J|A|C|S COMMUNICATIONS

Published on Web 02/20/2007

Targeted Labeling of DNA by Methyltransferase-Directed Transfer of Activated Groups (mTAG)

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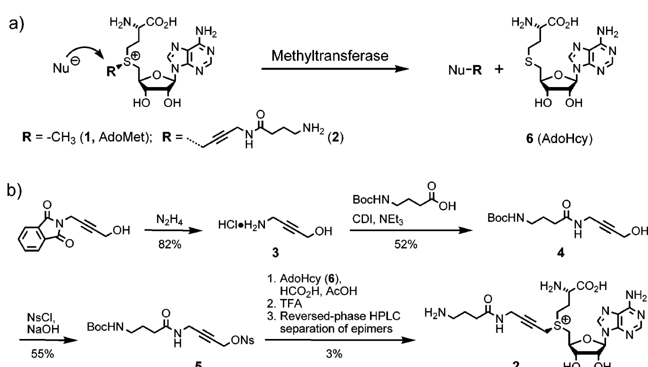
Methyltransferases (MTases) catalyze highly specific transfers of methyl groups from the ubiquitous cofactor *S*-adenosyl-L-methionine (AdoMet, **1**) to various nucleophilic positions in biopolymers like DNA, RNA, and proteins (Scheme 1a). Being the second most ubiquitous cofactor after ATP, AdoMet (**1**) is involved in numerous essential biochemical processes in all living organisms.[1] Despite the high importance of transmethylation reactions in biology, the naturally transferred methyl group has a very limited utility for practical applications. But the ability of MTases to catalyze highly specific covalent modifications of biopolymers makes them attractive molecular tools provided that transfer of larger chemical entities can be achieved. Recently, we have demonstrated that allylic and propargylic side chains can be efficiently transferred by DNA MTases with high sequence- and base-specificity.[2]

Here we describe the chemical synthesis of a prototype AdoMet analogue with an extended propargylic side chain carrying a primary amino group. Such an amino function can be used for regiospecific chemoligation reactions with a variety of amine-reactive probes to attach desired reporter groups onto DNA or RNA.[3] The synthesis of AdoMet analogue **2** (Scheme 1b) started from 4-phthalimidobut-2-yn-1-ol,[4] which was converted into 4-aminobut-2-yne-1-ol (**3**) by hydrazinolysis. The product was extended by carbodiimide (CDI) coupling with *N*-Boc-protected γ-aminobutyric acid. Triflate activation of the resulting N-protected aminoalcohol **4** proved unsuitable because of a high reactivity of the internal amide, and a milder activator, 4-nitrophenylsulfonyl chloride (NsCl), was found to be effective. The activated N-protected aminoalcohol **5** was directly coupled with AdoHcy **6** under acidic conditions,[2] the Boc protecting group was removed by acid treatment, and the epimers at the sulfonium center were separated by reversed-phase HPLC.

The novel cofactor analogue **2** was tested using representatives of two classes of DNA MTases, namely the DNA cytosine-5 MTase M.HhaI (target sequence GCGC) and the DNA adenine-N6 MTase M.TaqI (TCGA). Enzymatic transalkylations were monitored using a DNA protection assay, which takes advantage of the resistance of MTase-modified target sites to cleavage with cognate restriction endonucleases.[2] As in the case of the natural cofactor AdoMet **1**,[1] only one of the two sulfonium epimers was active with the MTases (Supporting Figure 1). On the basis of the stereochemical assignment of AdoMet **1** to the (*S*)-configuration at sulfur,[5] we assign the active cofactor analogue **2** to the (*S*)-epimer, and all further experiments were performed with this compound. Substoichiometric amounts of both M.HhaI (variant Q82A/N304A) and M.TaqI are sufficient for complete modification of the target sites in pBR322 DNA in 1 h, demonstrating that the transfer reactions are catalytic (Supporting Figure 2). Moreover, the plasmid stayed in its native supercoiled form after such a treatment (not shown). The structure of the modified nucleosides that are produced with the new cofactor analogue **2** and the two MTases was verified by HPLC−MS analysis[2] confirming that the enzymatic transalkylation of DNA occurred in a base- and sequence-specific manner (Supporting Figure 3). Altogether our data demonstrate that methyltransferase-directed transfer of activated groups (mTAG) is a convenient and robust technique for routine laboratory use.

Scheme 1 [a]



[a] Reaction a: methyltransferases (MTases) naturally catalyze the transfer of a methyl group (R = CH_3) from AdoMet (**1**) to numerous nucleophiles (Nu) in DNA, RNA, proteins, or small biomolecules Transfer of a primary amino group is achieved using the synthetic AdoMet analogue **2** in which the methyl group is replaced by an extended propargylic side chain. Reaction b: synthesis of AdoMet analogue **2.**

The utility of AdoMet analogue **2** is readily demonstrated by labeling the amino-functionalized DNA with reporter groups using amine-reactive probes. For example, the pBR322 DNA premodified with M.HhaI variant Q82A/N304A or M.TaqI in the presence of cofactor **2** was further derivatized with a cyanine-5 fluorophore by treatment with a commercial NHS-ester. Figure 1a shows that the fluorescence intensity distribution in four pBR322-GsuI fragments is fully consistent with the positions and numbers of the M.HhaI and M.TaqI sites in the original plasmid (see also Supporting Table online). This observation confirms sequence-specific and quantitative incorporation of the fluorophore. Sequence-specific MTase-dependent covalent labeling of native DNA can also be achieved with aziridine cofactor mimics,[6] which lead to coupling of the whole cofactor to DNA. However, strong product inhibitors are generally formed with these cofactors, and the DNA MTases have to be used in stoichiometric amounts with respect to their recognition sequences.

In addition, we used the mTAG technique for DNA methylation detection. pBR322 was treated with AdoMet analogue **2** and M.TaqI, and the amino-functionalized plasmid was labeled with NHS-biotin. In parallel, pBR322 was premethylated with the natural cofactor AdoMet **1** and the BseCI DNA MTase (M.BseCI), then

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10.1021/ja0691876 CCC: $37.00 © 2007 American Chemical Society

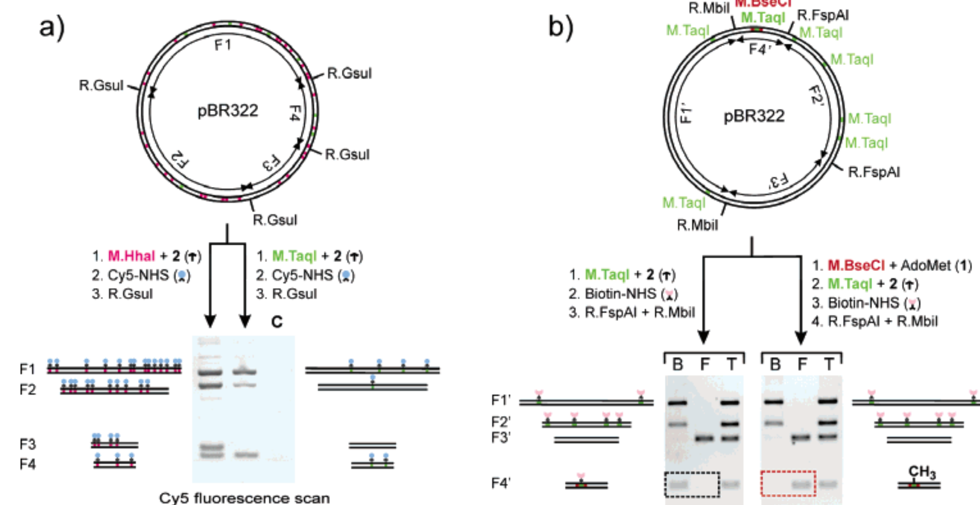

Figure 1. Sequence-specific two-step labeling of plasmid DNA. (a) pBR322 DNA was amino-modified with M.HhaI or M.TaqI in the presence of cofactor **2** and then treated with a cyanine-5 *N*-hydroxysuccinimide ester (Cy5-NHS). Labeled DNA was fragmented with R.GsuI endonuclease to produce fragments F1−F4 and analyzed by agarose gel electrophoresis. Imaging of the Cy5 fluorescence was performed using a 635 nm laser scanner. A DNA fragment (F3) containing no M.TaqI sequences is not visible in the scan. Part C shows unmodified pBR322 DNA treated with Cy5-NHS followed by R.GsuI fragmentation. (b) pBR322 DNA was amino-modified with M.TaqI in the presence of cofactor **2** and then treated with biotin-NHS. Labeled DNA was fragmented with R.FspAI and R.MbiI endonucleases to produce fragments F1′−F4′. The left panel shows an analysis by agarose gel electrophoresis of all DNA fragments (T = total), fragments not retained on streptavidin-coated magnetic beads (F = flow through), and fragments recovered from streptavidin-coated magnetic beads by extraction with phenol/chloroform (B = bound). The right panel shows the same analysis except that pBR322 DNA premethylated with M.BseCI and AdoMet **1** was used. The changed distribution of fragments shows that M.TaqI-specific biotinylation is blocked at the premethylated M.BseCI site.

amino-functionalized with M.TaqI and treated with NHS-biotin. M.BseCI methylates the second adenine residue within the hexanucleotide 5′-ATCGAT-3′ sequence, which overlaps with one of the seven M.TaqI sites in pBR322. Both plasmids were cleaved with the R.FspAI and R.MbiI endonucleases, and the fragments were passed over streptavidin beads to selectively capture biotin-containing fragments (Figure 1b). Indeed our experiment shows that quantitative M.TaqI-specific biotinylation is achieved. Most importantly, a different distribution pattern is observed with the M.BseCI-premethylated plasmid: the shortest fragment (F4′) is no longer retained on streptavidin-coated beads but is detected in the flow-through fraction. Clearly, enzymatic methylation by M.BseCI blocks the single M.TaqI site on this fragment for amino-functionalization by M.TaqI and subsequent biotinylation, and therefore methylated and unmethylated target sites can be quantitatively discriminated. This result demonstrates the utility of mTAG labeling to query the methylation status of specific sequences in DNA, paving the way to novel approaches for methylation profiling of genomic DNA.

In conclusion, we demonstrate that the novel synthetic AdoMet analogue **2** in combination with DNA MTases can serve as a versatile laboratory tool for highly specific labeling of large natural DNAs. The covalent amino-functionalization offers a wide diversity of reporters that can be attached to DNA via NHS ester chemistry including fluorophores and affinity probes. Furthermore, the synthetic scheme is readily adapted for attaching other widely used abiotic functionalities, such as terminal alkyne, azide, ketone or maleimide[7] (in preparation). Another advantage of this chemistry is that linear chains are introduced as linkers, which is expected to cause minimal steric perturbance to the labeled biomolecules. For example, no interference with the DNA polymerase action at amino-modified cytosine residues was detected when such DNA was used as a sequencing template (not shown). On the other hand, many more DNA, RNA, and protein MTases are active with this class of synthetic AdoMet analogues (in preparation). REBASE[8] currently lists DNA MTases with over 200 different DNA recognition sequences, offering unprecedented experimental control over sequence-specific manipulation of DNA. This new class of molecular tools envisions many potential applications[9] ranging from probes for genetic screening to molecular building blocks in DNA-based nanobiotechnology.

Acknowledgment. The authors thank M. Krenevičienė for recording NMR spectra, J. Šlyžiūtė for assistance with preparing M.HhaI mutants, and Prof. M. Kokkinidis for a gift of M.BseCI. This work was supported by grants from the Internationale Büro des BMBF, the Ministry of Education and Science of Lithuania, and the Lithuanian State Science and Study Foundation.

Supporting Information Available: Supporting figures, methods for the synthesis of AdoMet analogue **2**, DNA labeling, and analysis of labeled DNA. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA0691876