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Site-specific cleavage of duplex DNA by a semisynthetic nuclease via triple-helix formation

(staphylococcal nuclease/triple-helical DNA)

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ABSTRACT **A Lys-84 → Cys mutant staphylococcal nuclease was selectively linked to the 5′ and/or 3′ terminus of a thiol-containing polypyrimidine oligonucleotide via a disulfide bond. The oligonucleotide-staphylococcal nuclease adduct is capable of binding to a homopurine-homopyrimidine region of Watson–Crick duplex DNA by the formation of a triple-helical structure. Upon the addition of Ca^{2+}, the nuclease cleaves DNA at sites adjacent to the homopurine tract. Specific double-strand cleavage occurred predominantly at A+T-rich sites to the 5′ side of the homopurine tract for both the 5′-derivatized and the 5′,3′-diderivatized nucleases; the 3′-derivatized nuclease gave no cleavage. The cleavage pattern is asymmetric and consists of multiple cleavage sites shifted to the 5′ side on each strand, centered at the terminal base pair of the binding site. Microgram amounts of plasmid pDP20 DNA (4433 base pairs) containing a homopurine-homopyrimidine tract were selectively cleaved by a semisynthetic nuclease with greater than 75% efficiency at room temperature within 1 hr. Cleavage reaction conditions were optimized with respect to pH, temperature, reaction times, and reaction components. Semisynthetic nucleases of this type should provide a powerful tool in chromosomal DNA manipulations.**

Restriction endonucleases are important tools for molecular cloning, genetic mapping, and other manipulations of nucleic acids. However, the recognition site sizes (4–8 bases) and sequence specificities of the naturally occurring enzymes limit their usefulness for many applications. The development of artificial or semisynthetic nucleases capable of highly specific DNA cleavage at any desired sequence would greatly facilitate DNA manipulations, especially physical chromosomal mapping (1). Strategies for generating nucleases of this type include the coupling of oligonucleotides or DNA-binding proteins to either naturally occurring nucleases (2, 3), or oxidative or photoactive DNA cleaving moieties (4–9).

Adducts of oligonucleotides covalently linked to a cleaving agent have been delivered to complementary sequences within duplex DNAs by both Watson–Crick base-pairing (D-loop formation) (2, 10) and Hoogsteen base-pairing interactions (triple-helix formation) (4, 5, 8, 9). Although there appears to be no sequence limitation to D-loop formation, its use is currently limited to supercoiled substrates. Triple-helix formation, on the other hand, is limited largely to homopurine–homopyrimidine sequences, but it does not require supercoiled substrates (11). Previous examples of the triple-helix-directed cleavage of DNA have required a large excess of oligonucleotide-linked cleaving agents, resulting in oxidative cleavage of DNA with efficiencies generally below 25% (4, 5, 8, 9). We report here the coupling of staphylococcal nuclease, an enzyme that efficiently but relatively nonspecifically hydrolyzes both single-stranded and double-stranded DNAs (12–14), to a homopyrimidine oligonucleotide. The resulting semisynthetic nuclease can bind to DNA via triple-helix formation and selectively hydrolyze both strands of the target duplex DNA. In addition, cleavage of preparative quantities of a plasmid DNA can be carried out in high yield.

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MATERIALS AND METHODS

Synthesis of 1-*O*-(4,4′-Dimethoxytrityl)-3,3′-dithiodipropanol (Compound 2). To a stirred solution of 3,3′-dithiodipropanol (1) (15) (0.80 g, 4.5 mmol) in anhydrous pyridine (20 ml) containing dimethylaminopyridine (0.028 g, 0.22 mmol) and triethylamine (TEA) (0.61 g, 6.0 mmol) was added dropwise dimethoxytrityl chloride (Aldrich) (1.02 g, 3.0 mmol) in anhydrous pyridine (20 ml) over 1 hr. The mixture was stirred at room temperature for an additional hour. The solvent was removed by rotary evaporation and then coevaporation with toluene (three 5-ml portions) to afford a brown-red oil. The crude product was purified by chromatography on a silica gel column (20 × 1.5 cm) (MeOH/TEA/CH_2Cl_2, 2:1:97, vol/vol) to give a pale yellow oil (0.7 g, 48% yield): R_f (MeOH/TEA/CH_2Cl_2, 2:1:97) = 0.33; ^1H NMR (C^2HCl_3): δ 1.85 (m, 4 H), 2.76 (m, 4 H), 3.14 (t, J = 6.1 Hz, 2 H), 3.69 (t, J = 4.3 Hz, 2 H), 3.80 (s, 6 H), 6.79 (d, J = 7.1 Hz, 4 H), 7.10–7.40 (m, 9 H); IR (neat): 3450, 3040, 2940, 2840, 1610, 1510, 1460, 1300, 1180, 1030, 830 cm^{-1}; MS (positive fast atom bombardment): m/e 485 (MH^+, 10), 303 (dimethoxytrityl, 100), 165 (15).

Synthesis of Thiolated Controlled-Pore Glass (CPG). Long-chain alkylamine CPG (500 Å; Biosearch) was derivatized with compound 2 according to standard procedures (16). The loading of the compound 2 was approximately 12 μmol/g of support.

Synthesis of Oligonucleotides. All oligonucleotides were synthesized on a Biosearch model 8600 DNA synthesizer using the phosphoramidite method. CPG derivatized with thymidine containing a 3′-sulfhydryl was synthesized as previously described (17, 18). Standard CPG-nucleoside supports were from Milligen–Biosearch; all other DNA synthesis reagents were purchased from Cruachem (Sterling, VA). Oligonucleotides containing a 3′-thiol were synthesized either on thiolated CPG (3) or on CPG derivatized with 3′-thiolated thymidine (17, 18), deprotected and purified as described (17). Oligonucleotides containing a 5′-thiol were synthesized using *S*-trityl-*O*-(β-cyanoethoxy)diisopropylaminophosphinyl-6-mercaptohexanol (C_6-thiol modifier; Cruachem) in the final coupling reaction. The base-labile protecting groups were removed by concentrated ammonium hydroxide at 55°C for 6 hr and the 5′-*S*-trityl group was removed with silver nitrate as described by Connolly (19). The resulting thiolated oligonucleotide was applied to a Pharmacia FPLC fast desalting column, eluted with 40 mM

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Tris·HCl, pH 8.5, and collected into a tube containing 2 mg of 2,2′-dithiodipyridine (Aldrich) in acetonitrile (300 μl). This solution was mixed and allowed to incubate overnight at room temperature. The resulting *S*-thiopyridyl adduct was purified (17) by reverse-phase HPLC (Whatman C_{18} column) using a linear gradient of 0–43% acetonitrile in triethylammonium acetate (0.1 M, pH 7.5) over 30 min at 3 ml/min. A 5′- or 3′-thiopyridylated 17-nucleotide (nt) oligonucleotide has a retention time of approximately 23 min. Oligonucleotides containing both 3′- and 5′-thiols were obtained as described above except that after removal of the trityl group, an equal volume of buffer (0.5 M Tris·HCl, pH 8.0) and dithiothreitol at 5 mg/ml were added to reduce the 3′-disulfide. The 3′,5′-dithiopyridylated 17-nt oligonucleotide has a retention time of 27 min on reverse-phase HPLC.

Construction of Semisynthetic Nucleases. The K84C (Lys-84 → Cys) staphylococcal nuclease mutant was generated by site-directed mutagenesis by the method of Kunkel (20), using the primer 5′-AGAACTGATTGCTATGGACGTGGC -3′ (18, 21). Semisynthetic nucleases with the enzyme attached to either the 5′ or 3′ terminus of an oligonucleotide were constructed via disulfide exchange reactions between the thiopyridylated oligonucleotide and thiolated staphylococcal nuclease (21, 22). Semisynthetic nucleases with enzyme attached to both the 3′ and 5′ termini of an oligonucleotide were synthesized similarly except that the 3′,5′ dithiopyridylated oligonucleotide was added slowly to the reduced staphylococcal nuclease solution to ensure that the yield of the diderivatized oligonucleotide was maximized. Typically, one equivalent of a dithiopyridylated oligonucleotide (30–50 μM) was added to two equivalents of reduced K84C staphylococcal nuclease (≈0.1 mg in 300 μl of 50 mM Hepes/2 mM EGTA/300 mM NaCl, pH 7.6) in 10 portions over 30 min, and reaction was continued for another 30 min. The reaction was monitored by following thiopyridyl release at 343 nm ($\varepsilon = 7060\ M^{-1}\cdot cm^{-1}$). The semisynthetic nucleases were purified by anion-exchange chromatography on a Pharmacia Mono Q column (HR 5/5), eluted with 20 mM Tris·HCl, pH 7.5/2 mM EGTA and a linear gradient of 100–700 mM KCl over 20 min. The 5′,3′-diderivatized oligonucleotides eluted at significantly lower salt concentrations than those with only one enzyme attached to either the 5′ or 3′ terminus of the oligonucleotides. For example, a 5′,3′-diderivatized 17-nt oligonucleotide eluted at 290 mM KCl, whereas the 17-nt oligonucleotide with staphylococcal nuclease attached to either the 5′ or the 3′ terminus eluted at 390 mM KCl. The yields for the diderivatized oligonucleotides in disulfide exchange reactions are generally greater than 80%.

The concentrations of semisynthetic nucleases were estimated by determining the absorbance of the pyrimidine oligonucleotide at 260 nm, using extinction coefficients of 7400 $M^{-1}\cdot cm^{-1}$ and 9100 $M^{-1}\cdot cm^{-1}$ for thymidine and cytidine, respectively (ref. 23, p. 449). In the case of the 3′,5′-diderivatized oligonucleotides, the absorbance at 260 nm due to the enzyme was subtracted (≈10%) by comparing relative extinction coefficients of the oligonucleotide and the enzyme of known concentrations at 260 nm.

Preparation of DNA Substrates. Short DNA substrates (62 and 70 nt) were synthesized and purified according to standard protocols and labeled at the 5′ termini with [γ-^{32}P]ATP (Amersham; ≈3000 Ci/mmol; 1 Ci = 37 GBq) by using T4 polynucleotide kinase (ref. 23, p. 122). Unincorporated ATP was removed by applying the reaction mixture to a Sephadex G-50 column (5 × 1 cm) eluted with 10 mM Tris·HCl, pH 8.0/1 mM EDTA (TE buffer) and collecting the first radioactive fraction (1.5 ml). To form duplex substrates, equimolar amounts of each complementary strand (≈16 pmol) were mixed in 200 μl of TE buffer. The mixture was heated to 70°C in a water bath and then allowed to gradually cool to room temperature. The 70-base-pair (bp) DNA fragment (see Fig. 2 *Lower*) was inserted into *Hin*dIII-digested plasmid pBR322. The resulting plasmid, pDP20 [the homopyrimidine strand is the (+) strand], was used to transform *Escherichia coli* JM101 cells. DNA was then isolated from the transformed cells in their early stationary phase and purified by centrifugation in a CsCl gradient column (ref. 23, p. 86). The sequence of the DNA was confirmed by dideoxy sequencing using the double-stranded plasmid DNA as the template (ref. 24, chap. 7). The circular plasmid DNA was linearized by restriction endonuclease *Sty* I prior to use.

DNA Cleavage Reactions. Cleavage of the 70-bp DNA was carried out in a reaction mixture (final volume 10 μl) containing 5′-end-labeled DNA (0.024 μM), 25 mM Tris acetate (pH 5.7), 100 mM NaCl, 2 mM spermine, 20% (vol/vol) ethylene glycol, and semisynthetic nuclease (0.040 μM). After preincubation of the reaction mixture at room temperature (23°C) for 10 min, the reaction was initiated by the addition of 1 μl of a 100 mM $CaCl_2$ stock solution and the reaction was terminated after 10 min by the addition of 12 μl of formamide–dye mixture (formamide/H_2O/bromophenol blue 95:5:0.01, vol/vol) containing 10 mM EGTA, pH 7.5. The quenched mixture was then heated to 90°C for 3 min and loaded immediately onto a 40 × 30 × 0.04 cm high-resolution denaturing 15% polyacrylamide gel (1:20 crosslinked, 7 M urea). Electrophoresis was carried out at 1400 V until the bromophenol blue dye migrated four-fifths of the gel length. Autoradiography of the gels was carried out at −80°C overnight on Kodak XAR5 x-ray film. Cleavage of the 62-bp DNA fragment was carried out similarly, with slightly lower substrate DNA concentration (0.015 μM) and longer cleavage reaction time (20 min). Cleavage of *Sty* I-linearized pDP20 DNA was also carried out similarly, except that the reaction mixture was incubated for 1 hr at 23°C prior to the addition of $CaCl_2$, cleavage reaction time was 30 min, and 10 μg of poly(rA) (Pharmacia) was added to each reaction mixture. After the reaction was terminated, the DNA fragments were precipitated with ethanol, resuspended in 30 μl of formamide loading buffer, and analyzed by electrophoresis on a 1% agarose gel. The gel was stained with ethidium bromide and fragments were visualized by UV light.

RESULTS AND DISCUSSION

Cleavage of Plasmid DNA. The ability of semisynthetic nucleases to selectively cleave relatively large duplex DNAs was examined by using the plasmid pDP20 (4433 bp). Plasmid pDP20 is a pBR322 derivative with a 70-bp synthetic fragment (see Fig. 2 *Lower*) inserted at the *Hin*dIII site. The 70-bp DNA fragment contains a homopurine–homopyrimidine sequence (5′-AAAGAGAGAGAGAGGGA-3′) as well as three T+A-rich regions, two of which are directly adjacent to the homopurine tract (Fig. 2 *Lower*). *Sty* I-linearized pDP20 DNA (2 μg, 0.08 μM) and semisynthetic nuclease (0.2 μM) were combined in 25 mM Tris acetate, pH 5.7/100 mM NaCl/2 mM spermine/20% (vol/vol) ethylene glycol solution containing poly(rA) (10 μg, 3.3 mM in nucleotides). The reaction mixture was incubated for 1 hr at 23°C prior to initiation of the cleavage reaction. Cleavage reactions were initiated by the addition of Ca^{2+} (10 μM) and quenched after 30 min at room temperature by the addition of 12 μl of 10 mM EGTA [EGTA chelates the Ca^{2+} required for the nuclease activity (25)]. Cleavage products were analyzed on 1% agarose gels (Fig. 1).

Initially, a semisynthetic nuclease (**4**, Table 1) was constructed in which staphylococcal nuclease was linked to both termini of a 17-nt oligonucleotide (5′-TTTCTCTCTCTCTC-CCT-3′) complementary to the target site. For staphylococcal nuclease to cleave single-stranded DNA, the template must be aligned in the correct 3′ to 5′ orientation with respect to the enzyme active site (26). We therefore expected that

Table 1. Structures of semisynthetic nucleases

Semisynthetic nuclease	Structure 5′→3′	Length of the oligonucleotide, nt
4	Enz-S-S-$(CH_2)_6$-O-P(O_2)-O-TTTCTCTCTCTCTCCCT-S-S-Enz	17
5	Enz-S-S-$(CH_2)_6$-O-P(O_2)-O-TTTCTCTCTCTCTCCCT-OH	17
6	HO-TTTCTCTCTCTCTCCCT-S-S-Enz	17
7	Enz-S-S-$(CH_2)_6$-O-P(O_2)-O-TTTTCCCTCTCTCTCTC-O-P(O_2)-O-$(CH_2)_3$-S-S-Enz	17
8	HO-CCTTTTCTCTCTCTCTCCCT-S-$(CH_2CH_2O)_4$-CH_2CH_2-S-S-Enz	20

All the semisynthetic nucleases were constructed from the K84C mutant of staphylococcal nuclease. Enz, enzyme.

each nuclease would cleave only one strand of duplex DNA at the 3′ and 5′ sides of the binding site to afford a double-stranded cleavage product with "sticky" ends. Semisynthetic nucleases with the enzyme linked at the 3′ or 5′ terminus of the oligonucleotide (**6** and **5**, respectively, Table 1) were also synthesized for comparison.

The semisynthetic nucleases consisting of staphylococcal nuclease linked to the 5′ terminus (**5**) or both the 5′ and 3′ termini (**4**) of the 17-nt oligonucleotide cleaved the plasmid DNA into two discrete fragments in greater than 50% yield (Fig. 1, lanes 5 and 6, respectively). Comparison of the cleavage products with molecular weight standards generated by *Hin*dIII/*Sty* I digestion of pDP20 (3023 and 1410 bp) indicated that the cleavage occurred specifically at the target site adjacent to the homopurine-homopyrimidine tract. In control experiments, reduced free K84C staphylococcal nuclease (4 μM) was incubated with the plasmid DNA (4 μg, 0.16 μM) under the same reaction conditions. Nonspecific cleavage was observed both in the presence and absence of the 17-nt cognate oligonucleotide at 0.85 μM (Fig. 1, lanes 2 and 3, respectively). Cleavage efficiency could be increased by carrying out repeated hybridization and cleavage reactions as follows: A reaction mixture was preincubated for 30 min at room temperature, the reaction was initiated with Ca^{2+} and quenched after 15 min at room temperature by the addition of 1 μl of 100 mM EGTA, and then the mixture was heated to 65°C for 2 min to disrupt the triple-helix complex. The 30-min preincubation and 15-min cleavage periods were then repeated at room temperature and the reaction was again quenched with 12 μl of 10 mM EGTA. Under these conditions, semisynthetic nuclease **4** (0.4 μM) converted the linearized pDP20 DNA (2 μg) into desired products in greater than 75% yield (Fig. 1, lane 7).

The semisynthetic nuclease **6**, in which staphylococcal nuclease is linked to the 3′ terminus of the 17-nt oligonucleotide, showed no detectable cleavage of the plasmid DNA under standard reaction conditions (Fig. 1, lane 4). To determine whether the inability of the 3′ nuclease to efficiently cleave duplex DNA is due to the short tether (consisting of only a —S—S— bond) between the enzyme and the oligonucleotide, we constructed a semisynthetic nuclease (**8**, Table 1) in which staphylococcal nuclease is linked to the 3′ terminus of a 20-nt oligonucleotide (complementary to the binding site in the 70-bp insert) via a pentaethylene glycol tether. Nuclease **8** showed no specific cleavage of either the isolated 70-bp DNA fragment or plasmid pDP20 DNA (data not shown), suggesting that the short tether in nuclease **6** is not responsible for the lack of cleavage by this nuclease. Further experiments are necessary to explain the inability of the 3′ nuclease to cleave DNA.

Cleavage of Short DNA Substrates. The specificity of duplex DNA cleavage by semisynthetic nucleases was examined at nucleotide resolution by carrying out cleavage reactions with the 70-bp synthetic DNA fragment labeled at the 5′ terminus with ^{32}P and analyzing cleavage products on a high-resolution denaturing 15% polyacrylamide gel (Fig. 2). Surprisingly, semisynthetic nucleases **4** and **5** afforded the

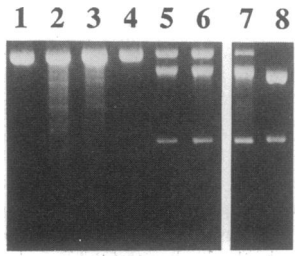

FIG. 1. A 1% ethidium bromide-stained agarose gel showing cleavage of plasmid pDP20 DNA. Lane 1, *Sty* I-linearized pDP20 DNA; lane 2, linearized pDP20 DNA (4 μg, 0.16 μM) treated with free staphylococcal nuclease (4 μM, room temperature, 15 min); lane 3, same as in lane 2 but in the presence of the cognate 17-nt oligonucleotide (0.85 μM); lane 4, pDP20 DNA (2 μg, 0.08 μM) treated with semisynthetic nuclease **6** (0.2 μM); lane 5, same as in lane 4 but with semisynthetic nuclease **5**; lane 6, same as in lane 4 but with semisynthetic nuclease **4**; lane 7, pDP20 DNA (2 μg, 0.08 μM) treated with semisynthetic nuclease **4** (0.4 μM) in two reaction cycles; lane 8, *Sty* I/*Hin*dIII digest of pDP20 DNA.

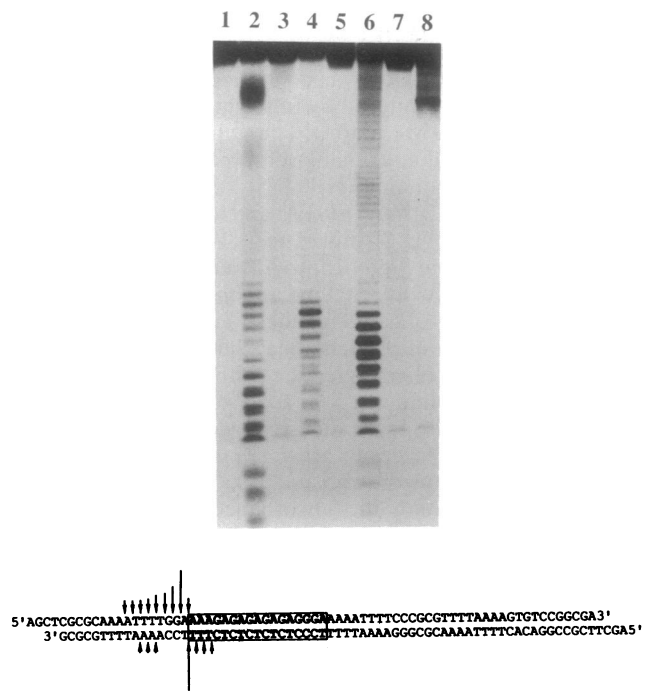

FIG. 2. (*Upper*) Autoradiogram of a denaturing 15% polyacrylamide gel, showing cleavage of 5′-end-labeled 70-bp DNA. In lanes 1–4 the 70-bp DNA was labeled at the 5′ terminus of the purine strand, and in lanes 5–8 DNA was labeled on the 5′ terminus of the pyrimidine strand. Lanes 1 and 5, undigested 70-bp DNA; lanes 2 and 6, K84C staphylococcal nuclease (4 μM) digestion (room temperature, 5 min) of the 70-bp DNA (0.05 μM); lanes 3 and 7, digestion of the 70-bp DNA (0.024 μM) with semisynthetic nuclease **6** (0.04 μM); lanes 4 and 8, digestion of the 70-bp DNA (0.024 μM) with semisynthetic nuclease **5** (0.04 μM). (*Lower*) Histogram of cleavage of the 70-bp DNA in *Upper*. The upper arrows represent the cleavage in lane 4, and the lower arrows represent the cleavage in lane 8. The heights of the arrows indicate the relative cleavage intensities. The boxed sequence is the semisynthetic nuclease binding site.

same cleavage pattern, with nuclease **4** having a slightly higher efficiency (data not shown). Both nucleases cleaved the purine strand at nine phosphodiester bonds directly adjacent to the 5′ terminus of the homopurine binding site, with the most cleavage occurring at an adenosine directly adjacent to the binding site (Fig. 2 *Upper*, lane 4; Fig. 2 *Lower*). The nucleases cleaved the homopyrimidine strand predominantly at the thymidine directly adjacent to the 3′ terminus of the homopyrimidine tract, with minor cleavage spread over three flanking thymidines and adenosines (Fig. 2 *Upper*, lane 8; Fig. 2 *Lower*). Semisynthetic nuclease **4** showed no cleavage at the 3′ side of the homopurine binding site. In control experiments, free K84C staphylococcal nuclease cleaved both purine and pyrimidine strands at all the phosphodiester bonds 5′ to thymidines and adenosines (Fig. 2 *Upper*, lanes 2 and 6, respectively). As expected, the 3′-derivatized semisynthetic nuclease (**6**, crosslinked directly with a disulfide bond) did not show significant cleavage, either at the target sequence or at other A+T-rich sites (Fig. 2 *Upper*, lanes 3 and 7).

Cleavage reactions were also carried out on a 62-bp DNA with semisynthetic nuclease **7** (Table 1) for comparison (Fig. 3). The 62-bp DNA fragment contains a homopurine-homopyrimidine binding site (5′-AAAAGGGAGAGAGAGAG-3′) and four T+A-rich sequences (Fig. 3 *Lower*). Two of the

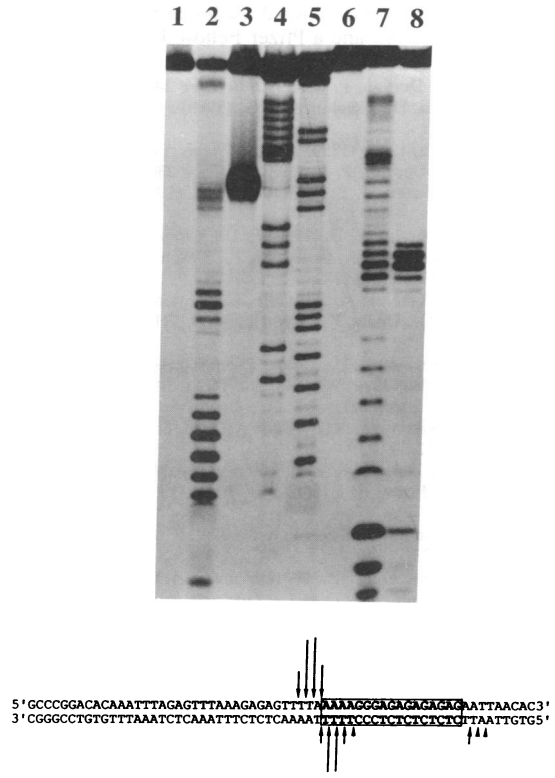

FIG. 3. (*Upper*) Autoradiogram of a denaturing 15% polyacrylamide gel, showing cleavage of 5′-end-labeled 62-bp DNA. Lanes 1–4 contain DNA labeled on the purine strand, while lanes 5–8 have DNA labeled on the pyrimidine strand. Lanes 1 and 6, undigested 62-bp DNA; lanes 2 and 7, K84C staphylococcal nuclease (1 μM) digestion (room temperature, 10 min) of the 62-bp DNA (0.03 μM); lanes 3 and 8, digestion of the 62-bp DNA (0.015 μM) with semisynthetic nuclease **7** (0.04 μM); lane 4, Maxam–Gilbert G reaction of the 62-nt purine strand; lane 5, Maxam–Gilbert C reaction of the pyrimidine strand. (*Lower*) Histogram of cleavage of the 62-bp DNA in *Upper*. The upper arrows represent the cleavage in lane 3 (determined on an underexposed film where the bands are apparent), and the lower arrows represent the cleavage in lane 8. The heights of the arrows indicate the relative cleavage intensities. The boxed sequence is the binding site of semisynthetic nuclease **7**.

T+A-rich sites are adjacent to the two termini of the binding site; the other two are located to the 5′ side of the homopurine binding site. Nuclease **7** was constructed by linking staphylococcal nuclease to both termini of a 17-nt oligonucleotide (5′-TTTTCCCTCTCTCTCTC-3′). In this case the 3′-terminal tether consisted of three methylene groups and a phosphate group. The cleavage pattern produced by nuclease **7** is qualitatively similar to that generated by nuclease **5** on the 70-bp substrate, despite the presence of additional T+A-rich sites and different target sequences in the two fragments. Cleavage of the purine strand occurred over four phosphodiester bonds at the T+A-rich sequence directly adjacent to the 5′ terminus of the nuclease binding site (Fig. 3 *Upper*, lane 3; Fig. 3 *Lower*). Cleavage of the pyrimidine strand took place at five phosphodiester bonds at the 3′ terminus of the pyrimidine binding site, with the most cleavage at the last two thymidines of the binding site (Fig. 3 *Upper*, lane 8; Fig. 3 *Lower*). No cleavage was observed at the two additional T+A-rich sites to the 5′ side of the binding site. A small amount of nicking also occurred at the T+A-rich site to the 3′ side of the binding site (Fig. 3 *Upper*, lane 8; Fig. 3 *Lower*).

For both DNA substrates, the predominant cleavage sites for the semisynthetic nucleases are the four to nine nucleotides centered at the 5′ terminus of the purine binding site. The nuclease linked to the 5′ termini of the oligonucleotides is responsible for the double-strand cleavage. Presumably, after cleaving the first strand of a duplex DNA, the tethered nuclease can rotate approximately 180° and cleave the second strand. The x-ray crystal structure of a staphylococcal nuclease·pTp complex (26) shows that Cys-84 is located near the nuclease binding cleft. It appears that the oligonucleotide attached to Cys-84 can be oriented either parallel or antiparallel to the bound substrate DNA and this is consistent with the observed double-strand cleavage. The asymmetric cleavage patterns (major cleavage sites shifted to the 5′ side of the terminal base of the binding site) in both cases are very similar to the pattern generated by oligonucleotide-EDTA·Fe(II) (4) complexes. It has been suggested that this asymmetric cleavage pattern results from oxidation of the deoxyribose ring by diffusible hydroxyl radicals delivered selectively to the major groove of DNA. Our results are consistent with the previous proposal that triple-helix formation occurs in the major groove of DNA (4). The fact that a nondiffusible nuclease here can produce essentially the same type of cleavage pattern raises an interesting question whether the diffusibility of hydroxyl radicals is necessarily responsible for the cleavage pattern. Multiple cleavage bands may result from conformational flexibility of the cleaving agents or from the superimposition of overlapping binding sites.

The semisynthetic nuclease also overcame the natural preference of staphylococcal nuclease for cleaving DNA at thymidines and adenosines (12–14). In the 70-bp DNA fragment, two G·C base pairs were inserted into the T+A-rich target sequence at the 5′ side of the binding site (Fig. 2 *Lower*). Semisynthetic nucleases **4** and **5** efficiently cleaved the phosphodiester bonds 5′ to the two guanosines; the cleavage is even more efficient than that at the thymidine next to the two guanosines (Fig. 2 *Upper*, lane 4; Fig. 2 *Lower*). This is in contrast to our earlier observation that semisynthetic nucleases retained the natural specificity of staphylococcal nuclease in single-strand DNA cleavage reactions (18). The relative rigidity of duplex DNA compared to single-stranded DNA probably restricts the nuclease in the proximity of the two guanosines, significantly lowering cleavage at the T+A-rich sequences further away from the binding site. This property of semisynthetic nucleases increases the number of sequences suitable for semisynthetic nuclease cleavage reactions.

Reaction Conditions. Cleavage reactions were carried out under a variety of conditions to examine the effects of pH, temperature, preincubation and cleavage times, and reaction components on cleavage specificity and efficiency. *Sty* I-linearized pDP20 DNA and semisynthetic nuclease **4** were used in these reactions. Optimal conditions are considered to be those that yield highest conversion from starting material to desired products without detectable nonspecific cleavage products.

The cleavage reaction is pH dependent with an optimum at pH 5.7, although significant amounts of specific cleavage were observed over the pH range from 5.3 to 7.0. At pH values lower than 5.3, the semisynthetic nuclease is inactive. At higher pH (>7.0), triple-helix formation is disfavored, since protonation of cytosine N3 is required for Hoogsteen base pairing, and staphylococcal nuclease itself has high activity [free staphylococcal nuclease cleaves DNA most efficiently between pH 8.6 and 10.3 (12)], leading to increased nonspecific cleavage (18, 27).

Another factor which affects the efficiency of the cleavage reactions is temperature. The semisynthetic nucleases are relatively inactive at low temperature (0°C). At elevated temperature (65°C), significant amounts of nonspecific cleavage began to appear, presumably due to the disruption of the triple-helix complex at high temperatures. Room temperature to 37°C appeared to be the optimal temperature range for semisynthetic nuclease reactions.

Preincubation and cleavage reaction times also affect DNA cleavage efficiency and specificity. No specific cleavage was observed when the cleavage reaction was carried out at 23°C without preincubation of substrate and the nuclease prior to the activation of the enzyme with Ca^{2+}. The yield of specific cleavage products steadily increased with increasing preincubation times of up to 1 hr, beyond which the amount of specific cleavage did not appear to change. The yield of specific cleavage products also increased with increasing reaction times (5 sec to 1 hr). Reaction times longer than 1 hr lead to an increase in nonspecific products. The requirement for preincubation may indicate that triple-helix formation is kinetically slow.

Polyamines such as spermine are required for specific cleavage (4). Without 2 mM spermine in the reaction mixtures, semisynthetic nuclease **4** cleaved pDP20 DNA nonspecifically and inefficiently. Spermine concentrations higher than 2 mM did not significantly increase the amount of specific cleavage. Ethylene glycol is not required for specific cleavage, but the presence of 20% (vol/vol) ethylene glycol in reaction mixtures slightly increased the cleavage efficiency. It has previously been shown that the duplex substrate in a triple-helix is in an A′ RNA-like conformation (28) and organic solvents such as ethylene glycol favor the B-to-A conformational transition (29), stabilizing the triple-helix complex.

Semisynthetic nuclease cleavage reactions were typically carried out with 2 molar equivalents of the nuclease relative to DNA substrate. Reactions with 2 to 5 equivalents of the nuclease afforded slightly higher yields of desired products. Higher semisynthetic nuclease concentrations (>10 equivalents) resulted in significant amounts of nonspecific hydrolysis. Reactions with only one equivalent of the nuclease resulted in a very low yield of cleavage product (<20%). Reactions with pDP20 DNA (1.6 pmol) and substoichiometric quantities of nuclease (0.4 pmol) at various temperatures (23°C, 37°C, 53°C, and 65°C) failed to show a significant yield of specific cleavage product, indicating that these nucleases are not catalytic.

Inhibition of Specific Cleavage by the Cognate Oligonucleotide. The ability of the 17-nt oligonucleotide to inhibit DNA cleavage by the cognate semisynthetic nucleases was determined by carrying out pDP20 DNA cleavage with semisynthetic nuclease **4** at 23°C in the presence of various concentrations of the 17-nt oligonucleotide. The 17-nt oligonucleotide was added to reaction mixtures, the mixture was allowed to incubate at 23°C for 15 min, and the semisynthetic nuclease was then added, followed by another 15-min incubation. The reaction was initiated by the addition of Ca^{2+} and terminated after 30 min and products were analyzed as described above. The presence of 0.085 μM 17-nt oligonucleotide completely inhibited the cleavage of pDP20 DNA (1 μg, 0.04 μM) by the semisynthetic nuclease (0.10 μM). This indicated that the oligonucleotide alone is sufficient to form the stable triple helix under the conditions tested, and binding of the semisynthetic nuclease to the target site is essential for cleavage.

Conclusion. We have demonstrated that staphylococcal nuclease linked to the 5′ terminus of an oligonucleotide can efficiently hydrolyze duplex DNA in a sequence-specific fashion. Cleavage reactions can be readily carried out over a relatively wide range of reaction conditions. Semisynthetic nucleases hydrolyze the phosphodiester bonds of DNA and generate products with intact termini which are suitable for subsequent enzymatic manipulations (27). They can cleave microgram quantities of plasmid DNA with greater than 75% efficiency with less than 5 equivalents of the nucleases. These nucleases should serve as powerful tools for molecular cloning.

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