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Reduced cleavage by sodium hydroxide of methyladenine in DNA sequencing

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ABSTRACT

When the Maxam and Gilbert DNA sequencing method which is modified by Bencini et al.(Biotechniques Jan/Feb pp4-5, 1984) is applied to DNA containing methylated adenine in a GATC sequence, the cleavage reaction by sodium hydroxide is found to be greatly reduced in comparison to that of non-methylated adenine. Thus, a faint band in A>C reaction suggests a methyl adenine and can be used for its detection. That the faint band corresponds to a methyladenine was confirmed by Sanger sequencing of the same fragment and further by Maxam and Gilbert sequencing of the complementary strand of DNA, which was replicated in an E. coli strain either having or lacking methylation enzymes.

INTRODUCTION

In addition to four common bases, rare bases such as N^6- methyladenine and 5-methylcytosine have been found in DNA from many species of bacteria and eukaryotic cells (see 1,2). The adenine in the sequence 5'GATC3' is methylated by dam (DNA adenine methylase), and the internal cytosine in the sequence 5'CC(A/T)GG3' is methylated by dcm (DNA cytosine methylase)(3,4). Previously, Ohmori et al.(5) have found that 5-methylcytosine is less reactive to hydrazine than cytosine and thymine in DNA sequences, resulting in disappearance of the band corresponding to that base in the Maxam and Gilbert sequencing method.

To detect methylated adenine, a set of isoschizomer enzymes can be used. For example, DpnI restricts the GATC sequence only if the adenine is methylated, while MboI cannot restrict the same sequence with methylated adenine. On the other hand, Sau3AI can recognize the sequence irrespective of methylation (see 2). In addition, specific antibody raised against N^6-methyladenine can be used (6,7).

In this paper we report that this modified adenine does not react appreciably in the sodium hydroxide reaction employed in the Maxam and Gilbert sequencing method for the A>C cleavage. The result is the appearance of a

faint band corresponding to a methylated adenine, signifying a greatly reduced signal intensity. It can be shown that the modified adenine exhibiting this unique banding intensity is due to N^6-methyladenine in the GATC sequence by comparing the sequence patterns in DNA replicated in E. coli JM101 and GM48, having or lacking DNA methylase, respectively.

MATERIALS AND METHODS

1. Biochemicals: Ampicillin sodium salt was purchased from Sigma. Endonucleases NruI and Sau3AI, T4 DNA ligase, and M13 cloning kit were from New England Biolabs. E. coli DNA polymerase I (Klenow fragment) and MboI were from Bethesda Research Labs; and PvuII, HindII, DpnI, and alkaline phosphatase were from Boehringer Mannheim. Polynucleotide kinase was from P-L Biochemicals. Terminal deoxyribonucleotidyl transferase and dephosphorylated SmaI restricted M13mp8 were from Amersham. γ^{32}P-ATP was purchased from New England Nuclear, and α^{35}S-dATP and α^{32}P-ddATP were from Amersham.

2. Preparation of DNA: Methylated b2 fragment of Rts1(8,9) that extends from the PvuII to HpaI site was prepared by propagating the plasmid pNK165(b+pBR322)(Okawa, manuscript in preparation) in E. coli JM101(10). Unmethylated b2 fragment was prepared from the host strain deficient in DNA methylases, GM48(11). The b2 fragment (538 bp) of Rts1 was prepared by digesting pNK165 with HindII, NruI, and PvuII, and isolated from the agarose band by using NA45 membranes (Schleicher and Schuell). For obtaining single stranded DNA, M13mp8 with the b2 fragment inserted was propagated in JM101.

3. Nucleotide sequence determinations: About 3 μg of the b2 fragment was end-labeled with either γ^{32}P-ATP(3,000 Ci/mmol) and polynucleotide kinase(12), or α^{32}P-ddATP(3,000 Ci/mmol) and terminal deoxynucleotidyl transferase(13). Labeled complementary strands were separated by electrophoresis on an 8% polyacrylamide slab gel (30 X 40 X 0.16 cm) at 320 V for 13 hr. DNA was then transferred to NA45 membranes and eluted with high salt (Schleicher and Schuell). For DNA sequencing, the chemical degradation and rapid butanol extraction procedure of Bencini et al.(14) was followed. The C reation shown in Fig.1a was separately run using our modification of the original Maxam and Gilbert method(12). The sequence ladders were obtained by electrophoresis on 15% or 6% polyacrylamide gels (30 x 40 x 0.04 cm, 18 x 85 x 0.04 cm). The b2 fragment was also sequenced by the Sanger method(15), using ^{35}S-dATP (600 Ci/mmol, Amersham) and E. coli DNA polymerase I.

RESULTS

Fig.1 shows partial sequence patterns of one strand of the b2 fragment of Rtsl DNA. As seen in Fig.1a, a faint band appears at the position indicated by an arrow in the A>C lane. The corresponding position in the G+A lane shows strong signal. Since the signals for normal A's are considerably stronger than this band, it is difficult to identify the base represented by this band. In an attempt to clarify the identity of this band, Sanger sequencing(15) was also run; the results showed that this base is A rather than G(Fig.1b). Our final decision that this base must be A was made after carrying out further sequencing of the complementary strand. The corresponding complementary base was found to be T(Fig.2a). This ambiguity in identifying base A was further noted at two other positions in the course of our DNA sequencing. In fact, cursory examinations of the sequence ladders reported by Garoff and Ansorge(see Fig.4, p455, reference 16) reveal the same weak signal in base A. In all of these cases, this pattern of a reduced A signal appeared reproducibly, and this occurred only with A's in 5'GATC3' sequences. These results suggested to us that this phenomenon might be due to methylation of the adenine residue in the DNA sequence.

The only modification that is known to occur in adenine in DNA is N^6-methyladenine(1,2). Backman(17)reported that all normal E. coli strains possess the methylase that methylates the adenine in the 5'GATC3' sequence. To verify that methylation is responsible for the observed alteration of the A signal in the A>C reaction, E. coli GM48 which is deficient in dam was used as host to obtain b2 DNA. When this DNA was subjected to sequencing reactions, we found that the same A bases in the tetranucleotide sequences exhibited normal signal intensity equivalent to other A's(Fig.2b). Control DNA obtained from the dam^+ strain JM101 showed a consistently reduced A signal in the sequence ladder(Fig.2a). From these observations, we conclude that methylated A in the sequence GATC should react less than unmethylated A with sodium hydroxide in the A>C reaction, so that the band signal for methylated A is greatly reduced. Depending on the state of methylation, those GATC sites should show differential sensitivities to the isoschizomer enzymes. Indeed, DNA obtained from JM101 was cut by DpnI and Sau3AI, but not by MboI, whereas DNA from GM48 was restricted by MboI and Sau3AI but not by DpnI, consistent with the expectation that the adenine band showing a reduced cleavage reation is N^6-methyladenine.

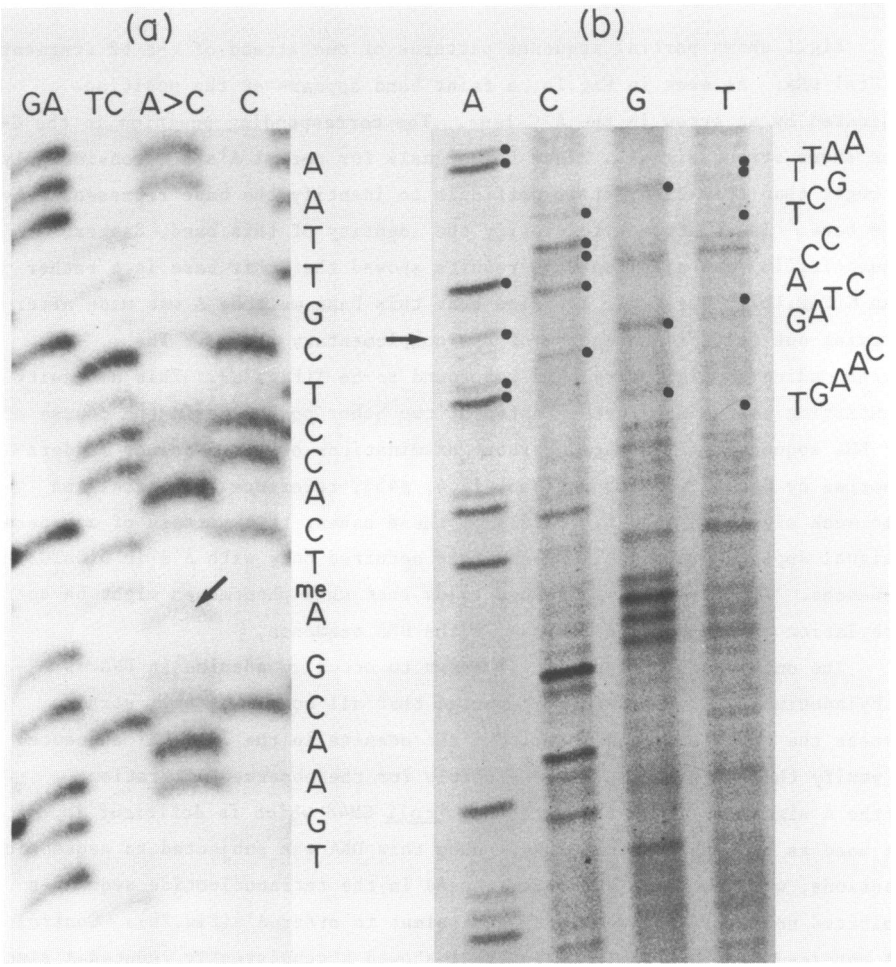

Fig.1. Autoradiograph of partial sequence ladders of the b2 fragment of Rtsl DNA. a; The arrow in the A>C lane indicates a faint band due to methylated adenine (^{me}A) in the 5'GATC3' sequence. The Bencini modification(14) of Maxam and Gilbert sequencing of 5'(^{32}P)-labeled DNA. Electrophoresis was run for 4.5 hr at 70 W in an 85 cm gel. b; Sanger sequencing of the same strand as in Fig.1a. DNA was labeled by α^{35}S-dATP. The arrow indicates the same base A as in Fig.1a and the solid dots show the sequence ladders flanking the GATC sequence. Electrophoresis was 4 hr at 50 W and the gel length was 40 cm.

DISCUSSION

The mechanism of the N^6-methyladenine reaction with sodium hydroxide is difficult to understand. When the electron donor property of the C6 position in a purine is as strong as in guanine, nucleophilic attack on the C8 position

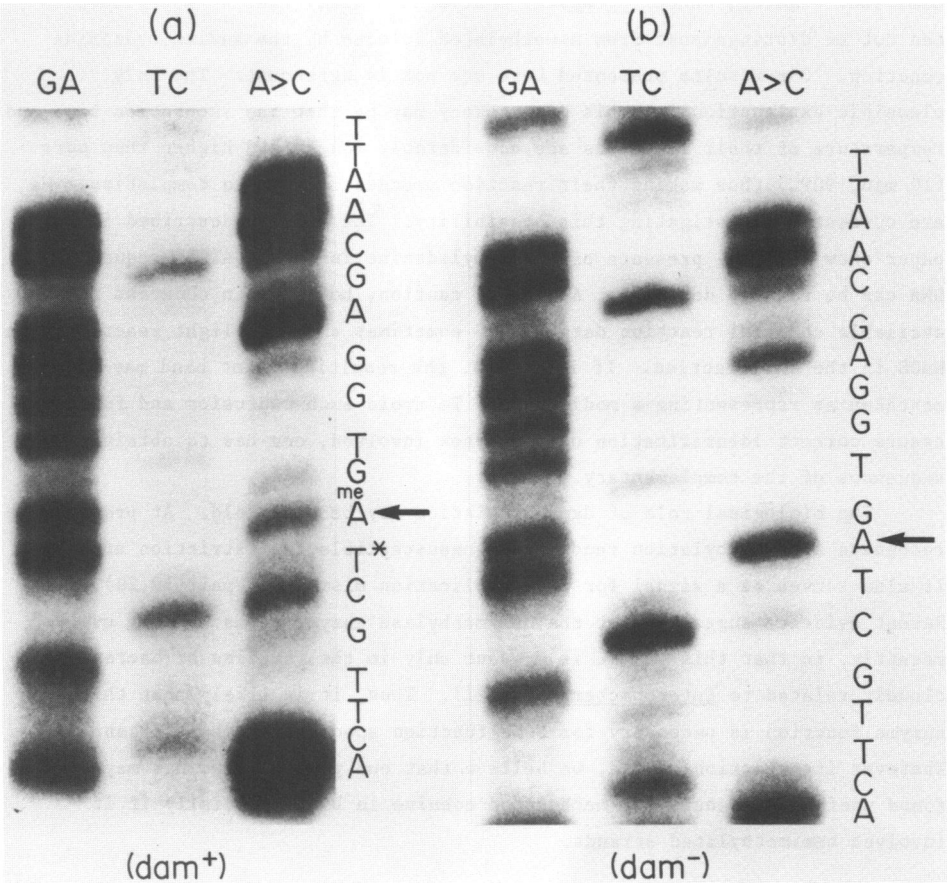

Fig.2. Comparison of DNA sequencing patterns surrounding the 5'GATC3' sequence of methylated(a) and unmethylated(b) DNAs. The b2 fragment was labeled with ^{32}P at the 3' end and the strands were separated. Two fast moving complementary strands of the ssDNA shown in Fig.1 were subjected to sequencing by the method of Bencini et al.(14). Electrophoresis was run for 3 2/3 hr in a 40 cm gel at 50 W. The letters on the ladders indicate the corresponding bases. The arrows indicate adenine in the 5'GATC3' sequence. *;The complementary base T of the methyladenine in Fig.1a.

is suppressed. On the other hand, if the oxo-group of guanine is replaced by a weaker electron donor group such as $-NH_2$ in adenine, cleavage results at the C8 position as in the A>C reaction(18). It is not immediately apparent why methylation at the N^6 position of adenine would sufficiently increase the electron donor capacity of C6, so that cleavage at the C8 position should

diminish. Indeed, Ohmori et al.(5) mentioned briefly that N^6-methyladenine can not be distinguished from unmethylated adenine by the sodium hydroxide reaction. Our results presented here are not in agreement. The only plausible explanation for this discrepancy may be that the incubation time and temperature of their reactions are considerably longer and higher than ours (10 min, 90°C),thus making their reaction proceed further to completion. We are currently investigating this possibility. The results described in this paper show that the presence of N^6-methyladenine in the 5'GATC3' sequence of DNA can be readily detected. A word of caution, however; in contrast to the available chemical reaction data(18), G sometimes shows a slight reaction with NaOH in the A>C reaction. If it occurs, the resulting faint band may be mistaken as representing a modified A. To avoid such confusion and further assure correct identification of the bases involved, one has to obtain sequences of the complementary strands.

The biological role of dam methylation may be many fold. At present, we recognize that methylation renders DNA unsusceptible to restriction attack. It also serves as a signal for post-replication mismatch repair(19,20). Recent evidence suggests that the dam methylase enzyme seems to have evolved recently, in that this enzyme is present only in the families of bacteria closely related to Enterobacteriaceae(21). Thus, it is likely that this enzyme function is necessary for some function specific to these organisms. Whatever its function may be, we believe that our present findings may be found useful in identifying methylated adenine in DNA, especially if it involves hemimethylated strands.

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