, a single activity peak was detected at approximately 120 ± 10 kDa with no evidence of activity in the 60- or 240-kDa region of the gel (Fig. 1B). These results indicate that an essential catalytic polypeptide for methyl transfer has a molecular size of approximately 120 kDa for human placental DNA methyltransferase. These results are consistent with several possibilities. Human DNA methyltransferase is a monomeric protein of 120 kDa, in accordance with the findings of Zucker et al. (3), or as the gel transfer assay only detects catalytic subunits involved in methyl transfer, the enzyme could have a more complex quaternary structure of catalytic and/or regulatory subunits as proposed by Pfeifer et al. (4).

To distinguish between these possibilities, the native enzyme was separated by high-performance gel exclusion chromatography (HPSEC) on Superose 12 in low (0.1 M NaCl) and moderate ionic strength buffers (0.8 M NaCl). These results are shown in Fig. 2, A and B, respectively. In 0.1 M NaCl, the maintenance activity (assayed with hemimethylated M13 DNA) and the de novo activity (assayed with poly(dI-dC)) coeluted as a single peak just following the catalase marker (250 kDa). The elution position is consistent with a protein having a molecular mass of 220 ± 15 kDa. Characterization of the substrate specificity for the 220-kDa activity is presented in Table I. The most efficient substrate was the alternating double-stranded copolymer poly(dI-dC), as reported by Pedra-Mony and Weissbach (5) for the HeLa enzyme, while the highest activity for DNA substrates with normal bases was obtained with hemimethylated, followed by unmethylated, substrates. In 0.8 M NaCl, however, approximately 15% of the activity has shifted to a higher molecular weight form, eluting approximately at 440 ± 20 kDa. This is consistent with the aggregation of two 220-kDa species. The molecular size experiments by HPSEC suggest a much larger size of native placental DNA methyltransferase operating in the cellular environment and not a monomeric protein of 120 kDa, as a monomer peak could be detected by HPSEC in the 120-kDa range.

To further resolve the issue of the size of the DNA methyltransferase in placenta nuclear extracts, radiation inactivation target analysis with γ rays was employed to analyze this parameter independently. This method gives reliable weight form, eluting approximately at 440 kDa. Determinations of relative activity were stopped after 1.5 h and processed as described under “Experimental Procedures.”

To achieve a high degree of accuracy not dependent on absolute beam calibration for each run, lyophilized DNA methyltransferase samples were prepared with internal standards: β-galactosidase, calf intestine alkaline phosphatase, and ace-tocholinesterase. The inclusion of these proteins did not alter the catalytic response of the lyophilized DNA methyltransferase preparation with various types of DNA substrates listed in Table I and thus would serve as valid internal calibration standards.

In Fig. 3 the radiation inactivation plots for human placental DNA methyltransferase are shown with the internal calibration standards: ace-tocholinesterase, calf intestine alkaline phosphatase, and β-galactosidase. The inactivation follows, within errors incurred by the slight of nonuniformity in pellet deposition, first order one hit inactivation kinetics. A linear regression of these points could be fitted by least squares analysis from which the D50 values could be extrapolated. The DNA methyltransferase inactivation kinetics assayed with poly(dI-dC) in Fig. 3 for four different preparations was consistently less radiosensitive than β-galactosidase and considerably more so than calf intestine alkaline phosphatase. The D50 in megarads as well as in the ratio of the slopes between DNA methyltransferase and either β-galactosidase or calf intestine alkaline phosphatase gave consistent results with a target size of approximately 230 kDa (Table II).

In Fig. 4 we examined whether or not the target size shows any functional dependence on the type of methylation mode (de novo versus maintenance) or DNA configuration (single-stranded versus double-stranded). After each radiation time point, the duplicate samples were reconstituted and split into 8 different aliquots. Each aliquot was assayed in duplicate with either poly(dI-dC), hemimethylated M13 DNA, or double-stranded M. luteus DNA and single-stranded M13 DNA.

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Table I

<table>
<thead>
<tr>
<th>Substrate*</th>
<th>% of control</th>
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<tbody>
<tr>
<td>M. luteus DNA (heat-denatured control)</td>
<td>100*</td>
</tr>
<tr>
<td>M. luteus DNA (native)</td>
<td>45</td>
</tr>
<tr>
<td>M13 mp 9, replicative form double-stranded DNA</td>
<td>57</td>
</tr>
<tr>
<td>M13 mp 9, single-stranded phage DNA</td>
<td>62</td>
</tr>
<tr>
<td>M13 mp 9, hemimethylated</td>
<td>590*</td>
</tr>
<tr>
<td>poly(dI-dC), double-stranded</td>
<td>1070</td>
</tr>
<tr>
<td>poly(dC-dG), double-stranded</td>
<td>290</td>
</tr>
</tbody>
</table>

* The concentration of all substrates was 2 μg with 2.5 μM [3H]AdoMet in buffer A in a 140-μl reactive volume. The reactions were stopped after 1.5 h and processed as described under “Experimental Procedures.”

** 100% activity was 19,000 dpm/h.

** Kinetics were nonlinear. At 30 min relative activity of hemimethylated M13 was nearly the same as poly(dI-dC).
the procedure. The identical responses to these substrates to cholinesterase that renaturation into a dimer is required for functional activity. or single-stranded configuration. This suggests that there are involved in the methylation of DNA in either double-stranded DNA methyltransferase by HPSEC and analysis is consistent with an enzyme molecule of molecular size of human placental DNA methyltransferase determined with hemimethylated DNA methyltransferase, 2.8 for DNA methyltransferase was calculated from the relation:

\[ M_r = 6.4 \times 10^3/(\text{D}_{57}) \]

and, alternatively, from the ratio of slopes relationship with proteins of known molecular weight and target size (15).

The radiation inactivation kinetics for each of these four substrates overlapped and was within experimental error for the procedure. The identical responses to these substrates suggest that there is no separate functional entity involved in maintenance methylation versus de novo methylation of DNA. Interestingly, the radiation inactivation kinetics of hemimethylated and single-stranded DNA where only one strand was the target indicated that the whole 230-kDa protein is involved in the methylation of DNA in either double-stranded or single-stranded configuration. This suggests that there are no independently active catalytic sites on each subunit and that renaturation into a dimer is required for functional activity.

**DISCUSSION**

The molecular size of human placental DNA methyltransferase by HPSEC and γ radiation inactivation target size analysis is consistent with an enzyme molecule of molecular mass in the 220–230 ± 15-kDa range. As previously reported for eukaryotic DNA methyltransferases, this enzyme species apparently catalyzes both de novo and maintenance function in vitro in the same molecule (1–4). Renaturation of the SDS-PAGE-separated polypeptides blotted to nitrocellulose revealed a single component that had catalytic activity. Comparison of the size of this component (120 ± 10 kDa) to the native size suggests that the enzyme is composed of identical subunits, which either retained their catalytic activity separately upon renaturation or as a result of oligomerization as shown for the formation in high salt (0.8 M NaCl) of a dimer of the native enzyme with molecular size in the 440-kDa range. This is supported by the fact that no 120-kDa component could be detected in solution or in the lyophilized state and is consistent with the observation that the native enzyme from nuclear extracts presented a similar target size for de novo methylation of single-stranded or double-stranded DNA substrates as for maintenance methylation. Each function is thus catalyzed nonindependently on the native enzyme. If this were not the case, one would expect to find evidence of this as a smaller target size (e.g. 120 kDa) for sites residing on each subunit capable of catalyzing methyl transfer to both strands simultaneously on double-stranded DNA or each subunit having independent equivalent sites involved in methyl transfer on a single-stranded DNA molecule. Second, as a 220-kDa target size for maintenance methylation of hemimethylated M13 DNA was observed, where effectively only one strand can accept methyl groups, this strongly argues that the enzyme operates as a functional dimer. It cannot be determined with the present analysis if there are separate catalytic sites for each function residing on the molecule or a single type of site that can catalyze both functions.

The present results are incompatible with the idea that human placental DNA methyltransferase is a monomeric protein although the present subunit size measurement of 120-kDa is comparable with the size of the major purified polypeptide on SDS-PAGE reported by Zucker et al. (3). The immunopurified human placental DNA methyltransferase described by Pfeifer et al. (4) has a total molecular mass of approximately 260 kDa if the 64-kDa polypeptides are considered to be artifactual proteolytic degradation products. In this case, the native enzyme consists of dissimilar subunits of 158 and 104 kDa, which is slightly larger than the range of

**TABLE II**

<table>
<thead>
<tr>
<th>Enzyme (assayed with)</th>
<th>Target size and D&lt;sub&gt;57&lt;/sub&gt; of human DNA methyltransferase with different DNA substrates and standard enzymes</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Galactosidase</td>
<td>M&lt;sub&gt;r&lt;/sub&gt; = 105,000, M&lt;sub&gt;r&lt;/sub&gt; = 105,000, M&lt;sub&gt;r&lt;/sub&gt; = 105,000</td>
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<tr>
<td>Calf intestine alkaline phosphatase</td>
<td>M&lt;sub&gt;r&lt;/sub&gt; = 140,000, M&lt;sub&gt;r&lt;/sub&gt; = 140,000, M&lt;sub&gt;r&lt;/sub&gt; = 140,000</td>
</tr>
<tr>
<td>Acetylcholinesterase</td>
<td>M&lt;sub&gt;r&lt;/sub&gt; = 230,000, M&lt;sub&gt;r&lt;/sub&gt; = 230,000, M&lt;sub&gt;r&lt;/sub&gt; = 230,000</td>
</tr>
<tr>
<td>DNA methyltransferase, (poly(dI-dC) native double stranded)</td>
<td>M&lt;sub&gt;r&lt;/sub&gt; = 230,000, M&lt;sub&gt;r&lt;/sub&gt; = 230,000, M&lt;sub&gt;r&lt;/sub&gt; = 230,000</td>
</tr>
<tr>
<td>DNA methyltransferase, (hemimethylated M13 mp9)</td>
<td>M&lt;sub&gt;r&lt;/sub&gt; = 230,000, M&lt;sub&gt;r&lt;/sub&gt; = 230,000, M&lt;sub&gt;r&lt;/sub&gt; = 230,000</td>
</tr>
<tr>
<td>DNA methyltransferase, (M13 mp9 phage DNA)</td>
<td>M&lt;sub&gt;r&lt;/sub&gt; = 230,000, M&lt;sub&gt;r&lt;/sub&gt; = 230,000, M&lt;sub&gt;r&lt;/sub&gt; = 230,000</td>
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<sup>a</sup>*M<sub>r</sub> was extrapolated from a linear regression by least squares of the data and the known D<sub>57</sub> for the standard protein. Target size (M<sub>r</sub>) for DNA methyltransferase was calculated from the relation: M<sub>r</sub> = 6.4 × 10<sup>3</sup>/D<sub>57</sub> (11) and, alternatively, from the ratio of slopes relationship with proteins of known molecular weight and target size (15).
molecular size determined herein by HPSEC and radiation inactivation. If heterodimerization is a precondition for active enzyme formation, as supported by the data herein on dimerization, then there may be some discrepancy in subunit size measurements, and the simplest interpretation is that the active enzyme is a homodimer composed of identical 120-kDa subunits.

Acknowledgments—We wish to thank Dr. Art Boyer for his assistance in setting up the radiation inactivation experiments and Marjorie Nortin and Betty Martz for the preparation of the manuscript.

Addendum—Pfeifer and Drahovsky (21) have recently immunoprecipitated a single polypeptide of 190 kDa with monoclonal anti-DNA methyltransferase antibody from human Raji and K562 cells using a high pH procedure to circumvent problems of endogenous proteolysis.

REFERENCES