Methylation of either cytosine in the recognition sequence CGCG inhibits *ThaI* cleavage of DNA

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ABSTRACT

ThaI (CGCG) sites which overlap HhaI (GCGC) sites in Φ X174 and pBR322 DNA were methylated *in vitro* with HhaI methylase and S-adenosylmethionine to yield CG^mCG, ^mCGCG or ^mCG^mCG (5-methylcytosine, ^mC). Methylation of either cytosine in the ThaI recognition sequence rendered the DNA resistant to ThaI cleavage. Rat pituitary cell genomic DNA was digested with ThaI or 2 other known methylation-sensitive enzymes, AvaI or XhoI. After electrophoresis and ethidium bromide staining of the DNA, all 3 enzymes showed the infrequent DNA cleavage characteristic of methylation-sensitive enzymes. Comparison of pituitary growth hormone (GH) genes bearing strain-specific degrees of methylation showed the less methylated gene to be more frequently cut by either AvaI or ThaI. ThaI resistant sites in GH genes were cleaved by ThaI after exposing cells to 5-azacytidine, an inhibitor of DNA methylation. We conclude that ThaI is a useful restriction enzyme for the analysis of ^mC at CGCG sequences in eukaryotic DNA.

INTRODUCTION

ThaI is a type II restriction enzyme isolated from the thermophilic mycoplasma Thermoplasma acidophilum (1). This enzyme cleaves DNA in the middle of the sequence CGCG. As most of the 5-methylcytosine residues in mammalian DNA occur in CpG dinucleotide sequences (2), ThaI may be a useful probe for DNA containing methylated cytosines. A large number of restriction enzymes fail to cut DNA at recognition sequences that have been modified by the conversion of adenine to 6-N-methyladenine or cytosine to 5-methylcytosine (3). The failure of a methylation-sensitive restriction enzyme to cut genomic DNA at a known recognition sequence can be detected after electrophoresis using Southern blotting and DNA hybridization assays (4), and is evidence that a methylated base occurs within that sequence.

Initial characterization of ThaI showed it did not digest T. acidophilum DNA (1). This is frequently indicative of an existing host restriction-modification system (5). We have therefore investigated the ability of ThaI to cut 5-methylcytosine modified DNA.

MATERIALS AND METHODS

Cell culture: The GH₃ cell line was established from a rat pituitary adenoma (6). We originally obtained this cell line from Dr. Armen Tashjian of Harvard University. These cells possess GH genes with extensive 5-methylcytosine modification at HpaII, AvaI and XhoI restriction enzyme recognition sites (7) and are therefore referred to as GH₃^m cells here. A subclone of GH₃^m cells which we have isolated exhibits a more moderate level of 5-methylcytosine at these same restriction sites (7); these cells are referred to as GH₃ in our reports. GL16 clone F is one of several clonal lines of somatic cell hybrids formed by fusion of GH₃^m and transformed mouse fibroblasts (LB82) (8). All these clones contain rat GH genes completely resistant to digestion by ThaI; GL16 clone F was used in the 5-azacytidine experiments described in this paper. The isolation and characterization of all of these cell lines, including detection of 5-methylcytosine in the rat GH genes, are described in detail elsewhere (7,8). All cells were grown in Dulbecco's Modified Eagle's Medium (Biofluids, Inc.) supplemented with 2% fetal calf serum (Biofluids, Inc.), 5% horse serum (Gibco, Inc.) and 2mM glutamine. Cell cultures were maintained at 37°C in a humidified, 95% air-5% CO₂ atmosphere.

5-Azacytidine treatment: 5-azacytidine was purchased from Sigma and stored dry at -20°C. Clone F hybrid cells were plated in 100mm plastic dishes in complete culture medium. Sterile 1000X stock solutions of 5-azacytidine were prepared in phosphate-buffered saline daily, and added immediately to logarithmically growing cells in fresh culture medium on 3 successive days. Control cells were treated with phosphate-buffered saline alone. After 3 days, the culture medium containing 5-azacytidine was removed and replaced with fresh 5-azacytidine-free medium. Cells were propagated 10 days in the absence of 5-azacytidine before isolating the genomic DNA.

DNA preparation and analysis: Genomic DNA was prepared from the cell culture lines according to the method of Steffen and Weinberg (9). Replicative form I ϕ X174 DNA was purchased from New England Biolabs, Inc. Plasmid pBR322 DNA was isolated from E. coli strain HB101 following the Triton-lysis procedure (10) and banded twice in ethidium bromide-caesium chloride gradients. Restriction enzyme digested genomic DNA was electrophoresed in 0.7% agarose gels using 89mM Tris-89mM borate-2mM EDTA, pH 8.2 buffer. Electrophoresis was conducted at 30 volts for 15 hours. Southern blotting and detection of rat GH genes in the genomic DNA by DNA hybridization were performed as described previously (11). ϕ X174 or pBR322

DNA and their enzyme digestion products were electrophoresed in 4% or 4.5% acrylamide gels for 40 minutes at 45 mA using the buffer described above. The DNA was visualized by illumination with 300nm UV light after staining the gels for 30 minutes in $\mu\text{g/ml}$ ethidium bromide in water.

Restriction enzyme reactions: AvaI, HhaI, HindIII and XhoI were purchased from New England Biolabs, Inc. ThaI was obtained from Bethesda Research Labs, Inc. and HpaII from Boehringer Mannheim Biochemicals. Genomic DNA (20 μg) was digested for 18 hours in 400 μl reaction volumes containing a 2-4 unit excess of enzyme and the buffers recommended by the enzyme suppliers. ThaI reactions were conducted at 60°C; all other digestions were at 37°C. Reactions were terminated by the addition of an equal volume of recrystallized phenol (Bethesda Research Labs, Inc.) equilibrated with 0.1M Tris, pH 8. Phenol-extracted DNA was ethanol precipitated then redissolved in 25 μl of water by heating at 50°C for 5 minutes. Concentrated electrophoresis buffer, glycerol and bromophenol blue were added to a final concentration of 1X electrophoresis buffer, 10% glycerol and 0.1% bromophenol blue before electrophoresis in 0.7% agarose gels.

DNA methylase reactions: HhaI methylase was purchased from New England Biolabs, Inc. HhaI methylase reactions with ϕX174 and pBR322 DNA were performed as per the protocol supplied by New England Biolabs, Inc. One μg of DNA was methylated in a 10 μl reaction containing 1 unit of HhaI methylase, the recommended buffer, and 80 μM S-adenosylmethionine (chloride salt, Sigma) prepared as a 10X stock in dilute sulfuric acid, pH 4. Reactions were conducted at 37°C for 1.5 hours. The samples were then diluted 5-fold with 1X HhaI restriction enzyme buffer and digested with 10 units of HhaI at 37°C for 1 hour, or diluted 10-fold with 1X ThaI restriction enzyme buffer and digested with 25 units of ThaI at 60°C for 1 hour. Samples were co-precipitated with 8 μg of tRNA in ethanol at -20°C overnight, redissolved in 20 μl of water and treated with 2 μg of heat-inactivated pancreatic RNase (Sigma) for 30 minutes at 37°C. Concentrated electrophoresis buffer, glycerol, and bromophenol blue were added as described above; samples were electrophoresed in 4% or 4.5% acrylamide gels.

HhaI methylase treatment was shown in control experiments to specifically inhibit ThaI enzyme activity. PstI digestion of either ϕX174 or pBR322 was unchanged by pretreatment with HhaI methylase. Furthermore, the addition of an unmethylated substrate (either pBR322 or ϕX174) to methylation reactions involving a different substrate (ϕX174 or pBR322) immediately following heat-inactivation of the HhaI methylase, resulted in selective digestion of the unmethylated substrate by ThaI.

TABLE 1
Effect of HhaI Methylase on
ΦX174 Digestion by ThaI

Recognition Site 1st bp ¹			Methylated Sequence ²	Predicted changes in <u>ThaI</u> cleavages if the methylated <u>ThaI</u> sequence is not cleaved	
				<u>lose</u>	<u>gain</u>
Region A	<u>HhaI</u>	772	<u>CG^mCGC</u>	1050	1069
	<u>ThaI</u>	771		19	
Region B	<u>HhaI</u>	2363	<u>G^mCGCG</u>	496	597
	<u>ThaI</u>	2364		101	
Region C	<u>HhaI</u>	1548, 1550	<u>G^mCG^mCGC</u>	259	386
	<u>ThaI</u>	1549		127	

¹ Denotes the first bp in the recognition sequence for HhaI or ThaI. Position "O" is the site of PstI cleavage. Sequence data is from (13) and personal communication.

² The sequence of the overlapping HhaI and ThaI recognition sequences is presented. The ThaI recognition sequence is underlined.

RESULTS

Inhibition of ThaI activity by in vitro methylation of DNA templates: A methylase specific for ThaI recognition sequences has not been identified. However, sequences of alternating GC are not infrequent in natural DNAs, and can result in overlapping ThaI and HhaI recognition sequences. Methylation of some ThaI sites can therefore be achieved in vitro using HhaI methylase which specifically modifies the sequence GCGC to G^mCGC (5). In ΦX174 DNA, ThaI and HhaI restriction sites overlap in the 3 regions denoted A,B, and C in Table 1. The ThaI restriction digest pattern of ΦX174 DNA is known precisely from its DNA sequence (13). From this data the changes in ThaI fragment sizes resulting from 5-methylcytosine inhibition of ThaI cleavage at regions A,B and C were predicted (Table 1).

Methylation of ΦX174 at HhaI restriction sites by HhaI methylase is demonstrated in Figure 1, lanes 2-4. Undigested ΦX174 DNA (lane 2) and most HhaI digested ΦX174 molecules pre-treated with HhaI methylase (lane 4) are large and fail to enter the gel. The single band migrating into the

Figure 1. Effect of HhaI methylase modification of ϕ X174 DNA on cleavage by ThaI. Lane 1, HaeIII digested ϕ X174 molecular weight markers; lane 2, undigested ϕ X174; lane 3, HhaI digested ϕ X174; lane 4, HhaI digestion of HhaI methylase-treated ϕ X174; lane 5, ThaI digested ϕ X174; lane 6, ThaI digestion of HhaI methylase-treated ϕ X174. DNA was electrophoresed in a 4% polyacrylamide gel.

gel in lane 4 probably reflects some non-specific endonuclease activity in the HhaI. This faint band co-migrates with linear ϕ X174. Treatment of ϕ X174 DNA with methylase alone, with or without the heat-inactivation step, does not generate any DNA which co-migrates with linear ϕ X174 DNA.

Furthermore, the product of methylase-treated ϕ X174 DNA digested with HhaI and PstI was a linear fragment identical to the linear ϕ X174 molecule generated by treating methylated (or unmethylated) ϕ X174 DNA with PstI alone. This experiment (data not shown) rules out the possibility that the faint band migrating in lane 4 is due to cleavage at a specific site. Lane 3 contains HhaI digested unmethylated ϕ X174 DNA; all expected fragments are present.

The effect of cytosine methylation on ThaI digestion of ϕ X174 DNA is shown in Figure 1, lanes 5 and 6. Thirteen of 14 expected ThaI digestion products of unmethylated ϕ X174 are evident in lane 5; only the 19bp fragment is not apparent.

Following HhaI methylase, discrete and predictable changes were observed in the ThaI digestion products of ϕ X174 DNA (lane 6). Four fragments (496, 259, 127 and 101 bp) disappeared, and 2 new fragments (597 and 386 bp) appeared. These are exactly the changes predicted if ThaI activity is inhibited by methylation in regions B and C (Table 1). No other changes in ThaI fragments were observed. The predicted changes in the

TABLE 2
Effect of HhaI Methylase on
pBR322 Digestion by ThaI

Recognition Site			Methylated ² Sequence	Predicted Changes in ThaI cleavages if <u>only</u> CGCG is not cleaved	Predicted Changes in ThaI cleavages if CGCG and CGCG are not cleaved		
1st bp ¹				lose	gain	lose	gain
Region A	HhaI	3102	CG ^m CGC	no change		581	911
	ThaI	3101				330	
Region B	HhaI	3925 ³	CG ^m CGC	no change		493	1287
	ThaI	3924				332	
Region C	HhaI	4257	CG ^m CGC	no change		452	
	ThaI	4256					
Region D	HhaI	700	G ^m CGCG	355	599	355	626
	ThaI	701		115		115	
Region E	HhaI	815	G ^m CGCG			129	
	ThaI	816					
Region F	HhaI	946	CG ^m CGC	no change		27	
	ThaI	945					
Region G	HhaI	2075	G ^m CGCG	no detectable change due to proximity to Region H		no detect-	513
	ThaI	2076				able change due to proximity to Region H	69
Region H	HhaI	2075	CG ^m CGC	no change			
	ThaI	2074					
Region I	HhaI	2178	G ^m CGCG	103	444	103	
	ThaI	2179		341		341	

¹ Denotes the first bp in the recognition sequence for HhaI or ThaI. Position "0" is the site of EcoRI cleavage. Sequence data is from (14).

² The sequence of the overlapping HhaI and ThaI restriction sites is presented. The ThaI recognition sequence is underlined.

³ Brackets indicate where overlapping HhaI/ThaI sites are contiguous with other overlapping HhaI/ThaI sites. When 2 contiguous sites are not cleaved, 3 fragments are lost and one is gained. When 3 contiguous sites are not cleaved, 4 fragments are lost and one is gained. Predicted changes in fragments derived from contiguous sites are clarified by the dashed and solid boxes.

sizes of the ThaI digestion products derived from Region A following HhaI methylase treatment were too small to evaluate in these gels.

We conclude that inhibition of ThaI may result wherever the external cytosine is methylated, which occurs in regions B and C of ϕ X174.

To test whether inhibition of ThaI also occurs when the internal cytosine is methylated, overlapping ThaI and HhaI sites in pBR322 DNA were methylated with HhaI methylase then digested with ThaI. There are 9 regions (Table 2, regions A-I) in pBR322 DNA where ThaI and HhaI sites overlap. Predicted changes in the ThaI digest products of methylated pBR322 compared to unmethylated pBR322 are summarized in Table 2. Less than one new fragment is generated for each site where ThaI cleavage is inhibited, because in pBR322, many of the overlapping ThaI and HhaI sites are contiguous. Inhibition of ThaI by methylation at 2 contiguous sites results in the loss of 3 individual fragments and the generation of one larger fragment whose size is the sum of all of these. Similarly, inhibi-

Figure 2. Effect of *HhaI* methylase modification of pBR322 DNA on cleavage by *ThaI*. Lane 1, *HaeIII* digested ϕ X174 DNA as molecular weight markers; lane 2, *ThaI* digested pBR322; lane 3, *ThaI* digestion of *HhaI* methylase-treated pBR322. DNA was electrophoresed in a 4.5% polyacrylamide gel.

tion of *ThaI* by methylation at 3 contiguous sites results in the loss of 4 fragments and the generation of one larger fragment whose size is the sum of all of these. Wherever this occurs is indicated in Table 2.

The external *ThaI* cytosine is methylated by *HhaI* methylase in regions D,E,G and I. If these are the only regions where the *ThaI* cleavage pattern will be altered following methylation of pBR322 with *HhaI* methylase, 2 new fragments will appear (599 and 444 bp) and 5 will disappear (Table 2). However, if methylation of either cytosine inhibits *ThaI*, cleavage of pBR322 at all 9 regions will be altered. Four new and distinct fragments will appear (1287, 911, 626 and 513 bp), and 12 fragments will disappear compared with the *ThaI* digested unmethylated pBR322, although not all of these fragments will be distinguishable due to similarities in size.

The results of the pBR322 methylation experiment are shown in Figure 2. Lane 1 contains *HaeIII* digested ϕ X174 DNA molecular weight markers. Lanes 2 and 3 show the *ThaI* digest products of unmethylated and methylated pBR322, respectively. *ThaI* digestion of the methylated pBR322 template results in 4 unique DNA fragments corresponding in size to those predicted if methylation at either cytosine in the *ThaI* recognition sequence inhibits *ThaI* cleavage. A comparison of lanes 2 and 3 also shows that the

Figure 3. Comparison of rat GH DNA digestion products following HindIII, ThaI, XhoI or AvaI digestion of rat genomic DNA. Lanes 1-7 contain GH₃^m DNA and lanes 8-14 contain GH₃ DNA. HindIII digests, lanes 1 and 8; ThaI digests, lanes 2 and 9; HindIII and ThaI digests, lanes 3 and 10. XhoI digests, lanes 4 and 11; HindIII and XhoI digests, lanes 5 and 12. AvaI digests, lanes 6 and 13; HindIII and AvaI digests, lanes 7 and 14. DNA was digested for 18 hours with the appropriate enzyme and buffer system at 37°C, then electrophoresed in 0.7% agarose gels and stained with 1 ug/ml ethidium bromide in H₂O. Southern blotting and hybridization reactions were performed as described in Materials and Methods.

appropriate smaller DNA fragments are absent from lane 3. Therefore, all 5 ThaI sites in pBR322 which were methylated at only the internal cytosine (regions A,B,C,F and H) were resistant to ThaI digestion.

Infrequent ThaI cutting of rat genomic DNA and the rat GH gene related to 5-methylcytosine content: Digestion of GH₃ rat genomic DNA to completion by the methylation-insensitive enzyme HindIII results in a population of DNA fragments broadly distributed in size from less than 100 bp to 20 kbp, slightly less than the size of the undigested genomic DNA. In contrast,

when genomic GH₃ DNA is digested with restriction enzymes such as XhoI or AvaI which are inhibited by the presence of 5-methyl cytosine in their recognition sequence (3), most DNA remains the size of the undigested material; by ethidium staining, it appears that approximately 5-10% of the DNA is digested to fragment sizes no less than 20 kbp. The pattern of ThaI digestion of rat genomic DNA closely resembles that of XhoI and AvaI. We therefore tested whether inhibition of ThaI cleavage of eukaryotic DNA might be mediated via methylation of its recognition sequence.

In Figure 3, GH cDNA was hybridized with restriction enzyme digested rat genomic DNA to reveal the GH gene digestion products. DNA from the GH₃^m strain of pituitary cells bearing GH genes heavily modified by 5-methylcytosine (7) is in lanes 1-7. HindIII digestion yields a 5.6 kbp GH gene fragment (lane 1). The GH gene remains in the high molecular weight DNA after digestion with either ThaI (lane 2), XhoI (lane 4) or AvaI (lane 6). The 5.6 kbp fragment remains prevalent after digestion GH₃^m DNA with both HindIII and XhoI (lane 5) or HindIII and AvaI (lane 7). This indicates that essentially all GH₃^m DNA contains 5-methylcytosine in the single XhoI site, and about 50% of the GH₃^m DNA contains 5-methylcytosine at all five AvaI sites which lie in the 5.6 kbp HindIII fragment (7). Similarly, there is little cleavage of the GH gene in GH₃^m DNA by ThaI (lane 3). About 50% of the GH DNA remains in the 5.6 kbp fragment and 50% is present on a 4.2 kbp fragment indicative of cleavage at one of two ThaI sites in the 5.6 kbp HindIII fragment.

There is a different pattern of GH gene digestion fragments in the less extensively methylated GH₃ strain of cultured pituitary cells (7) (lanes 8-14). The 5.6 kbp GH fragment seen after digestion with HindIII alone (lane 8) is absent following digestion with HindIII and ThaI (lane 10) or HindIII and AvaI (lane 14). Smaller GH fragments are present, evidence of varying degrees of cleavage at all possible ThaI and AvaI sites. Thus, there is an association between more extensive ThaI digestion and the 5-methylcytosine content of these rat GH genes.

ThaI digestion of DNA isolated from 5-azacytidine treated cells: Unlike GH₃ or GH₃^m cells, clone F GH₃^m X LB82 hybrid cells contain a rat GH gene totally resistant to ThaI cleavage. HindIII digestion of clone F DNA generates a 5.6 kbp GH fragment which is unchanged by subsequent incubations with ThaI (Figure 4, lane 1). Previous analyses with other restriction enzymes indicated this GH gene is sensitive to restriction enzyme digestion and is structurally intact (7 and unpublished results, Strobl and

Figure 4. Effect of 5-azacytidine-treatment on *Tha*I cleavage of the rat GH gene in hybrid cell genomic DNA. Lane 1, control cell DNA; lane 2, DNA prepared from hybrid cells treated for 3 days with 5uM 5-azacytidine. DNA was digested with *Hind*III and *Tha*I then electrophoresed in 0.7% agarose gels. Southern blotting and hybridization reactions were performed as described in Materials and Methods.

Thompson). The expected *Tha*I cleavage fragments of the rat GH gene in clone F DNA were observed after *Tha*I digestion of DNA isolated from cells exposed to 5uM 5-azacytidine (Figure 4, lane 2). DNA methyltransferase activity, which is responsible for the post-replicative modification of cytosine to 5-methylcytosine, is inhibited by 5-azacytidine (12); consequently, DNA synthesized in the presence of 5-azacytidine remains unmethylated. The ability of *Tha*I to partially digest the clone F GH gene only following exposure of cells to 5-azacytidine is further evidence that cytosine methylation inhibits *Tha*I activity in eukaryotic DNA.

DISCUSSION

We conclude that the presence of 5-methylcytosine at either cytosine in the *Tha*I recognition sequence CGCG prevents *Tha*I cleavage of DNA. The only modified base identified in eukaryotic DNA is 5-methyl cytosine, and it is found primarily in the dinucleotide CpG (2). The *Tha*I recognition sequence contains 2 CpG dinucleotides, and therefore should be a useful addition to those restriction enzymes already available for assaying the presence of 5-methylcytosine (3). As predicted by this data, *Tha*I cleaves rat genomic DNA infrequently. Analyses of the rat GH gene showed *Tha*I cleavage to be inversely related to the rat GH gene methylation status.

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