

# Nonenzymatic sequence-specific methyl transfer to single-stranded DNA

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**ABSTRACT** 2'-Deoxyuridine 5'-triphosphate with a methylthioether moiety at the 5 position can be incorporated into a primer-template DNA complex with Klenow enzyme. Activation with CNBr at 25°C, pH 5.5, followed by treatment with piperidine produces sequence-specific cleavage on the template DNA predominantly at a single guanine position. The mechanism involves methyl-group transfer from sulfur on the modified deoxyuridine of the extended primer to N-7 of guanine on the template DNA. This raises the possibility for the design and synthesis of a nonenzymatic class of sequence-specific methyl-transferases for DNA.

Synthetic DNA and RNA hybridization probes equipped with reactive groups can cleave the complementary sequence within large single-stranded nucleic acid (1-9). The DNA cleavage reactions differ with regard to mechanism and target: oxidation of the deoxyribose (2-4), electrophilic modification of the bases (5-7), and hydrolysis of phosphodiester bonds (8) exemplify the diversity of reaction. Most share the common feature of affording *multiple* cleavages at the site of hybridization. This feature can be due to a diffusible reactive species, as in the case of hydroxyl radical (2-4), and/or nonoptimized placement of the reactive moiety on the hybridization probe, as in the alkylation (5, 6) and nuclease cleavage approaches (8).

We recently reported that DNA hybridization probes carrying a methylthioether moiety activated by CNBr cleave single-stranded DNA predominantly at a single guanine position after piperidine treatment (7). We report here that this reaction is due to specific methyl-group transfer to N-7 of guanine. In addition, a protocol for the nonenzymatic cleavage of large single-stranded DNA at guanine addressed by DNA hybridization probes is described.

5-[3-[[3-Methylthio]propionyl]amino]-*trans*-1-propenyl]-2'-deoxyuridine 5'-triphosphate (MT-dUTP) can be enzymatically incorporated into a primer-template region (18 base pairs) within 5386 nucleotides (nt) of single-stranded  $\phi$ X174 DNA by use of the large fragment of DNA polymerase I (Klenow enzyme). Activation with CNBr at 25°C (pH 5.5) followed by treatment with piperidine at elevated temperatures produces cleavage on the complementary strand at a guanine 2-nt positions to the 5'-side of the modified base.

## MATERIALS AND METHODS

<sup>1</sup>H nuclear magnetic resonance (NMR) spectra were recorded on a JEOL Fourier transform 400-MHz spectrometer and are reported in parts per million (ppm) from tetramethylsilane. UV-visible spectra were recorded on a Cary 219 spectrophotometer. Mass spectral determinations [high-resolution positive-ion fast atom bombardment (FAB)] were obtained from the Midwest Center for Mass Spectrometry at Lincoln, NB (National Science Foundation Regional Instru-

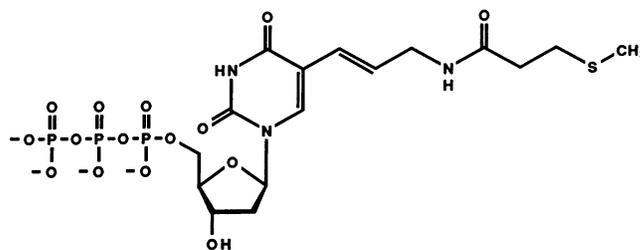


FIG. 1. MT-dUTP.

mentation Facility). Analytical HPLC was performed with a Hewlett-Packard 1090 liquid chromatograph with diode array detection and a Hewlett-Packard 79994A analytical workstation. Preparative ion-exchange HPLC was performed with a 250 × 4.6-mm i.d. Synchronpak Q300 (Synchron, Linden, IN) anion-exchange column using an Upchurch Uptight precolumn packed with Q300 material. Reverse-phase HPLC was performed with a 150 × 4.6-mm i.d. Vydac 201HS5415 C<sub>18</sub> column. Densitometry was carried out on an LKB Ultrascan XL laser densitometer.

Oligonucleotides were synthesized on a Beckman System 1 Plus DNA synthesizer and were as follows: oligonucleotide A, 5' ATTAAGCCACTTCTCCTC 3', oligonucleotide B, 5' TGACGACTACTGCTGAGA 3', and oligonucleotide C, 5' CTGTGTTCTAGTCTCAGCAGTAGTCGTCA 3'. Klenow enzyme and calf alkaline phosphatase were purchased from Boehringer Mannheim. All other enzymes and  $\phi$ X174DNA were from New England Biolabs. *N,N*-Dimethylformamide (HCONMe<sub>2</sub>) was distilled over CaH<sub>2</sub> at reduced pressure, and *N*-hydroxysuccinimide was recrystallized from ethyl acetate. All other chemicals were used without further purification.

**Synthesis of MT-dUTP (Fig. 1).** 3-Methylthiopropionic acid (26.4 mg, 0.22 mmol) was taken up in 5.0 ml of dry HCONMe<sub>2</sub> under argon along with 1,3-dicyclohexylcarbodiimide (49 mg, 0.24 mmol) and *N*-hydroxysuccinimide (38 mg, 0.32 mmol). The solution was allowed to stir at 25°C for 12 hr and was filtered. To the filtrate was added a solution of 5-(3-amino-*trans*-1-propenyl)-2'-deoxyuridine 5'-triphosphate (50 mg, 0.06 mmol) (9) in 5 ml of 0.1 M sodium borate buffer, pH 8.8, at 25°C. Aliquots (40  $\mu$ l) were periodically removed and subjected to a ninhydrin test for primary amines. The reaction was complete after 6 hr. The reaction mixture was diluted with 100 ml of water and loaded onto an 18 × 200-mm DEAE-Sephadex A-25-120 column pre-equilibrated with 0.05 M triethylammonium bicarbonate (Et<sub>3</sub>NHCO<sub>3</sub>) at pH 7.6. The product was eluted with a step gradient of 40 ml each of 0.1 M to 1.2 M Et<sub>3</sub>NHCO<sub>3</sub> in 0.1 M increments. Crude MT-dUTP eluted between 0.7 M and 0.8 M Et<sub>3</sub>NHCO<sub>3</sub> as the

Abbreviations: Et<sub>3</sub>NHCO<sub>3</sub>, triethylammonium bicarbonate; HCONMe<sub>2</sub>, *N,N*-dimethylformamide; MT-dU and MT-dUTP, 5-[3-[[3-(methylthio)propionyl]amino]-*trans*-1-propenyl]-2'-deoxyuridine and deoxyuridine 5'-triphosphate, respectively; nt, nucleotide(s); ddGTP, 2',3'-dideoxyguanosine 5'-triphosphate.

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last UV absorbing compound and was then lyophilized. The crude MT-dUTP was redissolved in water and purified by preparative HPLC on a Synchropak Q300 anion-exchange column, monitored at both 260 and 290 nm. MT-dUTP eluted at  $\approx 25$  min using a 30-min linear gradient of 0–0.3 M chilled ammonium bicarbonate  $[(\text{NH}_4)_2\text{CO}_3]$ , pH 7.6, and a flow rate of 0.75 ml/min. The appropriate fractions were pooled and lyophilized to yield the ammonium salt of MT-dUTP (23 mg, 55%), which was stored under vacuum. NMR ( $^2\text{H}_2\text{O}$ )  $\delta$  7.8 (1H, s,  $\text{H}_6$ ), 6.28–6.35 (1H, m, =CH-), 6.18–6.23 (2H, m, -HC=,  $\text{H}_1'$ ), 4.53–4.55 (1H, m,  $\text{H}_3'$ ), 4.08–4.12 (3H, m,  $\text{H}_5'$ ,  $\text{H}_4'$ ), 3.8 (2H, d,  $J = 5$  Hz,  $\text{CH}_2$ ), 2.66 (2H, t,  $J = 7$  Hz, - $\text{CH}_2$ -S), 2.49 (2H, t,  $J = 7$  Hz,  $\text{CH}_2$ ), 2.25–2.28 (2H, m,  $\text{H}_2'$ ), 1.99 (3H, s, S- $\text{CH}_3$ ). UV-Vis ( $\text{H}_2\text{O}$ ),  $\lambda_{\text{max}} = 239$  nm ( $\epsilon = 10,600$ ),  $\lambda_{\text{min}} = 267$  nm ( $\epsilon = 4100$ ),  $\lambda_{\text{max}} = 289$  nm ( $\epsilon = 7000$ ). IR (KBr) 1650, 1260, 1090, 1040, 800  $\text{cm}^{-1}$ .

**Primer-Template Extension with MT-dUTP and CNBr Cleavage Reactions on  $\phi\text{X174}$  DNA.** The 5'-end-labeled  $\phi\text{X174}$  DNA (4400 cpm) linearized at the *Xho* I site (10), was added to 1  $\mu\text{l}$  of  $10\times$  Klenow buffer (60 mM Tris-HCl, pH 7.4/500 mM NaCl/60 mM  $\text{MgCl}_2$ ) and 1  $\mu\text{l}$  of oligonucleotide A (10  $\mu\text{M}$ ). The solution was placed in an 80-ml water bath (60°C) and then allowed to cool to room temperature. One microliter of 50 mM dithiothreitol was added along with 3  $\mu\text{l}$  of a solution containing 3 mM each of dATP, dCTP, and 2',3'-dideoxyguanosine 5'-triphosphate (ddGTP). One microliter of 10 mM MT-dUTP (or TTP for the control reaction) was added followed by 2  $\mu\text{l}$  of Klenow enzyme (2 units/ $\mu\text{l}$ ), and the reaction was incubated at room temperature for 1 hr.

Seven and one-half microliters of 100 mM NaOAc/20 mM NaCl, pH 5.5 was added along with 1  $\mu\text{l}$  of deproteinized sonicated calf thymus DNA (1 mg/ml) and 9  $\mu\text{l}$  of 50 mM CNBr. The reaction was incubated in a sealed vial at room temperature for 18 hr, then 3  $\mu\text{l}$  of piperidine was added, and the solution was heated at 90°C for 20 min. The sample was frozen, lyophilized to dryness, and the residue was redissolved in 20  $\mu\text{l}$  of formamide loading buffer. The loading buffer solution was heated at 90°C for 3 min and then cooled in ice for 5 min, and 2  $\mu\text{l}$  was loaded onto an 8% denaturing polyacrylamide gel (1:20 crosslinked, 50% urea). The site-specific cleavage efficiency was found by densitometry to be 9%.

**Analysis of Oligonucleotide Cleavage Products.** A solution containing 75  $\mu\text{l}$  of 138  $\mu\text{M}$  oligonucleotide B and 150  $\mu\text{l}$  of 68  $\mu\text{M}$  oligonucleotide C labeled with  $^{32}\text{P}$  at the 5' end (22,000 cpm) was added to 75  $\mu\text{l}$  of  $10\times$  Klenow buffer. The solution was placed in an 80-ml water bath (60°C) and allowed to cool to room temperature. Three-hundred microliters of a solution with 3 mM (each) dATP, dGTP, and dCTP was added followed by 100  $\mu\text{l}$  of 10 mM MT-dUTP or 100  $\mu\text{l}$  of 10 mM TTP for the control reaction. Fifty microliters of Klenow enzyme solution (5 units/ $\mu\text{l}$ ) was added, and the reaction was incubated at 37°C for 25 min. Eighty-five microliters of glycerol loading buffer was added, and the sample was loaded on a 15% nondenaturing polyacrylamide gel (1:20 crosslink). The oligonucleotide was visualized with a short-wave UV light, and the uppermost band corresponding to the completely filled-in duplex was excised from the gel and crushed. Five hundred microliters of 0.2 M NaCl was added to the crushed gel, and the mixture was incubated at room temperature for 6 hr. The supernatant was removed, and another 600  $\mu\text{l}$  of 0.2 M NaCl was added to the gel fragments. After 8 hr the supernatant was again removed, and the combined supernatants were dialyzed against 4000 ml of water for 10 hr at 4°C. With this procedure 4.0 nmol of blunt-ended duplex containing 5-[3-{[3-(methylthio)propionyl]amino}-*trans*-1-propenyl]deoxyuridine (MT-dU) and 2.7 nmol of duplex with dT as control were recovered [based on  $\epsilon_{260} = 6700$  per phosphate for double-stranded DNA (11)].

The solution containing the oligonucleotide duplex was reduced in volume to 50  $\mu\text{l}$ ; then 25  $\mu\text{l}$  of 100 mM NaOAc (pH 5.5), and 25  $\mu\text{l}$  of 100 mM CNBr were added. The solution was incubated for 36 hr at 25°C. The solvent was evaporated with a stream of argon, and then the sample was placed under high vacuum for 3 hr. The pellet was redissolved in 100  $\mu\text{l}$  of water and heated at 90°C for 70 min to liberate any 7-methylguanine from the oligonucleotide duplex (12). Three-hundred microliters of ethanol was added, and after 15 min at room temperature, the mixture was spun at 12,000 rpm for 10 min. The supernatant was removed and evaporated to dryness. Residue from the supernatant was redissolved in 28  $\mu\text{l}$  of 10 mM ammonium acetate (pH 5.5).

Twenty-five microliters of the solution containing the residue from the ethanol supernatant was loaded onto a Vydac  $\text{C}_{18}$  column. The sample was eluted with 0.5 ml/min flow rate, and a 30-min linear gradient of 0–2% acetonitrile in 10 mM ammonium acetate (pH 5.5). The amount of released 7-methylguanine observed in the cleavage reaction was estimated by comparison with authentic standard samples of 7-methylguanine.

The oligonucleotide pellet obtained in the ethanol precipitation step of the large-scale CNBr cleavage reaction and product isolation was redissolved in 60  $\mu\text{l}$  of water. Three microliters of piperidine was added to 30  $\mu\text{l}$  (4400 cpm) of this solution. The reaction was heated at 90°C for 20 min, then frozen, and lyophilized, and the residue was redissolved in 20  $\mu\text{l}$  of formamide loading buffer. The solution was loaded onto a 20% denaturing polyacrylamide gel (1:20 crosslinked, 42% urea). The oligonucleotide-cleavage product has electrophoretic mobility identical to the same DNA fragment with a 3'-phosphoryl terminus. Amount of site-specific cleavage was 12% from densitometry of the autoradiogram.

## RESULTS

**Enzymatic Incorporation of MT-dUTP.** MT-dUTP is a substrate for the Klenow enzyme. Controls show that the Klenow enzyme was able to incorporate MT-dUTP onto the 3' end of the oligonucleotide primer using an oligonucleotide duplex containing a single adenine 5' overhang. Denaturing 20% polyacrylamide gel electrophoresis revealed that an oligonucleotide carrying the 3' terminal MT-dU had a slower mobility than the same oligonucleotide carrying a 3' terminal dT residue. Other controls demonstrated that in the absence of MT-dUTP or dTTP, a nucleotide triphosphate was not incorporated opposite the adenine in the overhang region of a partial duplex (13).

**Cleavage of Single-Stranded DNA.** The ability of MT-dUTP to sequence specifically methylate within large pieces of single-stranded DNA was investigated. Oligonucleotide primer A was hybridized to linearized single-stranded  $\phi\text{X174}$  DNA (5386 nt) at the complementary sequence seventy bases from the 5' end labeled with  $^{32}\text{P}$ . This primer was extended with dATP, dCTP, ddGTP, and MT-dUTP in the presence of Klenow enzyme. The ddGTP chain terminator (14) insured that the Klenow reaction stopped after adding eight residues so that a single residue of MT-dU was incorporated onto primer A (Fig. 2). CNBr was added to initiate the alkylation reaction. After incubation, piperidine was added to effect depurination and backbone cleavage. The site-specific cleavage was analyzed on a denaturing 8% polyacrylamide gel (Fig. 3). The autoradiogram of the sequencing gel reveals that cleavage on the  $\phi\text{X174}$  DNA occurs predominantly at a guanine residue 2 nt positions to the 5' side of the MT-dU position on the strand opposite the primer. The cleavage yield from densitometry was 9%. Cleavage was also observed at the adjacent guanine residue with one-thirteenth the efficiency relative to the major site of reaction.

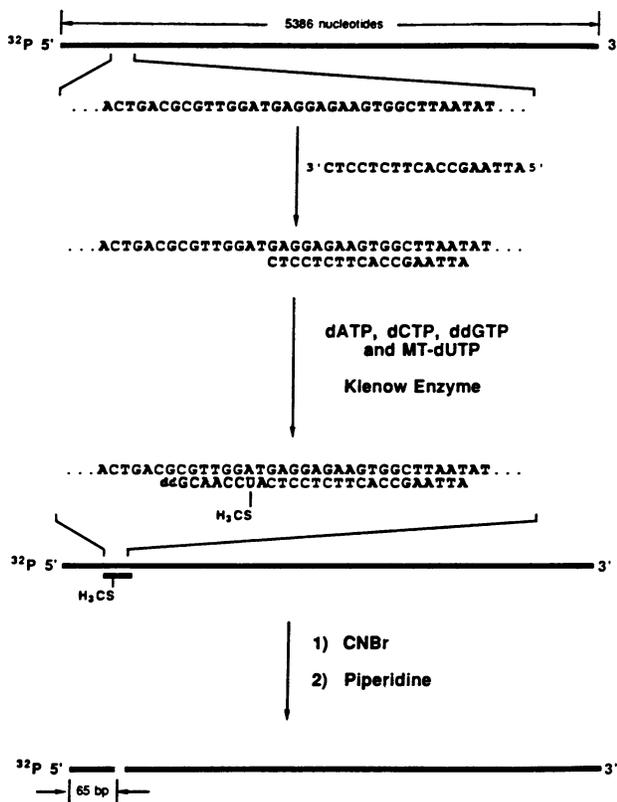


FIG. 2. Cleavage of large single-stranded DNA. The primer 3' CTCCTCTTCACCGAATTA 5' was hybridized to linearized single-stranded  $\phi$ X174 DNA labeled with  $^{32}$ P at the 5' end (*Xho* I restriction site). Primer extension (5'-3') was conducted with dATP, dCTP, MT-dUTP, and ddGTP (as terminator) in the presence of Klenow enzyme. After the Klenow enzyme reactions, NaCl and NaOAc (pH 5.5) solutions were added to final concentrations of 5 mM and 25 mM, respectively. One microgram of cytosine- and thymine-containing DNA was added, and the reactions were incubated at 25°C for 18 hr with or without 15 mM CNBr. Three microliters of piperidine was then added, and the samples were heated at 90°C for 20 min. The samples were lyophilized, and the residue was redissolved in formamide loading buffer and loaded onto a sequencing gel (see Fig. 3).

**Released Product Analysis.** The nature of the modified guanine species produced in the MT-dU/CNBr/piperidine cleavage reaction was investigated by carrying out a large-scale enzyme fill-in reaction using MT-dUTP and duplex DNA formed from oligonucleotides B and C. An assay was designed to identify by HPLC retention times and spectral analyses the methylated guanine base postulated to be a reaction product from the sequence-specific methylation/depurination reaction.

The mechanistic rationale for methylation at N-7 of guanine followed by depurination and cleavage upon piperidine workup is based on the work of Lawley and Brookes (15), who found that reaction of DNA with methyl methanesulfonate methylates N-7 of guanine. At pH values below 8.5, an acid-catalyzed hydrolysis yields 7-methylguanine as the released product. Above this pH, an alkali-catalyzed ring fission occurs to yield substituted 5-methylformamide-2,4-diamino-6-hydroxypyrimidine. Under the workup conditions used here for the analysis of base release, the pH is <8.5, and intact 7-methylguanine is the expected product for a methyl-transfer reaction. The target oligonucleotide was labeled with  $^{32}$ P to compare by autoradiography the relative yields of released 7-methylguanine to cleavage at that site.

The released products were analyzed by HPLC. Fig. 4a is a control showing the retention times (13–23 min) and

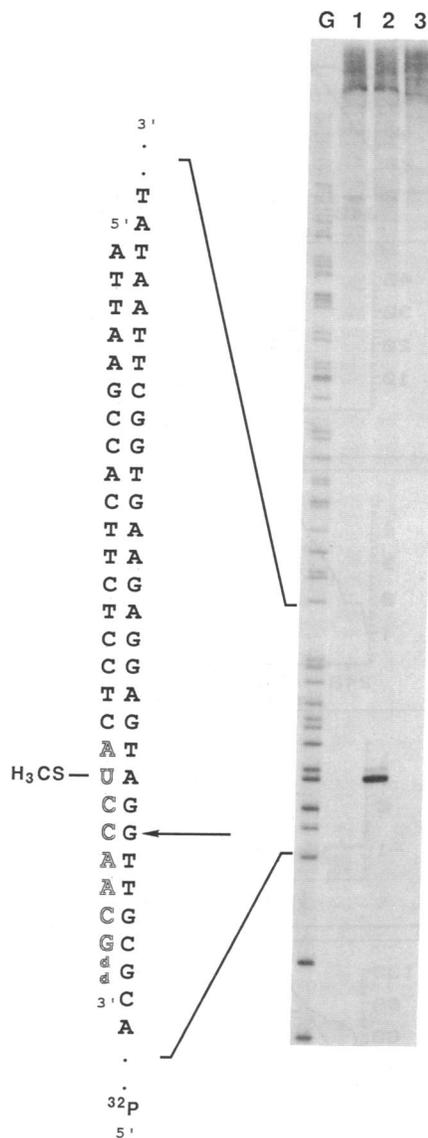


FIG. 3. Autoradiogram of a denaturing polyacrylamide gel for the protocol described in the legend to Fig. 2. Lane G, Maxam-Gilbert sequencing guanine reaction. Lane 1, Control; dTTP was used in place of MT-dUTP in the Klenow enzyme primer-extension reaction. Lane 2, Reaction conditions as described in the legend to Fig. 2. Lane 3, Control in which no CNBr was added. Duplex region shown at left is the hybridization site of the oligonucleotide primer. Outlined bases are the nucleotides extended by Klenow enzyme. The position of MT-dU is indicated. The arrow shows the observed major cleavage site.

corresponding UV-visible spectra from the HPLC analysis of authentic adenine and 7-methylguanine. Fig. 4b and c are the 13- to 23-min section of the HPLC chromatograms and the corresponding UV-visible spectra of those products found in the ethanol supernatants obtained in the large-scale cleavage reactions using oligonucleotide duplexes containing dT and MT-dU, respectively. A peak was detected at 16.3 min from the MT-dU-containing cleavage reaction mixture (Fig. 4c), which was not present in the corresponding control reaction mixture containing dT instead of MT-dU (Fig. 4b). By comparing the relative retention times and UV-visible spectra of the commercial standards with those of the peaks in Fig. 4b and c, the peak at 15.2 min (P1) in both b and c is identified as adenine (13), and the peak at 16.3 min (P2) in c is identified as 7-methylguanine. Therefore, the unique product detected by HPLC analysis of the ethanol/water super-

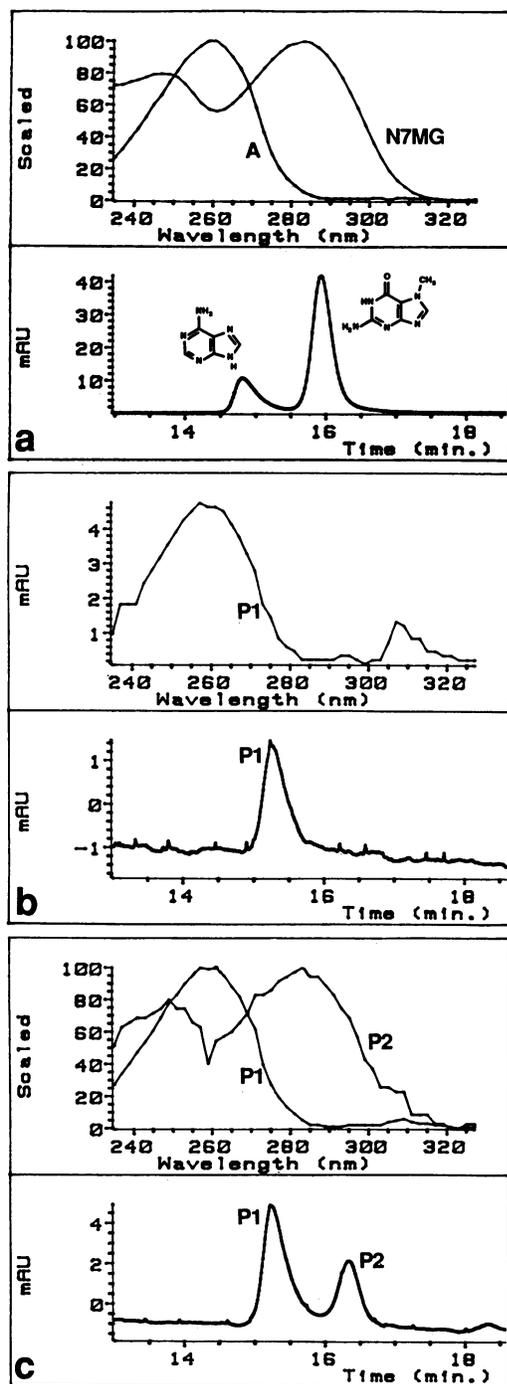


FIG. 4. Comparison of HPLC chromatograms and UV-visible spectra of authentic samples of adenine and 7-methylguanine with the released products from DNA-methylthioether/CNBr cleavage of DNA reaction, pH <8.5. Samples were eluted on reverse-phase column with a 30-min linear gradient of 0–2% acetonitrile in 10 mM ammonium acetate (pH 5.5) at a flow rate of 0.5 ml/min and monitored at  $270 \pm 30$  nm. (a) Control; authentic samples of adenine (A) and 7-methylguanine (N7MG). (b) Control; released product (P1) from ethanol precipitation supernatant in large-scale reaction with CNBr (pH 5.5) containing no MT-dU. Based on retention time and electronic spectrum the product P1 is assigned as adenine. (c) Released products P1 and P2 from large-scale cleavage reaction using MT-dUTP in the primer-extension reaction. The retention time and electronic spectrum of the new product P2 are identical to authentic 7-methylguanine.

nant from cleavage reaction using MT-dU complex and CNBr is 7-methylguanine.

To quantitate the amount of 7-methylguanine released in the methylthioether/CNBr methyl-transfer reaction, known amounts of authentic 7-methylguanine were injected onto the HPLC during calibration runs. Integration of the 16.3-min peak P2 in Fig. 4c indicated that this peak represents 0.3 nmol of 7-methylguanine. For comparison with the percent of released 7-methylguanine, cleavage efficiency on DNA was also analyzed. The precipitated oligonucleotide from the large-scale reactions was treated with piperidine and then loaded onto a denaturing 20% polyacrylamide gel. After electrophoresis, densitometry of the autoradiogram revealed that the single guanine residue on the complementary strand two bases to the 5' side of the residue of MT-dU was cleaved with 12% efficiency, which corresponds to  $\approx 0.48$  nmol of strand cleavage (from  $\approx 4.0$  nmol duplex  $\times 0.12$ ). This reveals that a lower limit of 63% (0.3/0.48) of the cleavage product is due to methyl transfer. Correction for recovery of 7-methylguanine found in controls (85%) and uncertainty in measuring the concentration of duplex could realistically put this value near unity.

## DISCUSSION

**Synthesis of DNA-Methylthioether Probes.** Ideally, modified DNA probes equipped with DNA-cleaving functions should be activated only after hybridization to the complementary target strand, thus avoiding the problems of inactivation during synthesis or autocleavage before hybridization. This constraint imposes limitations on the synthetic methods that can be used for the construction of DNA probes equipped with nucleic acid-reactive moieties. Three approaches have been described. The first methods used were modification of nucleic acid strands with heterobifunctional reagents—i.e., one end attaches the reagent to the DNA probe and, after hybridization, the other end modifies the complementary nucleic acid sequence (1). For example, Vlassov and coworkers (5, 6) attached 2-chloroethylamine derivatives to the 5' and 3' termini of oligonucleotides. Corey and Schultz (8) have attached a nuclease to an -SH-modified oligonucleotide. A second synthetic approach involves the incorporation of a modified base equipped with the masked cleaving function into an oligonucleotide by chemical methods. This allows the convenience of automated synthesis and affords control over the placement of the reactive moiety at discrete internal positions in the oligonucleotide strand. One example of this is DNA-EDTA probes constructed using the phosphoramidite of thymidine equipped with the triethyl ester of EDTA at the 5 position (2).

The method described here involves enzymatic incorporation of a modified deoxyridine triphosphate into a specific-oligonucleotide primer-hybridization site within large single-stranded DNA. The methylthioether function is not capable of cleavage in the absence of CNBr when incorporated into a DNA duplex. Once incorporated into the duplex region, the methylthioether is selectively activated to a presumed electrophilic cyanomethylsulfonium species by reaction with CNBr. This approach was inspired by the specific cleavage of peptides at methionine residues with CNBr, which is thought to result from the selective conversion to a similar sulfonium species (16).

**Nonenzymatic Modification of Single-Stranded DNA to Nucleotide Resolution.** Our results demonstrate that MT-dUTP is accepted as substrate for incorporation opposite adenine into a 5' overhang by Klenow enzyme. Assuming the DNA primer forms a B-like duplex structure, the presumed electrophilic sulfonium species generated by the reaction of the methylthioether and CNBr would be in the major groove proximal to the nucleophilic N-7 atoms on neighboring guanine bases (Fig. 5). A workup procedure using piperidine (90°C) cleaves the DNA backbone at the site of any guanine

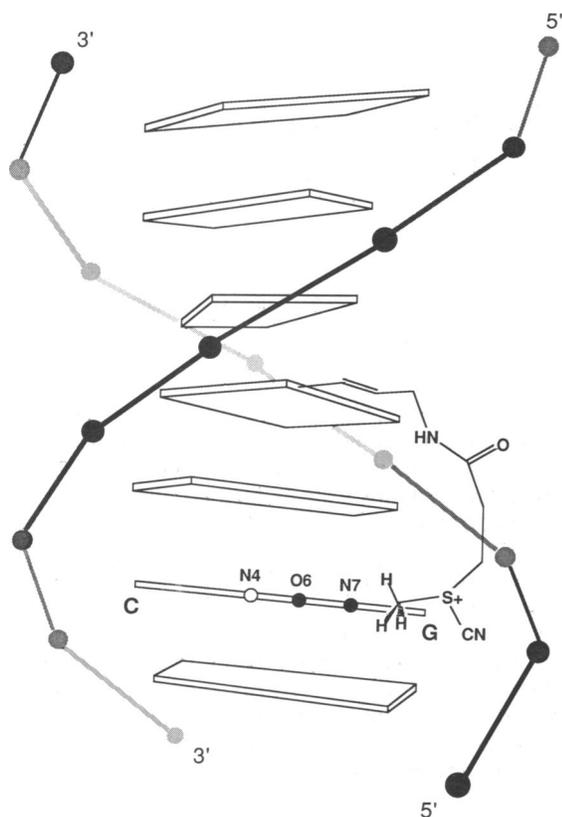


FIG. 5. Scheme for the nonenzymatic methyl group transfer from cyanomethylsulfonium attached to C-5 of uridine on one strand to the N-7 of guanine on the opposite strand.

alkylated at N-7 (17). To test the range of reactivity of the sulfonium species, the target site on the  $\phi$ X174 DNA contains two guanine residues to the 5' side of the adenine opposite the MT-dU base. Cleavage occurred predominantly at the guanine residue two bases to the 5' side of the MT-dU base. Cleavage at the neighboring guanine base occurred 13 times less efficiently.

**Mechanism of Sequence-Specific DNA Cleavage Involves Methyl Transfer to N-7 of Guanine.** The electrophoretic mobilities of the fragments produced indicate that the cleavage reaction affords 3'- and 5'-phosphate ends consistent with an alkylation-depurination mechanism, which results in excision of a base and sugar upon piperidine treatment (15, 17). The released-product analysis revealed that methyl transfer to N-7 of guanine is the predominant (and possibly exclusive) pathway of reaction responsible for depurination and cleavage of DNA. From model building the guanine cleaved is the one nearest the cyanomethylsulfonium cation if one assumes an  $S_N2$  geometry for transmethylation at N-7 (Fig. 5). Presumably the geometric requirements in the

transition state for an intrahelix nucleophilic substitution reaction (18, 19) are responsible for the observed specificity. No cleavage was observed when adenine was substituted in the complementary strand at the unique position of guanine cleavage. This is consistent with the relative reactivities of N-7 of guanine vs. adenine to methyl methanesulfonate (factor of 40) (12).

MT-dUTP analogues with one additional or deleted methylene unit between the amide carbonyl and methylthioether group were synthesized (19). When incorporated into DNA-methylthioether hybridization probes, the shorter analogue showed very little cleavage and the longer analogue showed specificity similar to MT-dUTP but lower efficiency of cleavage (13). Assuming that the linker arm at the 5 position of uridine is extended, this experimental result may provide some information on the cross-helix linker geometry required for  $S_N2$  interstrand reactions in nucleic acid duplexes. However, we cannot rule out that the different reactivity due to linker length reflects additional deactivation pathways, such as intramolecular decomposition of the cyanomethylsulfonium species.

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