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Non-random spontaneous chain breakages occur in DNA methylated with dimethyl sulfate

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The method of in vivo footprinting uses partial methylation of the DNA in living cells with dimethyl sulfate (DMS), cleavage of the DNA chains at modified guanosines with piperidine, and mapping of the site of cleavage by the genomic sequencing technique of Church and Gilbert. Here we report on a spontaneous breakage reaction of DMS-methylated DNA at guanosines as well as adenosines, which is highly non-random with respect to the DNA sequence. In our in vivo genomic footprinting studies at the chicken lysozyme promoter this reaction gave rise to additional adenosine-derived bands in the guanosine sequence ladder.

Footprinting, in vivo; Genomic sequencing; Methylguanosine; Methyladenosine

1. INTRODUCTION

In vivo genomic footprinting utilizing dimethyl sulfate (DMS) is a very straight-forward and often used approach to analyse protein-DNA interactions [1–6]. The method relies on the ability of DMS to enter living cells and to partially methylate N_7 residues of guanosines [1]. After methylation, purified and restricted DNA is reacted with piperidine to break the DNA backbone at positions of modified guanosines [7]. The DNA is then displayed by electrophoresis on sequencing gels and transferred to nylon membranes for hybridization with radioactive end-labeled probes [8]. It is generally assumed that strands are cleaved specifically at methylated guanosines in the piperidine reaction. During our studies on in vivo footprinting at the chicken lysozyme promoter we yet found that methylated DNA is spontaneously cleaved at methylated guanosines and adenosines as well, prior to the piperidine reaction, in a manner which is highly non-random with respect to the DNA sequence.

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2. MATERIALS AND METHODS

Nuclei were isolated from hen oviducts (Lohmann Selected Leghorn) in buffer A of Hewish and Burgoyne [9] as previously described [10]. Genomic DNA was then purified following the procedure of Becker et al. [5] by digestion with DNase-free RNase A and proteinase K, extraction with phenol-chloroform-isoamyl alcohol, and precipitation with ethanol. Naked genomic DNA (40 μg) was treated with DMS at 0.5% on ice for 10 min [1], digested with *Nco*I (40 U) by incubation for 16–17 h at 37°C in 10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, 50 mM NaCl, 50 mM KCl, and reacted with piperidine following the protocol of Maxam and Gilbert [7] (first type of experiment). In a second type of experiment naked DNA was first digested with *Nco*I, and then treated with DMS followed by reaction with piperidine. The third and fourth type of experiments were identical to the first and second type, respectively, except that the reaction with piperidine was omitted. Finally, *Nco*I-digested naked DNA was subjected to A + G reaction according to Maxam and Gilbert [7].

DNA fragments were electrophoretically resolved on sequencing gels and transferred to GeneScreen membranes following the procedure of Church and Gilbert [8]. The DNA hybridization probe was synthesized as described by Becker et al. [5] from the M13mp18 clone DN8, which contains the lower strand of the sequence from −284 to −173 (A. Dölle and W.H. Strätling, in preparation), except that the probe was electrophoretically

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separated on a 8.3 M urea 5% acrylamide gel, electroeluted using a Biotrap apparatus (Schleicher & Schüll), precipitated with ethanol, and dissolved in 4–5 ml hybridization buffer. Routinely, the probe was labeled to a specific radioactivity of about $1-2 \times 10^9$ cpm per μg of single-stranded DNA. Hybridization was performed as described by Church and Gilbert [8]. Exposure times were 7–14 days with intensifying screens (Agfa-Gevaert).

3. RESULTS AND DISCUSSION

Naked genomic chicken DNA was treated with DMS (D) [7], cut by digestion with *Nco*I (N) at position −284 relative to the main start site of transcription of the chicken lysozyme gene, and then reacted with piperidine to promote chain breakage at methylated guanosines (G) [7]. DNA fragments were displayed by electrophoresis on sequencing gels and transferred to GeneScreen membranes following the genomic sequencing method of Church and Gilbert [8]. The guanosine sequence ladder of the lower strand of the lysozyme promoter region extending from −210 to −50 was visualized by hybridization with a radioactively labeled, strand-specific DNA probe containing the sequence from −284 to −173. Lane 5 in fig.1 shows the result of such an experiment. A careful inspection of the sequence ladder yet revealed that, besides bands corresponding to guanosines (labeled with filled arrowheads), it contains bands (labeled with open arrows), which by comparison with the sequence (see fig.2) probably derive from cleavages at adenosines. These putative adenosine bands did not appear when the order of DMS treatment (D) and *Nco*I digestion (N) was reversed (lane 4). When naked DNA was digested with *Nco*I and treated with DMS but omitting the reaction with piperidine (lane 1), no bands are visible. However, when digestion with *Nco*I followed the DMS treatment (lane 2), again omitting the reaction with piperidine, numerous bands indicate that the strand was broken at multiple sites. Comparison of the bands in lane 2 with the A + G sequence in lane 3 and the G sequence in lane 4 shows that these breakages occurred at guanosines as well as adenosines. We infer from these results that incubation of methylated DNA in buffer for 16–17 h at 37°C, generated breaks in the DNA backbone. It has been known for a long time that methylated purines are lost from DNA at neutral pH at a significant rate by spontaneous chemical hydrolysis

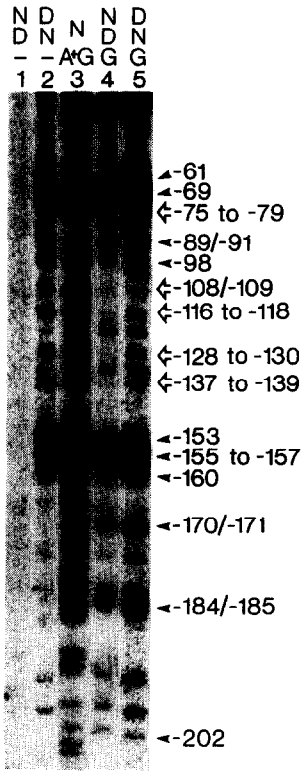

Fig.1. Spontaneous breakages in DMS-methylated DNA. Naked genomic chicken DNA was incubated with DMS (D) at 0°C for 10 min and subsequently digested with *Nco*I (N) for 16–17 h (lanes 2 and 5). In lanes 1 and 4, the order of DMS treatment and *Nco*I digestion was reversed. The samples then remained untreated (lanes 1 and 2), or were reacted with piperidine to cleave the DNA backbone at methylated G's (G) (lanes 4 and 5). In lane 3, naked DNA was digested with *Nco*I and subjected to chemical A + G sequencing reaction [7]. Following these treatments the DNA samples were electrophoretically resolved on 6% sequencing gels and transferred to GeneScreen membranes for hybridization with a radioactively labeled probe containing the sequence from −284 to −173 of the chicken lysozyme promoter. The positions of selected guanosines are indicated by filled arrowheads on the right side of the figure. The open arrows mark runs of adenosines, which give rise to additional bands in the guanosine sequencing ladder in lane 5 by a spontaneous breakage reaction.

[11]. m^3A, m^7A and m^7G are depurinated at pH 7 and 37°C with half-lives of ~26, 2.8 and ~155 h, respectively [12,13]. It has been further known that apurinic sites at neutral pH give rise to chain breakages at a measurable rate; this rate is increased by added amines and Mg^{2+} ions [14,15]. The half-life of a phosphodiester bond at an apurinic site at 37°C in the presence of 10 mM

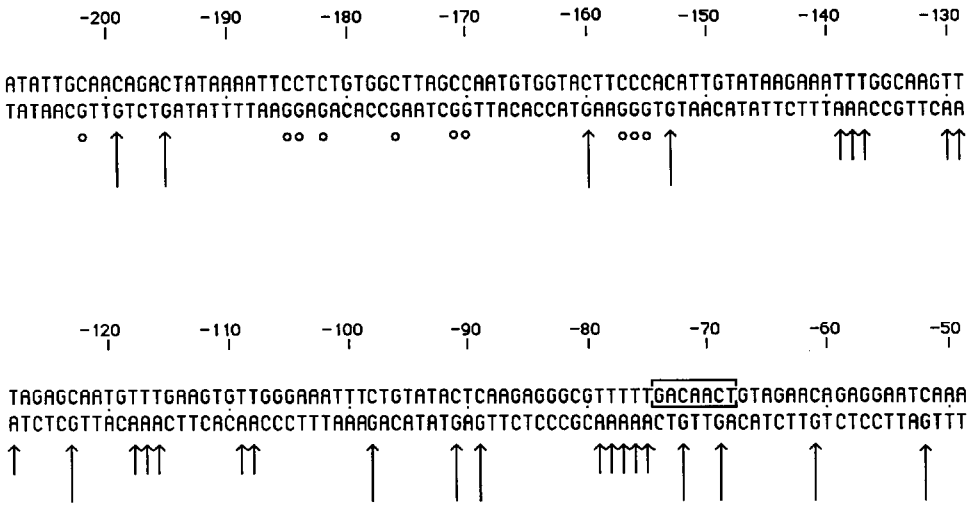

Fig.2. Spontaneous breakages are non-random with respect to the DNA sequence. The sequence of the chicken lysozyme promoter from −208 to −49 is shown [16]. The CCAAT-box equivalent sequence GACAACT is boxed. The positions of spontaneous breakages at guanosines (long arrows) and adenosines (short arrows) in the lower strand are marked. Not or weakly reactive guanosines are indicated by circles.

Mg^{2+} was determined as 190 h [15]. We thus conclude that spontaneous chain breakages occurred at guanosines and adenosines during long-term incubation of methylated DNA at 37°C in a Mg^{2+}-containing Tris-buffer in a reaction which probably consists of depurination followed by hydrolysis of the phosphodiester bond. We further conclude that the non-guanosine bands in lane 5 labeled with open arrows derive from such spontaneous breakages at methylated adenosines.

A detailed comparison of the pattern of spontaneous chain breakages in methylated DNA (fig.1, lane 2) with that of chemically promoted A+G cleavages (lane 3) and that of chemical G-specific cleavages (lane 4) further shows that the spontaneous breakages are distributed in a manner which is highly non-random with respect to the DNA sequence. A summary of our results is presented in fig.2. As the most striking example, the DNA strand was spontaneously cleaved at a high rate at G-153 and at G-160, while no spontaneous cleavage occurred at the 3 guanosines from G-155 to G-157 in between these (fig.1, lane 2). Further, spontaneous cleavage at 6 guanosines from G-170 to G-185, and at G-202, was very weak or non-detectable. Spontaneous cleavage at adenosines was mostly detectable at runs of 2–5 As. Yet cleavage at runs of adenosines in the upper strand was significantly weaker (not shown). The chemical basis of the non-random mode of spontaneous cleavage is presently unknown.

In summary, DMS-methylated DNA is spontaneously cleaved at guanosines as well as adenosines, in a manner which is highly non-random with respect to the DNA sequence. In our in vivo DMS footprinting studies at the chicken lysozyme promoter this reaction gave rise to adenosine-derived bands in the sequence ladder normally containing only guanosine-specific cleavages. Other sequences are most likely also subject to the reaction. It may be minimized by using short incubation periods during digestion of purified methylated DNA with restriction enzymes.

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