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A versatile non-radioactive assay for DNA methyltransferase activity and DNA binding

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ABSTRACT

We present a simple, non-radioactive assay for DNA methyltransferase activity and DNA binding. As most proteins are studied as GFP fusions in living cells, we used a GFP binding nanobody coupled to agarose beads (GFP nanotrap) for rapid one-step purification. Immobilized GFP fusion proteins were subsequently incubated with different fluorescently labeled DNA substrates. The absolute amounts and molar ratios of GFP fusion proteins and bound DNA substrates were determined by fluorescence spectroscopy. In addition to specific DNA binding of GFP fusion proteins, the enzymatic activity of DNA methyltransferases can also be determined by using suicide DNA substrates. These substrates contain the mechanism-based inhibitor 5-aza-dC and lead to irreversible covalent complex formation. We obtained covalent complexes with mammalian DNA methyltransferase 1 (Dnmt1), which were resistant to competition with non-labeled canonical DNA substrates, allowing differentiation between methyltransferase activity and DNA binding. By comparison, the Dnmt1^{C1229W} catalytic site mutant showed DNA-binding activity, but no irreversible covalent complex formation. With this assay, we could also confirm the preference of Dnmt1 for hemimethylated CpG sequences. The rapid optical read-out in a multi-well format and the possibility to test several different substrates in direct competition allow rapid characterization of sequence-specific binding and enzymatic activity.

INTRODUCTION

The modification of DNA by DNA methyltransferases is widespread and has a variety of biological functions (1). In bacteria, DNA methylation is involved in host defense mechanisms and strand discrimination during mismatch repair. In eukaryotic cells, DNA methylation is part of a highly complex epigenetic network regulating genome structure and activity (2,3). In contrast to the bacterial enzymes, eukaryotic DNA methyltransferases contain large regulatory domains that are involved in numerous intermolecular interactions and control enzyme activity through a largely unknown mechanism (4). The biochemical and cell biological characterization of DNA methyltransferases is pivotal for the understanding of epigenetic network regulation.

The basic biochemistry of the 5-methyl cytosine (5mC) methylation reaction is by now well understood. In a post-replicative reaction, DNA methyltransferases catalyze the transfer of a methyl group from *S*-adenosyl-L-methionine (AdoMet) to the C5 position of the nucleobase. During this multi-step reaction, the target cytosine is flipped out of the double helix (base flipping) and the recipient C5 position is activated by a transient, covalent complex formation with the enzyme at the C6 position (5,6). After methyl group transfer, the enzyme is released by β-elimination together with the proton at the C5 position. This last and crucial step of the enzymatic reaction can be exploited for a specific and mechanism-based inhibition with DNA substrates containing nucleotide analogs like 5-aza-dC or zebularine that are missing the essential proton at the C5 position (7–9). Although the catalytic mechanism of the 5mC DNA methyltransferases is known, the crucial question how eukaryotic enzymes recognize and discriminate target sites for methylation remains elusive.

Over the past decades, a variety of biochemical assays has been developed to determine the activity of DNA methyltransferases. The most commonly used methyltransferase activity assays measure the transfer of radioactively labeled methyl groups from the cofactor AdoMet to DNA substrates (10–14). Alternatively, DNA methylation by active methyltransferases can be monitored as protection against nucleolytic cleavage by restriction enzymes. The amount of methylated DNA can be measured as

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release or retention of terminal affinity probes of DNA substrates (15,16). Another indirect approach uses bisulfite treatment followed by incorporation and detection of hapten-labeled dCTPs at non-converted sites (17). Also direct detection of methylated cytosine residues by MALDI-TOF mass spectrometry (18) or monitoring of conversion of AdoMet to *S*-adenosyl-homocysteine (AdoHcy) by liquid chromatography and mass spectroscopy has been used (19). All these methods depend on either radioisotopes, expensive and demanding equipment, and/or multiple-step protocols.

Here, we present a simple, non-radioactive and versatile method to measure DNA methyltransferase activity. The assay measures methyltransferase activity as irreversible covalent complex formation with fluorescently labeled DNA substrates containing the mechanism-based inhibitor 5-aza-dC. The variation of DNA sequence and fluorescent label allows detection of DNA sequence specificity and discrimination of methyltransferase activity from DNA binding. We tested this assay using mammalian DNA methyltransferase 1 and mutants thereof.

MATERIALS AND METHODS

Expression vectors

The eukaryotic expression vectors for enhanced GFP (pEGFP-C1, Clontech, USA) and fusions with mouse Dnmt1 and its catalytically inactive mutant Dnmt1^{C1229W} were previously described (7). For GFP expression in bacteria, the pRSET-EGFP vector was generated. The GFP-coding sequence was amplified from pEGFP-C1 by PCR to add flanking XbaI/EcoRI restriction sites and a C-terminal His_6-tag. The PCR fragment was digested with XbaI and EcoRI and subsequently ligated into the bacterial expression vector pRSET (Clontech, USA).

Cell culture and transfection

Human embryonic kidney (HEK) 293T cells were cultured in DMEM supplemented with 10% fetal calf serum and 50 μg/ml gentamycine (PAA, Germany). HEK 293T cells were transiently transfected with expression plasmids for GFP, GFP-Dnmt1 and GFP-Dnmt1^{C1229W} using polyethylenimine as transfection reagent (Sigma, Germany) (20). After 48 h, about 80–90% of the cells were expressing GFP as determined by fluorescence microscopy. Cells were harvested, washed twice with PBS and stored at −80°C.

GFP purification

A 2 l culture of BL21 (DE3) *Escherichia coli* transformed with pRSET-EGFP was grown to OD 0.6 and induced with 1 mM IPTG for 20 h at RT. Bacteria were harvested and resuspended in 20 ml of binding buffer (500 mM NaCl, 20 mM imidazole, 1 mM PMSF in PBS). Lysis of *E. coli* was performed by sonification in the presence of 1 μg/ml lysozyme and 25 μg/ml DNase I. After centrifugation, 10 ml of soluble *E. coli* protein extract was loaded onto a His-Trap HP column containing 1 ml of Ni-NTA resin (GE Healthcare, Germany) using an ÄKTA purifier (GE Healthcare, Germany). After extensive washing of the bound material, the protein was eluted with elution buffer (500 mM NaCl, 250 mM imidazole in PBS) and 1 ml fractions were collected. Aliquots of elution fractions were subjected to SDS–PAGE and coomassie brilliant blue staining. Pure fractions of GFP were pooled and dialyzed three times against 1 l of PBS. The GFP concentration was determined by an analytical SDS–PAGE and coomassie brilliant blue staining with carbonic anhydrase as concentration standard.

Preparation of DNA substrates

DNA oligonucleotides were purchased from Metabion (Germany) or from IBA (Germany) and the sequences are listed in Table 1. Double-stranded DNA substrates were synthesized by primer extension using the large (Klenow) fragment of *E. coli* DNA polymerase I (Figure 1, Supplementary Figure 1A).

To prepare the DNA substrates, one upper (CG-up or MG-up) and one lower strand (Fill-In, Fill-In-550 or Fill-In-647N) oligonucleotide were denatured in NEB2 buffer (50 mM NaCl, 10 mM Tris–HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol) for 2 min at 95°C and annealed by slowly cooling down to 37°C. Upon addition of 0.05 u/μl Klenow fragment (NEB, Germany), dTTP, dGTP, dATP (PeqLab, Germany) at 1 mM final concentration, and either CTP at 1 mM, 5-aza-dCTP or 5-methyl-dCTP at 50 μM (Jena Bioscience, Germany), the Fill-In oligonucleotide was extended to produce either unmethylated, hemimethylated or fully methylated canonical DNA substrates or un- or hemimethylated suicide DNA substrates containing 5-aza-dC at the CpG site. 5-aza-dC containing suicide DNA substrates are referred to as 'trapping substrates' and DNA substrates not containing 5-aza-dC as 'binding substrates'. The design of the oligonucleotides allows the preparation of 15 different unlabeled, ATTO550 or ATTO647N labeled substrates with only five different oligonucleotides (Supplementary Figure 1B). Hemimethylated ATTO550 labeled and

Table 1. Sequences of DNA oligonucleotides used for preparation of double-stranded DNA substrates (M, 5-methylcytosine)

CG-up	5′-CTCAACAACTAACTACCATCCGGACCAGAAGAGTCATCATGG-3′
MG-up	5′-CTCAACAACTAACTACCATCMGGACCAGAAGAGTCATCATGG-3′
Fill-In-550	5′-ATTO550-CCATGATGACTCTTCTGGTC-3′
Fill-In-647N	5′-ATTO647N-CCATGATGACTCTTCTGGTC-3′
Fill-In	5′-CCATGATGACTCTTCTGGTC-3′

unmethylated ATTO647N labeled binding and trapping substrates were therefore prepared as described earlier, using MG-up and Fill-In-550 or CG-up and Fill-In-647N oligos. Unlabeled hemimethylated competitor DNA substrate was prepared using MG-up and Fill-In oligos.

Calibration measurements for GFP, ATTO550 and ATTO647N

Calibration curves for the fluorescent DNA substrates and proteins were determined by measuring the fluorescence signal of known concentrations of the DNA-coupled fluorophores and purified GFP and calculated by linear regression. For this, we used the PolarStarOptima fluorimeter (BMG, Germany) and the following excitation/emission band path filter sets: 485 ± 8 nm/520 ± 17 nm for GFP, 545 ± 5 nm/575 ± 5 nm for ATTO550 and 645 ± 5 nm/675 ± 5 nm for ATTO647N. The beads do not cause fluorescence background, and within the measurement error, no change of fluorescence intensity of the ATTO dyes was observed upon addition of beads. Interestingly, the GFP fluorescence signal is enhanced by binding to the GFP-binding protein (GBP), which is the active part of the GFP nanotrap. With the indicated filter set for GFP detection, the fluorescence signal is about 1.7 times enhanced (Supplementary Figure 3). This effect was taken into account for later conversion of the fluorescent signal into fluorophore concentration and calculation of binding and trapping rates as the ratio of ATTO and GFP signal.

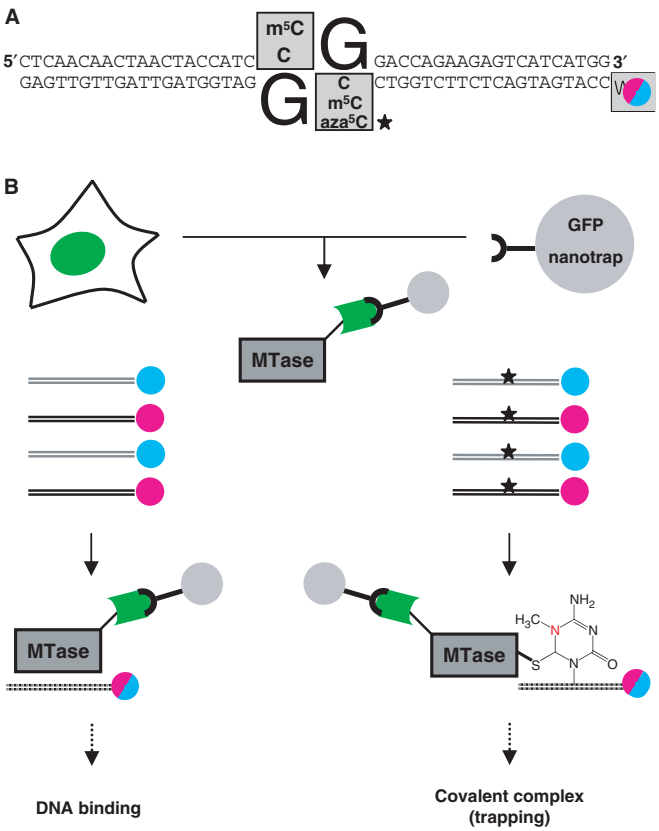

Figure 1. Outline of the binding and activity assay. The covalent complex formation is the first and crucial step of the methylation reaction. The incorporation of the mechanism-based inhibitor 5-aza-dC (depicted as a star) in DNA substrates leads to an irreversible complex formation with catalytically active DNA methyltransferase (trapping). Capture and detection of this reaction intermediate thus serves as a measure of enzyme activity. (**A**) Un-, hemi- or fully methylated canonical or 5-aza-dC containing double-stranded DNA substrates (binding and trapping substrates, respectively) are 42 base pairs long including one central CpG site and can be unlabeled, labeled with ATTO550 or labeled with ATTO647N. The asterisk marks 5-aza-dC. (**B**) The GFP fusion protein of interest, e.g. a DNA methyltransferase (MTase), is purified from cell lysates using a GFP nanotrap and incubated with binding or trapping DNA substrates. After pull-down of protein–DNA complexes, unbound DNA substrate is removed by two washing steps. Protein and DNA substrate amounts are calculated from fluorescence measurements of GFP, ATTO550 and ATTO647N, respectively.

Pull-down of GFP or GFP fusion proteins

Extracts from $\sim 1 \times 10^7$ cells were prepared by resuspension and incubation of the cell pellet in 200 µl lysis buffer (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 2 mM PMSF, 0.5% NP40, 1× mammalian protease inhibitor mix) for 30 min on ice. After centrifugation, supernatants were diluted to 500 or 1000 µl with immunoprecipitation buffer (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.5 mM EDTA). Extracts were incubated with 1 µg of a GBP coupled to agarose beads (GFP nanotrap; Chromotek, Germany) (21) for 1–2 h at 4°C with constant mixing. GFP or GFP fusion proteins were pulled down by centrifugation at 540*g*. The beads were washed twice with 1 ml of wash buffer (20 mM Tris–HCl pH 7.5, 300 mM NaCl, 0.5 mM EDTA). The amount of protein on the beads was determined with the PolarStarOptima fluorimeter after resuspension in 100 µl wash buffer or by western blot. In the latter case, beads were resuspended in 2× Laemmli buffer (22) and 25% was loaded onto a 6% SDS–PAGE. After blotting to a nitrocellulose membrane, GFP-Dnmt1 was detected with a specific antibody against Dnmt1 (kindly provided by Nowak, D. and Cardoso, M.C.) and an HRP-labeled secondary antibody.

Binding and trapping assay

The pull-down of GFP or GFP fusion protein was performed as described earlier. After the second washing step, beads were equilibrated with assay buffer (100 mM KCl, 10 mM Tris–HCl pH 7.6, 1 mM EDTA, 1 mM DTT). For determination of binding and trapping rates, the beads were resuspended in 500 or 1,000 µl of assay buffer supplemented with 160 ng/µl BSA and 100 µM *S*-adenosyl-L-methionine (AdoMet), and 0.1 µM binding or trapping DNA substrate, unless indicated otherwise. For qualitative determination of DNA methyltransferase activity, binding (with canonical-binding substrates) and trapping (with suicide trapping substrates) were performed at 37°C for 90 min, unless indicated otherwise. After washing twice with assay buffer to remove unbound substrate, beads were resuspended in 100 µl assay buffer and transferred into a 96-well microplate. The amounts of protein and DNA were determined by fluorescence measurements and comparison to a calibration curve.

Binding competition assay

Trapping and binding assays were performed as described earlier, except that for binding competition, referred to as binding or trapping with competitor, a further incubation step with 1 μM hemimethylated unlabeled binding DNA was performed for 45 min at 37°C to compete for binding of labeled non-covalently bound substrate in the binding and trapping sample. Before fluorescence measurement, two final washing steps with assay buffer were performed.

RESULTS AND DISCUSSION

Assay design

We previously generated a set of fluorescent Dnmt1 fusions and mutants thereof and characterized their cell-cycle dependent dynamics in living cells (23,24). To complement these data and to gain further insights into the structure, function and regulation of DNA methyltransferases, it is crucial to determine their sequence specific DNA binding and methyltransferase activity. For fast biochemical characterization of these GFP fusion proteins, we developed a simple, non-radioactive assay.

The assay is based on immunoprecipitation of fusion proteins with a GBP coupled to agarose beads [GFP nanotrap (21)]. Bound GFP fusion proteins were incubated with fluorescently labeled double-stranded DNA substrates. After removal of unbound substrate, the concentrations of fluorescent protein and bound DNA substrate were measured with a filter-based fluorescence spectrometer in a multi-well format (Figure 1).

The design of DNA oligonucleotides combined with a primer extension method allows preparation of a variety of substrates (Figure 1A, Supplementary Figure 1). Canonical DNA substrates (binding substrates) were used for binding studies and suicide DNA substrates containing 5-aza-dC at the CpG site (trapping substrates) for monitoring irreversible covalent enzyme–DNA complex formation as the first and crucial step of the DNA methylation reaction. The capture of these reaction intermediates serves as a measure of enzyme activity, although the final step of the methylation reaction, the methyl group transfer, is not detected. As DNA substrates can be labeled with different fluorophores, several different sequences, containing, e.g. un-, hemi- or fully methylated CpG sites, can be tested in direct competition. The fluorescence of protein and substrate allows direct determination of concentrations, molar ratios and specific activity.

Linear GFP-Dnmt1 pull-down with the GFP nanotrap

The GFP nanotrap allows fast and efficient one-step purification of GFP or GFP fusion proteins. For demonstration of linearity, we incubated a constant amount of the GFP nanotrap with different lysate volumes of GFP-Dnmt1 overexpressing HEK 293T cells and determined the concentration of GFP fusion protein bound by the beads. The amount of bound GFP-Dnmt1 did indeed increase linearly with the amount of lysate added, as quantified by fluorescence detection and western blot. Importantly, quantification with a fluorescence plate reader was very sensitive and showed a larger linear range than the corresponding western blot (Figure 2). This demonstrates the strength of the fluorescence-based readout of this assay. The exact quantification of the protein input allows the comparison of different samples and takes into account possible differences in pull-down efficiency.

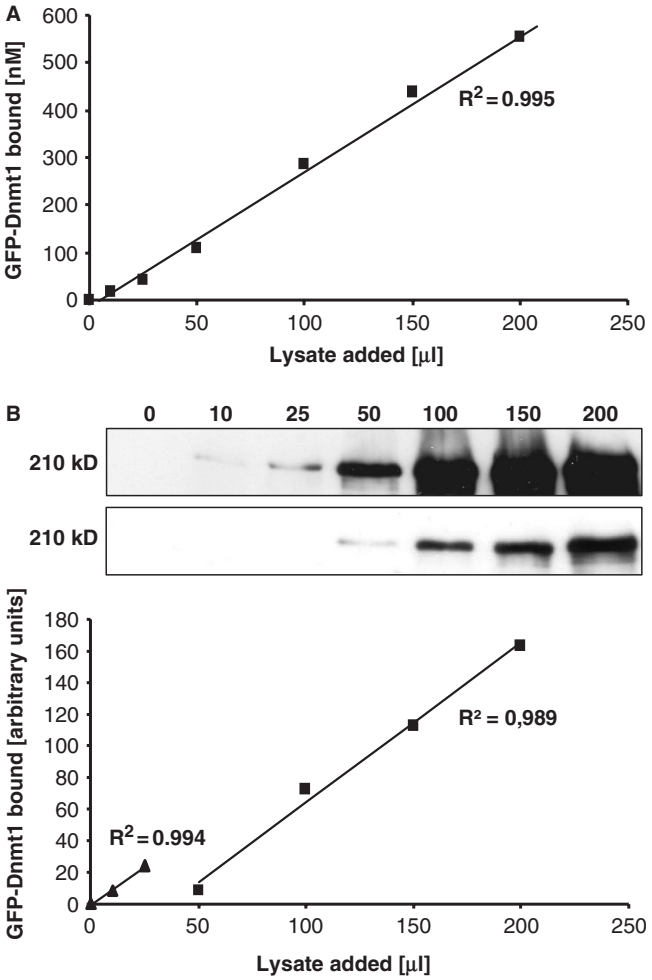

Figure 2. Linear GFP-Dnmt1 pull-down. Different amounts of cell lysate (0, 10, 20, 25, 50, 100, 150 and 200 μl) from GFP-Dnmt1 overexpressing HEK 293T cells were incubated with constant aliquots of the GFP nanotrap. (**A**) The concentration of precipitated GFP-Dnmt1 was calculated from the measured intensity of the GFP fluorescence signal. (**B**) Aliquots of the same samples were analyzed by western blot with an anti-Dnmt1 antibody. Shown are two different exposure times (2 min and 15 s). The band intensities were quantified with the Image J software using the higher exposure time for data points 0, 10, 25 and the lower exposure time for data points 50, 100, 150 and 200.

Characterization and optimization of assay conditions

To optimize assay conditions, we first determined the time course of DNA binding and irreversible covalent complex formation (trapping) of GFP-Dnmt1 with hemimethylated DNA substrates. The time course of GFP-Dnmt1 binding to hemimethylated substrate followed the classical-binding kinetics with an observed rate constant of $k = 0.034 \pm 0.002\,\mathrm{min}^{-1}$ (Supplementary Figure 2A).

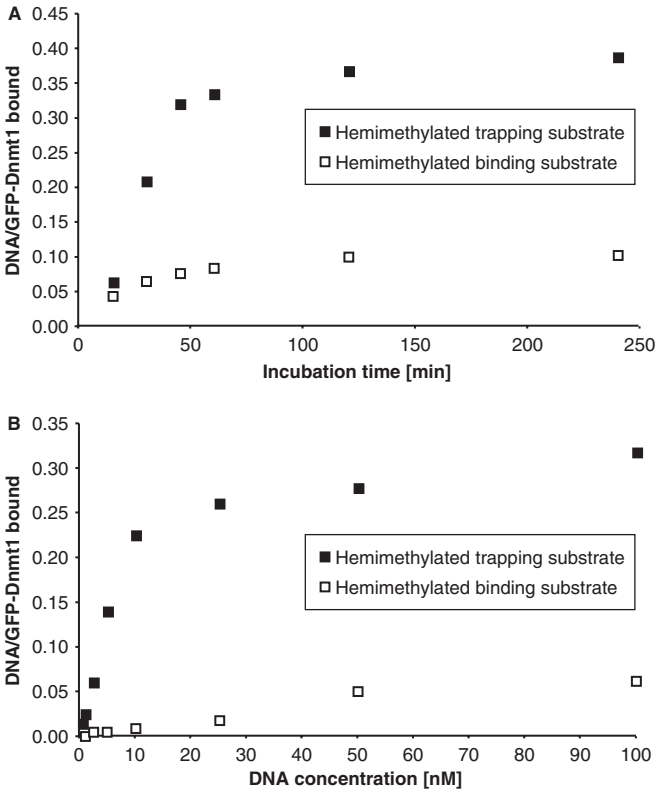

Figure 3. Optimization of trapping assay conditions. (**A**) Time course of binding and trapping reaction. GFP-Dnmt1 (25 nM) was incubated with 100 nM hemimethylated ATTO550 labeled binding (open square) or trapping substrate (filled square). The reactions were stopped by washing after 15, 30, 45, 60, 120 and 240 min, respectively. (**B**) Dependence of binding and trapping rate on the initial DNA substrate concentration. GFP-Dnmt1 (20 nM) was incubated with increasing amounts of hemimethylated ATTO550 labeled binding (open square) or trapping substrate (filled square). Binding and trapping rates are shown for initial substrate concentrations of 0.5, 1, 2.5, 5, 10, 25, 50 and 100 nM.

The trapping rate (ratio of bound suicide DNA substrate per protein) increased linearly within the first 50 min of reaction and reached a plateau at about 90 min (Figure 3A). For substrate specificity and qualitative methyltransferase activity assays, we chose 90-min incubation time to obtain maximal signals. For determination of initial reaction velocities, shorter incubation times were used to stay within the linear range of this assay.

To test the dependence of binding and trapping rate on the initial DNA substrate concentration, we incubated a constant amount of GFP-Dnmt1 with hemimethylated trapping substrate at different concentrations (Figure 3B). The fitting of binding data is shown in Supplementary Figure 2B. For substrate concentrations below the concentration of methyltransferase molecules, the trapping rate increased linearly with the substrate concentration until a plateau was reached at excess concentration of DNA substrate. Likewise, in the presence of an excess of DNA substrate, the concentration of bound fluorescent DNA increased with the amount of precipitated methyltransferase (Supplementary Figure 4), indicating that the trapping rate is constant in this range.

To test for unspecific DNA binding, we incubated a constant amount of the GFP nanotrap with increasing volumes of cell lysate from GFP overexpressing HEK 293T cells followed by incubation with trapping substrate. The concentration of precipitated GFP increased linearly with the amount of lysate added. In contrast, the minor unspecific binding of substrate was shown to be independent of the amount of precipitated protein (Supplementary Figure 5). The unspecific binding to the agarose beads was below the detection limit for DNA coupled ATTO647N (Supplementary Figure 5B and D) and negligible for DNA coupled ATTO550 (Supplementary Figure 5A and C), when compared with the values obtained for binding to GFP-Dnmt1 and its mutant GFP-Dnmt1^{C1229W}. Thus, the minor unspecific binding is attributable to the agarose beads rather than to the protein indicating that different amounts of precipitated GFP fusions can be compared reliably. The trapping rates were slightly dependent on the lysate preparation likely reflecting the percentage of active enzyme, but highly reproducible results were obtained with independent samples from the same experimental setup.

Discrimination of enzymatic activity-dependent trapping from DNA binding

To evaluate the possibility to distinguish between DNA binding and covalent complex formation, the crucial first step of the methyl transfer reaction, we incubated GFP-Dnmt1 and the catalytic site mutant GFP-Dnmt1^{C1229W} with DNA binding and trapping substrates and measured the fluorescence after precipitation (Figure 4A). Interestingly, wild-type and mutant protein showed similar specific DNA-binding activity. However, GFP-Dnmt1 showed a higher trapping than binding rate, whereas GFP-Dnmt1^{C1229W} did not. The difference between binding and trapping rate is due to the accumulation of covalent protein-DNA complexes over time, and thus confirms previously published results on Dnmt1 and its catalytic site mutant (7).

The trapping rate obtained for the active methyltransferase GFP-Dnmt1 after 90 min at excess initial substrate concentration reflects almost exclusively covalently bound DNA substrate. This was demonstrated by an additional competition step with unlabeled binding substrate to compete with non-covalently bound labeled substrate (Figure 4B). The maximal trapping rate after this binding competition step did not change, whereas the maximal binding rate decreased proportionally. These results show that the combination of DNA binding and trapping substrates with non-fluorescent competitors allows the distinction between DNA binding and enzyme activity dependent covalent complex formation of DNA methyltransferases.

Cofactor dependence of covalent complex formation

Covalent complex formation of cytosine methyltransferases with DNA has been shown to be independent from the cofactor AdoMet. In the absence of AdoMet, the activated cytosine undergoes hydrogen exchange

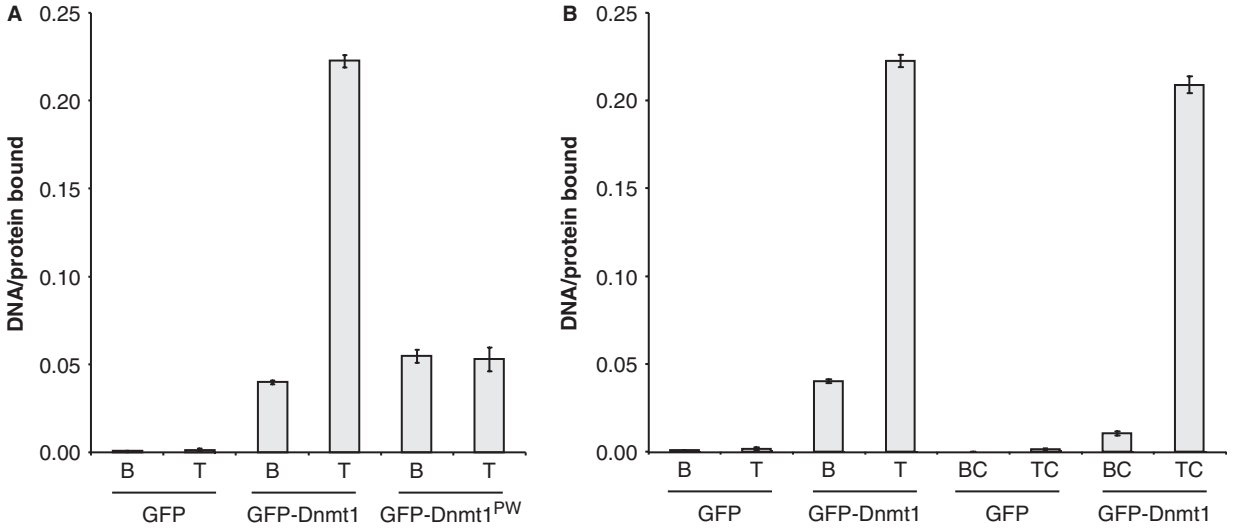

Figure 4. Binding and trapping assay with competitors. (**A**) Binding and trapping assays were performed with GFP-Dnmt1 and GFP-Dnmt1^{C1229W} and hemimethylated ATTO550 labeled DNA. Shown are the means of maximal binding and trapping rates with standard error bars from three independent experiments for the GFP control and GFP-Dnmt1 and two independent experiments for GFP-Dnmt1^{C1229W}. (**B**) Assays with substrates for binding [B], trapping [T], binding with competitor [BC] and trapping with competitor [TC] were performed with GFP-Dnmt1 as described earlier. Shown are the means with standard error bars from three independent experiments. GFP was used as negative control.

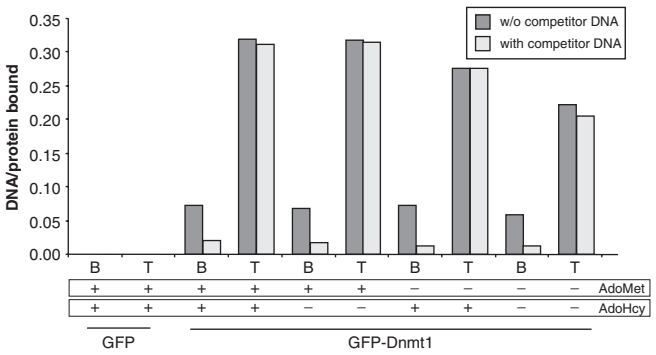

Figure 5. Covalent complex formation in dependence on AdoMet and AdoHcy. Maximal binding and trapping rate were determined for GFP-Dnmt1 and hemimethylated ATTO550 labeled DNA substrate with or without unlabeled competitor DNA. The assay buffer was supplemented with 10 μM AdoMet or AdoHcy as indicated. GFP was used as negative control.

instead of methylation at position 5. AdoMet as well as its analog and competitor *S*-Adenosyl-L-homocysteine (AdoHcy) significantly bind to the enzyme only after the DNA substrate is bound (25–27). We tested GFP-Dnmt1 binding and trapping with hemimethylated DNA substrate and compared maximal rates at different conditions (Figure 5). An additional competition step with unlabeled competitor DNA to compete for non-covalently bound labeled DNA was included to monitor irreversible covalent complex formation. In accordance with the prior biochemical studies (25–27), we found that GFP-Dnmt1 forms a covalent complex with DNA in the presence and absence of AdoMet and AdoHcy, albeit at different efficiencies. Similarly, this assay could be used for inhibitor studies and to screen for small molecules that prevent covalent enzyme-DNA complex formation.

Competition assay to directly determine substrate preference

A unique feature of this method is the possibility to compare different DNA substrates in direct competition. The trapping rates of GFP-Dnmt1 with either un- or hemimethylated DNA trapping substrate or with both substrates in direct competition clearly showed a preference for hemimethylated DNA (Figure 6A). This result demonstrates that substrate preference can be detected in a single measurement by direct competition. Interestingly, the preference for hemimethylated DNA was only pronounced in the rate of covalent complex formation (trapping assay) and not in the DNA-binding assay. The direct competition of un- and hemimethylated DNA-binding substrates revealed even a slight preference of GFP-Dnmt1 for unmethylated substrate (Figure 6B). The substrate preference of GFP-DNMT1 was tested in four independent experiments and revealed on average about 15-fold higher activity on hemimethylated than on unmethylated DNA substrate (Figure 6C). These results are consistent with data obtained with previous biochemical activity assays measuring the transfer of radioactively labeled methyl groups by purified Dnmt1 or GFP-Dnmt1 and catalytic site mutants (28–31).

In summary, we present a novel, non-radioactive assay for fast characterization of DNA methyltransferase activity and DNA binding. We show that the DNA binding, substrate specificity and activity of DNA methyltransferases fused with GFP can reliably be measured with this method. The simplicity and versatility of this assay allows fast and inexpensive screening of enzymes, complexes and mutants. By careful selection of fluorophores with distinct excitation and emission spectra, multiple fluorescent substrates can be analyzed simultaneously in direct competition. We applied the assay to the mammalian Dnmt1 and confirmed its preference for DNA

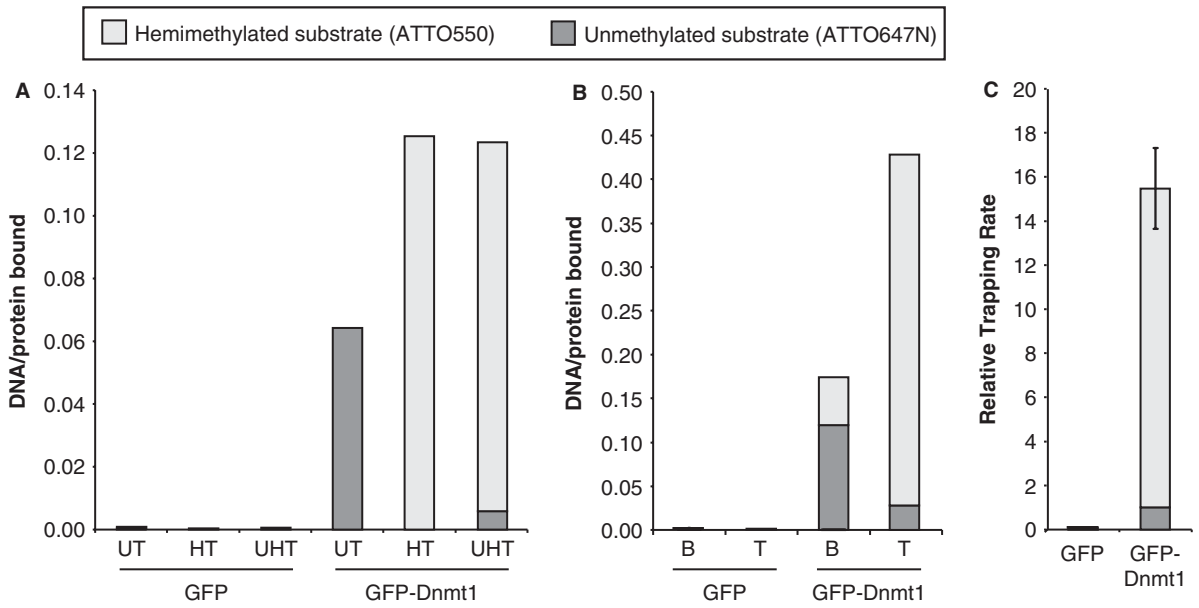

Figure 6. Substrate preference of GFP-Dnmt1. (**A**) Maximal trapping rates were determined by incubation of constant concentrations of GFP-Dnmt1 with unmethylated ATTO647N labeled DNA trapping substrate [UT], hemimethylated ATTO550 labeled DNA trapping substrate [HT] or unmethylated ATTO647N and hemimethylated ATTO550 labeled DNA trapping substrate in competition [UHT]. (**B**) Maximal binding and trapping rates for GFP-Dnmt1 are given for incubation with either unmethylated ATTO647N or hemimethylated ATTO550 labeled binding substrate (B), or unmethylated ATTO647N and hemimethylated ATTO550 labeled trapping substrate [T] in competition. (**C**) The trapping rates for GFP-Dnmt1 on unmethylated ATTO647N and hemimethylated ATTO550 labeled trapping substrate in competition were determined in four independent experiments. The value for unmethylated substrate was set to one and the relative rate for hemimethylated substrate was calculated accordingly. Means of the relative trapping rates are shown with standard error bars. GFP was used as negative control.

substrates containing hemimethylated CpG sites. In addition, we could show that the active site mutation (C1229W) abolishes covalent complex formation, but not DNA binding. The usage of GFP fusion proteins allows a direct link of biochemical data to cell biological data on subcellular localization and mobility of the very same molecule obtained by fluorescence microscopy and photobleaching experiments. However, endogenous DNA methyltransferases could analogously be assayed by incubation with fluorescent binding and/or trapping substrates and subsequent precipitation with specific antibodies. Alternatively, samples incubated with fluorescent trapping substrates could also be separated by SDS–PAGE and catalytically active methyltransferases could be detected in gel and identified by western blot or mass spectrometry. This assay can easily be adapted for general DNA- and RNA-binding studies providing a time-saving alternative to electrophoretic gel shift assays (32).

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. H.L. is a co-founder of Chromotek.

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