inates the residual ribonuclease activity, although the effect of this treatment on the subsequent experiments should be checked. An inhibitor of RNase, such as Rnasin, can also be added to further enzymatic reactions.

This study has described a degrading activity in RNA preparations from ovine, bovine, and porcine brain and ovine liver. Further experiments on these species need to be undertaken to verify whether the ribonuclease activity is present in other tissues or not. RNA extracted according to protocols other than those of Chomczynski and Sacchi should also be tested in artiodactyls as well as in species from other orders.

REFERENCES

1. Chomczynski, P. and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156-159.
2. Huang, Y.H., P. Leblanc, V. Apostolou, B. Steward, and R.B. Moreland. 1995. Comparison of Milli-Q®PF Plus water to DEPC-treated water in the preparation and analysis of RNA. *BioTechniques* 19:656-661.
3. Wilfinger, W.W., K. Mackey, and P. Chomczynski. 1997. Effect of pH and ionic strength on the spectrophotometric assessment of nucleic acid purity. *BioTechniques* 22:474-481.
4. Groppe, J.C. and D.E. Morse. 1993. Isolation of full-length RNA templates for reverse transcription from tissues rich in RNase and proteoglycans. *Anal. Biochem.* 210:337-343.
5. Santokh, S.G., R.A. Aubin, C.A. Bura, I.H.A. Curran, and T.I. Matula. 1996. Ensuring recovery of intact RNA from rat pancreas. *Mol. Biotechnol.* 6:359-362.
6. Gruffat, D., C. Piot, D. Durand, and D. Bauchart. 1996. Comparison of four methods for isolating large mRNA: apolipoprotein B mRNA in bovine and rat livers. *Anal. Biochem.* 242:77-83.

Address correspondence to Dr. Gilles Bruneau, UMR 6073 Physiologie de la Reproduction et des Comportements, (INRA/CNRS/Université François Rabelais de Tours), 37380 Nouzilly, France. e-mail: bruneau@tours.inra.fr

Received 23 December 2002; accepted 11 February 2003.

**Delphine Pillon and
Gilles Bruneau**
INRA/CNRS/Université
François Rabelais de Tours
Nouzilly, France

Methylation Sensitivity of Restriction Enzymes Interacting with GATC Sites

BioTechniques 34:924-930 (May 2003)

DNA methylation plays an important role in many species ranging from bacteria to man, by controlling the expression of genes and protection of the genome from selfish DNA like transposons and viruses (1,2). In *E. coli* and other γ -proteobacteria, methylation of adenine residues (dam, DNA adenine methylation) occurs at GATC sites. It serves to discriminate between parental and daughter strand during post-replicative mismatch repair, to coordinate cell cycle, DNA replication, and to regulate gene expression (2,3). It also controls the pathogenicity of different γ -proteobacteria (4,5). To investigate the methylation state of DNA from various bacteria, archaea as well as lower and higher eukaryotes, digestion of the DNA with GATC-interacting restriction enzymes is often used (6-13). In addition to its biological functions, dam methylation and GATC-specific restriction enzymes have several important applications in biotechnology and molecular biology. (i) One of the most commonly used methods for site directed mutagenesis employs a specific digestion of dam-methylated DNA by *DpnI* (14-18). In this application, *DpnI* is used to digest the template DNA after PCR, which almost completely removes the background of wild-type plasmid used as PCR template. (ii) Since dam methylation does not occur in eukaryotes, it can be used to follow the replication of bacterial DNA transfected into eukaryotic cells (19-27). (iii) Transcription factors can be fused to the dam methyltransferase and transfected into cells. The resulting pattern of dam methylation allows one to deduce the DNA binding specificity of the transcription factor (28,29). In addition, the pattern of dam methylation in eukaryotic cells overexpressing the bacterial dam enzyme can be used to map the chromatin structure, because the dam methyltransferase is inhibited by nucleosomes (30-32).

To understand the biological processes related to dam methylation and to employ dam methylation for biotechnological purposes, characterization of the exact methylation status of target DNA is necessary. There are different tools to map methylated bases in DNA; one of the most commonly used methods is to digest the endogenous DNA with restriction enzymes, which are characterized with respect to their methylation sensitivity. Data on the methylation sensitivity (and many other important properties) of restriction enzymes are compiled in REBASE (<http://rebase.neb.com/rebase/>) (33). Unfortunately, methylation sensitivity was not easily available for some enzymes interacting with GATC sites, or inconsistent data were published (Table 1). Therefore, we decided to reinvestigate the methylation sensitivity of restriction enzymes acting on GATC sites with respect to the effects of adenine and cytosine methylation. Since we intended to distinguish between the effect of methylation of only one and both DNA strands, we chose modified oligonucleotides as substrates. Usage of synthetic substrates also ensures that complete methylation is present, which is not the case if enzymatically methylated DNA is used.

We used synthetic 19-mer oligonucleotide substrates (5'-CGCGGCCG-GATCCCCGGGC-3') that differ in the methylation status of the GATC sequence being unmethylated, methylated in one strand of the DNA (hemimethylated), or methylated in both DNA strands (fully methylated). The HPLC-purified oligonucleotides were purchased from Thermohybid (Heidelberg, Germany). One strand of each substrate was phosphorylated with T4-PNK and [γ -³²P]ATP and annealed to the other strand by heating to 75°C and slowly cooling to ambient temperature. By using hemimethylated substrates labeled in the unmethylated or methylated strand, we can distinguish between cutting of both strands and nicking of only one. *DpnI*, *DpnII*, and *Sau3AI* were from New England Biolabs (Beverly, MA, USA), *NdeII* from Sigma (St. Louis, MO, USA), and *MboI* from MBI Fermentas (Vilnius, Lithuania). For the cleavage reaction, different amounts of enzymes (0.01-20 U) and 30 nM DNA

Benchmarks

were used. The cleavage reaction was performed in a reaction volume of 10 μ L at 37°C for 1 h with the buffer recommended by the company. The cleavage products were separated on denaturing polyacrylamide gels (19% acrylamide, 1% bisacrylamide) containing 8 M urea that were run in 0.5 $\times$ TTE (20 $\times$: 2 M Tris, 575 mM taurine, 20 mM EDTA).

We have determined the methylation sensitivity of five restriction enzymes interacting with GATC sites. For each enzyme, five different substrates were tested: unmethylated DNA, DNA fully methylated, or hemimethylated at the 5-position of cytosine residues (GAT^mC/ GAT^mC: “fully-C”; GATC/GAT^mC: “hemi-C”), and DNA fully methylated or hemimethylated at adenine residues (G^mATC/G^mATC, “fully-A”; GATC/ G^mATC, “hemi-A”). (Cytosine methylation can also occur at the N4-position, which is not investigated here. In many cases, enzymes interact similarly with substrates containing ^m5C and ^m4C.) With hemimethylated DNA substrates, cleavage of the methylated and unmethylated strands was analyzed separately. Table 1 summarizes the results of our experiments. The most important findings are as follows.

In contrast to what has been reported in REBASE, we demonstrate that

Table 1. Methylation Sensitivity of Restriction Enzymes Interacting with GATC Sites

Enzyme	Methylation Status	REBASE	This Work
<i>DpnI</i>	hemi-A	not cut/cleavage impaired	cleavage impaired (activity at least 100-fold lower, methylated strand is cleaved slightly faster)
	fully-A	cut	cut
	hemi-C	no data available	not cut
	fully-C	not cut	not cut
<i>DpnII</i>	hemi-A	not cut	not cut
	fully-A	not cut	not cut
	hemi-C	not cut	cut
	fully-C	no data available	cut
<i>NdeI</i>	hemi-A	no data available	not cut
	fully-A	not cut	not cut
	hemi-C	no data available	cut
	fully-C	cut	cut
<i>MboI</i>	hemi-A	not cut/impaired	not cut
	fully-A	not cut	not cut
	hemi-C	cleavage impaired	cut
	fully-C	cut/ cleavage impaired	cut
<i>Sau3AI</i>	hemi-A	cut/cleavage impaired (unmethylated strand not cut)	cut (methylated strand 2-fold faster)
	fully-A	cut	cut
	hemi-C	not cut	unmethylated strand impaired (activity 10-fold lower)
	fully-C	not cut	methylated strand not cut

“Cut” means that the cleavage efficiency of the enzyme is less than 10-fold lower; “not cut” means that it is lower by at least 1000-fold.

Figure 1. Cleavage of C-methylated GATC sites by *DpnII*. Each substrate was digested with three different amounts of enzyme as indicated. In each case, the upper strand of the DNA was labeled by ³²P, which is indicated by a *.

Benchmarks

DpnII efficiently cuts both DNA strands of hemi-C methylated substrates. This result is shown in Figure 1, which displays cleavage of unmethylated DNA by *DpnII* (C*/C panel) and cleavage of hemi-C methylated DNA by *DpnII* (panels labeled with C*/^mC and ^mC*/C). Panel C*/^mC shows cleavage of the unmethylated strand of the hemimethylated substrate; cleavage of the methylated strand is shown in panel ^mC*/C. In addition, fully-C methylated substrates are also cleaved with similar efficiency as unmethylated substrates (Figure 1, compare panels *C/C and ^mC*/^mC). This result shows that *DpnII* is ideally suited to map the specificity of DNA binding proteins by dam methylation in mammalian cells or to follow the fate of bacterial DNA transfected into mammalian cells, because this enzyme is not influenced by the endogenous cytosine methylation in the mammalian system.

DpnI has been reported not to cut hemi-A methylated sites (34). We show here that *DpnI* also cuts hemi-A methylated sites, albeit with reduced activity and with a preference for cutting the methylated strand of the oligonucleotide. Panel A*/^mA in Figure 2 shows cleavage of the unmethylated strand of hemi-A methylated DNA; panel ^mA*/A shows the cleavage of the methylated strand. This result is important, as *DpnI* has been often used to distinguish hemi-A methylated sites from fully methylated ones (20–23,26,35). Our data show that a very careful analysis of the DNA cleavage pattern with different amounts of *DpnI* is required to allow reliable distinction between

hemi-A and fully-A methylated DNA by *DpnI* digestion. This conclusion is supported by similar results of an independent and recently published work (36), which also shows that *DpnI* does cleave hemi-A methylated sites, albeit with reduced efficiency.

MboI has been reported to show impaired cleavage of hemi-C methylated substrates (37). We show here that the activity in cleavage is not reduced. The reason for this difference could be in problems with the purity of the chemically synthesized substrates by Ono and Ueda (37).

Sau3AI has been reported not to cut hemi-C methylated sites (37,38). We show here that this conclusion is only valid for the modified strand. The unmodified strand is cleaved, albeit with an about 10-fold lower activity supporting an earlier finding by another group (39).

We provide the first data with respect to the interaction of *DpnI*, *DpnII*, and *NdeII* with some substrates. Hemi-C methylated sites are cleaved by *DpnI*, like fully-C methylated sites. *DpnII* cleaves hemi-C and fully-C methylated sites. *NdeII* does not cleave hemi-A methylated sites, but it cuts hemi-C methylated sites.

Our data resolve conflicts in the literature that exist for some restriction enzymes and some substrates: *MboI* has been reported to cut fully-C methylated sites with normal (40) or impaired efficiency (41). We show here that no reduction in cleavage activity is observed. The reason for the reduced cleavage efficiency observed by Huang et al. (41) could be that DNA from bacteriophages was used that contains

many additional modifications. In addition, *MboI* has been reported to cleave hemi-A methylated DNA with low efficiency (37) or not at all (38,42,43). Our data confirm the latter result. *Sau3AI* has been reported to cleave hemi-A methylated DNA with normal (43) or reduced activity (37).

It was the main goal of our study to provide a set of results obtained with the same oligonucleotide substrates. Therefore, the results obtained with different enzymes can be directly compared.

DNA methylation at GATC sites has important biological roles. In addition, dam methylation and GATC-interacting restriction enzymes have several applications in biotechnology and molecular biology. Understanding of the biological roles and biotechnical applications requires a set of GATC interaction restriction enzymes that are well characterized with respect to the effect of adenine and cytosine methylation. Here, we have analyzed the effect of cytosine and adenine methylation in one or both strands of the DNA on DNA cleavage by five different restriction enzymes. The results presented will aid the design of future experiments that employ dam methylation for a biotechnical purpose or aim to study the biology of dam methylation in *E. coli* and other bacteria.

REFERENCES

1. Jones, P.A. and D. Takai. 2001. The role of DNA methylation in mammalian epigenetics. *Science* 293:1068-1070.
2. Jeltsch, A. 2002. Beyond Watson and Crick: DNA methylation and molecular enzymology of DNA methyltransferases. *ChemBiochem* 3:274-293.
3. Barras, F. and M.G. Marinus. 1989. The great GATC: DNA methylation in *E. coli*. *Trends Genet.* 5:139-143.
4. Garcia-Del Portillo, F., M.G. Pucciarelli, and J. Casadesus. 1999. DNA adenine methylase mutants of *Salmonella typhimurium* show defects in protein secretion, cell invasion, and M cell cytotoxicity. *Proc. Natl. Acad. Sci. USA* 96:11578-11583.
5. Heithoff, D.M., R.L. Sinsheimer, D.A. Low, and M.J. Mahan. 1999. An essential role for DNA adenine methylation in bacterial virulence. *Science* 284:967-970.
6. Blackburn, E.H., W.C. Pan, and C.C. Johnson. 1983. Methylation of ribosomal RNA genes in the macronucleus of *Tetrahymena thermophila*. *Nucleic Acids Res.* 11:5131-5145.

Figure 2. Cleavage of A-methylated GATC sites by *DpnI*. Each substrate was digested with three different amounts of enzyme as indicated. In each case, the upper strand of the DNA was labeled by ³²P, which is indicated by a *.

7. **Padhy, R.N., F.G. Hottat, M.M. Coene, and P.P. Hoet.** 1988. Restriction analysis and quantitative estimation of methylated bases of filamentous and unicellular cyanobacterial DNAs. *J. Bacteriol.* 170:1934-1939.
8. **Harrison, G.S., R.C. Findly, and K.M. Kar-rer.** 1986. Site-specific methylation of adenine in the nuclear genome of a eucaryote, *Tetrahymena thermophila*. *Mol. Cell. Biol.* 6:2364-2370.
9. **Edmonds, P., B.M. Hall, W.R. Edwards, and K.M. Hartline.** 1992. Presence of methylated adenine in GATC sequences in chromosomal DNAs from *Campylobacter* species. *J. Bacteriol.* 174:8156-8157.
10. **Soper, B.W. and K.J. Reddy.** 1994. Identification of a nuclease and host restriction-modification in the unicellular, aerobic nitrogen-fixing cyanobacterium *Cyanothece sp.* *J. Bacteriol.* 176:5565-5570.
11. **Hemavathy, K.C. and V. Nagaraja.** 1995. DNA methylation in mycobacteria: absence of methylation at GATC (Dam) and CCA/TGG (Dcm) sequences. *FEMS Immunol. Med. Microbiol.* 11:291-296.
12. **Arnold, H.P., U. Ziese, and W. Zillig.** 2000. SNDV, a novel virus of the extremely thermophilic and acidophilic archaeon *Sulfolobus*. *Virology* 272:409-416.
13. **Fantappie, M.R., E.R. Gimba, and F.D. Rumjanek.** 2001. Lack of DNA methylation in *Schistosoma mansoni*. *Exp. Parasitol.* 98:162-166.
14. **Weiner, M.P., G.L. Costa, W. Schoettlin, J. Cline, E. Mathur, and J.C. Bauer.** 1994. Site-directed mutagenesis of double-stranded DNA by the polymerase chain reaction. *Gene* 151:119-123.
15. **Li, S. and M.F. Wilkinson.** 1997. Site-directed mutagenesis: a two-step method using PCR and DpnI. *BioTechniques* 23:588-590.
16. **Fisher, C.L. and G.K. Pei.** 1997. Modification of a PCR-based site-directed mutagenesis method. *BioTechniques* 23:570-574.
17. **Kirsch, R.D. and E. Joly.** 1998. An improved PCR-mutagenesis strategy for two-site mutagenesis or sequence swapping between related genes. *Nucleic Acids Res.* 26:1848-1850.
18. **Jeltsch, A. and T. Lanio.** 2002. Site-directed mutagenesis by polymerase chain reaction. *Methods Mol. Biol.* 182:85-94.
19. **Li, J.J. and T.J. Kelly.** 1985. Simian virus 40 DNA replication in vitro: specificity of initiation and evidence for bidirectional replication. *Mol. Cell. Biol.* 5:1238-1246.
20. **Hughes, P., A. Landoulsi, R. Kern, and M. Kohiyama.** 1989. Dam methylated and hemimethylated oriC plasmids are replicated symmetrically; a novel and general test of replication symmetry. *Mol. Gen. Genet.* 217:278-280.
21. **Hsieh, C.L., R.P. McCloskey, E. Radany, and M.R. Lieber.** 1991. V(D)J recombination: evidence that a replicative mechanism is not required. *Mol. Cell. Biol.* 11:3972-3977.
22. **Ceccotti, S., E. Dogliotti, J. Gannon, P. Karran, and M. Bignami.** 1993. O6-methyl-guanine in DNA inhibits replication in vitro by human cell extracts. *Biochemistry* 32:13664-13672.
23. **Nielsen, T., D. Bell, C. Lamoureux, M. Zannis-Hadjopoulos, and G. Price.** 1994. A reproducible method for identification of human genomic DNA autonomously replicating sequences. *Mol. Gen. Genet.* 242:280-288.
24. **Patnaik, P.K., X. Fang, and G.A. Cross.** 1994. The region encompassing the procyclic acidic repetitive protein (PARP) gene promoter plays a role in plasmid DNA replication in *Trypanosoma brucei*. *Nucleic Acids Res.* 22:4111-4118.
25. **Shirakata, M. and K. Hirai.** 1998. Identification of minimal oriP of Epstein-Barr virus required for DNA replication. *J. Biochem. (Tokyo)* 123:175-181.
26. **Bragaglia, D., P. Heun, P. Pasero, B.P. Duncker, and S.M. Gasser.** 1998. Semi-conservative replication in yeast nuclear extracts requires Dna2 helicase and supercoiled template. *J. Mol. Biol.* 281:631-649.
27. **Lim, C., H. Sohn, D. Lee, Y. Gwack, and J. Choe.** 2002. Functional dissection of latency-associated nuclear antigen 1 of Kaposi's sarcoma-associated herpesvirus involved in latent DNA replication and transcription of terminal repeats of the viral genome. *J. Virol.* 76:10320-10331.
28. **van Steensel, B. and S. Henikoff.** 2000. Identification of in vivo DNA targets of chromatin proteins using tethered dam methyltransferase. *Nat. Biotechnol.* 18:424-428.
29. **van Steensel, B., J. Delrow, and S. Henikoff.** 2001. Chromatin profiling using targeted DNA adenine methyltransferase. *Nat. Genet.* 27:304-308.
30. **Singh, J. and A.J. Klar.** 1992. Active genes in budding yeast display enhanced in vivo accessibility to foreign DNA methylases: a novel in vivo probe for chromatin structure of yeast. *Genes Dev.* 6:186-196.
31. **Kladde, M.P. and R.T. Simpson.** 1994. Positioned nucleosomes inhibit Dam methylation in vivo. *Proc. Natl. Acad. Sci. USA* 91:1361-1365.
32. **Wines, D.R., P.B. Talbert, D.V. Clark, and S. Henikoff.** 1996. Introduction of a DNA methyltransferase into *Drosophila* to probe chromatin structure in vivo. *Chromosoma* 104:332-340.
33. **Roberts, R.J. and D. Macelis.** 2001. REBASE—restriction enzymes and methylases. *Nucleic Acids Res.* 29:268-269.
34. **Vovis, G.F. and S. Lacks.** 1977. Complementary action of restriction enzymes endo R-DpnI and Endo R-DpnII on bacteriophage f1 DNA. *J. Mol. Biol.* 115:525-538.
35. **Maas, R.** 2001. Change of plasmid DNA structure, hypermethylation, and Lon-proteolysis as steps in a replicative cascade. *Cell* 105:945-955.
36. **Lu, L., H. Patel, and J.J. Bissler.** 2002. Optimizing DpnI digestion conditions to detect replicated DNA. *BioTechniques* 33:316-318.
37. **Ono, A. and T. Ueda.** 1987. Synthesis of decaoxyribonucleotides containing N6-methyladenine, N4-methylcytosine, and 5-methylcytosine: recognition and cleavage by restriction endonucleases (nucleosides and nucleotides part 74). *Nucleic Acids Res.* 15:219-232.
38. **Nelson, M., C. Christ, and I. Schildkraut.** 1984. Alteration of apparent restriction endonuclease recognition specificities by DNA methylases. *Nucleic Acids Res.* 12:5165-5173.
39. **Streeck, R.E.** 1980. Single-strand and double-strand cleavage at half-modified and fully modified recognition sites for the restriction nucleases *Sau3A* and *TaqI*. *Gene* 12:267-275.
40. **Brooks, J.E. and R.J. Roberts.** 1982. Modification profiles of bacterial genomes. *Nucleic Acids Res.* 10:913-934.
41. **Huang, L.-H., C.M. Farnet, K.C. Ehrlich, and M. Ehrlich.** 1982. Digestion of highly modified bacteriophage DNA by restriction endonucleases. *Nucleic Acids Res.* 10:1579-1591.
42. **McClelland, M.** 1981. The effect of sequence specific DNA methylation on restriction endonuclease cleavage. *Nucleic Acids Res.* 9:5859-5866.
43. **McClelland, M. and M. Nelson.** 1985. The effect of site specific methylation on restriction endonuclease digestion. *Nucleic Acids Res.* 13:r201-r207.

This work was supported by grants from the Deutsche Forschungsgemeinschaft (JE 252/1-2 and JE 252/2-3) and the Fonds der Chemischen Industrie. Address correspondence to Dr. Albert Jeltsch, Institut für Biochemie, FB 8, Justus-Liebig-Universität, Heinrich-Buff-Ring 58, 35392 Giessen, Germany. e-mail: albert.jeltsch@chemie.bio.uni-giessen.de

Received 7 November 2002; accepted 21 February 2003.

**Andrea Hermann and
Albert Jeltsch**
*Justus-Liebig-Universität
Giessen, Germany*