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Generation of a Catalytic Sequence-Specific Hybrid DNase†

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ABSTRACT: Hybrid nucleases consisting of an oligonucleotide fused to a unique site on the relatively nonspecific phosphodiesterase staphylococcal nuclease have been shown to sequence specifically cleave DNA. We have introduced mutations into the binding pocket of the nuclease which lower the k_{cat}/K_m of the enzyme. Hybrid nucleases generated from these mutants sequence selectively hydrolyze single-stranded DNA in a catalytic fashion, and under a much wider range of conditions than was previously possible. One such hybrid nuclease (Y113A, K116C) was able to site selectively cleave single-stranded M13mp7 DNA (7214 nt), primarily at one phosphodiester bond. Another hybrid nuclease (Y113A, L37A, K116C) catalyzed the hydrolysis of a 78-nt DNA substrate with a k_{cat} of 1.2 min^{-1} and a K_m of 120 nM. The effects of variations in the length and sequence of the oligonucleotide binding region were examined, as were changes in the length of the tether between the oligonucleotide and the enzyme. Cleavage specificity was also assayed as a function of substrate DNA primary and secondary structure and added poly(dA).

One approach to the development of biological catalysts with novel specificities involves the introduction of new binding domains into enzymes. We have previously applied this strategy to the development of sequence-specific nucleases consisting of the relatively nonspecific phosphodiesterases staphylococcal nuclease and RNase S selectively fused to an oligodeoxyribonucleotide of defined sequence via a disulfide linkage. The resulting hybrid nucleases site specifically hydrolyzed both single-stranded DNA and single-stranded RNA adjacent to the oligonucleotide binding site (Corey & Schultz, 1987; Zuckermann et al., 1988; Zuckermann & Schultz, 1988, 1989). These agents, along with those which use other DNA cleaving moieties such as EDTA–Fe(II) (Stroebel et al., 1988; Dervan, 1986), Cu(II)–phenanthroline (Chen & Sigman, 1987), or class IIS restriction enzymes (Kim et al., 1988), are the first steps toward developing restriction enzyme mimics that can cleave RNA or DNA at any desired sequence. Such sequence-specific cleaving agents should have applications in gene cloning, nucleic acid structure studies, and chromosomal mapping.

We now report the effects of alterations in the binding site of staphylococcal nuclease, the length and sequence of the oligonucleotide binding site, and the tether length on the properties of these nucleases. Cleavage specificity as a function of substrate DNA primary and secondary structure and added poly(dA) was also examined. These studies have afforded sequence-specific nucleases *that catalytically cleave single-stranded DNAs* and that function under a wide range of reaction conditions. Moreover, these hybrid nucleases are capable of site specifically hydrolyzing the naturally occurring single-stranded DNA M13mp7. It should be possible to apply a similar combination of chemical and biological mutagenesis to modify the specificity of other classes of enzymes, such as proteases or glycosidases.

Experimental Procedures

General. IR spectra were recorded on a Mattson-Polaris Fourier transform infrared spectrophotometer, and all absorptions were reported in wavenumbers (cm^{-1}). UV spectra were recorded on a Hewlett-Packard diode array 8452A

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spectrophotometer. ^{1}H NMR spectra were determined at 250 MHz on Fourier transform spectrometers in the College of Chemistry, University of California, Berkeley. Chemical shifts were reported in δ values, positive values indicating shifts downfield of the reference (internal TMS). Significant data were tabulated in order: multiplicity (s, singlet; d, doublet; t, triplet; m, multiplet); number of protons. Mass spectra were recorded on an AE1 M415 mass spectrometer. Oligodeoxyribonucleotides were synthesized by standard methods on a Biosearch Model 8600 DNA synthesizer using reagents obtained from Cruachem. 5′-End-labeling was performed with T4 polynucleotide kinase as described (Maniatis et al., 1982) unless otherwise noted.

Mutagenesis. Plasmid pONF1, which contains the gene for staphylococcal nuclease, was obtained from John Gerlt, University of Maryland. The K116C mutation was generated by the method of Kunkel (1985). The *Hin*dIII/*Sal*I fragment, which encodes amino acid residues 102–149 and contains the K116C mutation, was inserted into M13mp18 and mutagenized by the method of Eckstein (Nakamaye & Eckstein, 1986), using the primer 5′-AAAGTTGCTGCTGTTTACTGCCC-3′, to produce the Y113A, K116C double mutant. This fragment was subsequently inserted back into *Sal*I/*Hin*dIII-linearized pONF1 to produce pDC8. Similarly, the *Hin*dIII/*Xba*I fragment from pONF1 was inserted into M13mp19 and then mutagenized with the primer 5′-TTCAGAC-TAGCATTGGTTGAT-3′. This fragment was then inserted into *Hin*dIII/*Xba*I-linearized pDC8 to produce plasmid pDC9 coding for the L37A, Y113A, K116C triple mutant. The presence of the mutations was confirmed by Sanger dideoxy sequencing (Sanger et al., 1977) of the entire *Hin*dIII/*Xba*I and *Hin*dIII/*Sal*I fragments.

Enzyme Purification and Assay. Plasmids pDC8 and pDC9 were transformed separately into *Escherichia coli* DH 1. Isolation of staphylococcal nuclease was carried out as described (Shortle, 1985); 4.0 mg of purified enzyme was obtained per liter of cells. The nuclease was concentrated via vacuum dialysis to give stock solutions of approximately 1 mg/mL protein in 50 mM NaCl, 2.0 mM Na-HEPES,[1] pH 6.8 buffer. Enzyme was judged to be greater than 95% homogeneous by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Laemmli, 1970). The enzyme was isolated as a disulfide-linked dimer and was reduced as described prior to use (Corey & Schultz, 1987). The K_m and V_{max} values for the various enzymes were determined with salmon sperm DNA as a substrate (Serpersu et al., 1987; Cuatrescasas et al., 1967). All enzymes, including wild type, were treated with 0.05 M dithiothreitol for 8 h at 37 °C prior to assay.

Synthesis of 3′-Mercapto-5′-O-(4,4′-dimethoxytrityl)thymidine (**2**). To a stirred solution of sodium ethoxide (87 mmol) in 50 mL of ethanol, 120 mL of dry dimethylformamide, and thiolacetic acid (5.50 mL, 80 mmol) was added 5′-*O*-(4,4′-dimethoxytrityl)-2,3′-anhydrothymidine (**1**) (5.4 g, 10 mmol) (Zuckermann et al., 1987). The solution was maintained at 80 °C overnight and then neutralized by pouring it into vigorously stirring ice-cold 1 M ammonium acetate buffer (1.5 L, pH 7.0). The mixture was extracted with $CHCl_3$ (3 × 300 mL), and the organic layers were pooled. After the solvent was removed under reduced pressure, the resulting brown oil was washed exhaustively with ice-cold water. The residual solvent was removed in vacuo, and 6.8 g of a brown solid was obtained.

The crude solid product was redissolved in 5 mL of CH_2Cl_2 to which sodium borohydride (0.40 g, 10 mmol) in 50 mL of absolute ethanol was added. The mixture was allowed to stir for 30 min at room temperature and was quenched by the dropwise addition of water. The solvent was removed under reduced pressure, and the resulting amorphous brown solid was dissolved in 150 mL of CH_2Cl_2 and washed with ice-cold saturated sodium bicarbonate solution (3 × 100 mL). The organic layer was collected and evaporated under reduced pressure to afford 5.9 g of a brown solid. The crude product was purified by chromatography on a silica gel column (4.0 × 45 cm) eluted with 2.5% MeOH in CH_2Cl_2. The solvent was removed under reduced pressure and the product dried in vacuo, affording 1.8 g (32%) of a light yellow amorphous solid: IR (KBr) 3200, 3050, 2900, 2790, 2560, 2350, 1690, 1550, 1250, 1040, 840 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.50 (s, 3 H), 1.58 (d, 1 H), 2.40 (m, 2 H), 2.64 (m, 1 H)8 3.36–3.76 (m, 2 H), 3.82 (s, 6 H), 3.92 (m, 1 H), 6.20 (t, 1 H), 6.85 (d, 4 H), 7.20–7.50 (m, 9 H), 7.75 (s, 1 H), 8.60 (s, 1 H); MS-FAB 1117 (dimer), 559 [(M − H)$^+$], 125.

Synthesis of 3′-[(Hydroxyethyl)dithio]-5′-O-(4,4′-dimethoxytrityl)thymidine (**3**). To a stirred solution of *S*-(2-pyridylthio)-2-mercaptoethanol (1.5 g, 8 mmol) (Zuckermann et al., 1987) in ethanol (30 mL) containing triethylamine (1.5 mL) was added dropwise over 1 h a solution of **2** (1.7 g, 3 mmol) in 80% MeOH in $CHCl_3$. After the reaction was complete (6 h), the solvent was removed under reduced pressure. The resulting oil was dissolved in CH_2Cl_2 (20 mL) and washed with ice-cold 10% citric acid (3 × 15 mL) and water (3 × 30 mL). The organic layer was dried under reduced pressure and purified on a silica gel column (2.0 × 30 cm) with 5% MeOH in CH_2Cl_2 as eluant to yield 0.77 g (38%) of a light yellow foam: IR (KBr) 3450, 3200, 3060, 2940, 2060, 1700, 1610, 1510, 1470, 840 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.40 (s, 3 H), 2.40–2.60 (m, 3 H), 2.75 (t, 2 H), 3.35 (d, 2 H), 3.60–3.75 (m, 3 H), 3.80 (s, 6 H), 4.10 (m, 1 H), 6.20 (t, 1 H), 6.80 (d, 4 H), 7.20–7.40 (m, 9 H), 7.70 (s, 1 H), 8.42 (s, 1 H); MS-FAB 635 [(M − H)$^+$], 591 [(M − CH_2CH_2-OH)$^+$], 559 [(M − SCH_2CH_2OH)$^+$], 125.

Derivatization of Controlled Pore Glass (CPG) (**4**). Controlled pore glass (**4**) containing the 3′-thymidine was prepared by derivatizing long-chain alkylamine CPG (Biosearch, 500 Å) with the modified nucleoside (**3**) as described (Atkinson & Smith, 1984). The loading of the nucleoside was approximately 18 μmol/g of support, comparable to that of commercially available supports.

Synthesis of 3′-Thiopyridylated Oligonucleotides. Oligonucleotides of defined sequence were synthesized with the thymidine disulfide controlled pore glass (**4**) by the phosphoramidite method. Approximately 40 mg (0.7 μmol) of support was used for each synthesis. The 5′-(dimethoxytrityl) group was retained, and the fully protected oligonucleotide was cleaved from the support after the synthesis was completed. The oligonucleotide was deprotected and purified by HPLC as described (Zuckermann et al., 1987).

Construction of Oligonucleotide–Nuclease Adducts. The construction of the hybrid nucleases (Table I) was carried out as previously described (Corey & Schultz, 1987; Zuckermann et al., 1988). The concentration of hybrid nuclease was estimated by determining the absorbance of the oligonucleotide binding domain at 260 nm, assuming 20 μg/mL per absorbance unit (OD) (Maniatis et al., 1982).

Preparation of DNA Substrates. Short oligonucleotide substrates were synthesized as described above, but with commercially available supports (Biosearch). The oligomers

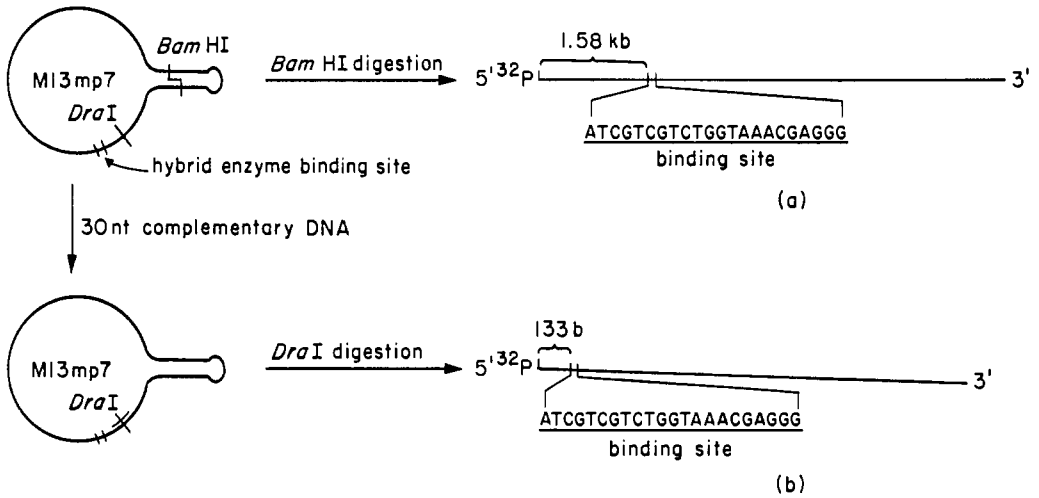
[1] Abbreviations: nt, nucleotide; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Table I: Structures of Nuclease–Oligonucleotide Adducts[a]

adduct	enzymatic domain	tether	oligonucleotide binding domain
5	L37A, Y113A, K116C	-S-S-	3′-TCGCCGAACACGCCC-5, 15 nt
6	Y113A, K116C	-S-S-	3′-TCGCCGAACACGCCC-5′, 15 nt
7	Y113A, K116C	-S-S-	3′-TCGCCGAACACGCCCTTAA-5′, 19 nt
8	Y113A, K116C	-S-S-	3′-TCGCCGAA-5′, 8 nt
9	K116C	-S-$(CH_2)_6$-S-	3′-TCGCCGAACACGCCC-5′, 15 nt
10	K116C	-S-S-	3′-TCGCCGAACACGCCC-5′, 15 nt
11	K116C	-S-S-	3′-TAGCAGCAGACCATTTGCTCCC-5′, 22 nt
12	Y113A, K116C	-S-S-	3′-TAGCAGCAGACCATTTGCTCCC-5′, 22 nt
13	Y113A, K116C	-S-S-	3′-TGGCCGAACACGCCC-5′, 15 nt
14	Y113A, K116C	-S-S-	3′-TCGCCGAAGACGCCC-5′, 15 nt
15	Y113A, K116C	-S-$(CH_2)_6$-S-	3′-TCGCCGAACACGCCC-5′, 15 nt

[a] All nuclease–oligonucleotide adducts constructed as described under Experimental Procedures. A, alanine; C, cysteine; K, lysine; L, leucine; Y, tyrosine. For example, Y113A denotes a tyrosine 113 to alanine 113 mutation.

Scheme I: Preparation of Polynucleotide Substrates a and b from Circular Single-Stranded M13mp7 DNA



were purified (Corey & Schultz, 1987) and then 5′-end-labeled with [γ-^{32}P]ATP. Unincorporated ATP was removed by applying the reaction mixture to a Sephadex G-50 column (5 × 1 cm) and collecting the first radioactive fraction (1 mL).

Single-stranded M13mp7 DNA was prepared by transforming *E. coli* TG1 cells with RF M13mp7 DNA (Amersham) according to the method of Messing (1983). To prepare substrate a (Scheme I), the resulting closed circular single-stranded DNA was linearized with *Bam*HI (Been & Champoux, 1983). Approximately 30 μg of circular DNA in 80 μL of buffer (0.2 M NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA) was heated for 2 min at 95 °C and then for 5 min at 65 °C. After being cooled to room temperature, 3 μL of 0.2 M $MgCl_2$ and 6 μL (72 units) of *Bam*HI were added to the mixture followed by incubation overnight at 37 °C. The DNA was purified on a 0.8% agarose gel and dephosphorylated with calf intestinal phosphatase (BRL) (Maniatis et al., 1982). The 5′-terminus was then labeled with ^{32}P as described previously. The 3′-terminus of the *Bam*HI-linearized DNA was labeled by a modified method (Challberg & Englund, 1980; Been & Champoux, 1983). Approximately 4 μg of *Bam*HI-digested DNA in 50 μL of buffer containing 20 mM Tris-HCl (pH 7.4), 7 mM $MgCl_2$, 50 mM NaCl, 10 mM DTT, 0.05 mM dGTP, 0.25 μM [α-^{32}P]dATP (Amersham, 800 Ci/mmol), and 10 units of Klenow fragment of DNA polymerase I was incubated for 30 min at 11 °C. The DNA was ethanol precipitated twice in order to remove unincorporated dATP.

A second substrate (b) (Scheme I) was prepared by annealing the oligonucleotide, 5′-CCCCTCAAATGCTTTAAACAGTTCAGAAAA-3′, to nucleotides 460–489 of the circular single-stranded DNA. The double-stranded region was then cleaved with *Dra*I. Approximately 20 μg of circular DNA in 45 μL of a buffer containing 4 μM 30-nt oligonucleotide, 10 mM NaCl, 10 mM Tris-HCl (pH 8.0), and 10 mM $MgCl_2$ was heated to 70 °C and then slowly cooled to 37 °C followed by the addition of 5 μL (200 units) of *Dra*I. The reaction was incubated overnight at 37 °C. The DNA was purified on a 0.8% agarose gel, dephosphorylated with calf intestinal phosphatase, and labeled at its 5′-terminus as described above.

Site-Selective Cleavage Reactions of M13mp7 DNA. A reaction mixture (total volume of 10 μL) containing approximately 12 nM ^{32}P-end-labeled DNA and 14 nM hybrid nuclease in 50 mM Tris-HCl (pH 7.0), 50 mM NaCl, and 1 mM EDTA was heated for 2 min at 65 °C and then adjusted to the desired temperature. The reaction was initiated by the addition of 1 μL of 100 mM $CaCl_2$ and was quenched by the addition of 10 μL of formamide–dye mixture containing 10 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) after a specified reaction time. The quenched mixture was heated in a 90 °C bath for 3 min followed by cooling on ice. The resulting DNA fragments were analyzed by electrophoresis on 5% polyacrylamide gels containing 7 M urea (1:20 cross-linking) or on 1% agarose gels, followed by autoradiography at −80 °C with Kodak XAR 5 X-ray film.

Reactions with 63, 70, and 78-nt substrates were carried out under the same conditions as those described above except that slightly higher concentrations of substrates and nuclease were used as noted and 1 μL of 1.0 mg/mL bovine serum albumin (BSA) was added to each reaction mixture.

Determination of V_{max} and K_m. The k_{cat} and the K_m for hybrid nuclease (**5**) (Table I) composed of a 15-nt (3′-TCGCCGAACACGCCC-5′) binding domain cross-linked to

the L37A, Y113A, K116C mutant nuclease were determined by performing cleavage reactions using a fixed concentration of the hybrid nuclease (1.3 nM) and varying amounts of 5′-end-labeled 78-nt substrate (24, 48, 72, 96, 150, 230, 310, and 380 nM). All reactions contained 0.1 μg/μL BSA and 50 mM Bis-Tris-HCl, 50 mM NaCl, and 0.1 mM EDTA, pH 6.5. Stock solutions of the hybrid nucleases were diluted as required in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 0.1 μg/μL BSA. Reaction mixtures were incubated at 65 °C for 5 min prior to the addition of 2.5 mM $CaCl_2$ (final concentration). Reactions were carried out at 65 °C. At 5, 10, 15, 25, and 35 min after addition of $CaCl_2$, 10-μL aliquots of the reaction mixtures were withdrawn and added to a quench solution consisting of 25 mM EGTA in 90% formamide, 0.01% bromophenol blue, and 0.01% xylene cylanol. Aliquotted reaction mixtures were analyzed by electrophoresis on a 15% (1:40 cross-linking) denaturing polyacrylamide gel followed by autoradiography with Kodak XAR 5 X-ray film. Band intensities were determined by excising the desired bands from the gel and counting them in 7 mL of scintillation cocktail with an LKB (Model 1209) scintillation counter.

Turnover as a Function of Temperature. Reaction mixtures were prepared with 5′-^{32}P-end-labeled 78-nt substrate (35 nM) with a 1:100 ratio of hybrid nuclease to DNA for those enzymes which had either 15-nt (**6**, **13**, **14**) or 19-nt (**7**) binding domains (0.35 nM) and a 1:400 ratio of enzyme to DNA for the enzyme with an 8-nt (**8**) binding domain (0.080 nM). The same buffers were used in these reactions as in those described above. Each aliquot was heated to 65 °C for 5 min prior to being cooled and incubated at a given temperature. Cleavage reactions were performed as described. The reactions were then analyzed with a 15% denaturing polyacrylamide gel (1:40 cross-linking). The amount of specific cleavage was assayed by scintillation counting as described above.

Stability of the Oligonucleotide–Nuclease Adduct. The hybrid nuclease (**7**) consisting of a 19-nt (3′-TCGCCGAA-CACGCCCTTAA-5′) binding domain cross-linked directly to the Y113A, K116C nuclease was phosphorylated at the 5′-hydroxyl of the oligonucleotide with [γ-^{32}P]ATP in the absence of dithiothreitol. The labeled hybrid nuclease (20 nM) was incubated with 2.5 mM $CaCl_2$ in 80 μL of 50 mM Bis-Tris-HCl (pH 6.5), 50 mM NaCl, and 0.1 mM EDTA buffer at 65 °C, and aliquots were withdrawn at various times (1, 2, 5, 15, and 30 min) prior to quenching and analysis as described above.

Results and Discussion

The ability to rationally design catalysts with tailored specificities should provide useful tools for biology and chemistry as well as new therapeutic agents. For example, the development of catalysts that sequence specifically hydrolyze DNA or RNA at defined recognition sites would facilitate structure–function studies of nucleic acids, recombinant DNA manipulations, and genomic mapping. One approach to the generation of such catalysts involves the generation of peptides and oligonucleotides that have been chemically modified with EDTA–Fe(II) (Dervan, 1986; Stroebel et al., 1988; Mack et al., 1987) or other synthetic oxidative DNA cleaving functionalities (Chen & Sigman, 1987). Alternatively, the specificity of enzymes can be altered via chemical modification (Kaiser & Lawrence, 1984) or site-directed mutagenesis (Estell et al., 1986; Cronin et al., 1987).

We have used a combination of the latter techniques to generate sequence-specific single-stranded DNA and RNA phosphodiesterases. A surface cysteine (K116C) was introduced into staphylococcal nuclease by site-directed mutagenesis (Corey & Schultz, 1987; Zuckermann et al., 1988; Zuckermann & Schultz, 1989). The K116C mutation did not significantly alter the catalytic properties of the enzyme but allowed the nuclease to be selectively cross-linked to oligonucleotides via disulfide exchange to generate hybrid nucleases with new specificities. The hybrid nucleases selectively hydrolyzed single-stranded DNA and RNA at predefined target sites. This approach offers several potential advantages over oxidative cleavage strategies, most notably the high efficiency of the enzymatic cleavage reaction and the fact that hydrolysis generates termini that can be used in subsequent enzymatic reactions (Zuckermann & Schultz, 1989).

While these hybrid nucleases sequence specifically hydrolyzed single-stranded DNA and RNA, they functioned only as stoichiometric agents and only under a limited set of reaction conditions. Outside of this range, the inherent nonselective hydrolytic activity of the enzyme dominated the oligonucleotide-directed cleavage. In order to increase the utility of these enzymes as molecular biological tools, we have studied the effects of (1) mutations in the nuclease binding site, (2) variations in the length of the tether between the oligonucleotide and the nuclease, and (3) alterations in the oligonucleotide binding domain's length and composition on the properties of the hybrid nuclease.

Introduction of the Y113A and L37A Mutations. The cleavage specificity of the hybrid nucleases reflects the binding properties of both the attached oligonucleotide and staphylococcal nuclease itself. Consequently, selective cleavage at the target site can be accompanied by cleavage at other T-rich sites as a result of the enzyme's native binding specificity. By construction of mutants with decreased values of k_{cat}/K_m, the rate of nonspecific (oligonucleotide independent) hydrolysis by the hybrid nuclease should be decreased. On the other hand, cleavage at sites adjacent to the oligonucleotide binding site should be relatively unaffected since hybridization increases the effective concentration of the nuclease at the target sequence.

The mutation Y113A was introduced into the binding site of the mutant K116C staphylococcal nuclease. This mutation eliminates a π stacking interaction between the tyrosine and the pyrimidine or purine ring of a bound polynucleotide substrate. The mutant was found to have a V_{max} of 0.011 Δabs_{260}/min (μg of enzyme) and K_m of 17 μg of DNA/mL for salmon sperm DNA which compare to wild-type values of 2.1 Δabs_{260}/min (μg of enzyme) and 4.1 μg of DNA/mL, respectively. Another substitution, L37A, afforded the L37A, Y113A, K116C enzyme, which had a V_{max} of 0.015 Δabs_{260}/min (μg of enzyme) and a K_m of 57 μg of DNA/mL. The wild-type nuclease has a strong preference for hydrolyzing single-stranded DNA to the 5′ side of thymidine (T > A ≫ C, G). This preference is preserved in both mutants despite their enlarged binding pockets (Figure 1).

Alteration of Tether Length. Previous studies involved hybrid nucleases with tethers of 6–10 Å between the enzyme and the oligonucleotide. Shortening this distance should decrease the flexibility of the catalytic domain of the hybridized enzyme which in turn might decrease cleavage of substrate DNA at sites distant from the hybridization site. A shortened tether might also limit cleavage to one phosphodiester bond. The nucleoside analogue 3′-mercapto-5′-(4,4′-dimethoxytrityl)thymidine was synthesized and attached to controlled pore glass. Oligonucleotides synthesized from this support have been coupled to both the K116C (**10**) and the Y113A, K116C (**6**) mutants to generate enzymes directly linked via a 3′-di-

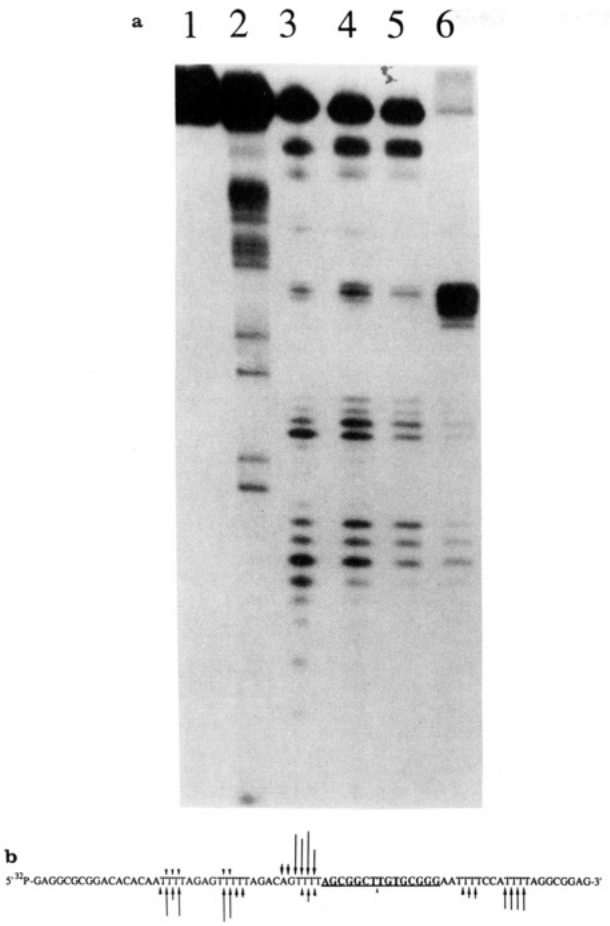


FIGURE 1: (a) Comparison of cleavage by underivatized mutant staphylococcal nucleases and by a hybrid nuclease. Lane 1: 78-nt DNA. Lane 2: Maxam–Gilbert G reaction. Lane 3: Cleavage by 100 nM K116C staphylococcal nuclease. Lane 4: Cleavage by 1.0 μM Y113A, K116C nuclease. Lane 5: Cleavage by 1.0 μM L37A, Y113A, K116C nuclease. Lane 6: Specific cleavage by 0.050 μM hybrid nuclease Y113A, K116C (**6**). All reactions were carried out as described under Experimental Procedures and contained 0.035 μM substrate DNA. (b) Histograms of cleavage DNA cleavage patterns in (a). The heights of the arrows show the relative cleavage intensities at the indicated phosphodiester bonds. The upper arrows represent the cleavage in lane 6, while the lower arrows represent the cleavage in lane 4. The underlined bases are the hybrid nuclease binding sequence.

sulfide bond to the oligonucleotide.

When these hybrid nucleases were assayed with a 5′-end-labeled 78-nt substrate, only slight differences were observed in the cleavage patterns produced by these enzymes relative to those with longer tethers. The hybrid nuclease (**9**) constructed from the Y113A, K116C mutant with a 10-Å linkage and a 15-nt binding domain cleaved the relatively unstructured 78-nt DNA at two phosphodiester bonds while the corresponding enzyme directly linked to the 3′-thiol of the oligonucleotide hydrolyzed substrate at three phosphodiester bonds. However with other substrates, the cleavage patterns were identical, or the enzyme with the direct linkage afforded slightly more specific cleavage. These results may indicate that the nuclease–DNA complex has considerable flexibility and that the structure of the substrate is the major factor in determining the exact nature of cleavage, an observation which has been made previously (Zuckermann et al., 1989).

Effect of the Y113A and L37A Mutations on Cleavage Specificity of the Hybrid Nuclease. Cleavage of single-stranded DNA by a hybrid nuclease (**6**) constructed from the

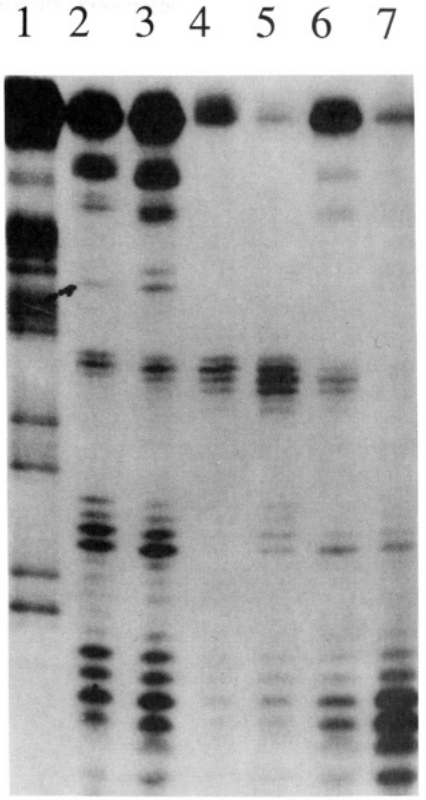


FIGURE 2: Comparison of cleavage by the Y113A, K116C hybrid nuclease (**6**) and the K116C hybrid nuclease (**10**) at 0 and 65 °C. Lane 1: Maxam–Gilbert G reaction. Lane 2: Cleavage by 1.0 μM K116C, Y113A underivatized nuclease. Lane 3: Cleavage by 100 nM K116C underivatized nuclease. Lanes 4 and 5: Cleavage by 20 nM K116C, Y113A hybrid nuclease (**6**), at 0 and 65 °C, respectively. Lanes 6 and 7: Cleavage by 20 nM K116C hybrid nuclease (**10**) at 0 and 65 °C, respectively. Reactions were carried out for 5 s prior to termination as described under Experimental Procedures. All reactions contained 35 nM 78-nt DNA (see Figure 1 for sequence).

Y113A mutant was compared with a hybrid nuclease (**10**) that possessed an identical 15-nt oligonucleotide binding domain but lacked the Y113A mutation. Nuclease and substrate were hybridized at 65 °C for 5 min followed by gradual cooling. Reaction of the 78-nt substrate with the K116C nuclease (**10**) at 0 °C for 2 s led to cleavage largely at secondary sites rather than at the target site (Figure 2, lane 6). In contrast, the Y113A, K116C hybrid nuclease (**6**) hydrolyzed the DNA predominantly at the target site under the same conditions (Figure 1, lanes 4 and 6, Figure 2, lane 4). Furthermore, when the reactions were carried out at 65 °C, the difference in specificity between the single and double mutant was even more significant. The K116C nuclease (**10**) cleaved the substrate nonspecifically with no cleavage observed at the target site (Figure 2, lane 7), whereas the Y113A, K116C nuclease (**6**) continued to hydrolyze the substrate with high specificity (Figure 2, lane 5). Similar differences in cleavage selectivity were observed when the reaction times were lengthened from 5 s to 1 min or when substrate concentrations were raised from 10 to 100 nM. Analogous results were also observed for nucleases **9** and **15**, which were constructed with a 10-Å tether. These results demonstrate that the relative importance of the oligonucleotide in determining the specificity of the hybrid enzyme can be increased by site-directed mutagenesis.

Substrate Turnover. The ability of the Y113A hybrid nuclease to selectively cleave DNA at elevated temperatures and long reaction times suggested that cleavage could occur near the T_m of the hybrid nuclease-substrate complex. Since

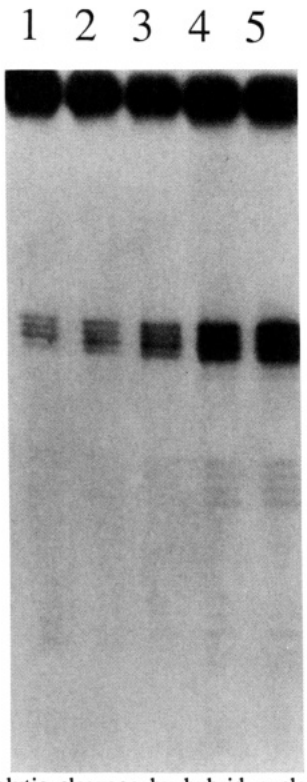


FIGURE 3: Catalytic cleavage by hybrid nuclease. Lanes 1–5: Cleavage of 28 nM 78-nt DNA (for substrate sequence see Figure 1b) at 65 °C by 0.22 nM L37A, Y113A, K116C hybrid nuclease (**5**) as a function of reaction time at 5, 10, 15, 20, and 30 min, respectively.

under these conditions the free hybrid nuclease is in rapid exchange with bound nuclease, the nuclease should catalytically cleave the target DNA. Hybrid nucleases containing a 15-nt binding domain and either the Y113A, K116C mutant (**6**) or the L37A, Y113A, K116C mutant (**5**) were assayed for their ability to catalytically hydrolyze the 78-nt DNA substrate at 65 °C for 30 min at a ratio of 1:200 enzyme:DNA, as described under Experimental Procedures. The calculated T_m for the hybrid nuclease–substrate complex is 62 °C.[2] Under these conditions, with either hybrid nuclease, approximately one substrate molecule was cleaved per nuclease per minute, and the amount of product continued to increase over 30 min until substrate was depleted (Figure 3). Cleavage was observed only if 0.1 μg/μL bovine serum albumin was present both in the reaction buffer and in the buffer used to dilute the enzyme.

The kinetic parameters for nuclease **5** which contains the L37A, Y113A, K116C mutant were determined at a fixed concentration of nuclease (1.3 nM) and varied concentrations of the 78-nt substrate (24–380 nM). Reactions were maintained at 65 °C, and aliquots were withdrawn at 5, 10, 15, 25, and 35 min after the addition of Ca^{2+} (final concentration of 2.5 mM). Cleavage products were separated by a denaturing polyacrylamide gel, and the amount of specific cleavage versus total cleavage was quantified by scintillation counting. Cleavage by the nuclease increased over time, and the rate of cleavage was dependent on substrate concentration. A Lineweaver–Burke analysis of the initial rates afforded a K_m of 120 nM and a k_{cat} of 1.2 min^{-1}. The value of K_m approximates that of K_D for the dissociation of the nuclease–substrate complex. The low value of k_{cat} is consistent with the notion that dissociation of the enzyme–substrate complex is the rate-determining step of the reaction. Some nonspecific hydrolysis did occur but did not exceed 30% of total cleavage. In contrast, cleavage by a hybrid nuclease with a noncognate binding site afforded 92% nonspecific cleavage. When cleavage by the hybrid nuclease was carried out over a range of temperatures between 25 and 80 °C, turnover occurred only between 52 and 68 °C, with an optimum at 60–62 °C. At temperatures above 68 °C the amount of selective cleavage decreased abruptly. No major difference in the amount of specific or nonspecific cleavage was observed with the double mutant compared to the triple mutant.

[2] Calculated by the program of S. Lipson, which is based on the work of Breslauer (Breslauer et al., 1986).

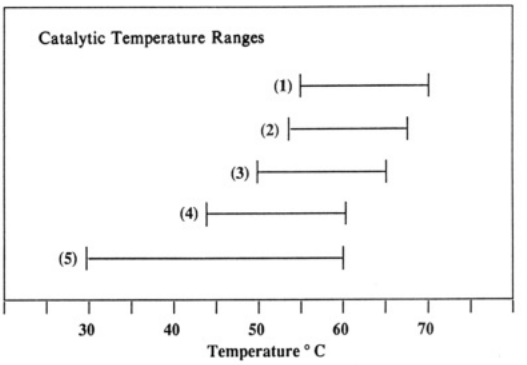


FIGURE 4: Temperature dependence of catalysis. Hybrid nuclease with (1) 19-nt binding domain (**7**), (2) 15-nt binding domain (**6**), (3) 15-nt binding domain with a 3′-terminal mismatch (**13**), (4) 15-nt binding domain with an interior mismatch (**14**), and (5) 8-nt binding domain (**8**). Sequences are as described in text; reaction conditions are as described under Experimental Procedures. Substrate was the 78-nt DNA (see Figure 1b).

Introduction of the Y113A and L37A mutations allows the hybrid nuclease to selectively cleave DNA, even at temperatures above the T_m, where a significant fraction of the nuclease is free in solution. This result demonstrates that a combination of chemical mutagenesis (introduction of an oligonucleotide) followed by biological mutagenesis (binding site mutations) can be used to rationally modify the specificity of an enzyme while still maintaining catalytic turnover.

Variation in Length and Sequence of the Oligonucleotide Binding Domain. Hybrid nucleases were constructed with oligonucleotides of 8 (3′-TCGCCGAA-5′) (**8**), 15 (3′-TCGCCGAACACGCCC-5′) (**6**), or 19 (3′-TCGCCGAACACGCCTTAAA-5′) (**7**) nucleotides (each from the Y113A, K116C mutant). No differences were observed in cleavage specificity by these three nucleases when cleavage reactions were carried out at temperatures below 25 °C. The nucleases were then assayed at temperatures ranging from 25 to 80 °C as described under Experimental Procedures. Each was found to be catalytic over a different range of temperatures (Figure 4). The 15- and 19-nt enzymes were catalytic between 54 and 68 °C and between 55 and 72 °C, respectively. The 8-nt hybrid nuclease was catalytic over the broadest temperature range, 30–60 °C, but was more prone to nonspecific cleavage (55% of total cleavage) than the 15- or 19-nt enzymes (30% of total cleavage). The 8-nt nuclease also had the highest turnover number (k_{cat} = 30 min^{-1}) at enzyme:DNA ratios as low as 1:1200. The high yield of nonspecific cleavage by the 8-nt nuclease can be reduced to 40% of total cleavage by the addition of a 100-fold excess (by weight) of noncognate competing DNA. These results are consistent with the notion that dissociation of the nuclease–DNA complex is rate limiting, and consequently, the T_m of the oligonucleotide for its target site determines the temperature range over which the hybrid nuclease is catalytic.

Hybrid nucleases were prepared with mismatches in a 15-nt

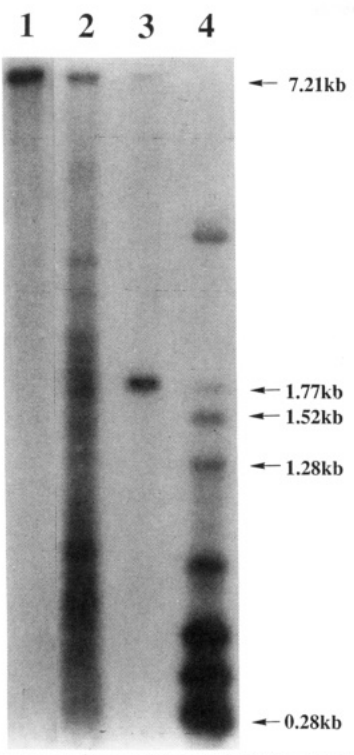


FIGURE 5: Cleavage of 5′-end-labeled M13mp7 DNA. Lane 1: 5′-^{32}P-end-labeled DNA (12 nM). Lane 2: Nonspecific cleavage of the 60 nM M13mp7 DNA by 10 nM underivatized K116C mutant staphylococcal nuclease at 0 °C, 1 s. Lane 3: Specific cleavage of 12 nM DNA by 14 nM K116C, Y113A hybrid nuclease (**12**) at 65 °C with reaction time of 1 s. Lane 4: 5′-^{32}P-end-labeled single-stranded RNA molecular weight markers.

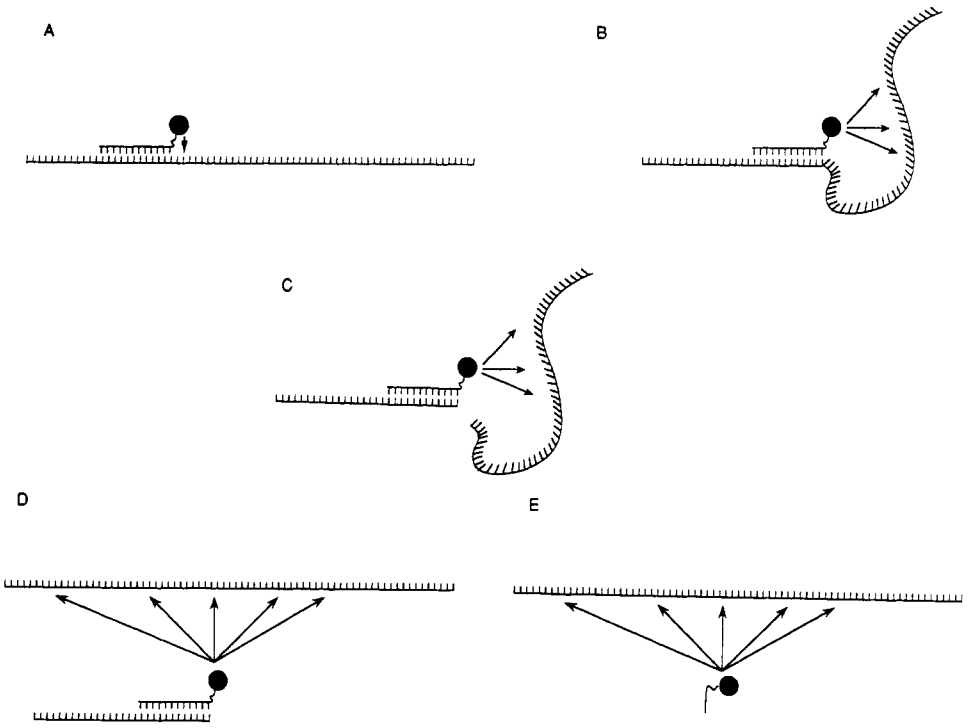
binding domain (Y113A, K116C mutant). Substitutions of G for C were placed either near the 3′ terminus (3′-TGGCCGAACACGCCC-5′) (**13**) or in the interior of the oligonucleotide (3′-TCGCCGAAGACGCCC-5′) (**14**). Neither of these alterations had any effect on either the specificity of the hybrid nuclease or the cleavage pattern at the site adjacent to the oligonucleotide binding site. Both hybrid nucleases were assayed for cleavage between 25 and 75 °C and were found to be catalytic over lower temperature ranges than the analogous fully complementary 15-nt nuclease (**6**) (Figure 4). The hybrid nuclease with the 3′-terminal mismatch was catalytic over a range of 50–64 °C while the nuclease with the interior mismatch was catalytic between 44 and 60 °C. These values compare to the temperature range of 54–68 °C for the nuclease with the fully complementary binding domain. The effects of mismatches on the catalytic properties of the hybrid nucleases reflect the reported effects of mismatches on DNA hybridization.

Stability of Oligonucleotide–Nuclease Adduct to Autodigestion. The ability of the hybrid nucleases to catalytically cleave DNA in a selective fashion may appear somewhat surprising given that the cross-linked oligonucleotide can be digested by Ca^{2+}-activated staphylococcal nuclease. Consequently, the stability of the oligonucleotide–nuclease adduct toward autolysis at elevated temperatures and over extended reaction times was investigated by 5′-end-labeling the 19-nt binding domain (3′-TCGCCGAACACGCCCTTAA-5′) of a hybrid nuclease (**7**) (Y113A, K116C mutant) with ^{32}P. The labeled adduct was incubated with 2.5 mM Ca^{2+} and 0.1 mg/mL BSA at 65 °C in the absence of substrate, and the course of the incubation was monitored by electrophoresis. No degradation was observed after 30 min. Resistance to intramolecular autolysis may be due to an inability of the oligonucleotide to align itself in the correct 5′ to 3′ orientation for cleavage in the enzyme active site.

FIGURE 6: Autoradiogram of a 5% denaturing polyacrylamide gel showing cleavage of *Dra*I-linearized, 5′-^{32}P-labeled single-stranded M13mp7 DNA [Scheme I (b)]. Lane 1: 5′-^{32}P-end-labeled M13mp7 DNA (12 nM). Lanes 2 and 3: Cleavage of 12 nM DNA by 14 nM Y113A, K116C hybrid nuclease (**12**) at 65 and 0 °C, respectively, with a reaction time of 2 s and reaction volume of 10 μL. Lane 4: Single-stranded RNA marker (0.16 kb). Shown on the left is the histogram of the cleavage patterns of reactions at 65 °C (left arrows) and 0 °C (right arrow). The heights of the arrows indicate the relative cleavage intensities.

Cleavage of Single-Stranded M13 DNA. The increased specificity of hybrid nucleases containing the Y113A mutation suggested that the nuclease might cleave large single-stranded DNAs such as M13mp7 bacteriophage DNA. This 7214-nt single-stranded DNA substrate contains many T-rich sites that are susceptible to nonspecific cleavage. M13 DNA has been engineered with a short hairpin region containing a number of restriction sites (Messing, 1983). The single-stranded DNA was linearized by *Bam*HI digestion and labeled at the 5′ terminus with ^{32}P. Two hybrid nucleases (**11** and **12**) were constructed by directly fusing a 22-nt oligonucleotide (3′-TAGCAGCAGACCATTTGCTCCC-5′) to the K116C and the Y113A, K116C mutant staphylococcal nucleases, respectively. The latter nuclease selectively cleaved M13mp7

Scheme II: Mechanisms for Specific and Nonspecific Cleavage



at one site to generate two fragments, 1.58 and 5.63 kb in length, in approximately 50% yield at 65 °C (Figure 5). In contrast, the K116C nuclease (**11**) cleaved M13mp7 nonspecifically at 65 C and primarily at two sites at 0 °C. Similar results were obtained when the M13mp7 substrate was labeled at the 3′ terminus.

In order to examine the cleavage site at single-base resolution, a 30-nt oligonucleotide was annealed to the circular single-stranded M13mp7 DNA to generate a short double-stranded region which was subsequently cleaved with *Dra*I restriction endonuclease. In the resulting linear single-stranded DNA, the nuclease binding site is only 133 bases away from the 5′ terminus (Scheme I). Consequently, the cleavage products could be analyzed on a 5% denaturing polyacrylamide gel. The autoradiogram of the cleavage gel revealed that the Y113A, K116C hybrid nuclease (**12**) hydrolyzed the M13mp7 fragment predominantly at one phosphodiester bond two nucleotides away from the 5′ end of the oligonucleotide binding site at 0 °C (Figure 6). At an elevated temperature (65 °C) cleavage occurred over several bonds but at only one site adjacent to the hybrid nuclease binding site (Figure 6). Because the hybrid nucleases are capable of site specifically cleaving large structural single-stranded DNAs, they should prove useful tools for manipulating and studying the structure of such DNAs.

Effect of Primary and Secondary Substrate Structure on Cleavage. The ability of hybrid nuclease (**6**) to specifically cleave substrates with secondary structures was investigated over a range of reaction temperatures. A 63-nt substrate was synthesized containing a self-complementary sequence, 3′-TTTTGCCCCTGCAAAA-5′, directly adjacent to the oligonucleotide binding site (Figure 7c). At reaction temperatures below 45 °C little specific cleavage was observed (Figure 7a). Presumably secondary structure renders the thymidines in the target site inaccessible, and nonspecific cleavage at secondary sites predominates. However at 65 °C, above the calculated T_m of the six base pair hairpin, very little nonspecific hydrolysis was observed relative to specific cleavage at the target site (Figure 7a).

Free staphylococcal nuclease preferentially cleaves substrates at T-rich regions regardless of whether the L37A, Y113A, or K116C mutations are present. Cleavage at C or G is especially disfavored. In order to determine whether hybridization of the oligonucleotide–nuclease adduct can overcome this base preference, a 70-nt DNA substrate was prepared with the sequence GCCGCGG adjacent to the oligonucleotide binding site. Four 15-nt hybrid nucleases (**6**, **9**, **10**, **15**) were assayed with this substrate. None of these nucleases cleaved within the C/G-rich region under any conditions. However, cleavage did occur at T-rich sites to the 5′ side of the hybridization site, primarily at the site nearest to the bound hybrid nuclease (80% of total cleavage) but also at sites even further away (20% of total cleavage) (Figure 8). Again these results support the notion that the oligonucleotide–nuclease adduct has considerable flexibility when hybridized to the target sequence.

Effect of Poly(dA) on Cleavage Specificity. Although the double mutant (Y113A, K116C) and triple mutant (L37A, Y113A, K116C) hybrid nucleases cleave DNA both catalytically and under a wide range of conditions, nonspecific hydrolysis, ranging from 10% to 60% of total cleavage, is still associated with the reactions. Understanding the nature of nonspecific cleavage should aid in engineering hybrid nucleases that approach restriction enzymes in their specificity and cleavage efficiency. Nonspecific cleavage associated with the nuclease can occur via a number of mechanisms (Scheme II). The nuclease can bind and cleave DNA in an intermolecular reaction which is independent of the oligonucleotide domain (Scheme IIE) and which can occur either before or after hybridization and cleavage of the target sequence (Scheme IID). Nonspecific cleavage can also occur in an intramolecular fashion, in which hybridization first localizes the nuclease at the target site. Conformational flexibility or secondary structure in the substrate at the target site may then make upstream 5′ T-rich sites accessible to cleavage by the nuclease (Scheme IIB). Alternatively, cleavage at the target site can be followed by secondary cleavage at other T-rich sites by a processive mechanism in which the enzyme does not dissociate from substrate (we have determined that a significant com-

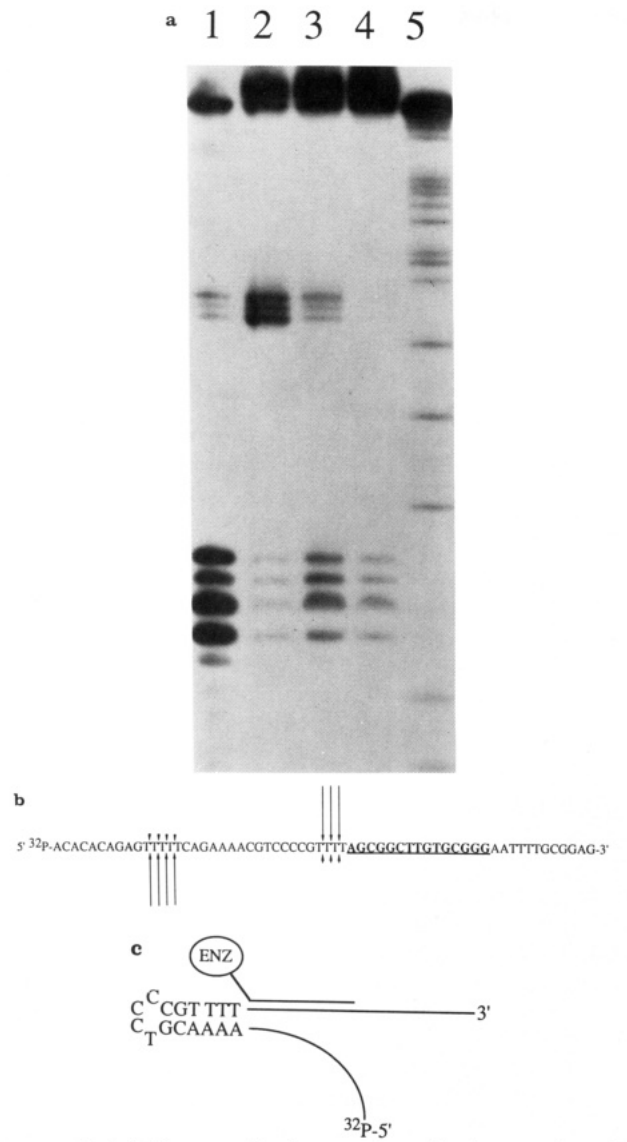


FIGURE 7: (a) Cleavage of a short, structured substrate. Lane 1: Cleavage by 100 nM underivatized Y113A, K116C nuclease at 37 °C. Lanes 2–4: Cleavage by 35 nM Y113A K116C hybrid nuclease (**6**) at 65, 37, and, 0 °C, respectively. Lane 5: Maxam–Gilbert G reaction. All reactions contained 35 nM 63-nt substrate DNA and were incubated with 2.5 mM Ca^{2+} for 5 s prior to quenching as described under Experimental Procedures. (b) Histograms of cleavage of 63-nt substrate in (a) (lanes 1 and 2). The upper arrows represent the cleavage in lane 2, while the lower arrows illustrate the cleavage in lane 1. The heights of the arrows indicate the relative cleavage intensities. The underline bases are the hybrid nuclease binding sequence. (c) Predicted structure of the hairpin loop formed by the 63-nt DNA substrate.

FIGURE 8: Histogram of cleavage of a 70-nt substrate with a G/C-rich region adjacent to the oligonucleotide binding site. The upper arrows represent specific cleavage by 20 nM Y113A, K116C hybrid nuclease (**6**), while the lower arrows show cleavage by 10 nM underivatized Y113A, K116C nuclease. The heights of the arrows indicate the relative cleavage intensities. Reactions contained 35 nM 70-nt substrate DNA and were carried out as described under Experimental Procedures. The underlined bases are the hybrid nuclease binding sequence.

ponent of single-stranded DNA cleavage by free staphylococcal nuclease does occur via a processive mechanism) (Scheme IIC).

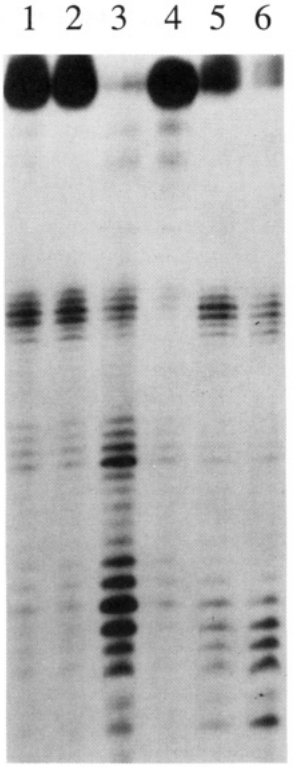


FIGURE 9: Effect of poly(dA) on DNA cleavage by cognate and noncognate hybrid nucleases. Lane 1: Reaction of 78-nt substrate (see Figure 1) with 30 nM Y113A, K116C hybrid nuclease (**6**) containing a fully complementary binding domain, at 20 °C for 5 s. Lane 2: Same as lane 1 with 2 μg of poly(dA) DNA added. Lane 3: Reaction of 100 nM Y113A, K116C hybrid nuclease (**12**) containing a 22-nt noncognate binding domain at 37 °C for 15 s prior to quenching. Lane 4: Same as lane 3 except that 2 μg of poly(dA) DNA was added. Lane 5: Reaction with 20 nM K116C hybrid nuclease (**10**) containing the fully complementary 15-nt binding domain used in lanes 1 and 2 at 0 °C for 5 s with 2 μg of poly(dA) added. Lane 6: Same as lane 5 except that no poly(dA) DNA was added. Each reaction was carried out with 30 nM 78-nt substrate as described under Experimental Procedures.

In order to distinguish whether inter- or intramolecular cleavage by the oligonucleotide-directed nuclease was responsible for this nonspecific hydrolysis, an excess of an unlabeled competitive substrate, poly(dA), was added. Poly(dA) should depress intermolecular cleavage to a greater degree than intramolecular cleavage. Cleavage reactions were performed at 37 °C for 5 s, with and without a 200-fold (by weight, relative to 78-nt substrate) excess of poly(dA). Equimolar amounts of the 15-nt Y113A nuclease (**6**) and the 5′-^{32}P-end-labeled 78-nt substrate were used. Nonspecific cleavage was between 15% and 20% of total cleavage in both the presence and the absence of poly(dA) (Figure 9, lanes 1 and 2). When a Y113A nuclease (**12**) containing a 22-nt (3′-TAGCAGCAGACCATTTGCTCCC-5′) noncognate oligonucleotide (i.e., one which is not complementary to the substrate) was used, the addition of poly(dA) significantly decreased the amount of nonspecific hydrolysis (Figure 9, lanes 3 and 4). When the K116C enzyme (**10**) which lacks the binding pocket mutation was assayed, nonspecific cleavage was also greatly reduced by the addition of poly(dA) DNA (Figure 9, lanes 5 and 6). These results suggest that nonspecific cleavage by Y113A hybrid nucleases is due largely to intramolecular cleavage and that intermolecular cleavage contributes significantly to the nonspecific cleavage associated with oligonucleotide-directed nucleases lacking the binding pocket mutations.

The effect of poly(dA) on nonspecific cleavage at elevated

temperatures where the oligonucleotide-directed nuclease is catalytic was also examined. Reactions were carried out at 65 °C at a 100:1 ratio of 78-nt substrate to 15-nt Y113A nuclease (**6**). The addition of a 100-fold excess of poly(dA) DNA to substrate consistently reduced the amount of nonspecific cleavage from 60% to 40% of specific cleavage. Thus reaction conditions which allow substrate turnover also increase nonspecific intermolecular cleavage, since the oligonucleotide–nuclease adduct spends a greater proportion of the reaction time in an unbound state.

Further experiments are necessary to distinguish the nature of the nonspecific intramolecular cleavage reaction associated with the Y113A hybrid nuclease. It is clear that the flexibility of the tether does not have a dramatic effect on the ratio of selective to nonselective cleavage. Nonspecific cleavage, however, is considerably reduced when structured DNA or RNA substrates are used and therefore appears to be associated with the secondary structure of substrate (Zuckermann & Schultz, 1989).

Conclusion. We have optimized the cleavage of single-stranded DNA by oligonucleotide-directed staphylococcal nuclease through site-directed mutagenesis of the protein's binding pocket. The altered hybrid nucleases selectively hydrolyze DNA under a wide range of conditions and function catalytically. These modifications greatly increase the potential of these nucleases as tools for manipulating and studying naturally occurring nucleic acids. Moreover, the success of this strategy in rationally modifying enzyme specificity suggests that the splicing of biomolecular domains to produce hybrid molecules may prove a general approach to the design of tailored catalysts.

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