Purification and Characterization of Mammalian DNA Methyltransferases by Use of Monoclonal Antibodies*

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Previously, we have derived murine hybridomas producing monoclonal antibodies against DNA methyltransferase from mouse P815 mastocytoma cells, was used for the immunooaffinity purification of mouse and human DNA methyltransferases. In sodium dodecyl sulfate-polyacrylamide gels and in immunoblotting studies, the immunooaffinity-purified mouse DNA methyltransferase revealed 5–6 polypeptides of molecular masses 150–190 kDa. The immunooaffinity-purified human placental DNA methyltransferase was characterized by a polypeptide of 158 kDa, presumably representing the native enzyme molecule and by polypeptides of 105–108 kDa and 50–68 kDa, probably generated by a limited proteolysis of the native enzyme molecule. The immunooaffinity-purified DNA methyltransferases preferred hemimethylated DNA substrates over unmethylated ones, and among all unmethylated substrates tested, poly[(dG-dC)-(dG-dC)] had the highest methyl-accepting activity. DNA polymers of at least 90 base pairs in length were required for the binding reaction of the immunooaffinity-purified human DNA methyltransferase, and this initial binding was apparently independent of the nucleotide composition of the DNA polymer and of the presence of S-adenosyl-L-methionine.

The biological role of 5-methylcytosines, which have been detected in mammalian DNA more than three decades ago (1, 2), is still a subject of speculation. The experimental evidence links hypermethylation to gene silencing and undermethylation to the expression of certain genes (for recent reviews see Refs. 3–7). 5-Methylcytosine, the only modified base in mammalian DNA, arises by transfer of methyl groups from S-adenosyl-L-methionine (AdoMet) to the carbon 5 of the cytosine ring in a DNA polymer. More than 90% of the 5-methylcytosines are found in CpG dinucleotides (8). The enzymatic methylation reaction is carried out by DNA methyltransferases-DNA methyltransferases prefer hemimethylated DNA substrates over unmethylated ones, and among all unmethylated substrates tested, poly[(dG-dC)-(dG-dC)] had the highest methyl-accepting activity. DNA polymers of at least 90 base pairs in length were required for the binding reaction of the immunooaffinity-purified DNA methyltransferase, and this initial binding was apparently independent of the nucleotide composition of the DNA polymer and of the presence of S-adenosyl-L-methionine.

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1 The abbreviations used are: AdoMet, S-adenosyl-L-methionine; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

cytosine 5-methyltransferases (DNA methyltransferases, EC 2.1.1.37). Two types of DNA methyltransferase activities have been postulated. The maintenance one transfers methyl groups to hemimethylated sites generated shortly after the DNA replication and is responsible for the inheritance of specific DNA methylation patterns; the de novo activity of the DNA methyltransferase introduces methyl groups to previously unmethylated sites. Experiments with isolated enzymes suggested that both these activities are carried out by the same DNA methyltransferase (9, 10). DNA methyltransferases have been isolated from a number of tissues and cells (9–20). At present, however, the information about the molecular structure of mammalian DNA methyltransferases is rather scarce.

Previously, we succeeded in the construction of hybridomas producing monoclonal antibodies against DNA methyltransferase from human placenta (21). These monoclonal antibodies also undergo immune complex formation with DNA methyltransferase from mouse P815 mastocytoma cells. In this report, we have used these antibodies for the immunooaffinity purification and further characterization of DNA-methylating enzymes from human placenta and mouse P815 cells.

**EXPERIMENTAL PROCEDURES**

**Purification of DNA Methyltransferases—DNA methyltransferase from terminal human placenta was purified as previously described (9) with some modifications. Nuclei were isolated by suspending the minced tissue in 10 mM Tris-HCl, (pH 7.5), 0.25 M sucrose, 3 mM CaCl₂, 0.2 mM phenylmethylsulfonyl fluoride (PMFSF) (buffer A) and homogenizing in a Waring Blendor for 20 s. The homogenate was filtered successively through two layers of gauze and two layers of nylon cloth. The filtrate was centrifuged at 1000 g for 10 min. The resulting pellet was washed twice in buffer A and then suspended in buffer A containing 0.5% Triton X-100. The suspension was homogenized in a motor-driven glass homogenizer, and nuclei were pelleted by centrifugation at 1000 × g for 10 min and finally washed three times in buffer A.

Isolated nuclei were extracted in 50 mM Tris-HCl (pH 7.5), 0.8 M KCl, 1 mM EDTA, 1 mM dithioerythritol, 0.2 mM PMSF, 1 μg/ml chymostatin, 0.5% Triton X-100, 10% (v/v) glycerol (buffer B). After the extraction, the salt concentration was lowered to 0.5 M by dilution with 10 mM Tris-HCl (pH 7.8). Reconstituted chromatin was removed by centrifugation at 20,000 × g for 20 min, and the dialyzed supernatant was subjected to chromatography on DEAE-cellulose as previously described (9). The enzymatically active fractions obtained after the DEAE-cellulose chromatography were dialyzed against 20 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.5 mM dithioerythritol, 0.2 mM PMSF, 0.2% (v/v) glycerol (buffer C), and subjected to chromatography on heparin-agarose. The column (15 × 1.6 cm) was washed with buffer C containing 0.1 M NaCl, and enzymatic activity was eluted with a linear gradient from 0.1–0.6 M NaCl in buffer C.

DNA methyltransferase from mouse P815 mastocytoma cells was isolated and purified essentially as previously described (10). Nuclei were extracted in buffer B, and the nuclear extract was further purified by chromatographies on DEAE-cellulose and heparin-agarose.
Immunoaffinity Purification of Mammalian DNA Methyltransferases

Monoclonal Antibodies against Human DNA Methyltransferase Cross-react with DNA Methyltransferase from Mouse

puriﬁed enzyme was stable for at least 3 weeks. DNA methyltransferase was eluted from the column containing 0.3 M NaCl. DNA methyltransferase was eluted from the DEAE-cellulose. The obtained immunoglobulins were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The purity of the monoclonal IgG fractions was >98%.

Immunoaffinity Purification of DNA Methyltransferases—The puriﬁed monoclonal antibody M2B10 was covalently linked to a N-hydroxysuccinimide ester-activated cross-linked agarose gel support (Affi-Gel 10) by a procedure suggested by the supplier (Bio-Rad). Approximately 4 mg of IgG were bound to 1 ml of the gel.

Prior to the immunoafﬁnity step, partially puriﬁed DNA methyltransferases (heparin-agarose fractions) were dialyzed against 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 2.5 mM MgCl2, 1 mM dithioerythritol, 0.2 mM PMSF, 10% (v/v) glycerol (buffer D) and applied at a ﬂow rate of 20 ml/h to a M2B10-Affi-Gel 10 column (4 × 0.9 cm) equilibrated in buffer D at 2 °C. The ﬂowthrough material was reapplied three times at the same ﬂow rate on the same column. Then, the column was washed extensively with buffer D containing 0.25% Triton X-100 (buffer E) and then with buffer E containing 0.3 M NaCl. DNA methyltransferase was eluted from the antibody column with 1.5 M NaCl in buffer E. The eluate was dialyzed for 5 h against buffer C. All enzymatically active fractions were stored at −80 °C. Under these conditions, the activity of the immunoafﬁnity-puriﬁed enzyme was stable for at least 6 months.

SDS-Polyacrylamide Gel Electrophoresis—Denaturing polyacrylamide gel electrophoresis was performed in 8.75 and 10% SDS-polyacrylamide gels, respectively, essentially as described by Laemmli (22). Protein samples were reduced with 1.5% 2-mercaptoethanol prior to loading. The polypeptides were visualized by silver staining essentially as described by Merril et al. (23). Dilute protein samples were concentrated for electrophoresis by precipitation in 10% (w/v) ammonium sulfate (50% saturation) and further purified by chromatography on DEAR-cellulose. The obtained immunoglobulins were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Immunoafﬁnity Purification of Mammalian DNA Methyltransferases—The immunoafﬁnity-puriﬁed human DNA methyltransferase fraction was estimated by denaturation scanning of Coomassie Blue-stained SDS-polyacrylamide gels. The protein concentration of the immunoafﬁnity-puriﬁed mouse DNA methyltransferase fraction was estimated by denaturation scanning of silver-stained SDS-polyacrylamide gels using β-galactosidase as standard.

Immunoblot Analysis of DNA Methyltransferase Proteins—5 μg of the immunoafﬁnity-puriﬁed DNA methyltransferase were subjected to SDS-PAGE 8.75% SDS-polyacrylamide gels. The separated polypeptides were electrotransferred to nitrocellulose (Schleicher & Schuell, BA 85) according to the method of Towbin et al. (25). After the transfer, the nitrocellulose ﬁlters were pretreated with 3% bovine serum albumin in 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 0.02% NaN3, 0.05% Nonidet P-40 (buffer F) for 1 h at 4°C. The filter was then incubated with 1 μg of the puriﬁed monoclonal antibody in 15 ml of buffer F at 37 °C for 5 h. The ﬁlters were washed in buffer F without bovine serum albumin and subsequently reacted with 5 × 106 cpm of 35S-labeled protein A (New England Nuclear, speciﬁc activity 8.4 μCi/μg) for 16 h at room temperature. The ﬁltered immunobiopeptides were ﬁnally visualized by autoradiography on Kodak XAR 5 ﬁlms at −80 °C.

Glyceraldehyde 3-Phosphate Dehydrogenase—Glyceraldehyde 3-phosphate dehydrogenase (Table I; Fig. 1) for 15 min at 37 °C in the absence of or with 10 μM of dCTP, dGTP, dATP, and dTTP (0.1 mM each) at 37 °C for 5 h. Replacement of d-TTP by dCTP in the same reaction mixture provided the unmethylated substrate for the demonstration of DNA methyltransferase activity. Under these conditions, 10–20% of the DNA bases were replaced.

Ligation of DNA Linkers and Nitrocellulose Binding Assay—Linker fragments (EcoRI, BamHI, and PstI, Boehringer Mannheim) were initially labeled as follows. 10 μg of linkers were incubated with 125 μCi of 32P-ATP (Amersham Buchler, 3000 Ci/mmol) in 60 μl of a solution containing 70 mM Tris-HCl (pH 7.6), 10 mM MgCl2, 50 mM dithioerythritol with 40 units of T4 polynucleotide kinase (Bethesda Research Laboratories) for 15 min at 37 °C. Then, the volume was adjusted to 100 μl followed by the addition of cold ATP to a ﬁnal concentration of 1 mM and 40 units of T4 polynucleotide kinase. After incubation for a further 30 min, the reaction was terminated by freezing in an ice-water bath.

A ladder of ligated linkers was produced by diluting the kinased linkers in 10 volumes of 66 mM Tris-HCl (pH 7.6), 5 mM MgCl2, 5 mM dithioerythritol, 1 mM ATP, and addition of 7 units of T4 ligase (Boehringer Mannheim). After 3 h at room temperature, the reaction was stopped with EDTA (final concentration 20 mM). The solution was extracted with phenol/chloroform and the polymerized linker fragments were precipitated by the addition of 15 volumes of cold ethanol. The linkers were dissolved in adsorption buffer (20 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.5 mM dithioerythritol, 5% glycerol) and used in nitrocellulose filter-binding assays as follows. 0.3 μg of ligated linkers, in 25 μl of adsorption buffer, were incubated with 4 μl (about 40 μg of protein) of DNA methyltransferase (fraction VB, Table I; Fig. 1) for 15 min at 37 °C in the absence or presence of AdoMet (10 μM). The mixture was then ﬁltered through prewetted nitrocellulose (BA 85, Schleicher & Schuell) under mild suction, and the ﬁlters were extensively washed with adsorption buffer. The DNA bound to the filters was then dissolved in 45 mM Tris borate (pH 9.5), 1.25 mM EDTA, 0.1% SDS, and electrophoresed through 10% polyacrylamide gel. The gels were then dried and autoradiographed at −80 °C for 6 h.

RESULTS

Immunoafﬁnity Purification of Human and Mouse DNA Methyltransferases

Monoclonal Antibodies against Human DNA Methyltransferase Cross-react with DNA Methyltransferase from Mouse
Immunofinity Purification of Human Placental DNA Methyltransferase—Previously, we have reported purification procedures for the isolation of DNA methyltransferases from terminal human placenta (9) and P815 mouse mastocytoma cells (10). Three different column chromatographies were incorporated into these procedures, and the final protein fractions showed a specific activity of about 2000 units/mg of protein. In the purification protocol described here, human and mouse DNA methyltransferases were prepurified from high salt nuclear extracts by chromatographies on DEAE-cellulose and heparin-agarose. The enzymes eluted from DEAE-cellulose as single peaks of activity at 150 mM NaCl. Human placental DNA methyltransferase frequently showed two activity peaks on heparin-agarose, a major one (about 80%) eluted at 0.3 M NaCl and a minor one (about 20%) eluted at 0.5 M NaCl. The heparin-agarase fractions were subjected to immunoaffinity chromatography using immobilized murine monoclonal anti-human DNA methyltransferase antibody M2B10.

The purification procedure for the human placental DNA methyltransferase is summarized in Table I. The DNA methyltransferase activity found in the 0.3 M NaCl peak of the heparin-agarose fractionation (fraction IVA, Table I) was applied to the M2B10 antibody column. About 30–50% of the applied DNA methyltransferase activity (depending on the enzyme preparation) was always recovered in the flowthrough volume (fraction VA, Table I), even if reapplied several times onto unused columns of the same immobilized antibody. This portion of DNA methyltransferase also failed to undergo immune complex formation with monoclonal antibodies M2B10, M8F73A, and M11F1 in the liquid phase.

The bound DNA methyltransferase activity was eluted from the antibody column with 1.5 M NaCl, 0.25% Triton X-100 (fraction VB, Table I). The presence of Triton X-100 was essential for the elution of DNA methyltransferase protein. The DNA methyltransferase activity in the minor 9.5 M NaCl heparin-agarase peak failed to complex with monoclonal antibodies M2B10, M8F73A, and M11F1 and was not subject to further purification.

The immunoaffinity-purified DNA methyltransferase was subjected to SDS-PAGE, and the resulting polypeptides were visualized by silver staining. Fig. 1 shows the purified DNA methyltransferase from human placenta obtained after the purification outlined in Table I (fraction VB). The pattern is characterized by polypeptides of 158, 150, 105–108, 56, and 54 kDa. The 105–108 kDa band represents a doublet which was not resolved under these conditions (see also Fig. 2). Depending on the preparation 1–3 additional polypeptides were found in the area of 50–68 kDa (Figs. 2 and 3). In some preparations, the 150 kDa polypeptide was not detected (Fig. 2). Common to all so far prepared immunoaffinity-purified human placental DNA methyltransferase fractions were the polypeptides of 158, 108, 105, 56, and 54 kDa.

The reactivity of the monoclonal antibodies M2B10, M8F73A, and M11F1 toward the individual polypeptides of the immunoaffinity-purified human placental DNA methyltransferase was examined by immunoblotting. The polypeptides were separated by SDS-PAGE, transferred to nitrocellulose, and reacted with the monoclonal antibodies. The immune complexes were visualized by specific protein A and autoradiography (Fig. 2). The three monoclonal antibodies give the same pattern. They react with the polypeptides of 158, 108, and 105 kDa and also with the polypeptides in the area of molecular mass 50–68 kDa. Thus, the epitopes recognized by the monoclonal antibodies are present on all polypeptides obtained after immunoaffinity purification.

In glycerol gradients, the activity of the immunoaffinity-purified DNA methyltransferase sedimented in a broad peak at about 6.9 S. When the individual glycerol gradient fractions were subjected to SDS-PAGE, the presence of the polypeptides of 158, 150, and 105–108 kDa and of the smaller polypeptide of 56 kDa was found to correlate with the DNA methyltransferase activity (Fig. 3). The distribution of the other polypeptides of 50–68 kDa did not parallel the enzyme activity in the glycerol gradient. However, these polypeptides were also found in the enzymatically active gradient fractions and were recognized in immunoblotting (Fig. 2). The spreading of these polypeptides over the gradient is as yet unexplained. Further purification of fraction VB (Table I) on single-stranded DNA-heparin agarose did not result in elimination of

### Table I

<table>
<thead>
<tr>
<th>Step</th>
<th>Fraction</th>
<th>Total activity</th>
<th>Protein</th>
<th>Specific activity</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear homogenate</td>
<td>I</td>
<td>160,000 (100)</td>
<td>23,740</td>
<td>6.74</td>
<td>1</td>
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<tr>
<td>Chromatin free nuclear extract</td>
<td>I</td>
<td>141,200 (88)</td>
<td>4,455</td>
<td>31.7</td>
<td>4.7</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>III</td>
<td>60,800 (38)</td>
<td>125</td>
<td>487.6</td>
<td>75.3</td>
</tr>
<tr>
<td>Heparin-agarose 0.3 M NaCl</td>
<td>IVA</td>
<td>38,377 (24)</td>
<td>21.4</td>
<td>1,793.3</td>
<td>266.0</td>
</tr>
<tr>
<td>Heparin-agarose 0.5 M NaCl</td>
<td>IVB</td>
<td>8,640 (54)</td>
<td>2.5</td>
<td>3,756.5</td>
<td>557.3</td>
</tr>
<tr>
<td>M2B10 Affi-Gel 10° flowthrough</td>
<td>VA</td>
<td>19,514 (12)</td>
<td>21.3</td>
<td>793.6</td>
<td>135.9</td>
</tr>
<tr>
<td>M2B10 Affi-Gel 1.5 M NaCl eluate</td>
<td>VB</td>
<td>4,880 (3.0)</td>
<td>0.07</td>
<td>69,714.3</td>
<td>10,343.3</td>
</tr>
</tbody>
</table>

* Quantities are expressed per 4 kg wet weight of placental tissue. One unit of DNA methyltransferase activity is the amount of enzyme that incorporates 1 pmol of methyl groups into double-stranded *M. luteus* DNA in 1 h at 37°C under standard assay conditions.

* Fraction IVA was applied to M2B10 Affi-Gel 10°.
any of the polypeptides from the enzymatically active fractions.

**Immunoaﬃnity Purification of Mouse P815 DNA Methyltransferase**—The cross-reactivity of the monoclonal antibody M2B10 provided the possibility to purify also the DNA methyltransferase from mouse P815 cells by immunoaﬃnity chromatography. The mouse DNA methyltransferase activity which eluted as a single peak at 0.38 M NaCl from heparin-agarose was applied to M2B10 Afﬁ-Gel 10. The puriﬁcation steps were the same as for the human placental enzyme and are summarized in Table II. In contrast to the human placental DNA methyltransferase it was possible to bind more than 95% of the mouse P815 DNA methyltransferase activity to the M2B10 antibody column. The reasons for these diﬀerences are unknown as yet.

SDS-PAGE of the immunoaﬃnity-puriﬁed DNA methyltransferase from mouse P815 cells (1.5 M NaCl eluate, fraction V, Table II) revealed 5–6 polypeptides in the molecular weight range 150,000–190,000 (Fig. 4, lane 1). In contrast to the DNA methyltransferase preparation obtained from human placenta, polypeptides of lower molecular weight were absent.

In Fig. 4, lanes 2–4, the immunoblot analysis of mouse DNA methyltransferase is shown. The DNA methyltransferase polypeptides obtained after immunoaﬃnity puriﬁcation (fraction V, Table II) were separated by SDS-PAGE, transferred to nitrocellulose, and reacted with three diﬀerent anti-DNA methyltransferase antibodies, M2B10, M8F73A, and M11F1. The polypeptides in the molecular weight range 150,000–190,000 reacted to a similar extent with each of the three monoclonal antibodies.

**Properties of the Immunoaﬃnity-puriﬁed DNA Methyltransferases**

The specific activities of the immunoaﬃnity-puriﬁed DNA methyltransferases were about 70,000 units/mg of protein for the human enzyme and 66,000 units/mg of protein for the mouse enzyme. While being stable at -80 °C for at least 3 weeks, the highly puriﬁed DNA methyltransferases seem to be rather unstable at 37 °C. However, after addition of other (indifferent) proteins to the incubation mixture, the activity of the immunoaﬃnity-puriﬁed DNA methyltransferases could be increased up to 10-fold. Such an eﬀect was seen, for example, with hemoglobin, bovine lactate dehydrogenase, or goat immunoglobulins, but not with bovine serum albumin.

When these proteins were added to reaction mixtures containing partially puriﬁed DNA methyltransferase (heparinagarose fractions) little or no stimulating eﬀect was observed.

The only product of the methylation reactions catalyzed by the immunoaﬃnity and partially puriﬁed DNA methyltransferases was 5-methylcytosine, which was detected after methylation of double-stranded M. luteus DNA with [methyl-3H] AdoMet, isolation and hydrolysis of the DNA, and separation of the bases by thin layer chromatography. More than 98% of total incorporated radioactivity cochromatographed with 5-methylcytosine (data not shown).

The immunoaﬃnity-puriﬁed DNA methyltransferases transferred methyl groups to both double- and single-stranded natural DNAs; the methyl-accepting activity of the DNAs was correlated to their GC content, and homologous DNAs were poor substrates. A sequence requirement for the methylation reaction was tested by use of cytosine containing synthetic DNA polymers (Table III). Human and mouse DNA methyltransferases showed about the same substrate speciﬁcity. They did not transfer methyl groups to the homopolymer poly[(dG)-(dC)] and into the alternating copolymer poly[(dA-dG)-(dC-dT)]. A very low rate of methylation was observed with poly[(dA-dC)-(dG-dT)], and clearly the highest methyl-accepting ability showed poly[(dG-dC)-(dG-dC)]. These observations are compatible with a preferred methyl group transfer to CpG sequences, as it was also described for other previously isolated mammalian DNA methyltransferases (13, 16–19).

A plasmid DNA that contained hemimethylated sites was constructed by nick translation in the presence of dATP,
**Immunoaffinity Purification of Mammalian DNA Methyltransferases**

**FIG. 3** (left). Glycerol gradient centrifugation of human placental DNA methyltransferase. Sedimentation of immunoaffinity-purified DNA methyltransferase in a 10–30% glycerol gradient was performed as described under “Experimental Procedures.” The gradient fractions were assayed for DNA methyltransferase activity and subjected to electrophoresis in a 10% SDS-polyacrylamide gel. Protein markers run in parallel gradients were catalase (CAT), lactate dehydrogenase (LDH), and hemoglobin (Hb). The size of marker proteins in SDS-PAGE is given in kDa. Polypeptides were visualized by silver staining.

**FIG. 4** (right). Polypeptide composition of DNA methyltransferase from P815 mouse mastocytoma cells. Polypeptides of fraction V (Table II) were separated on an 8.75% SDS-polyacrylamide gel and visualized by silver staining (lane 1). The separated polypeptides were transferred from the gel to nitrocellulose and reacted with monoclonal antibody M2B10 (lane 2), M8F73A (lane 3), and M11F1 (lane 4), respectively. Immune complexes were revealed by reaction with 125I-protein A and autoradiography. The size of molecular weight markers is given in kDa.

**TABLE II**

<table>
<thead>
<tr>
<th>Step</th>
<th>Fraction</th>
<th>Total activity*</th>
<th>Protein</th>
<th>Specific activity</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear homogenate</td>
<td>I</td>
<td>190,220 (100)</td>
<td>45,400</td>
<td>4.2</td>
<td>1</td>
</tr>
<tr>
<td>Chromatin free nuclear extract</td>
<td>II</td>
<td>173,100 (91)</td>
<td>11,200</td>
<td>15.5</td>
<td>3.7</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>III</td>
<td>150,400 (79)</td>
<td>585</td>
<td>257.0</td>
<td>61.2</td>
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<tr>
<td>Heparin-agarose</td>
<td>IV</td>
<td>64,930 (34)</td>
<td>30.8</td>
<td>2,108.0</td>
<td>501.9</td>
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<tr>
<td>M2B10 Affi-Gel 10</td>
<td>V</td>
<td>4,988 (2.6)</td>
<td>0.075</td>
<td>66,240.0</td>
<td>15,771.4</td>
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</table>

* Quantities are expressed per $1.3 \times 10^{11}$ cells. One unit of DNA methyltransferase activity is the amount of enzyme that incorporates 1 pmol of methyl groups into double-stranded *M. luteus* DNA in 1 h at 37 °C under standard assay conditions.
### Table III

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Human placenta</th>
<th>Mouse P815 cells</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>pmol CH₃ transferred/µg DNA/h</td>
<td></td>
</tr>
<tr>
<td>Human placenta DNA</td>
<td>1.03</td>
<td>ND</td>
</tr>
<tr>
<td>Mouse P815 DNA</td>
<td>ND</td>
<td>0.57</td>
</tr>
<tr>
<td><em>Clostridium perfringens</em> DNA</td>
<td>1.24</td>
<td>0.58</td>
</tr>
<tr>
<td><em>E. coli</em> B DNA</td>
<td>6.08</td>
<td>5.83</td>
</tr>
<tr>
<td><em>M. luteus</em> DNA, native</td>
<td>14.20</td>
<td>11.67</td>
</tr>
<tr>
<td><em>M. luteus</em> DNA, heat denatured</td>
<td>12.86</td>
<td>12.20</td>
</tr>
<tr>
<td>Poly[(dA-dC)-(dG-dT)]</td>
<td>0.21</td>
<td>0.13</td>
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<tr>
<td>Poly[(dG-dC)-(dG-dC)]</td>
<td>27.76</td>
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<tr>
<td>Poly[(dA-dG)-(dC-dT)]</td>
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<td>0</td>
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<tr>
<td>Poly[(dG)-(dC)]</td>
<td>0</td>
<td>0</td>
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<tr>
<td>pUR222 DNA, nick translated in presence of dCTP, native</td>
<td>13.26</td>
<td>11.54</td>
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<td>pUR222 DNA, nick translated in presence of dCpT, heat denatured</td>
<td>13.35</td>
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<td>pUR222 DNA, nick translated in presence of d5-mCTP, native</td>
<td>45.33</td>
<td>36.26</td>
</tr>
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<td>pUR222 DNA, nick translated in presence of d5-mCTP, heat denatured</td>
<td>8.02</td>
<td>6.71</td>
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* ND, not determined.

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**DISCUSSION**

Monoclonal antibodies prepared against DNA methyltransferase from human placenta cross-react with DNA methyltransferase from mouse P815 mastocytoma cells. This may suggest a certain degree of evolutionary conservation of these epitopes on mammalian DNA-methylating enzymes. DNA methyltransferases from human placenta and mouse P815 cells have been immunoaffinity purified on immobilized anti-DNA methyltransferase antibodies 10,000-15,000-fold over the nuclear homogenate. The specific activities of the immunoaffinity-purified enzymes were very similar (approximately 70,000 units/mg of protein) and exceeded more than 10 times those of DNA methyltransferases previously purified by conventional methods (9-20).

**SDS-PAGE** of the immunoaffinity-purified mouse DNA methyltransferase fractions tested, including the immunoaffinity-purified ones, transferred methyl groups to both types of DNA substrates; the hemimethylated substrate was methylated more than 3 times as high as the unmethylated one (Table III). When the unmethylated DNA substrate was heat denatured, the methyl-accepting activity of this DNA remained nearly unchanged. Heat denaturation of the hemimethylated DNA resulted in a loss of more than 80% of its methyl-accepting activity (Table III). This indicates that the methylation of hemimethylated sites was involved in the native substrate. These results are consistent with the preferene for hemimethylated DNA substrates as compared to unmethylated ones, which seems to be a property of all mammalian DNA-methylating enzymes (9, 10, 14, 17-20).

The presence of maintenance and de novo activities in the immunoaffinity-purified DNA methyltransferase preparations suggests that either both activities are carried out by the same enzyme molecule or that the enzyme molecules responsible for these two types of activities carry the same M2B10 epitope. The DNA methyltransferase fractions IVB and VA from human placenta (Table I) showed the same substrate specificity as the antibody-bound fraction VB (data not shown).

The binding reaction of the immunoaffinity-purified human DNA methyltransferase to DNA was analyzed in a nitrocellulose filter-binding assay. We have applied this method to determine the size and nucleotide requirement of DNA which interacts with DNA methyltransferase. Three different octamer linkers, *BamHI* (sequence 5'-GGGATCCC-3'), EcoRI (sequence 5'-GGAATTCC-3'), and *PstI* (sequence 5'-CCTGCGAGG-3') were terminally labeled and ligated to polymers of different size. The ligated linkers were incubated with the immunoaffinity-purified DNA methyltransferase, and DNA-protein complexes were isolated by use of nitrocellulose filters. The bound DNA fragments were then eluted from the filters, subjected to polyacrylamide gel electrophoresis, and visualized by autoradiography (Fig. 5). The obtained patterns clearly show that (a) the size of double-stranded DNA capable to undergo a complex formation with DNA methyltransferase is at least about 90 base pairs, (b) the enzyme-DNA interaction is independent of the presence of AdoMet, and (c) DNA methyltransferase-DNA complex formation is virtually independent of the nucleotide composition of the DNA involved. It should be noted that Cpg sequences are absent from polymerized *PstI* linkers, which also interact with DNA methyltransferase. This is compatible with previous observations that binding of DNA methyltransferase to a DNA polymer is independent of the presence of methylatable sites (20, 26). Following initial random binding, the enzyme may scan the DNA helix for methyl-accepting Cpg sites in a processive mode of action (11, 12).
methyltransferase revealed the presence of 5–6 polypeptides of molecular mass 150–190 kDa, which reacted with three different monoclonal DNA methyltransferase antibodies in immunoblotting studies. The polypeptide composition of the immunoaffinity-purified DNA methyltransferase from human placenta differed from that of the mouse enzyme inasmuch as additional polypeptides of molecular mass 105–108 kDa and 50–68 kDa were present. Two polypeptides of 158 and 150 kDa (the latter being not always observed) of the placental enzyme preparation were of similar size as those of the mouse enzyme preparation. These two high molecular weight polypeptides as well as the polypeptides of 108, 105, and 56 kDa paralleled the enzyme activity in glycerol gradients.

All polypeptides obtained after immunoaffinity purification of the placental enzyme reacted with three different monoclonal antibodies in immunoblotting. On the basis of these data, we propose that the native human placental DNA methyltransferase molecule corresponding to about 158 kDa is partially proteolytically degraded during the physiological involution of the term placenta or in the course of the purification procedure, and thus revealed under reducing conditions in SDS-PAGE bands corresponding to 108, 105, and 50–68 kDa. However, the possibility that a part of DNA methyltransferase molecules, which carry M2B10 epitopes, is composed of subunits of 105–108 and 50–68 kDa or 50–68 kDa only cannot be excluded at present. Another kind of heterogeneity of the placental DNA methyltransferase enzyme, which was not observed with the P815 enzyme, was the frequent appearance of a second activity peak on heparin-agarose chromatography and the presence of a DNA methyltransferase fraction which did not bind to the column of immobilized antibodies. We were unable to detect functional differences between these different DNA methyltransferase fractions in human placenta. The observed heterogeneity may reflect a real in vivo situation or may be an artificial one produced by proteolytic processes, which split the epitopes recognized by the monoclonal antibodies but retain the catalytic center of the enzyme. At present we cannot discriminate between these two situations. In an effort to avoid this possible proteolytic degradation, several additional protease inhibitors of different specificities have been included in buffers for enzyme isolation. However, they were found to be not useful as they dramatically decreased the extractable DNA methyltransferase activity.

The biological significance of the multiple high molecular weight polypeptides in the range 150,000–190,000, which were obtained after the immunoaffinity purification of the mouse P815 DNA methyltransferase, is unexplained at present. They may represent functionally different DNA methyltransferase molecules. However, the existence of a large precursor and several intermediate forms of the mature enzyme and/or post-translational modification processes cannot be excluded. The separation of two DNA methyltransferases from Friend murine erythroleukemia cells on Cibacron blue agarose has been reported (12). Our inability to reproduce this separation with DNA methyltransferase from P815 cells may be due to the different mouse cells used for enzyme isolation. Nevertheless, the molecular mass of Friend cell DNA methyltransferases of 175 and 150 kDa, respectively (12), as well as the presence of 158- and 150-kDa polypeptides in the immunoaffinity-purified DNA methyltransferase from human placenta and of 150–190-kDa polypeptides in the mouse P815 enzyme preparation implies that mammalian DNA methyltransferase molecules consist of single polypeptide chains with molecular masses ranging from 150–190 kDa.

The novel approach to DNA methyltransferase purification reported in this study provides a basis to resolve the questions of number and properties of DNA methyltransferases in eukaryotic cells.

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