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Base-specific reactions useful for DNA sequencing: methylene blue – sensitized photooxidation of guanine and osmium tetraoxide modification of thymine

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ABSTRACT

Exposure of DNA to methylene blue and visible or ultraviolet light causes guanine-specific modification, and subsequent treatment with piperidine leads to chain cleavage at each guanine residue. Treatment of DNA with osmium tetraoxide in dilute pyridine leads to thymidine-specific modification, and subsequent treatment with piperidine leads to chain cleavage at the modified thymidine residues. Both reactions can be used in conjunction with other base specific modifications described by Maxam and Gilbert (1) for the determination of the nucleotide sequence in DNA.

INTRODUCTION

The method of DNA sequencing recently described by Maxam and Gilbert (1) relies on limited, base-specific modification of DNA followed by chain cleavage at modified sites. Some of the reagents used for base modification are somewhat toxic, and their specificity is sensitive to reaction conditions. We have undertaken a search for additional base specific reagents in order to improve the specificity of the reactions, especially that for thymidine. This report describes the use of methylene blue-sensitized photooxidation for the guanine-specific modification (2-4), and osmium tetraoxide (5) for thymidine-specific modification of DNA, and the use of these reagents for DNA sequencing.

MATERIALS AND METHODS

Polyoma component I DNA was extracted and purified from infected 3T6 cells as described elsewhere (7). The supercoiled DNA was digested with the sequence-specific endonu-

clease Hpa II in Hin buffer (6.6 mM Tris, pH 7.6, 6.6 mM $MgCl_2$, 0.1 mM dithiothreitol, and 50 mM NaCl), and the eight known Hpa II fragments (8) separated on a 5-15% polyacrylamide gel in TBE buffer (90 mM Tris, pH 8.3, 90 mM boric acid, 0.1 mM EDTA) after electrophoresis for 20 hours at 250 volts. The fragments were visualized by staining with 0.5 μg/ml ethidium bromide in TBE buffer, and the individual bands cut from the gel. The fragments were electroeluted in 1/5 TBE buffer containing 0.1% SDS, precipitated at -20° with 2½ volumes ethanol after the addition of one tenth volume 3M NaAc, pH 5.2. The pelleted DNA was washed once with ethanol, vacuum dried, redissolved in water and stored at -20°.

Hpa II fragment 5 was labelled at its 5' ends by reaction with ^{32}p-γ-ATP in the presence of polynucleotide kinase by a modification of the method described by Maxam and Gilbert (1). The reaction was stopped by extraction with phenol followed by ether extraction to remove the phenol and ethanol precipitation at -20°. The DNA was pelleted by centrifugation at 12,000 xg for 4 minutes, vacuum dried, and redissolved in Hin buffer. The labelled fragment was digested with Hha I, and the resulting 2 fragments separated on a 5% polyacrylamide gel in TBE buffer after electrophoresis at 300 volts for 4-5 hrs. The fragments were located by autoradiography and eluted with 0.5 M NH_4Ac, 10 mM $MgAc_2$, 0.1 mM EDTA, 0.1% SDS at 67° for 12 hours. After removal of acrylamide pieces by filtration through glass wool, the fragments were precipitated and washed once with ethanol, vacuum dried and stored at -20°.

Aternatively, polyoma DNA was cleaned with Eco R_1, and labelled with ^{32}P-α-dATP and ^{32}P-α-dTTP in Hin buffer in the presence of DNA polymerase (Klenow). The labelled DNA was cleaved with Hae III, and the two resulting fragments separated on a 5% polyacrylamide gel as above. The larger fragment was used in subsequent sequences studies.

SEQUENCE DETERMINATION

The nucleotide sequence of the labelled fragments were determined by the method of Maxam and Gilbert (1), using the

methylation reaction for G and G+A, the alkali reaction for A, and the hydrazine reactions for C+T and for C. The samples were analyzed on a 12% polyacrylamide gel with 7M urea in TBE buffer, and electrophoresis was carried out for 7 hours, at 1000 volts, 35 ma.

ALTERNATIVE METHYLENE BLUE REACTION

An aliquot of the labelled fragment 5b containing 20 µg sonicated calf thymus DNA in 5 µl water was added to 20 µl of 0.1% methylene blue in water on a non-wettable surface. The mixture was exposed to direct or room sunlight or to a 75 watt light bulb at a distance of 12 inches for 15 minutes, or to a short wave UV source (UV Products, Inc., San Gabriel, California) delivering 160 $\mu W/m^2$ for 15 minutes. The sample was diluted and redissolved in 0.2 ml 0.3 M NaAc, pH 5.2, containing 50 µg yeast tRNA, precipitated with 700 µl ethanol at -70°, and centrifuged at 12,000 xg for 3 minutes. The pellet was washed once with ethanol, vacuum dried, redissolved in 10% redistilled piperidine and heated for 30 minutes at 90° in a sealed glass capillary. The sample was vacuum dried, redissolved in 20 µl water and dried again. Finally, the sample was dissolved in 98% formamide containing 0.05% xylene cyanol and 0.05% bromphenol blue, heated at 90° for 3-4 minutes and applied to a 40 cm x 20 cm x 1.5 mm 12% polyacrylamide gel in TBE buffer containing 7M urea. Electrophoresis was for 7 hours at 1000 volts. The gel was exposed to Fuji Medical X-ray film flashed to an optical density of approximately 0.2, using an Ilford tungsten intensifying screen at -70°.

The identical procedure was used for irradiation in the presence of rose bengal.

OSMIUM TETRAOXIDE REACTION

Labelled fragment in 5 µl water was added at 0° to 10 µl 5% osmium tetraoxide in water and 1 µl pyridine. The resulting precipitate was agitated and kept at 0° for 15 minutes. Thereafter 0.2 ml 0.3 M NaAc, pH 5.2, containing 50 µg yeast to RNA were added, and the entire sample precipitated with 2½ volumes ethanol. The sample was centrifuged at 12,000 xg for 3 minutes, redissolved in 0.3 M

NaAc, pH 5.2 and precipitated again with ethanol. After recentrifugation, the sample was dried in vacuum, redissolved in 10% piperidine, and heated at 90° for 30 minutes. After vacuum drying, the sample was dissolved in 98% formamide, heated at 90° for 3-4 minutes, and examined on acrylamide gels as described above.

RESULTS

Figure 1 demonstrates the use of the methylene blue reaction together with the standard reactions of the direct chemical DNA sequencing method. The nucleotide sequence of this portion of Hpa II-5 has been determined independently and confirmed by sequence determination of both strands, and this data will be presented elsewhere.

Figure 2 shows the sequence determination at the $EcoR_1$ site in polyoma, extending clockwise along the genetic map, and demonstrates the effect of the concentrations of osmium tetraoxide.

DISCUSSION

It has been known for some time that irradiation in the presence of methylene blue and other dyes including rose bengal causes guanine-specific damage to DNA (2-4). It is known that singlet oxygen can be generated during visible light irradiation of aqueous solutions of methylene blue in the presence of oxygen (9,10) and that cyclo-addition can occur at various positions in the purine ring (11-15). Exhaustive treatment of guanosine leads to ribose and ribosylurea (4). The product from guanine residues under mild conditions has not been identified but its formation doen not lead to chain scission in the polynucleotide (16). We thought, however, that it might render DNA susceptible to base catalysed cleavage.

Photooxidation is brought about by direct exposure to diffuse or direct sunlight, to a tungsten light bulb or to an ultraviolet light source. As expected, the reaction did not lead to strand scission directly but after piperidine treatment, specific cleavage products were seen on gel electrophoresis.

With a single exposure to sunlight for 15 minutes, we

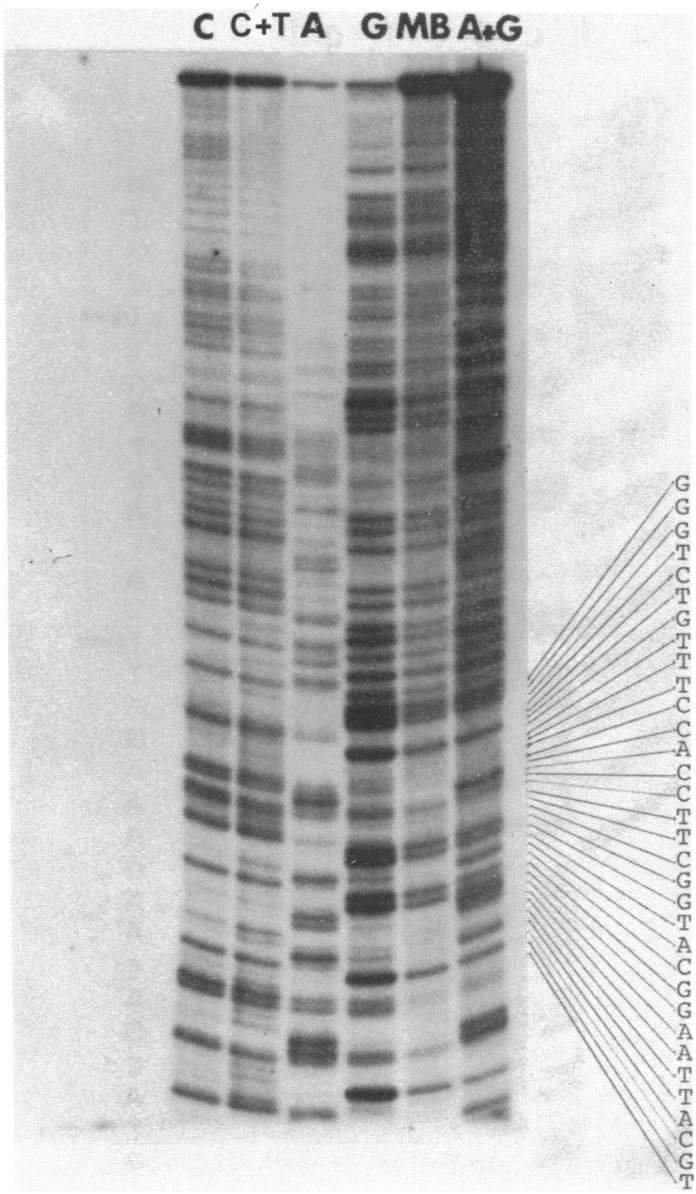

Fig. 1. Polyacrylamide gel electrophoresis of standard sequencing reactions for G, alternate A, T + C, C, and A + G. as well as the reaction for G using methylene blue and visible light. A portion of the sequence is given adjacent to the electrophoretic pattern, reading in a 5' to 3' direction upward from the bottom of the gel. The channels for the standard G and for the methylene blue G are essentially indistinguishable.

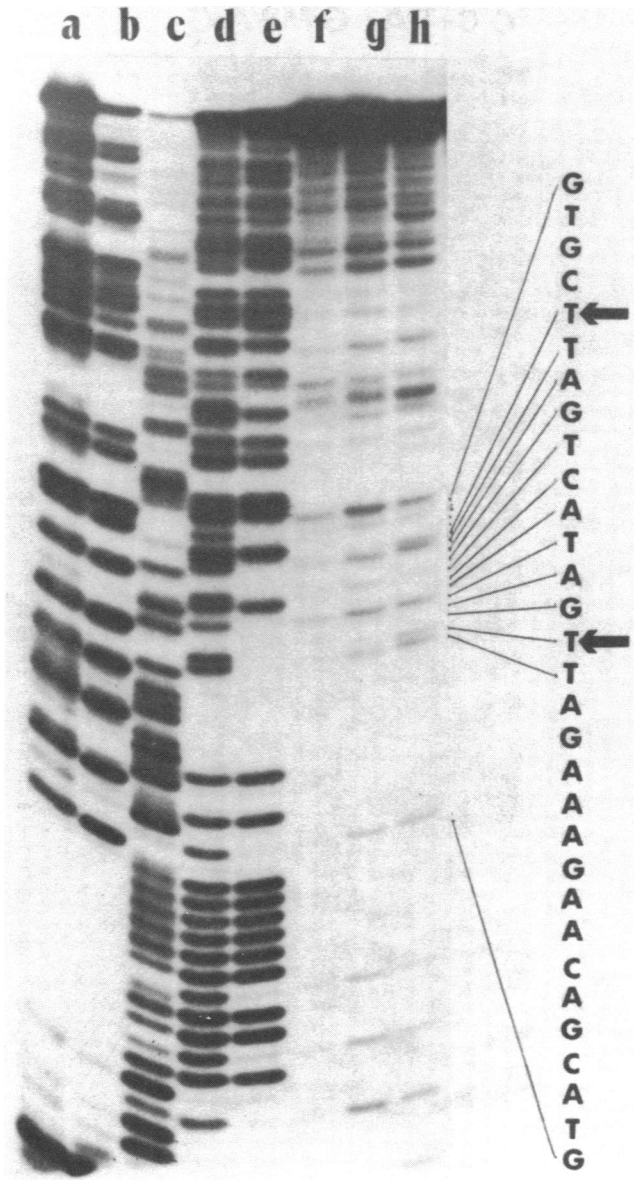

Fig. 2. Polyacrylamide gel electrophoresis of a portion of the polyoma fragment Eco R_1/Hae III 8. The concentrations of osmium tetraoxide were 0.05% for f, 0.5% for g and 5% for h. Channels a - e are A + G, methylation G, alternate A, C + T and C, as described by Maxam and Gilbert. At least two of the T residues (arrows) are relatively unreactive in the lower concentrations of osmium tetraoxide.

have been able to identify guanine residues in sequences up to 140-150 nucleotides in length. The present procedure with methylene blue does not offer major advantages over the usually reliable methylation reaction, although the band intensity has been somewhat more uniform in the photooxidative reactions for G.

Indistinguishable results have recently been obtained with photooxidation in the presence of 0.1% rose bengal, followed by ultraviolet irradiation and piperidine treatment as described above.

Photooxidation of formylmethionine tRNA in the presence of methylene blue has been used previously to identify a guanine in the acceptor stem required for acceptor activity (17). Only two guanosine residues in the whole molecule were modified, the rest presumably being protected by the secondary structures of the tRNA. This kind of protection does not seem to occur in DNA, since all guanine residues become sites of strand scission after irradiation and piperidine treatment.

Since the hydrazine reaction for pyrimidines, especially that for T, has been somewhat less reliable in our laboratory, the development of a T-specific reagent is of potentially greater usefulness. Osmium tetraoxide is known to interact at alkaline pH specifically with the T residues of DNA, and recent work has shown that DNA modification by OsO_4 can be used to identify the positions of T residues in short synthetic oligonucleotides by DEAE cellulose chromatography (6).

Concentrations of osmium tetraoxide from 0.05% to 5% are all effective, although the higher concentrations are necessary for efficient reaction with a small number of T residues. It seems probable that features of the secondary structure of the DNA at those regions make those residues less accessible to the bulky osmium reagent.

The reaction of osmium tetraoxide with thymidine residues is thought to result in the formation of 5, 6 dihydroxy thymidine as the major product (5), a change that in itself does not result in chain cleavage, but rather leads to labilization of the polynucleotide chain at the position of modified T residues. The chain lengths of cleavage products

of OsO_4- modified DNA are identical to the products produced by the standard hydrazine reactions described by Maxam and Gilbert.

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