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A new procedure for determining thymine residues in DNA sequencing. Photoinduced cleavage of DNA fragments in the presence of spermine

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ABSTRACT

A new procedure for T specific cleavage of DNA fragments utilizing photoreaction with spermine has been described. Irradiation of 3'-[^{32}P]-end-labeled DNA fragments for 10-20 min with a germicidal lamp emitting mainly 254-nm light in the presence of 1 M spermine in distilled water resulted in a T specific cleavage of the DNA chains. This method does not require piperidine treatment. By contrast, when the DNA fragments were irradiated in the presence of methylamine under similar conditions, both G and T bands with the intensity of G > T have appeared. A similar but less selective T cleavage has also been observed in the irradiation of 5'-[^{32}P]-end-labeled DNA fragments in the presence of spermine followed by brief heating of the photolysate in a loading buffer for gel electrophoresis. The T specific photoreaction with spermine and the G > T reaction with methylamine described here may be conveniently used in combination with the standard Maxam-Gilbert's reactions to provide independent confirmatory readings.

INTRODUCTION

While the Maxam-Gilbert method based on base selective chemical reactions of end-labeled DNA has been widely used for sequencing DNA (1), there still needs a simple and reliable method for T specific cleavage of DNA. The pyrimidine specific reactions using hydrazine in the Maxam-Gilbert method are somewhat less reliable than those used for purines, and their specificity is sensitive to reaction conditions. Occasionally, incomplete suppression of T reaction by adding NaCl or weakened T cleavage in the C + T reaction occurs in hydrazine reactions, and this may cause the difficulty to distinguish C and T in chemical DNA sequencing (2,3,4). To attain a T specific reaction, oxidative modifications using potassium permanganate (4) and osmium tetroxide (2) have recently been reported. While these methods lead to a T selective cleavage of DNA fragments, the variability in band strength at T residues has also been reported in both cases (2,4).

Recently, Simoncsits and Török reported that irradiation of 5'-[^{32}P]-labeled DNA fragments in the presence of primary alkylamines followed by

piperidine treatment (90 °C, 30 min) led to the cleavage of the DNA chains at T residues, being accompanied by a less intensive G cleavage together with faint C- and A reactions (5). We report herein a more convenient method for T specific cleave of DNA fragments by utilizing a T selective photoreaction with spermine. Our method is easy to perform without hazardous reagents and gives bands of almost equal strength at all T residues. A more significant advantage of our method is to eliminate the step of time-consuming piperidine treatment.

MATERIALS AND METHODS

Restriction enzymes *Hae*III, *Taq*I and *Hinf*I, and T4 polynucleotide kinase were obtained from Takara Shuzo Co. Ltd., the Klenow fragment of DNA polymerase I of *Escherichia coli* was from Bethesda Research Laboratories GmbH, and calf intestine alkaline phosphatase was from Boeringer Manheim GmbH. [α-^{32}P]dCTP and [γ-^{32}P]ATP (specific activity of about 3000 Ci/mmol) were perchased from New England Nuclear a Du Pont Co., and Amersham International plc. Commercially available spermine, cyclohexylamine and methylamine were used without purification as 2 M stock solutions in distilled water. Irradiation was made with a commercial 15 W germicidal lamp (Toshiba GL 15).

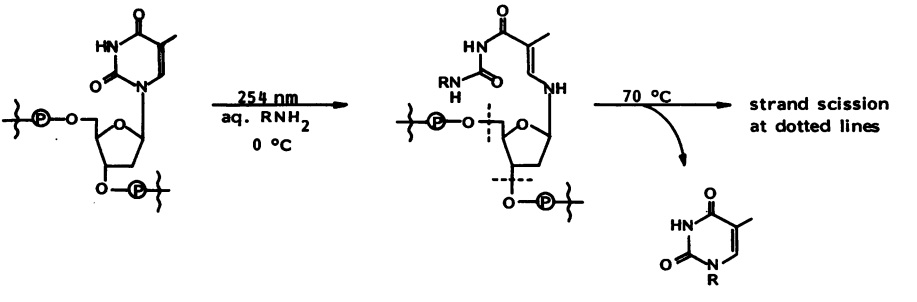
Three DNA fragments of defined sequences were obtained from bacteriophage ϕX174 replicative form DNA. Double-stranded ϕX174 replicative form DNA was prepared as previously described (6) and digested with *Hae*III, and 194 and 234 base pair fragments [Z_8 and Z_7 fragments in the map reported by Sanger (7)] were purified. Fragment Z_7 was digested with *Taq*I and labeled by extension of the 3' termini with Klenow polymerase in the presence of [α-^{32}P]dCTP (8). Resulting 3'-end labeled 56 and 178 base pair fragments (C_{436}-C_{491} and C_{492}-C_{669} in the map reported by Sanger, respectively) were purified by electrophoresis on a 6% polyacrylamide gel. 5'-end labeled DNA fragments were obtained by incubation of the Z_7 or Z_8 fragment with [γ-^{32}P]ATP and T4 polynucleotide kinase. After digestion with *Taq*I or *Hinf*I, 5'-end labeled 55 and 139 base pair fragments (C_{436}-T_{490}, C_{980}-G_{1118}) were purified by electrophoresis on a 6% polyacrylamide gel.

10 µL of the 3'-end-labeled DNA fragment was mixed with 10 µL of 2 M spermine in a 1.5 mL Eppendorf tube. The open sample tube was cooled with ice-water at 0 °C and exposed to UV light at a distance of 25 cm for 10-20 min. Then 230 µL of 0.3 M sodium acetate buffer containing 0.1 mM EDTA and 25 µg/mL of *t*RNA and 750 µL of 95% ethanol was added to the solution at 0 °C. The mixture was chilled for 10 min at -70 °C and centrifuged at 12,000 g for

10 min. The pellet was resuspended in 250 µL of 0.3 M sodium acetate buffer (pH 5.2), and DNA was precipitated with ethanol again. The pellet was rinsed in 70% ethanol, dried and dissolved in 5 µL of 80% formamide-10 mM NaOH loading buffer for gel electrophoresis. The solution was heated for 1 min at 90 °C and loaded on 10% or 16% polyacrylamide sequencing gel. In the case of the 5'-end labeled DNA fragment, the pellet was dissolved in 5 µL of 80% formamide-10 mM NaOH loading buffer, heated at 90 °C for 10 min and loaded on polyacrylamide sequencing gel.

RESULTS AND DISCUSSION

Our earlier work has demonstrated that irradiation of a dinucleotide, *e.g.*, thymidylyl-(3'-5')-2'-deoxyadenosine, in the presence of methylamine produces a ring-opened adduct of the pyrimidine ring which on subsequent heating at 70 °C leads to an efficient cleavage of the 3'-5'-phosphodiester linkage via β-elimination (9,10). We considered that the sequence of these reactions might be used for T specific cleavage of DNA fragments. Our previous work has also suggested that the G cleavage observed by Simoncsits and Török (5) is probably due to the photooxidation of G residues which can be suppressed by addition of a singlet oxygen quencher (10), whereas the minor C cleavage is assumed to result from the alkaline-sensitive lesions at pyrimidine-cytosine sequences recently observed by Haseltine and coworkers in 254-nm irradiation of human DNA (11,12). We know from the model experiments that the T cleavage induced by the photoreaction with primary alkylamines does not require piperidine treatment (10). Based on these considerations, we devised a new method for highly T selective cleavage of DNA fragments by utilizing a photoreaction with spermine which is known to strongly bind to DNA and may act as an inhibitor for G photooxidation.



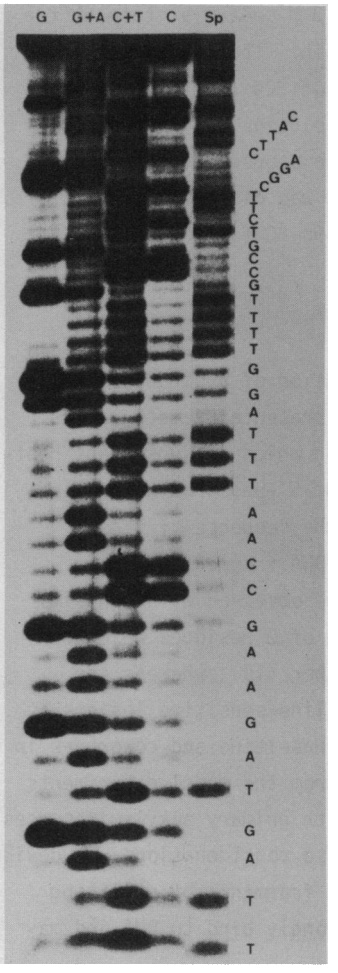


Figure 1. Autoradiograph of sequencing gel of short DNA fragment (56 bp) showing both the Maxam-Gilbert reactions (G, G + A, C + T, C) and spermine photoreaction (Sp).

Figures 1 and 2 show the autoradiographs of the sequencing gels of UV-irradiated two 3'-[^{32}P]-end-labeled DNA fragments of defined sequences together with the standard sequencing reactions of the Maxam-Gilbert method. Irradiation for 10∿20 min with a commercial germicidal lamp emitting mainly 254-nm light in the presence of 1 M spermine in distilled water resulted in a T specific cleavage of the DNA chains of short (56 bp) and longer (178 bp) DNA fragments without piperidine treatment. The longer the irradiation time the more intensive bands at T residues appeared as seen in Figure 2. It is noteworthy that only the T bands appeared uniformly within 20 min irradiation, whereas prolonged irradiation (>30 min) resulted in minor side-reactions

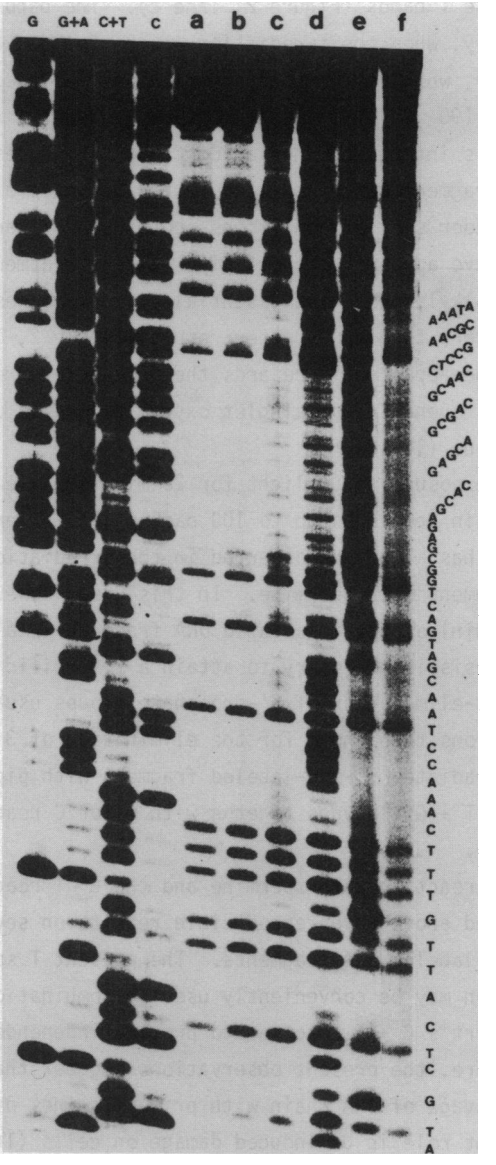


Figure 2. Photoreaction of a long DNA fragment (178 bp) in the presence of various amines. In lane a, b and c, the irradiation was performed in the presence of spermine for 10, 20 and 30 min, respectively. In lane d, 20 min irradiation with spermine was followed by treatment of 1 M piperidine (90 °C, 30 min). In lane e and f, the irradiation was performed for 20 min in the presence of methylamine and cyclohexylamine, respectively.

together with intense T bands (Figure 2, lane c). The band pattern was not altered significantly, when the irradiation was performed under nitrogen atmosphere. However, when the same irradiation was followed by treatment with 1 M piperidine (90 °C, 30 min), the strong bands at T residues were accompanied with less intensive bands at G, C and A residues (Figure 2, lane d). When the DNA fragment was irradiated in the presence of methylamine in place of spermine under similar conditions, both G and T bands with the intensity of G > T have appeared without piperidine treatment (Figure 2, lane e). Other primary alkylamines gave essentially the same result, although cyclohexylamine appears to give a better result (Figure 2, lane f). Unlike methylamine, spermine appearently retards the G reaction presumably by chemical or physical quenching of singlet oxygen generated under the irradiation conditions (10,13).

With a single exposure to UV light for 20 min, we have been able to identify T residues in sequences up to 100 base pairs in length. A similar T specific cleavage has also been observed in the irradiation of a 5'-[^{32}P]-end-labeled DNA fragment with spermine. In this case, however, a prolonged heating (90 °C, 10 min) of the irradiated DNA fragment in a loading buffer for gel electrophoresis is necessary to attain a T specific reaction, presumably because β-elimination of 5'-phosphate groups usually requires more drastic conditions than those for the elimination of 3'-phosphate groups. Treatment of the irradiated 5'-end-labeled fragment with piperidine (90 °C, 30 min) resulted in T + G cleavage together with minor C reactions as already reported (5).

The T specific reaction with spermine and the G > T reaction with methylamine described above gave reproducible results on several short and longer 3'-[^{32}P]-end-labeled DNA fragments. The present T specific reaction and the G > T reaction may be conveniently used in combination with the standard Maxam-Gilbert's C + T reaction to provide independent confirmatory readings. Furthermore, the present observations suggest that a similar type of photoinduced cleavage of DNA chain with primary amines of natural origin may play an important role in UV-induced damage on cells (14).

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