Synthesis and properties of oligodeoxynucleotides with an AP site at a preselected position

Gregory R. Stuart and Robert W. Chambers

Department of Biochemistry, Dalhousie University, Halifax, Nova Scotia B3H 4H7, Canada

Received July 31, 1987; Revised and Accepted August 24, 1987

ABSTRACT
A general synthesis of a deoxyoligonucleotide with an AP site at a preselected sequence is described. Deoxyuridine is introduced during routine oligonucleotide syntheses of d(TTTUTTTT) and d(AAAAGTTUAAAACAT). Treatment with uracil DNA-glycosylase produces d(TTTmTT), where r = deoxyribose, and d(AAAAGTTprpAAAACAT). KM and Vmax are: d(TTTUTTTT), 7.3 x 10^-9 M and 2.0 x 10^-9 μmol / min; d(AAAAGTTUAAAACAT), 1.5 x 10^-8 M and 6.4 x 10^-9 μmol / min. Both d(AAAAGTTprpAAAACAT) and d(TTTpTTTT) undergo rapid β-elimination in 1 M piperidine at 25° giving two oligonucleotide fragments, d(AAAAGTTtpAAAACAT) and d(pAAAACAT), where r' = -O-CH2-CHOH-CH=CH-CHO (or its hemiacetal form). The fragment, d(AAAAGTTtp'), which can be isolated by reverse phase chromatography, is resistant to the 3'→5' exonuclease activity of snake venom phosphodiesterase. Endonucleolytic hydrolysis of the penultimate phosphodiester occurs removing pTpr' and generating a normal 3'-OH end. In 1 M piperidine at 90° two β-eliminations occur producing the oligonucleotides d(AAAAGTTp) and d(pAAAACAT) from d(AAAAGTTtpAAAACAT); d(TTTp) and d(pTTTT) from d(TTTpTTTT).

INTRODUCTION
Removal of a purine or pyrimidine moiety from DNA leaves a deoxyribose residue and creates a so-called "AP site". These sites are formed spontaneously under mildly acidic conditions by removal of adenine or guanine. They are also produced enzymatically as the first step in repair of purine and pyrimidine derivatives formed when DNA reacts with a variety of carcinogens. The repair process is not always successful, however, and base substitutions arise when a deoxyribose residue is encountered by the replication machinery of the cell. Although a great deal has been learned about the mutagenic activity of AP sites, some important questions remain unanswered. For example, in bacteria it is necessary to turn on the error-prone repair system in order to observe mutations from AP sites in vivo, but it is not clear why this is so since various DNA polymerases produce mutations when they copy AP sites in vitro. There is even less information about the behavior of AP sites in animal cells, though there is evidence that this kind of damage is both mutagenic and carcinogenic. For these reasons it seems desirable to study AP sites at single preselected positions in biologically active DNA. One way to achieve this is to synthesize the desired DNA enzymatically by the primer extension method using a synthetic oligonucleotide containing the deoxyribose residue at the desired position as a primer.
The first chemical synthesis of such an oligonucleotide d(TIPP) where T = deoxyribose, has been reported\textsuperscript{10}. An alternative procedure that utilizes the enzyme, uracil DNA-glycosylase, to remove uracil that has been incorporated as deoxyuridine during a routine oligonucleotide synthesis has also been described\textsuperscript{11}. In this paper we will describe the use of this latter method to synthesize two oligonucleotides, d(AAAAGTTUAAAACAT) and d(AAAAGTTpIPAAAACAT), that are complementary to the first 15 nucleotides in gene G of ΦX174. The first oligonucleotide carries the sequence complementary to (TAA)\textsuperscript{ochre} at the third codon; the second carries an AP site (f) in the second position of this codon. We will describe the Michaelis constants for the reaction of uracil DNA-glycosylase with d(AAAAGTTUAAAACAT) and record some unusual properties of d(AAAAGTTpIPAAAACAT) that bear on the repair of AP sites. We also report the synthesis and properties of d(TTTUTTTT), which is a convenient substrate for assaying the enzyme. Finally, we discuss the conditions for cleaving the phosphodiester bonds at an AP site in oligonucleotides or DNA.

RESULTS

Synthesis and Properties of d(AAAAGTTUAAAACAT)

5'-O-Dimethoxytrityldeoxyuridine was converted to the appropriate phosphorochloridite derivative and incorporated into the oligonucleotide d(AAAAGTTUAAAACAT) during a routine synthesis. The analysis of the product for its nucleoside composition by treatment with snake venom phosphodiesterase and \textit{Escherichia coli} alkaline phosphomonoesterase at pH 8.0 for two hours at 45° is shown in Scheme 1. Generation of an AP site with uracil DNA-glycosylase, as shown in Scheme 1, can be followed by measuring the release of uracil or the disappearance of starting material using reverse phase HPLC. Unless the uracil is labelled, the first method is not very sensitive. In the second method, separation of the AP oligonucleotide product, 2, and the starting material, 1, is not complete under the conditions we have explored. A more satisfactory analysis is achieved by making use of the alkaline lability of an AP site\textsuperscript{12,13,14} to generate the two fragments, 3 and 4, shown in Scheme 1. In 1M piperidine at 25° this elimination reaction is complete in less than 5 minutes, though we routinely used 30 min for the reaction. The fragments, 3 and 4, separate easily from the starting material and from each other as shown in Fig 1. Thus, by removing aliquots, adding piperidine and analyzing the mixture chromatographically, the enzymatic reaction can be followed easily and quickly.

On a preparative scale isolation of the AP oligonucleotide, 2, proved more difficult than expected. The AP site is so labile that attempts to lyophilize 2 in the buffer used for the enzymatic reaction (HEPES, DTT, EDTA, NaCl and BSA) produced considerable cleavage. To avoid this it was necessary to separate these solutes from the oligonucleotide by gel filtration (see EXPERIMENTAL). When handled in this way, there was <1% cleavage of oligonucleotide 2 as measured by the method shown in Fig. 1 (data not shown).
When the desalted AP oligonucleotide, 2, was analyzed for its nucleoside composition by digestion with snake venom phosphodiesterase and bacterial alkaline phosphomonoesterase at pH 8.0 for 2 h at 45°, the analysis shown in Scheme 1 was obtained. The accuracy of this analysis is approximately ±5% (see EXPERIMENTAL). The values for dCyd and dAdo were within experimental error, but the value for dGuo was 19% too high and for dTyd 24% too low. It appeared that the 3',5'-exonuclease was having difficulty cleaving the terminal phosphodiester bond when it reached d(AAAAGTTPtPr) in d(AAAAGTTPpAAAACAT). To examine this further the AP oligonucleotide, 2, was dissolved in 1 M piperidine at 25° to cleave the phosphodiester backbone as shown in Scheme 1. The individual products, 3 and 4, were isolated by HPLC (Fig. 1) and analyzed for their nucleoside composition. The results are shown in Scheme 1. The material from the first peak gave the correct analysis for the 3'-fragment, 4. Analysis of the material from the other peak, which should be the 5'-fragment, 3, was anomalous.
Figure 1. Chromatographic separation of the heptanucleotides formed by treating 120 pmol d(AAAAGTTUAAAACAT) with uracil DNA-glycosylase followed by 1M piperidine at 90° for 30 min. The oligonucleotides were eluted with a 3-27% linear gradient of CH3CN in 0.1 M triethylammonium acetate (TEAA), pH 7.0, developed over 20 min at a flow rate of 1 ml/min (average pressure 1,000 psi). The first peak (A) is d(pAAAACAT), Rf= 8.8 min; the second (B) is d(AAAAGTTTpr'), Rf= 9.6 min; the third (C) is d(AAAAGTTUAAAACAT), Rf= 10.7 min.

Snake venom phosphodiesterase is a 3',5'-exonuclease that works best on oligonucleotides with a free 3'-hydroxyl group\textsuperscript{15}, which is lacking in d(AAAAGTTTpr'). The analysis of this oligonucleotide, like that of the longer oligonucleotide from which it was derived, suggested the enzyme has difficulty catalyzing hydrolysis of the terminal phosphodiester to release pr' and generate a free 3'-OH. This was investigated further as follows: After treating 1 with uracil DNA-

\[
\text{d(AAAAGTTTprpAAAACAT)}
\]

\[
\begin{array}{c}
1 \beta\text{-elimination (1 M piperidine, 90°)}
\end{array}
\]

\[
\text{d(AAAAGTTTp) + r'' + d(pAAAACAT)}
\]

\[
\begin{array}{c}
5 \quad 4
\end{array}
\]

Theory: \(d_{G1.00} \quad d_{C1.00} \quad d_{A9.00} \quad d_{T3.00}\)

Found: \(d_{G1.05} \quad d_{C1.03} \quad d_{A8.94} \quad d_{T2.98}\)

\(r'' = 9 \text{ or } 10\) (see Scheme 3)

Scheme 2

7454
Figure 2. Isolation of d(AAAAGTTp) + d(pAAAACAT), (A), after heating 400 pmol of d(AAAAGTTAAAACAT) with 1 M piperidine for 30 min at 90°. The column was as described in Fig. 1. Elution was isocratic with 7% CH$_3$CN in 0.1 M TEAA, pH 7.0 until 5.1 min after injection, when the concentration of CH$_3$CN was increased from 7.0 to 10.0% over 3.0 min. (B) is the intact, dU-containing starting oligonucleotide.

glycosylase to produce 2, the reaction mixture was treated with 1 M piperidine at 90° for 30 min to cleave both the phosphodiester bonds at the AP site and produce the two heptanucleotides, 4 and 5, shown in Scheme 2.

As shown in Fig. 2, fractionation of the reaction mixture by reverse phase HPLC showed a minor peak corresponding to the intact uracil-containing oligonucleotide, 1, which we showed was stable in 1 M piperidine at 90° for 1 h. Two major peaks believed to contain the two heptanucleotides, 4 and 5, were detected. They did not separate well from each other so their identity was confirmed by analyzing the heptanucleotide mixture for its nucleoside composition using snake venom phosphodiesterase and phosphomonoesterase as before. The results are shown in Scheme 2. The data are within the experimental error of the analysis and are consistent with the conclusion that the venom phosphodiesterase skips the phosphodiester bond at Tp in both d(AAAAGTTpAAAACAT) and d(AAAAGTTp+) removing pTpr, and leaving d(AAAAGT), which is hydrolyzed by the 3'→5' exonuclease activity of the enzyme in the normal manner. There is precedence for this kind of behavior. Snake venom phosphodiesterase has difficulty catalyzing the hydrolysis of certain phosphodiester bonds$^{16,17}$. For example, digestion of irradiated DNA with large amounts of the phosphodiesterase gives dNMP's and d(pNpD), where
Figure 3. Evaluation of $K_M$ and $V_{\text{max}}$ for 5'-[32P]d(pAAAAGTTUAAAACAT). (A) Assay for uracil DNA-glycosylase activity. Various concentrations of the substrate, 5'-[32P]d(pAAAAGTTUAAAACAT), were incubated with enzyme for 20 min. An aliquot was treated with 1.35 M piperidine at 90° for 30 min to cleave the product, d(pAAAAGTTUAAAACAT), into two fragments, which were separated from the starting material by gel electrophoresis. The reaction rate was determined by densitometer tracings as described in the Experimental section. The trace shown is for lane 2 which is for $[S] = 5.2 \times 10^{-10}$ M. (B) A plot of the data obtained as in (A). See experimental section for details.

$D =$ thymine dimer. [It should be noted that we used an enzyme isolated from *Crotalus durissus* where all the previous studies were done with enzyme isolated from *Crotalus adamanteus*. It appears that these two venom enzymes act similarly.]

$K_M$ and $V_{\text{max}}$ for the Release of Uracil from d(pAAAAGTTUAAAACAT) and d(pTTTUTTTT) by Uracil DNA-glycosylase

As shown above, treating aliquots of the enzymatic reaction mixture with 1 M piperidine cleaves the AP oligonucleotide 2 into fragments that are easily separated from the starting material either by HPLC (Fig. 1) or by polyacrylamide gel electrophoresis. To increase the sensitivity of the assay, we chose to use 5'-[32P]-labelled material. Since only one of the cleavage products, 3, is labelled, it is easy to measure both the remaining starting material and the products after
Table 1. Kinetic Constants for Uracil DNA-glycosylase

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_M$ (M)</th>
<th>$V_{max}$ (µmol/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>d(pAAAAGTTUAAAACAT)</td>
<td>1.5 x 10^{-8}</td>
<td>6.4 x 10^{-9}</td>
</tr>
<tr>
<td>d(pTTTUTTTT)</td>
<td>7.3 x 10^{-9}</td>
<td>2.0 x 10^{-9}</td>
</tr>
<tr>
<td>PBS1 DNA</td>
<td>4.0 x 10^{-8}</td>
<td></td>
</tr>
</tbody>
</table>

fractionation by polyacrylamide gel electrophoresis. A typical assay and a plot of the kinetic data obtained using 6 x 10^{-8} units of enzyme, assayed with d(TTTUTTTT), are shown in Fig. 3. The constants are given in Table 1. For comparison, the $K_M$ for release of uracil from [3H]-labelled PBS1 DNA using the standard assay is reported^{18}.

In a similar assay, the kinetic constants for 5'-[32P]-d(pTTTUTTTT), synthesized, characterized and phosphorylated in a manner similar to that described for oligonucleotide 2, were measured. The values are shown in Table 1. d(pTTTUTTTT) is easily prepared and is a convenient substrate for measuring uracil DNA-glycosylase activity. One unit measured with PBS2 DNA is equal to about 3 units measured with the oligonucleotide. Phage PBS2 is a clear-plaque variant of phage PBS1^{19}.

DISCUSSION

The synthesis of an oligonucleotide carrying a deoxyribose residue (AP site) at a preselected position in the sequence described here is general, and it does not require any chemical expertise. Using this approach deoxyuridine, which is now commercially available as its phosphoramidite, can be introduced at any desired site in DNA. This DNA should be stable. It should be a valuable substrate for studying the effects of dU repair in DNA in vivo at single preselected sites in biologically active DNA. The dU residue in the DNA can, in turn, be converted enzymatically to an AP site. The reaction can be monitored easily since cleavage of DNA carrying the AP site occurs under mild conditions. However, for studying site-specific mutagenesis and the effect of DNA repair on the mutation frequency, it may be desirable to convert the dU residue to a well-characterized AP site in the oligonucleotide, such as the one described here, which can be used to prime the enzymatic synthesis of DNA. Our data indicate that both the AP oligonucleotide and the resulting DNA can be handled provided certain precautions are taken to avoid premature cleavage at the AP site.

Syntheses of two oligonucleotides are described in this paper. The first, d(AAAAGTTUAAAACAT), corresponds to a (-) strand sequence at the start of gene G in ΦX174 RF DNA. It carries a sequence complementary to (TAA)^ochre at the third codon. The second is derived from the first and carries an AP site in the second position of the third codon. The potential use of this sequence for studying the mutagenic effects of a specific AP site and the effect of DNA repair on the mutation frequency in vivo has been described^{20}. 

7457
Some observations concerning cleavage of the phosphodiester bonds at the AP site are in order. The reactions shown in Scheme 3 are a critical part of the DNA sequencing method developed by Maxam and Gilbert\textsuperscript{14}. Both reactions occur at 90° in the presence of 1 M piperidine, but they occur under much milder conditions (0.05 M NaOH, 37°, 2 h) as well\textsuperscript{21}. As we have shown here, the second reaction is rate limiting. A product like 8 accumulates in 1 M piperidine at 25°.

There is solid evidence that the first reaction is a β-elimination\textsuperscript{21,22,23}. In piperidine, an aldamine (Schiff’s base) is probably involved\textsuperscript{13,14}, but this has not been demonstrated experimentally; an aldamine is not essential since the reaction occurs in the absence of an amine. It is interesting that endonuclease III, an enzyme involved in the repair of certain kinds of thymine damage in DNA, also catalyzes this reaction. This enzyme acts first as a glycosylase removing the damaged thymine and then as an AP endonuclease. Convincing evidence has been presented that the AP endonuclease activity of this enzyme is that of a β-eliminase and not that of a phosphodiesterase\textsuperscript{21}. This mode of action creates a potential block for repair because endonuclease III does not catalyze the second β-elimination shown in Scheme 3, and other 3'–5' exonucleases tested, including that of \textit{E. coli} DNA polymerase, are unable to act on the terminal phosphodiester bond\textsuperscript{24}. However, the minor AP endonuclease (endonuclease IV) and the major AP endonuclease (endonuclease VI activity of exonuclease III) both act as phosphodiesterases cleaving the phosphodiester bond at AP sites by hydrolysis rather than by β-elimination\textsuperscript{25}. These enzymes remove the α,β-unsaturated aldehyde as its phosphomonoester by hydrolysis generating a 3'-terminal OH, which can be repaired in the usual manner\textsuperscript{1,2}. 

\textbf{Scheme 3}
By vinology, the proton at C4 in the α,β-unsaturated aldehyde should be acidic, and elimination should occur in alkali as indicated in Scheme 3. The evidence for the second elimination is less rigorous than for the first. Neither loss of a proton from C4 nor the formation of the unsaturated keto aldehyde product, 10, have been demonstrated, yet there is little doubt that the reaction occurs. Assisted hydrolysis through a cyclic phosphate is ruled out on the basis of rates and the nature of the products. Unassisted hydrolysis of a phosphodiester bond does not occur either in 1 M piperidine at 90° or in 0.05 M NaOH at 37°, yet the cleavage reaction occurs under both of these conditions. Taken together these observations provide strong evidence that the second cleavage reaction also occurs by β-elimination as shown in Scheme 3.

Bailly and Verly observe a doublet on their electrophoresis gels when d(pTgprpTn) is treated either with 0.05 M NaOH at 37° for 2 h with endonuclease III. One of the bands is the β-elimination product (8 in Scheme 3). They suggest the other might be the 2-oxocyclopent-1-enyl derivative that has been observed when DNA containing AP sites was heated for 1 h at 100° in 1 M NaOH but they do not present any evidence that this compound forms under the conditions used. We have observed a similar doublet under a variety of conditions, always involving an oligonucleotide having a 5'-terminal phosphate. We have traced the doublet to an electrophoresis artifact produced by the secondary phosphate ionization and the pH of the solution at the time the gel is loaded. This would also explain the doublet seen by Bailly and Verly.

Until now most experiments dealing with the chemistry or enzymology of AP sites have been done with either poorly defined substrates (i.e. DNA carrying randomly generated AP sites) or simple compounds such as d(pTgpr Tn). The synthesis described here provides a general route to virtually any AP oligonucleotide of defined sequence one might wish to study. Knowledge of the properties described here should enable the synthesis to be extended so that a single AP site can be inserted at a preselected position in biologically active DNA.

EXPERIMENTAL

All common reagents and solvents were from commercial sources unless specified otherwise and were used without further purification. Deoxyuridine was from Sigma. This was converted to its 5'-O-dimethoxytrityl derivative and then to its phosphorochloridite derivative by standard procedures. The other nucleoside phosphoramidite derivatives were from American Bionuclear or Beckman Instruments. Since completion of this work the dU phosphoramidite has become commercially available.

Crotalus durissus venom phosphodiesterase was from Boehringer Mannheim. Uracil DNA-glycosylase and [3H]-PBS2 DNA were gifts from Dr. Bruce Duncan.

Effluents from reverse phase HPLC columns were monitored at 254 and 280 nm with a dual wave length detector.

The oligonucleotides were synthesized, purified and characterized as described previously. Nucleoside analysis was carried out as described previously. The amount of
each nucleoside (in pmol) was measured from standard curves constructed with purified nucleosides. The analytical column was calibrated each day with a standard mixture. The nucleoside composition was calculated by summing the pmol of each nucleoside found, dividing this sum by the number of nucleoside residues (the AP site was not counted), and normalizing the pmol of each nucleoside with this quotient. This method of calculation avoids normalizing the data to any particular nucleoside and reduces the error. (We are indebted to Dr. Siu Sing Tsang for suggesting this method of normalizing the data.) The accuracy of the analysis is ±5-8% for a single determination and ±5% for duplicate analyses.

The concentrations of the oligonucleotides were calculated initially from the average pmol given by nucleoside analyses. The hypochromicity was calculated from absorbance measurements made before and after hydrolysis with snake venom phosphodiesterase. Thereafter, concentrations were calculated from absorbance measurements at 260 nm and a calculated molar extinction coefficient at 260 nm corrected for the hypochromic effect, which for d(AAAAGTTUAAAAACAT) was 31% and for d(TTTUTTTT) was 5%. When appropriate, phosphorylation at the 5'-position was carried out with polynucleotide kinase in the usual manner.

Initially the enzymatic assays were carried out essentially as described by Lindahl et al.\(^1\) The final concentrations in the incubation mixture were: NaHEPES, pH 8.0, 67 mM; Na\(_2\)EDTA, pH 8.0, 1 mM; DTT (fresh), 1 mM; BSA (DNase-free), 200 µg/ml; \([\text{3H}]\)PBS2 DNA, 8 µg/ml or oligonucleotide as indicated in legends to figures. Stock solutions of the enzyme were prepared immediately before use with dilution buffer [NaCl, 300 mM; NaHEPES, pH 8.0, 50 mM; Na\(_2\)EDTA, pH 8.0, 1 mM; DTT (fresh), 1 mM; BSA (DNase-free), 100 µg/ml]. The incubation mixture (minus BSA and DTT) was heated in a boiling water bath for 10 min to denature the DNA and cooled rapidly on ice. BSA and DTT were added. The reaction was started by addition of appropriately diluted enzyme. Incubation was at 37° for the times indicated in the text. The incubations were terminated by placing the tube in ice and adding 1 drop of 2% (w/v) sodium dodecyl sulfate. The reaction was followed by the release of \([\text{3H}]\)-uracil from labeled PBS2 DNA.

On a preparative scale (2 n mole) removal of uracil from the oligonucleotide was followed by reverse phase HPLC on a 4.6 x 250 mm C\(_18\) LiChrosorb (10 µm particle) column eluted with 0.025 M KH\(_2\)PO\(_4\) for d(TTTUTTTT) or on a C\(_3\) reverse phase column (4.6 x 75 mm, 5 µm particle) for d(TTTUTTTT) and d(AAAAGTTUAAAAACAT), both eluted with a linear gradient of CH\(_3\)CN (3→27%) in 0.1 M triethylammonium acetate, pH 7.0, developed over 20 min. The enzyme buffer components were removed by gel filtration on a 0.9 x 25 cm column of Sephadex G-50 (superfine) in a siliconized glass column before proceeding. The void volume was 6.1 ml. The eluent was water run at 0.6 ml/min. The retention time for the oligonucleotide was 11 min; \(V_e\) = 6.6 ml. The appropriate fractions were collected and lyophilized. Recoveries were about 70-90%.

Enzyme kinetics were measured with 5'-\([\text{32P}]\)-oligonucleotides as follows: The substrate was treated with 6 x 10\(^{-8}\) units of enzyme measured as described above. Other conditions were as
described except that HEPES, which causes band broadening on electrophoresis, was replaced by Tris-HCl. This has no effect on the activity\textsuperscript{18}. The reaction volume was 30 \( \mu l \). Initial velocities were maintained for at least 20 min. After 10 or 20 min at 37\(^\circ\)C, depending upon the substrate concentration, a 10 \( \mu l \) aliquot was removed and added to 90 \( \mu l \) of 1.35 M piperidine in a 0.5 ml siliconized plastic centrifuge tube. The tube cap was sealed with a hot glass rod and the solution was heated at 90\(^\circ\)C for 30 min. Controls showed that under these conditions piperidine concentrations between 0.85 M and 1.35 M cleave the AP oligonucleotide produced by the enzymatic reaction, but have no effect on the dU-oligonucleotide. The solvent and piperidine were removed under reduced pressure. The pellet was redissolved in water and re-evaporated. The final pellet was dissolved in 2.5 \( \mu l \) of electrophoresis loading buffer. Electrophoresis was carried out on a 20\% polyacrylamide gel (containing 8 M urea) in TBE buffer, pH 8.3, for 1.5 h at 20 watts. The bands were visualized by autoradiography at -70\(^\circ\)C without an intensifying screen. A typical pattern is shown in figure 3(A). The intensities of the bands were measured with a scanning microdensitometer. The densities were proportional to the dpm provided the film was not overexposed. Initial velocities (\( \mu \)mol/min) were calculated from these data. The kinetic constants were determined from a linear regression using an Apple microcomputer and the StatView program (BrainPower Inc. Calabasas, CA). The error bars are based on the standard error.

ACKNOWLEDGEMENTS

This work was supported by a Terry Fox Special Initiatives Award from the National Cancer Institute (Canada).

REFERENCES