Restriction enzyme digestion of hemimethylated DNA

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ABSTRACT

Hemimethylated duplex DNA of the bacteriophage ØX 174 was synthesized using primed repair synthesis in vitro with E. coli DNA polymerase I followed by ligation to produce the covalently closed circular duplex (RFI). Single-stranded ØX DNA was used as template, a synthetic oligonucleotide as primer and 5-methyldeoxycytidine-5'-triphosphate (5mdCTP) was used in place of dCTP. The hemimethylated product was used as substrate for cleavage by various restriction enzymes. Out of the 17 enzymes tested, only 5 (BstN I, Taq I, Hinc II, Hinf I and Hpa I) cleaved the hemimethylated DNA. Two enzymes (Msp I and Hae III) were able to produce nicks on the unmethylated strand of the cleavage site. Msp I, which is known to cleave at CCGG when the internal cytosine residue is methylated, does not cleave when both cytosines are methylated. Another enzyme, Apy I, cleaves at the sequence CC$^{A}_{T}$GG when the internal cytosine is methylated, but is inactive on hemimethylated DNA in which both cytosines are methylated. Hemimethylated molecules should be useful for studying DNA methylation both in vivo and in vitro.

INTRODUCTION

Most of the known restriction enzymes are inactive when specific methylated cytosine or methylated adenine residues are present in their recognition site. Other enzymes are active whether or not their recognition site in the DNA is methylated, and a third small group of enzymes require DNA methylation for cleavage (1). The use of restriction enzymes in molecular genetics, including their recent use in the analysis of eukaryotic DNA methylation, prompted us to survey the response of various commonly used restriction enzymes to methylated cytosine residues in their cleavage sites. The present paper summarizes the results of such a survey which was carried out using in vitro synthesized hemimethylated ØX 174 duplex DNA as substrate.

MATERIALS AND METHODS

The following enzymes were purchased from New England Biolabs Co:

Sac II, Mbo II, Msp I, Hae III, Taq I, BstN I, T4 DNA ligase and E. coli DNA polymerase I. We obtained Hpa II, Alu I and Pst I from Boehringer Mannheim Co. and the enzymes Ava I, Hpa I, EcoR II, Hinc II and Hinf I were obtained from Bethesda Research Laboratories Products. The restriction enzyme Apy I was kindly provided by R. Delauro. $\alpha[^{32}P]$ dNTPs were obtained from New England Nuclear and non radioactive dNTP from Sigma Co. 5-methyl dCTP was kindly provided by Dr. Gary Felsenfeld and the ØX 17 mer oligonucleotide primer was chemically synthesized as previously described (2). The bacteriophage ØX 174 and unlabelled as well as $[^{32}P]$ labelled ØX DNA were prepared as described (3).

Restriction Conditions. All restriction reactions were performed for 1 hr using the buffers and conditions recommended by the manufacturers in mixtures containing lng hemimethylated DNA, 1µg double stranded ØX RF DNA and 2 units of enzyme. Restricted DNA was analyzed by gel electrophoresis on agarose (Seakem Co.) slab gels followed by autoradiography. Single strand DNA nicks were detected by alkaline agarose gel electrophoresis in 30 mM NaOH, 2 mM EDTA. The digestion of single stranded DNA was carried out on DNA denatured for 15 min in 30 mM NaOH and subsequently neutralized under conditions of minimal reannealing.

Preparation of Hemimethylated DNA. Hemimethylated DNA was synthesized in vitro using primed bacteriophage ØX 174 single stranded (ss) DNA as template (2). The reaction mixture (100 µl) contained 50 µM each of dATP, dTTP, 5mdCTP and $[^{32}P]$-α-dGTP (5 Ci/mmole), 10 mM dithiothreotol, 66 mM Tris-HCl, pH 7.4, 6.6 mM $MgCl_2$, 0.1 mM ATP, 1.5 µg ss ØX DNA, 0.5 µg 17 mer primer, 15 units E. coli DNA polymerase I and 1 unit T4 DNA ligase. Following incubation at 30°C for 1 hr the DNA was extracted with phenol and precipitated with alcohol. The ØX DNA was essentially completely converted to circular duplex ØX DNA as judged by analytical gel electrophoresis. It should be noted that under these reaction conditions the newly synthesized DNA strand is both methylated and labelled with $[^{32}P]$. For some experiments we used duplex ØX methylated in the newly synthesized minus strand and labelled with $[^{32}P]$ in the nascent plus strand. This was prepared as described above using unlabelled nucleotides and in vivo $[^{32}P]$ labelled ØX DNA.

RESULTS AND DISCUSSION

In order to test the effect of 5-methylcytosine (5mC) on the specificity of various restriction enzymes we made use of a DNA molecule containing one

strand in which every cytosine was replaced by 5-methylcytosine. This was accomplished by synthesizing the complementary strand of ØX in vitro using 5mdCTP instead of dCTP. This hemimethylated DNA molecule could then be used as a substrate for various restriction enzymes. In each case small quantities of labelled hemimethylated DNA were digested with the particular restriction enzyme in the presence of natural ØX RF. Thus the normal pattern of ØX restriction could be observed on ethidium bromide stained gels and the digestion of the hemimethylated molecule by autoradiography (Fig. 1). Out of 17 enzymes which have restriction sites on ØX DNA, twelve enzymes were unable to cleave the methylated molecule, indicating that 5mC interferes with their specificity. Another five enzymes digested the hemimethylated ØX DNA in the expected manner. These results are summarized in Table 1.

It should be noted that in cases where 5-methylcytosine is involved in restriction specificity, methylation in one strand was sufficient to inhibit

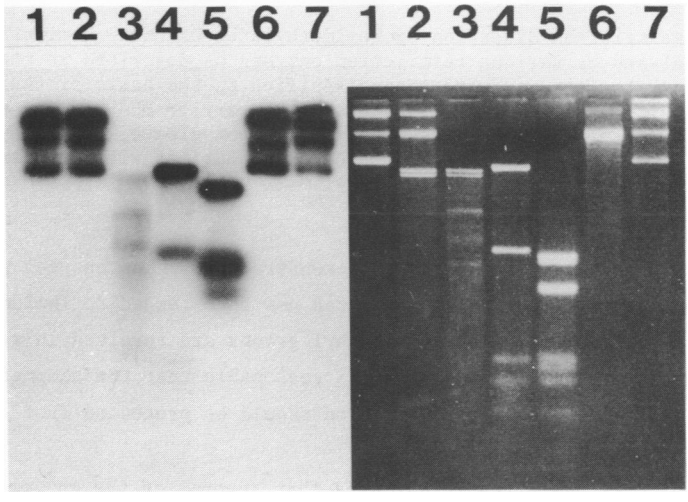

Fig. 1. Restriction digestion of hemimethylated ØX DNA. Hemimethylated [^{32}P]-DNA and natural ØX RF were digested by various restriction enzymes and analyzed by analytical agarose gel electrophoresis as described in Materials and Methods. The digestion pattern of the ØX RF was monitored by ethidium bromide staining (right side) and is compared to the cleavage of the labelled hemimethylated DNA as analyzed by autoradiography (left side). The various lanes include undigested DNA (1) and DNAs cleaved by Apy I (2), BstN I (3), Taq I (4), Mbo II (5), Sac II (6) and EcoR II (7). ØX RF is not cleaved by EcoR II due to the in vivo methylation of this restriction modification site. Apy I gave only partial digestion of ØX RF, in this experiment. However, in other experiments with 1000 fold excess of Apy I enzyme the same results were obtained with hemimethylated DNA as seen here (lane 2, left side).

INACTIVE		ACTIVE	
Hpa II	[CCGG]	Hpa I	[GTTAAC]
Msp I	[CCGG]	BstN I	[CC$^{A}_{T}$GG]
Hae II	[PuGCGCPy]	Taq I	[TCGA]
Hae III	[GGCC]	Hinc II	[GTPyPuAC]
Pst I	[CTGCAG]	Hinf I	[GANTC]
EcoR II	[CC$^{A}_{T}$GG]		
Apy I	[CC$^{A}_{T}$GG]		
Sac II	[CCGCGG]		
Alu I	[AGCT]		
Mbo II	[$^{GAAGA}_{CTTCT}$]		
Ava I	[CPyCGPuG]		
Hha I	[GCGC]		

Table I. Restriction digestion of hemimethylated ØX DNA. Hemimethylated DNA was analyzed by various restriction enzymes as described in the legend to Fig. 1 and the enzymes were then classified on the basis of their ability to cleave this DNA. In every case, the ØX RF carrier DNA gave an expected digestion pattern. To ensure that enzymes do not cleave, hemimethylated DNA (1ng) was incubated under standard reaction conditions without carrier DNA using a vast excess of enzyme (2 units).

the enzyme. This has previously been demonstrated for the enzymes Hpa II and Hha I(12) and Sau 3A(7). This conclusion can now be extended to include the enzymes shown in Table I. If these methyl groups are involved in restriction modification of the bacterial host it is reasonable that the hemimethylated DNA present during chromosome replication should be protected from restriction.

Previous data has already indicated that several of the enzymes tested are affected by 5mC in their restriction site (1). Many of these enzymes have been useful in the study of eukaryotic DNA, where methylation occurs principally at the dinucleotide sequence CpG (4). For some of the enzymes tested this is the first indication that 5mC is involved in restriction activity. Hae II contains several cytosines in its site and our data cannot pinpoint which cytosine is involved. If methylation at the internal cytosine causes restriction modification this enzyme may also be useful for the study of methylation in eukaryotic DNA. Mbo II is particularly interesting since only one strand of the restriction site contains cytosine. Since several of

the Mbo II sites in ØX RF have cytosine in the ØX plus strand, these sites were not methylated in vitro and are therefore cleaved normally by Mbo II. Sites in which the cytosine is in the in vitro synthesized strand are not cut by Mbo II. Examination of the sequence of ØX DNA shows that the digestion pattern obtained matches that expected of hemimethylated DNA. The fact that Pst I is inhibited by cytosine methylation may be useful in the study of higher plant methylation. In addition to methylation at CpG, plant DNA contains 5mC at CpA, CpT and CpC residues and recent experiments in our laboratory indicate that Pst I is unable to cleave this DNA.

The enzymes Hpa II and Msp I recognize and cleave the same restriction site, CCGG, but differ in their sensitivities to methylation (5). Whereas Hpa II is inhibited by methylation of the internal cytosine, Msp I is apparently blocked by methylation of the external cytosine (6 and T.A. Trautner, personal communication). Since both cytosines are modified in our hemimethylated DNA, neither enzyme was able to cleave.

Although most enzymes tested were unable to cleave the hemimethylated substrate, 5 enzymes gave normal patterns of digestion. As expected, Hpa I and Hinc II and Taq I were unaffected by cytosine methylation, since these enzymes are known to be influenced in vivo by 6-methyladenine in their restriction sites (1, 13). Taq I has already been shown to cleave normally in the presence of methylated cytosine (7) and this enzyme may be employed for studying methylation at CpG moieties in eukaryotic DNA since the 5mC can be detected following end labelling of Taq I restriction fragments. The methylation specificities of this enzyme as well as BstN I and Hinf I cannot be determined directly from this type of experiment, but one can exclude cytosine methylation as the mode of modification. Synthesis of a ØX molecule hemimethylated at adenine nucleotides would enable one to determine the involvement of this modification directly.

The enzymes EcoR II, Apy I and BstN I all recognize the same restriction site ($CC\overset{A}{\underset{T}{}}GG$). EcoR II has previously been shown to be blocked by the methylation of the internal cytosine and Apy I seems to have a preference for digesting at sites methylated at this cytosine (8). Our results show that Apy I may be inhibited by methylation at the external cytosine and that BstN I is not influenced by methylated cytosines.

Several enzymes, including Msp I, Hpa II, Hha I and Hae III, are known to be capable of cleaving single-stranded DNA (9). In fact, when single strand ØX was incubated with a large excess of these enzymes we observed the expected digestion pattern (results not shown). Since these enzymes recog-

nize their cleavage site in single strand DNA it was of interest to see if they would recognize and nick a hemimethylated site. To this end, hemimethylated ØX DNA labelled either in the plus strand or in the methylated strand, was incubated with these enzymes and the digestion products analyzed by alkaline gel electrophoresis (Fig. 2).

Of all of these enzymes only Hae III showed clear nicking activity on the unmethylated strand. Similar results have been observed for the enzyme Sau 3A (7). At very high enzyme to substrate ratios Msp I showed only partial nicking, while Hha I and Hpa II were unable to nick at all. It should also be noted that all four of these enzymes, while able to cleave single strand DNA, could not digest methylated single strand DNA, prepared by denaturation of the hemimethylated molecule (results not shown).

The single strand restriction activity of the enzyme Hpa II and Hha I may be useful for the analysis of specific genes in eukaryotic DNA. Experiments in several independent systems have demonstrated that there are speci-

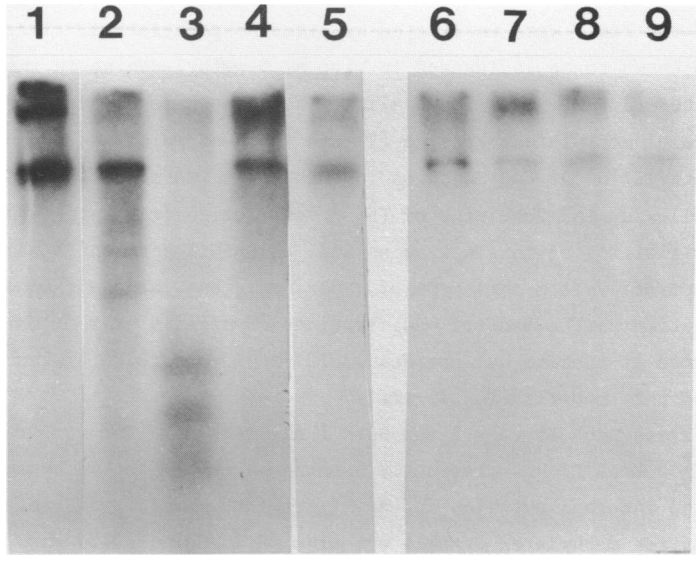

Fig. 2. Nicking activity of restriction enzymes on hemimethylated DNA. Hemimethylated ØX[^{32}P]DNA (1ng) labelled in vivo in the viral plus strand (lanes 1-5) or hemimethylated ØX[^{32}P]DNA (1ng) labelled in the in vitro synthesized minus strand (lanes 6-9) were incubated with various restriction enzymes (2 units per reaction). Following the reaction the digestion products were analyzed by alkaline agarose (2.5%) gel electrophoresis. Undigested DNA is shown in lanes 1 and 6; Msp I (lanes 2 and 7); Hae III (lanes 3 and 8); Hha I (lanes 4 and 9) and Hpa II (lane 5).

fic changes in the methylation pattern of particular genes which accompany differentiation. In all cases certain genes seem to be undermethylated in tissues in which these genes are expressed, as compared to other tissues and to sperm DNA (6, 10-11). One model which may explain the mechanism of this demethylation involves several rounds of DNA replication without concomitant methylation. If this is correct one would expect that sites in specific genes would pass through a stage in the differentiation program in which they would be hemimethylated. Hemimethylated DNA can be detected by cleaving both double stranded and single stranded DNA with Hpa II or Hha I. A hemimethylated site is revealed by the digestion of the single strand but not the double strand template.

The hemimethylated ØX molecule employed in this paper should be a useful tool for the study of DNA methylation. We have already introduced this DNA into mouse L-cells using DNA mediated gene transfer in order to analyze the specificity of the animal cell maintenance methylase. It can also be used as a substrate for studying methylation in vitro. Preliminary experiments have shown that hemimethylated DNA is the preferred template for animal cell methylase.

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REFERENCES

1. Roberts, R.J. (1981).Nucleic Acids Res. 9, 175-196.
2. Razin, A., Hirose, T., Itakura, K. and Riggs, A.D. (1978). Proc. Nat. Acad. Sci. USA 75, 4268-4270.
3. Razin, A., Sedat, J.W. and Sinsheimer, R.L. (1970). J. Mol. Biol. 53, 251-259.
4. Razin, A. and Riggs, A.D. (1980) Science 210, 604-610.
5. Cedar, H., Solage, A., Glaser, G. and Razin, A. (1979). Nucleic Acids Res. 6, 2125-2132.
6. Van der Ploeg, L.H.T. and Flavell, R.A. (1980). Cell 19, 947-957.
7. Streek, R.E. (1981). Gene, in press.
8. Razin, A., Urieli, S., Pollack, Y., Gruenbaum, Y. and Glaser, G. (1980). Nucleic Acids Res. 8, 1783-1792.
9. Godson, G.N. and Roberts, R.S. (1976). Virology 73, 561-567.
10. Mandel, J.L. and Chambon, P. (1979). Nucleic Acids Res. 7, 2081-2090.
11. Weintraub, H. and Groudine, M. (1981). Cell, in press.
12. Bird, A.P. (1978) J. Mol. Biol. 118, 49-60.
13. Sato, S., Nakazawa, K. and Shinomiya, T. (1980). J. Biochem. 88, 737-747.