Oligonucleotide-Directed Nucleic Acid Scission by Micrococcal Nuclease†

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ABSTRACT: "Sequence-dictated" scission of DNA can be achieved by tethering a fusion protein composed of glutathione *S*-transferase and attenuated micrococcal nuclease (MN) to a targeting oligonucleotide using Cibacron blue (CB) F3G-A. Deoxyoligonucleotides derivatized with this dye bind to the fusion protein in gel mobility shift assays. This binding scheme was successfully used to achieve site-specific scission of a single-stranded DNA substrate after hybridization with a CB-derivatized complementary oligonucleotide. Although covalently cross-linked hybrids of MN and oligonucleotides have been successfully used in the past to target nucleolytic activity, this novel scheme opens new possibilities for targeting and probing both DNA and RNA sequences by allowing the addition of the nuclease subsequent to hybridization.

The development of nucleic acid scission reagents that could be directed to selected sequences would facilitate the analysis and manipulation of chromosomal DNA and provide a method for mapping the three-dimensional conformation of a folded RNA in solution. In the analysis of chromosomal DNA, the possibility of achieving double-stranded scission at any nucleotide sequence would allow the measurement in base pairs of the distance between marker genes, map the size of a transcription unit, and define positions of chromosomal breakpoints. In addition, the methodology would facilitate the cloning of chromosomal fragments of interest. In the analysis of RNA conformation, tethering an efficient scission reagent to a hybridizing oligonucleotide would allow estimation of the physical distance between the sites of hybridization and scission.

One method of achieving "sequence-dictated scission" makes use of RNAs linked to the chemical nuclease (1,10-phenanthroline)copper (Chen et al., 1993a; Sigman et al., 1993a,b). Sequence recognition is achieved through R-loop formation; scission is accomplished by the oxidative chemical nuclease. Initial attempts at mapping RNA conformation with scission reagents used deoxyoligonucleotides complementary to single-stranded loops linked at the 5′ end to (1,10-phenanthroline)-copper (Chen et al., 1993b). Although both approaches with this nucleolytic activity show promise, one drawback is background scission generated as a result of the requirement for cupric ion, thiol, and hydrogen peroxide. In addition, the chemical nuclease is not as efficient in cleaving the phosphodiester backbone as hydrolytic biological catalysts. For that reason, we have investigated the adaptation of micrococcal nuclease (MN) for a "sequence-dictated" strategy. This enzyme was chosen because it is a relatively nonspecific nuclease capable of cleaving single- and double-stranded DNA as well as RNA at any nucleotide despite a reaction preference for cleavage at thymidines (Cuatrecasas et al., 1967). The reaction produces 3′-phosphomonoesters and 5′-hydroxyl groups. One additional advantage is its strong dependence on Ca^{2+}, which provides a convenient way to activate and deactivate the enzyme using Ca^{2+}-specific chelating reagents such as EGTA (Corey et al., 1989).

The mechanism and structure of MN have been extensively studied (Cuatrecasas et al., 1967, 1969; Anfinsen et al., 1971; Gerlt, 1992). In addition, the enzyme has been genetically and chemically modified in an attempt to convert it into a site-specific nuclease. For example, the enzyme has been linked to the DNA binding domain of the λ phage repressor (Pei & Schultz, 1990) and tethered to deoxyoligonucleotides (Corey & Schultz, 1987; Pei et al., 1990). For these experiments, a genetically modified derivative of MN was used in which a cysteine residue was introduced for covalent modification and the K_m of the enzymatic activity was increased by replacing both leucine 37 and tyrosine 113 with alanines (Corey et al., 1989).

In contrast, our approach for targeting MN involved the preparation of a fusion protein of an attenuated mutant of MN with a protein with a high-affinity binding site for small easily derivatized ligands. Sequence-dictated scission would then be achieved by chemical modification of a targeting oligonucleotide with the ligand and using it to target a preselected site of hybridization. Since the fusion protein could be added after the hybridization reaction, any method of hybridization [e.g., R-loops (Thomas et al., 1976) or D-loops (Rao et al., 1991)] driven by the formation of Watson–Crick base pairs could be used.

Glutathione *S*-transferase (GST) provided one possible approach to implement this strategy for two reasons. First, since the metabolic role of GST is to conjugate glutathione to a variety of xenobiotic compounds (Habig et al., 1974), it seemed likely that a tight binding ligand for tethering a oligonucleotide to MN via its catalytic site could be found. Second, GST fusion proteins have provided a reliable method for expressing a variety of proteins (Smith & Johnson, 1988). They are produced in high yield, are soluble, and are not toxic to cells. Moreover, fusion vectors are readily available in all three reading frames, and the desired protein can be purified on an affinity column using hexylglutathione as the GST-specific ligand if necessary. These procedures suggested that a glutathione derivative might serve as an appropriate ligand for tethering a deoxyoligonucleotide to MN via a binding site

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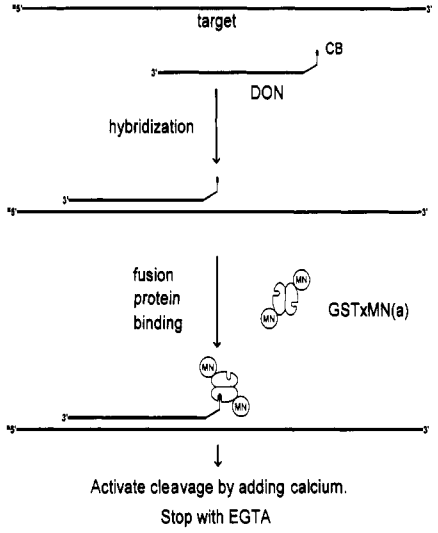

FIGURE 1: Targeted scission by glutathione *S*-transferase–micrococcal nuclease fusion protein using Cibacron blue (CB)-derivatized deoxynucleotides. The schematic emphasizes the two-step approach to targeting. The hybridization reaction is carried out with protein-free derivatized oligonucleotide. This would allow for a wide spectrum of hybridization techniques for double- or single-stranded DNA and RNA. The subsequent protein binding step is based on the high-affinity pocket for CB on GST.

contributed by GST.

In this paper, we demonstrate a reliable and adaptable method for targeting the hydrolytic activity of micrococcal nuclease using Cibacron blue F3G-A. This dye is a strong inhibitor of the GST activity for the free enzyme (Mannervik et al., 1985) as well as the fusion construct. It binds with high affinity, is readily available, and can be converted into iodoacetyl derivatives which can alkylate 5′-thiophosphorylated oligonucleotides. Sequence-specific scission has been achieved in two test systems using either a 121- or a 70-base oligonucleotide and a Cibacron blue-derivatized complementary deoxyoligonucleotide to direct cleavage via a Cibacron blue–GST tether (Figure 1).

EXPERIMENTAL PROCEDURES

The vector pONF-1 containing the wild-type micrococcal nuclease (Takahara et al., 1985) was a generous gift of Prof. John Gerlt, University of Maryland. pGEX-3X and pET-3a were purchased from Pharmacia, T4 polynucleotide kinase and IPTG were from BRL, Cibacron blue F3G-A-A was from Fluka, ATPγS was from Boehringer Mannheim, [^{35}S]ATPγS (1000 Ci/mmol) was from Amersham, terminal deoxynucleotide transferase was from U.S. Biochemicals, and succinimidyl 6-[(iodoacetyl)amino]hexonate (SIAX) was from Molecular Probes. Spectroscopic assays were carried out on a Hewlett-Packard 8452A diode array spectrophotometer. All oligonucleotides were synthesized on a Pharmacia Gene Assembler Special using standard phosphoramidite chemistry. All other chemicals were purchased from Sigma.

Construction and Purification of Glutathione S-Transferase (GST)–Micrococcal Nuclease (MN) Fusion Constructs. Micrococcal nuclease with wild-type activity was amplified by PCR from pONF-1. The N-terminal primer was modified in order to remove the omp-A leader sequence present in pONF-1 and to introduce an *Eco*RI site in frame with the glutathione *S*-transferase gene in pGEX-3X. The C-terminal primer introduces an *Eco*RI site following the stop codon present in pONF-1. In addition, alanine in position 1 was replaced with cysteine to provide the option of expressing MN that can be modified site-specifically since the wild-type enzyme does not contain cysteine. The final sequence of the junction between glutathione *S*-transferase and micrococcal nuclease in this construct was **Asn**-Ser-Cys-Thr-Ser-Thr-Lys-Lys-Leu-His (underlined sequence is micrococcal nuclease (A1C), **Asn** encoded by AAT of the *Eco*RI site). This fusion construct with wild-type nuclease activity is referred to as GST–MN.

The attenuated mutant (L37A, Y113A) was generated in two steps by PCR mutagenesis based on pONF-1. In the first step, two fragments were generated. One fragment contained the segment from the N-terminus to the mutation L37A. This fragment also contained an *Nde*I site to allow for cloning into pET-3a. The second fragment covered mutation Y113A and the C-terminus followed by a *Bam*HI site. Both fragments were used for a second cycle of PCR, and the full-length product was cloned into pET-3a. The correct insert was confirmed in this vector. Following expression, the protein was purified on a phosphocellulose column and its attenuated phenotype established by comparison with both the wild-type protein and an alternative attenuated mutant (K116C, Y113A), a generous gift of Dr. Corey (University of Texas, Dallas). A fusion protein in which an additional eight amino acid spacer was inserted into the junction of GST and MN was prepared by PCR using a C-terminal primer that placed an *Eco*RI site in the same position as above for GST–MN but introduced an eight amino acid spacer into the N-terminal primer. After cloning into the *Eco*RI site of pGEX-3X, the protein sequence of the junction in this fusion construct was **Asn**-Ser-Pro-Pro-Gly-Gly-Pro-Gly-His-Met-Thr-Ser-Thr-Lys-Lys-Leu-His (underlined sequence is micrococcal nuclease, **Asn** encoded by AAT of the *Eco*RI site). The His-Met sequence in this junction is the result of maintaining the *Nde*I site from pET-3a. This construct, composed of a linker and an attenuated nuclease, is referred to as GST–*x*–MN(a).

The proteins were expressed in *Escherichia coli* DH5α cells by induction with 0.5 mM isopropyl β-D-thiogalactoside (IPTG) at 0.6 OD_{600} for 1.5 h. The cell pellet was resuspended in 10 mL of 50 mM HEPES (pH 6.8) and lysed by sonification. The low-salt supernatant was applied to a MonoS cation-exchange column and was eluted with a linear 100-mL gradient from 100 to 500 mM KCl and 50 mM HEPES (pH 6.8) at 0.5 mL/min. The fusion proteins eluted around 300 mM KCl and were greater than 95% homogeneous as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (yield: 40–50 mg for 1 L of cells).

Enzyme Assay for Micrococcal Nuclease with Wild-Type Activity. The assay for micrococcal nuclease with wild-type activity can be measured by following the increase in absorbance at 260 nm resulting from the hydrolysis of nucleic acids (Cuatrecasas et al., 1967). Heat-treated calf thymus DNA (ctDNA) was used as the substrate and was incubated with MN in 1 mL of 50 mM Tris-HCl buffer (pH 8.8) and 50 mM $CaCl_2$, and the increase in absorbance was monitored spectroscopically. The K_m, as determined in this assay, is calculated in μg(ctDNA)/mL.

Qualitative Assay for Micrococcal Nuclease (Attenuated Mutant). One microgram of supercoiled pUC vector was diluted into 20 μL of Tris-HCl (pH 8.6) containing either 50 mM $MgCl_2$ and EGTA (50 mM) or 50 mM $CaCl_2$. Micrococcal nuclease was added at different concentrations and aliquots were removed after 1, 5, and 30 min. The reaction was then stopped with 60 mM EDTA and run on agarose gels. The conversion to relaxed circular, linear, and fragmented plasmid was visualized by ethidium bromide staining. The

relative activity was estimated by comparison with standards derived from diluted micrococcal nuclease with wild-type activity or free attenuated mutant at identical concentrations.

Enzyme Assay for Glutathione S-Transferase. The GST activity was determined by measuring the increase in absorbance at 340 nm that results from the conjugation of glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB) (Habig et al., 1974). The net reaction is GSH + CDNB → GS–DNB + HCl. The reaction is carried out in a final volume of 1 mL containing 10 mM phosphate buffer (pH 7.2). Measurements were started by the addition of enzyme, and the initial velocities were corrected for the small nonenzymatic background reaction. The kinetic parameters of the fusion protein are in good agreement with those of native GST. To determine the inhibition constant for various inhibitors, velocities and inhibitor concentrations were plotted using Dixon plots ($1/v$ vs [I]) and Lineweaver–Burk ($1/v$ vs $1/s$) plots.

Synthesis of Amino-C6–CB. Cibacron blue F3G-A was dissolved at a final concentration of 10 mM in an aqueous solution of 1,6-diaminohexane (300 mM) and was stirred overnight at room temperature. Excess hexanediamine was removed by precipitation of Cibacron blue in ethanol by adding 10 volume equiv of 95% ethanol and two volume equiv of 5 M potassium acetate (pH 4.8). The resulting pellet was washed three times with 10 volume equiv of ethanol (95%). The reaction was checked for completion by thin-layer chromatography on silica plates using CCl_4–CH_3OH–NH_4-OH (15:15:1) as the solvent. The reaction was complete with the exception of a very small quantity (estimated <5%) of material which contaminated the starting material and might be a Cibacron blue F3G-A derivative in which the chloro group had been hydrolyzed.

Synthesis of Iodoacetyl-LC–CB. Amino-C6–CB and succinimidyl 6-[(iodoacetyl)amino]hexonate (SIAX) were dissolved in DMF to a final concentration of 39 and 93 mM, respectively. The reaction was incubated at 37 °C for 30 min. The product was precipitated and washed as described above and analyzed on thin-layer chromatography. The yield for this reaction (according to TLC) was >90%, and the presence of the iodoacetyl group was confirmed by reacting a small aliquot of the product (100 mM) with 200 mM GSH. The expected product was completely and selectively converted into a slower running species. Since the remaining impurities (<10%) were inert toward sulfhydryl-containing reagents, the product was not purified further.

Synthesis and Derivatization of Oligonucleotides. Deoxyoligonucleotides (DONs) were synthesized on a Pharmacia Gene Assembler. The 121 nucleotide long target sequence (TAGGTGAGACTATAGAATACACGGAATTC-CAGTGAATGCGTAATCATGGTCATAGCT-GTTTCCTGTGTGAAATTGTTATCCGCTCA-CAATTCCACACAACATACGAGCCGGGCCCAA-GCTT) was gel purified on a 15% denaturing polyacrylamide gel prior to 5′ end labeling by T4 polynucleotide kinase. A 50-nucleotide DON (DON–50′mer) (ATTACGCAT-TCACTGGAATTCCGTGTATTCTATAGTGT-CACCTAAATCGT), complementary to positions 1–44 of the 121-nucleotide substrate, was used to direct the hydrolysis by the fusion protein. It was thiophosphorylated for 2 h at 37 °C in a 100-μL reaction containing 0.15 mM oligonucleotide, 0.3 mM ATPγS, 2.4 μM [^{35}S]ATPγS (1000 Ci/mmol), 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT), and 50 units of T4 polynucleotide kinase. After a phenol–chloroform extraction the solution was desalted on two successive G-50 spin columns. DTT was added (0.2 mM) and then IAc-Lc–CB (2.5 mM). The 200-μL reaction was incubated at 37 °C for 2 h. Excess IAc-LC–CB was removed on a G-50 spin column. The product was ethanol precipitated and applied to a 15% denaturing polyacrylamide gel together with an underivatized control. The product formed a single slowly migrating band which was excised and eluted. The ethanol-precipitated Cibacron blue-derivatized 50′mer (CB-50) was 3′ labeled to assay gel shifts, using [α-^{32}P]ddATP and deoxynucleotide terminal transferase following the protocol provided by U.S. Biochemicals. The purification and derivatization of the oligonucleotides for the second test system (70 nucleotide substrate and CB-23) were carried out in the same manner.

Gel Retardation Assays of Cibacron Blue-Derivatized Oligonucleotides. Cibacron blue-derivatized and 3′-[α-^{32}P]-labeled DONs (final concentration 1 nM) were incubated with 25 mM HEPES buffer (pH 7.6), 1 mM DTT, 1 mM EDTA, 100 ng/mL calf thymus DNA, and varying concentrations of fusion protein in a total volume of 20 μL. After 5 min at room temperature, glycerol was added to 7% w/v, and the reaction mixture was loaded onto a 5% acrylamide gel equilibrated with $^1/_2$× TBE and run at a constant current of 30 mA. Bromophenol blue was left out of the loading buffer because it was identified as a competitive inhibitor of GST activity and therefore blocked Cibacron blue binding to glutathione *S*-transferase. Gel shifts for cutting reactions were carried out in 50 mM HEPES, pH 7.6.

Gel Retardation of CB-Linked Duplexes by Fusion Proteins. The 5′-^{32}P-labeled 121-nucleotide single-stranded substrate was incubated with a 5-fold excess of complementary CB–50′mer in 100 mM NaCl and 10 mM HEPES (pH 7.6). Control reactions contained the identical concentration of non-CB-derivatized 50′mer. After 1 min at 95 °C, the reaction was left at room temperature for 10 min. The volume was increased to 20 μL with EDTA (final 1 mM), calf thymus DNA (final 100 ng/mL), water, and fusion protein at a final concentration of 200 nM. The gel shift was carried out as described above, and the gel-shifted band (approximately 20%) was located by autoradiography and excised.

Cutting Reactions in the Gel Slice. The cutting reaction was activated by adding an equal volume of 50 mM $CaCl_2$ in 25 mM HEPES, pH 7.6, at room temperature and stopped by adding $^1/_5$ volume of 500 mM EDTA. The product of the cutting reaction was eluted overnight at 50 °C with 2 volumes of elution buffer [1 M ammonium acetate (pH 7.1), 1 mM EDTA], ethanol precipitated, and analyzed on a 15% denaturing polyacrylamide gel.

Cutting Reaction in Solution. Hybridization and protein binding were carried out as described above. The cutting reaction was started by adding 1 μL of $CaCl_2$ to a final concentration of 20 mM. The reaction was stopped by removing aliquots and adding EDTA to a final concentration of 50 mM. Samples were ethanol precipitated and analyzed on 15% denaturing acrylamide gels.

Molecular Modeling. The computer modeling of CB binding and the structure comparison of CB with bromosulfophthalein (BSP) were done on a Silicon Graphics Computer using the Insight II program (Biosym). The CPK models for CB and BSP were generated using the Builder module of Insight II and were subsequently optimized using the same program. The crystal structure for the rat liver 3–3 isoform of glutathione *S*-transferase is based on the coordinates

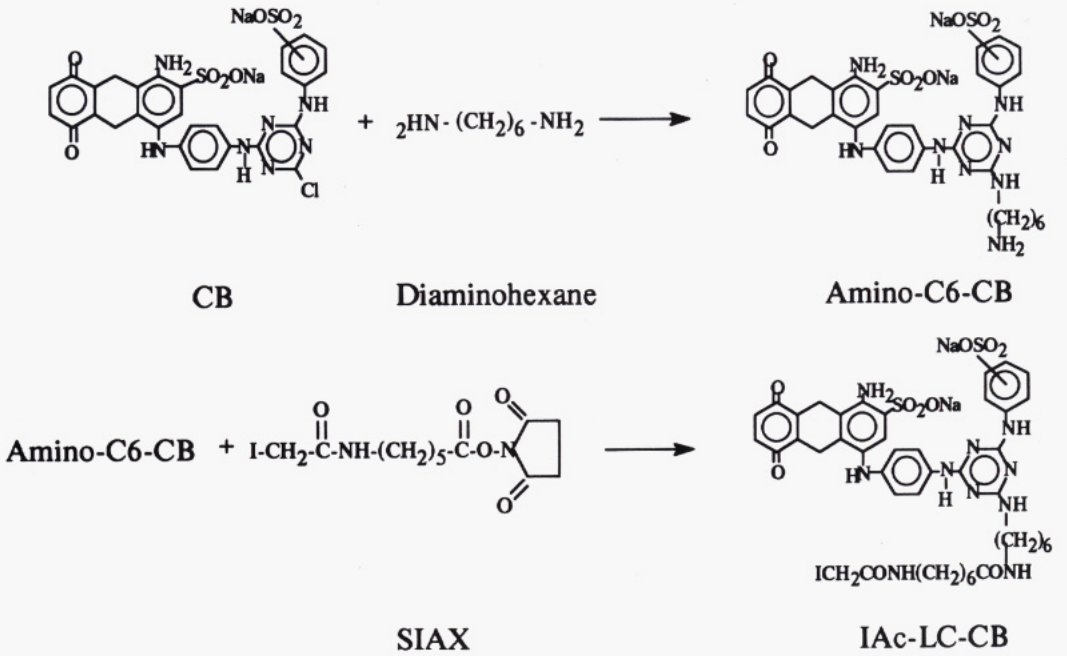

FIGURE 2: Synthesis of iodoacetyl-long chain–Cibacron blue F3G-A (IAc-LC–CB).

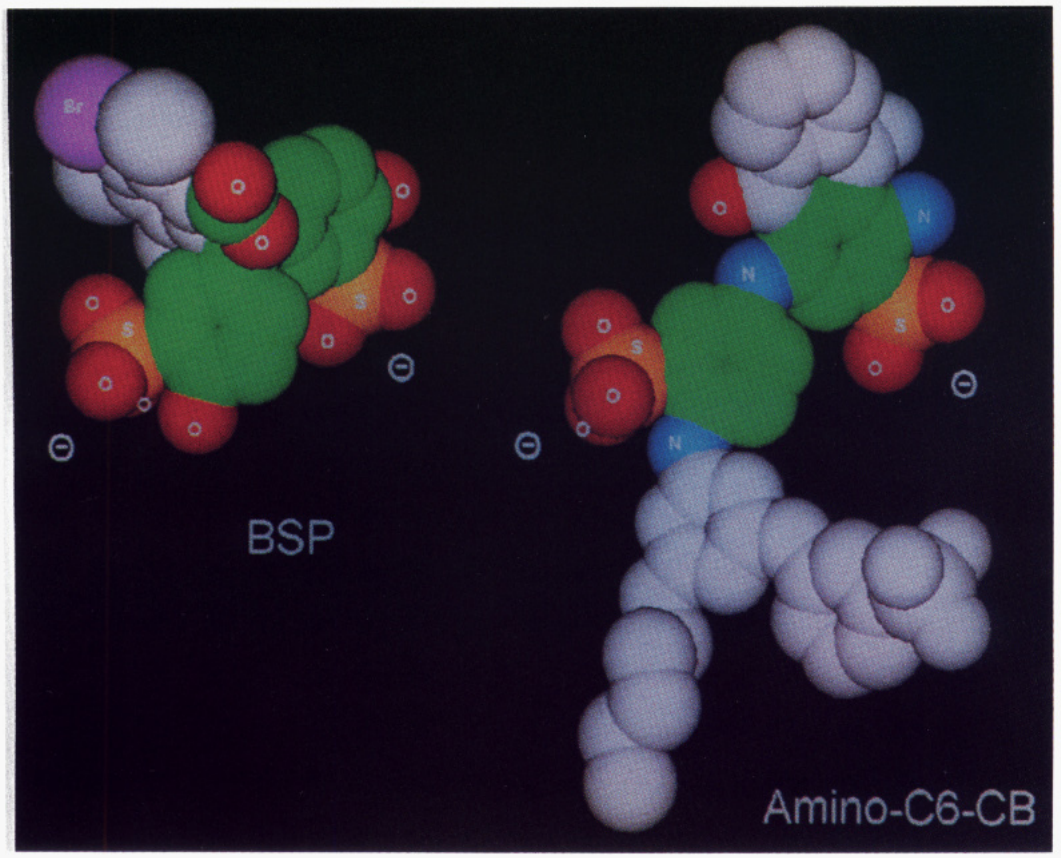

FIGURE 3: Comparison of amino-C6–CB and bromosulfophthalein. Both molecules were modeled and optimized using the builder module of Insight II (Biosym). The colored core region shows great similarity with respect to structure and distribution of functional groups and charges. The Br on BSP indicates the site for conjugation with GSH (Habig et al., 1974). The competitive inhibition mode of CB and the independence of the inhibition to modifications of the linker arm support the assumption that CB binds in a manner similar to BSP to the xenobiotic binding pocket of GST–*x*–MN.

deposited into the Brookhaven Data Bank by Ji et al. (1992) under the file name pdb1gst.ent.

RESULTS

Fusion Proteins of Micrococcal Nuclease and Glutathione S-Transferase. Two fusion proteins composed of MN and GST were prepared using the *Eco*RI site of pGEX-3X. The first fusion construct links glutathione *S*-transferase directly to micrococcal nuclease with wild-type activity (GST–MN). The second construct [GST–*x*–MN(a)] introduces an eight amino acid spacer between both proteins and uses a double mutant of MN (L37A, Y113A). The mutations at positions 37 and 113 of MN attenuated the catalytic activity (Corey

et al., 1989). These were introduced since the wild-type activity of MN is so efficient that it is difficult to control and would generate extensive background activity. Both proteins were overproduced and isolated by standard anion-exchange chromatography. SDS–PAGE analysis showed a single band with a molecular weight of approximately 44 900. Analysis of the native structure of the enzyme on a Superose-12 gel filtration column indicated that the protein exists as a dimer as do most free GST's (Mannervik et al., 1985). The glutathione transferase and wild-type nuclease activities were assayed spectrophotometrically. Both activities were comparable to those of the free enzymes (Habig et al., 1974; Cuatrecasas et al., 1967) (Table 1). The nuclease activity of the attenuated mutant was too low to be determined accurately using the same assay system. However, the calcium-dependent nuclease activity of the attenuated construct was confirmed on agarose gels using supercoiled circular plasmid DNA as the substrate. With this assay, the attenuated fusion construct was compared to the free attenuated nuclease at identical protein concentrations and proved to have a comparable activity.

Inhibitors of GST. An important advantage of fusing MN to GST is that the transferase activity of GST can be used to rapidly identify inhibitors that would be candidates for tethering oligonucleotides to the nuclease activity. This screen is based on the assumption that a low K_i reflects high-affinity binding to the free enzyme. In this case, the steady-state inhibition pattern of an appropriate candidate should have a significant slope effect in a double-reciprocal plot indicative of the reversible binding of the inhibitor to the same enzyme form as the varied substrate. Even though alkyl derivatives of glutathione are used as ligands in affinity chromatography (Smith & Johnson, 1988) their K_i values are not sufficiently low to be useful as tethers. The likely reason that they are effective ligands for affinity chromatography is that the resins are multivalent. Since the enzyme is dimeric, the high affinity is attributable to the binding of glutathione to each of the two catalytic sites.

The most potent monovalent inhibitor was Cibacron blue F3G-A (CB). The sensitivity of the enzyme activity to CB confirms that the GST of the fusion protein shares kinetic properties with the mammalian mu class isoforms (Mannervik et al., 1985). The GST cloned into pGEX-3X is derived from *Schizosoma japonicum* and has a 40% overall homology of the amino acid sequence to the mu isoform from rat liver and a 60% homology in the N-terminus which is used to classify different isoforms of GST (Smith et al., 1986). Dyes related to CB, including Cibacron brilliant red 3B-A, Cibacron brilliant yellow 3G-P, and Procion yellow H-E3G, were studied as inhibitors at 100 nM. None of these dyes were stronger inhibitors than Cibacron blue.

CB shows a competitive inhibition pattern with respect to GSH and CDNB. This inhibition can be explained by the interaction of the dye at the xenobiotic binding pocket so that it protrudes into the GSH binding site. The overlap of the binding site for CB with two substrate binding sites is also observed with lactate dehydrogenase. This enzyme has to be eluted from blue dextran–Sepharose columns with a combination of both substrates, NAD and pyruvate (Stellwagen, 1977). A similar mode of inhibition based on overlapping binding sites has been suggested for the inhibition of GST by triphenylmethane dyes (Clark, 1989).

In addition to providing an efficient method for identifying candidate tethering ligands, the transferase activity has been used to determine the pH, salt, and buffer conditions which enhance the binding of the inhibitor to the enzyme. In alkaline Tris buffer (100 mM Tris-HCl, pH 8.6), which is often used in assays for MN, Cibacron blue inhibits GST activity weakly. CB is most efficient as an inhibitor in phosphate buffer, pH 7.0, but this is incompatible with the activation of MN by $CaCl_2$. The buffer which provided the best compromise between MN activity and CB binding was HEPES buffer (25 mM, pH 7.6). As a result all scission reactions were carried out in 25 mM HEPES (pH 7.6) and 50 mM $CaCl_2$.

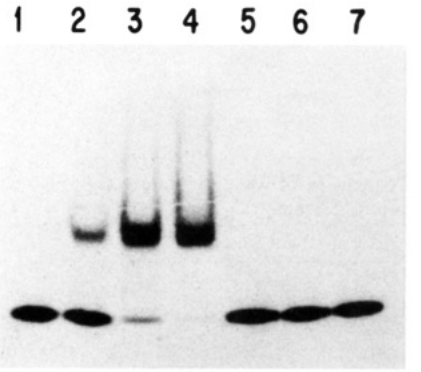

FIGURE 4: Autoradiogram of a gel mobility shift assay using 3′-^{32}P-labeled CB-derivatized and underivatized 50′mer (1 nM) and GST–*x*–MN(a). Lanes 1–4 (CB-derivatized 50′mer): no protein (1), 50 nM (2), 200 nM (3), and 500 nM GST–*x*–MN(a). Lanes 5–7 (underivatized 50′mer): 50 nM (50), 200 nM (6), and 500 nM (7) GST–*x*–MN(a).

Tethering of CB to Oligonucleotides. The chemistry and biochemistry of CB have been previously investigated because CB has high affinity for the nucleotide fold of many enzymes and has been used in the synthesis of affinity columns for the purification of kinases and NAD- and NADP-dependent dehydrogenases (Stellwagen, 1977). To convert CB into a tether for sequence-dictated scission, it was reacted successively with 1,6-diaminohexane and then succinimidyl 6-[(iodoacetyl)-amino]hexonate (SIAX). This provides a product with a long linker arm (Figure 2) that retains reactivity with thiophosphorylated oligonucleotides and should minimize unproductive steric clash between the dye and GST. Both modifications had no significant effects on the inhibitory characteristics of Cibacron blue toward the transferase activity. This finding is consistent with the assumption that the binding of CB to GST occurs mainly through the anthraquinone group. A comparison of CPK models of CB and bromosulfophthalein (BSP), a substrate for GST, shows similarity in size, distribution of hydrophobic elements, and charges between BSP and the portion of CB assumed to be important for the binding (Figure 3). On the basis of the structure of the rat liver 3–3 isoforms (Zhang et al., 1992; Ji et al., 1992) and a comparison of its structure with BSP, the binding of CB to the xenobiotic binding pockets would result in the linker arm pointing away from the protein dimer while bringing the nuclease close to the site of CB attachment.

Binding of CB-Linked Deoxyoligonucleotides (DONs) with GST–MN. Gel mobility shift assays were used to demonstrate the binding of CB DONs with both GST–MN and GST–*x*–MN(a). Underivatized DONs labeled at the 3′ terminus were used as a control for CB-linked DONs which had been purified on a 20% acrylamide sequencing gel. CB-derivatized DONs show specific gel shifts after addition of either of the two fusion proteins. In the case of GST–MN (Figure 4), the apparent K_d of 15–20 nM agrees with a K_i of 50 nM obtained

Chart 1

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                10        20        30        40        50        60
5'-TAGGTGAGAC TATAGAATAC ACGGAATTCC AGTGAATGCG TAATCATGGT CATAGCTGTT
   TCCTGTGTGA AATTGTTATC CGCTCACAAT TCCACACAAC ATACGAGCCG GGCCCAAGCT T-3'
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Table 1: Comparison of Kinetic Parameters of the Free Enzymes and the Fusion Constructs[a]

		GST–MN	MN	GST
GSH	K_m (M)	1.7×10^{-4}		1.9×10^{-4}, 2×10^{-4}[f]
CDNB	K_m (M)	5.1×10^{-4}		4×10^{-4}, 8×10^{-4}[f]
ctDNA	K_m (μg/mL)	9.5	12, 4.1[e]	
CB	K_i (M)	1.7×10^{-8},[b] 5.0×10^{-8}[c]		$(5–70) \times 10^{-8}$[g]
BSP	K_i (M)	5×10^{-6}[d]		$(5–10) \times 10^{-7}$[g]

[a] Literature data for the free GST are given for the rat liver isoform with highest homology to the GST from *S. japonicum*. [b] Variable substrate was GSH. [c] Variable substrate was CDNB. [d] Variable substrate was GSH. [e] Substrate: salmon sperm DNA; Corey et al., 1989. [f] Habig et al., 1974. [g] Mannervik et al., 1985.

in a double-reciprocal plot in which CDNB is the varied substrate. The K_d of 100 nM for GST–*x*–MN(a) is also close to the observed K_i of 150 nM. The CB-mediated affinity is therefore roughly 2 orders of magnitude greater than the nonspecific affinity of the catalytic site of MN(a). The K_m of MN(a) has been estimated as 57 μg/mL (Corey et al., 1989) using a 78-nucleotide substrate which is equivalent to 1.2 μM in units of molarity. This is reflected in a nonspecific gel shift caused by the GST–*x*–MN(a) fusion construct at 5–8 μM. Nonspecific binding was avoided by limiting the concentration of the fusion protein to 200–300 nM.

Sequence-Dictated Scission. The initial assay for CB–DON-mediated targeting by GST–MN involved (a) hybridization of the CB-linked DON to a complementary single-stranded DNA, (b) binding the GST–MN fusion protein to the duplex, (c) isolation of the GST–MN duplex by gel retardation, (d) activation of scission by addition of Ca^{2+}, (e) quenching the reaction by addition of EGTA, and (f) analysis of the products on a sequencing gel (Figure 1). The advantage of using an experimental design incorporating gel retardation is that it permits the factors associated with the binding of the fusion protein to the hybridized duplex to be distinguished from those associated with the hydrolytic reaction within the complex. The sequence of the 121-nucleotide target is given in Chart 1 with the region complementary to the CB-derivatized oligonucleotide underlined.

Both GST–MN fusion proteins were tested for binding and targeted scission. Each gave gel-shifted bands as anticipated from the data summarized in Table 1. Analysis of the unshifted target that had been incubated with the GST–MN construct revealed background scission even though the preliminary binding step was carried out in the absence of calcium. This is presumably due to traces of Ca^{2+} in the GST–MN preparation that are sufficient to activate the wild-type nuclease activity. As a result, studies were focused on the attenuated GST–*x*–MN(a) construct which did not give any background scission on the same time scale as the wild-type activity of GST–MN.

Following the separation of the complex composed of GST–*x*–MN(a) and the CB-linked duplex, the gel slice containing the complex was soaked in 50 mM $CaCl_2$ and HEPES (pH 7.6) buffer. The scission pattern after 2 h of incubation is presented in Figure 5. Scission occurs almost exclusively at positions 44 and 47, which correspond to the 5′ terminus of the hybridizing strand and a sequence in the target deoxyoligonucleotide three nucleotides downstream in the 3′ direction. Consistent with the reaction preference of MN, thymidine is the nucleotide at both sites of cleavage. The overall yield of cutting in a pH 7.6 HEPES buffer is 75%, roughly 3-fold greater than was obtained in a pH 8.6 Tris buffer. Although MN is a more efficient catalyst at high pH's, the yield of the targeted scission at pH 7.6 is greater because the CB–GST interaction is more stable in pH 7.6 HEPES buffer than in pH 8.6 Tris buffer.

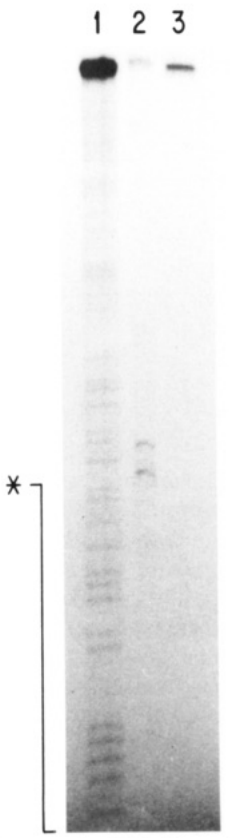


FIGURE 5: Autoradiogram of a 15% denaturing acrylamide gel of the 5′-^{32}P-labeled 121′mer substrate after hybridization to nonlabeled CB–50′mer, gel shift by GST–*x*–MN(a), and cutting in the gel slice. Cutting of the gel-shifted band was carried out by soaking the gel slice in scission buffer [25 mM HEPES (pH 7.6), 50 mM $CaCl_2$] for 2 h. Lanes: 1, G + A of the 121′mer; 2, gel-shifted band plus scission buffer; 3, non-gel-shifted band plus scission buffer.

Sequence-Dictated Scission in Solution. The successful demonstration of sequence-dictated scission following separation of the complex by gel retardation prompted assaying the reaction in solution. In this experiment, the DNA duplex is preformed by the addition of the complementary CB-linked DON to the 121-nucleotide target. The GST–*x*–MN(a) fusion protein is then added only to a concentration of 200 nM to avoid nonspecific binding. At these concentrations, the protein is present in excess relative to the target duplex. At short time points (1 min), the reaction containing the CB–DON (50′mer) shows pronounced targeted scission. The controls for this experiment are the target strand alone and the target strand with the underivatized DON (50′mer) (Figure 6). At longer time points, the targeted scission exhibits a higher yield of scission and characteristic cleavage at the site at or near the position of the CB tether. The yields of products

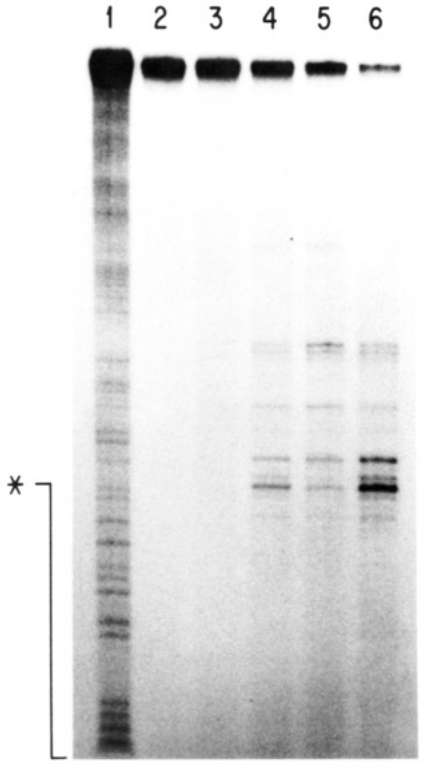


FIGURE 6: Autoradiogram of a 15% denaturing gel of 5′-^{32}P-labeled 121′mer substrate (1 nM) after hybridization with CB-derivatized or underivatized 50′mer (5 nM) and in-solution cutting by GST–*x*–MN(a) (200 nM). Lanes: 1, G + A of the 121′mer; 2, untreated control substrate; 3, 1-min cutting, underivatized 50′mer; 4, 1-min cutting, CB–50′mer; 5, 20-min cutting, underivatized 50′mer; 6, 20-min cutting, CB–50′mer. The asterisk indicates the 5′ end of the hybridizing 50′mer and the position of CB.

generated by GST–*x*–MN(a) in the presence of the CB–DON (50′mer) and underivatized DON (50′mer) are reported in panels A and B of Figure 7, respectively. The kinetic advantage of CB-targeted scission relative to the control reaction is apparent from the comparison of their cleavage rates (Figure 7C). After 1-min incubation, 21% of the target strand is cleaved. Although 70–80% of CB–DON is gel shifted by GST–*x*–MN(a) in the absence of other components, only 25% of the 121′mer target strand was present in a ternary complex with CB–DON and GST–*x*–MN(a) under the buffer conditions used for nucleic acid hybridization. The efficiency of scission by the tethered nuclease in a ternary complex is therefore 80% after 1 min.

However, the efficiency of CB–DON targeting is partially masked because the preferred sites of scission (lane 6, Figure 6, and Figure 7B) lie in a sequence region of the 121′mer that is modestly cleaved by the untargeted nuclease (lane 5, Figure 6, and Figure 7A). The observed rate enhancement of targeted scission therefore is only 2–5 fold greater than untargeted cutting, depending on sequence position (Figure 7C). To avoid this problem, an assay system was devised in which the targeted sites would be poorly cleaved by the free enzyme. The assay system given in Chart 2 was used. A 23-nucleotide DON complementary to the underlined sequence was prepared and derivatized with CB and used to target the scission of the indicated 70′mer nucleotide. This single-stranded DNA was identical in sequence to positions 1–70 of the 121′mer with the exception that sequence positions 46–49 were changed from CATG to TATA. These alterations are 27 nucleotides from the 5′ terminus of the targeting DON.

In this modified assay, reaction conditions were changed by decreasing the ratio of enzyme to DNA duplex. The

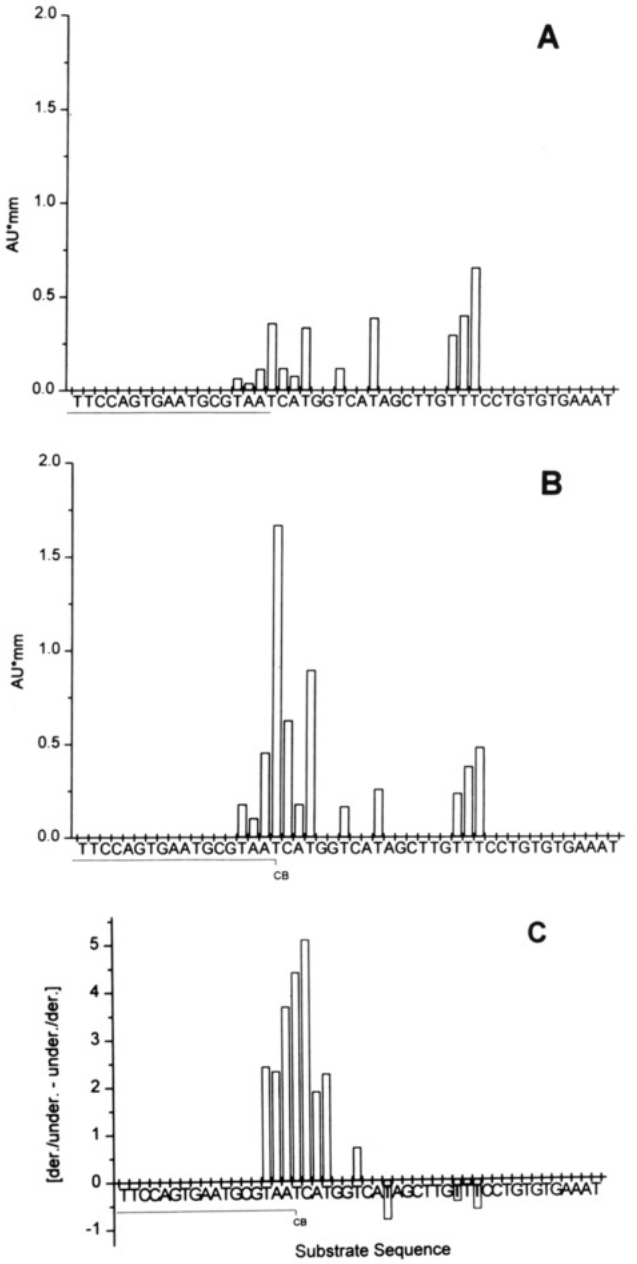
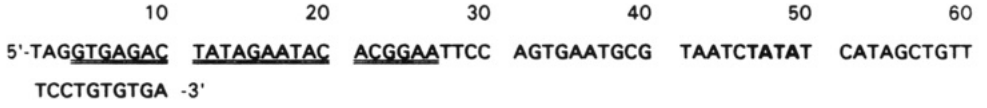


FIGURE 7: Histograms showing the intensities of cutting of the 121′mer after 20 min of in-solution cutting by GST–*x*–MN(a) following hybridization to (A) underivatized or (B) CB-derivatized 50′mer. (C) shows the relative change in the intensity of cutting as a result of the introduction of CB. The change is plotted as (derivatized/underivatized) – (underivatized/derivatized) to give an equal representation of increasing and decreasing intensities. The strongest changes can be observed around the site of CB attachment.

previous ratio of enzyme:CB–DON:substrate of 66:5:1 at 200 nM enzyme was changed to 10:5:1 at 300 nM enzyme. Scission was carried out in solution as described earlier. Figure 8 shows the results obtained with this second assay system after 4 and 16 min. As in the previous experiment, the substrate hybridized to CB–DON (lanes 5 and 6) is cleaved more efficiently relative to the appropriate control reactions (lanes 1–4). For example, substantial scission is observed at these sites after 4 min (lane 5) while the control reaction with underivatized DON (lane 3) has no detectable scission. Complete scission of the substrate in the targeted reaction is observed after 16 min while control reactions show only faint scattered cutting bands at the same time point. It is important to note that, within the limits of our experiment, scission of approximately 45–50% of the substrate occurs after 4 min at

Chart 2



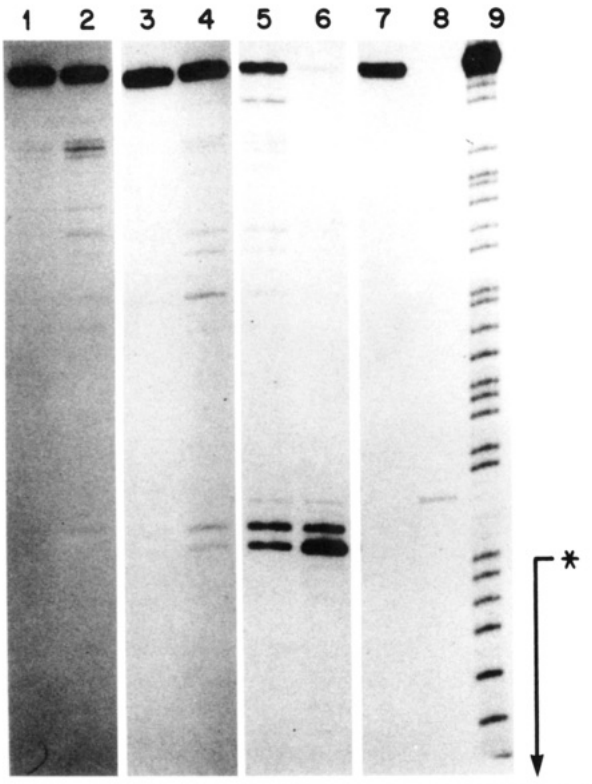


FIGURE 8: Autoradiogram of a 12% denaturing gel of 5′-^{32}P-labeled 70′mer substrate (30 nM) after hybridization with CB-derivatized or underivatized (under) 23′mer (150 nM) and in-solution cutting by GST–*x*–MN(a) (300 nM). Lanes: 1, 4-min cutting, free 70′mer; 2, 16-min, cutting, free 70′mer; 3, 4-min cutting, 70′mer + under 23′mer; 4, 16-min cutting, 70′mer + under 23′mer; 5, 4-min cutting, 70′mer + CB–23′mer; 6, 16-min cutting, 70′mer + CB–23′mer; 7, untreated 70′mer; 8, CB–23′mer (faint band visible due to the ^{35}S-label used during preparation and purification); 9, G + A of 70′mer. The asterisk indicates the 5′ end of the hybridizing 23′mer and the position of CB.

the two target sites while the remaining substrate remains full length. Since intermediate bands are almost absent, the primary bands are not the result of successive degradation of the starting material but rather a consequence of scission at the target sites. A comparison of the rate of scission of substrate hybridized to CB–DON relative to underivatized DON reveals a 50–100-fold increase in the rate of scission. This estimate is consistent with the difference in affinity of approximately 2 orders of magnitude in the binding of GST–*x*–MN(a) to CB-derivatized DONs relative to underivatized DONs.

DISCUSSION

Several approaches can be envisioned for targeting the scission of micrococcal nuclease by means of a fusion protein. Our choice of the glutathione *S*-transferase system was largely based on the availability of vectors, the good record of GST fusion constructs with respect to solubility and recovery of activity, and the ease of synthesis of the substrates and ligands involved. The GST–MN fusion proteins proved to be well behaved and could be purified on either affinity columns specific for GST or ion-exchange columns like phosphocellulose useful for MN. The fusion protein also self-associated to form a dimer comparable to naturally occurring GST's. The GST–MN was the second fusion protein that was studied. The first construct was a streptavidin–micrococcal nuclease protein fusion [based on the fusion vector pTSA-18F (Sano & Cantor, 1991; Sano et al., 1992)] that could have exploited the range of modification chemistry and the high affinity of the biotin–streptavidin system. However, the fusion product was insoluble and required resolubilization. The resolubilized protein did not oligomerize and form the tetramer essential for the high-affinity binding ($K_d = 10^{-14}$ M). Its dissociation constant was in the micromolar range, and therefore the binding was not tight enough for sequence-dictated scission. The following simple saturation scheme of affinity labeling applies to CB-targeted scission.

$$\text{CB duplex} + \text{GST–MN} \overset{K_d}{\leftrightarrow}$$

$$\text{(GST–MN)–(CB duplex)} \overset{k_{cat},Ca^{2+}}{\rightarrow} \text{scission products}$$

With a molar excess of fusion protein, the rate of scission is

$$dP/dt = k_{cat}(\text{CB duplex})(\text{GST–MN})/(K_d + \text{GST–MN})$$

where K_d is the dissociation constant of the CB–GST complex. For nonspecific scission where affinity is provided solely by the hydrolytic site of MN, the analogous expression is

$$dP/dt = k_{cat}(\text{CB duplex})(\text{GST–MN})/(K_m + \text{GST–MN})$$

K_m reflects the affinity of MN for a random cleavage site. The specificity factor of sequence-dictated scission/background scission (SF) therefore is

$$SF = (K_m + \text{GST–MN})/(K_d + \text{GST–MN})$$

At high fusion protein concentration, SF approaches 1 and targeted scission will not be observed. Specificity is optimal when $K_d \ll K_m$ and the concentration of fusion protein is equivalent to K_d. This straightforward treatment indicates that the achievable specificity depends directly on the binding affinity of the tethering ligand for GST. Tighter binding mutants of GST for CB will be sought using "phage-display methodology" (Hawkins et al., 1992).

In an extensive series of valuable studies, covalently linked deoxyoligonucleotides have been used to target MN activity (Corey & Schultz, 1987; Pei et al., 1990). In these cases, the deoxyoligonucleotide was linked to the enzyme via a disulfide bridge. The method of targeting reported here should be advantageous since it permits the tethering of the nucleotide subsequent to hybridization. For example, the relatively harsh conditions necessary for R-loop formation involving heating the nucleic acids in 70% formamide could be carried out before the addition of the enzyme (Rosbash et al., 1979). Similarly, a range of procedures have been developed for the RecA-mediated formation of D-loops (Ferrin & Camerini-Otero, 1991; Rigas et al., 1986). Since these procedures require high concentrations of hybridizing deoxyoligonucleotides, extensive nonspecific DNA degradation would be expected if deoxyoligonucleotides linked to MN were used during the

hybridization procedure. This problem is averted if the enzyme is added after the hybridization reaction.

These studies have demonstrated a general approach using oligonucleotides to target the catalytic activity of a genetic variant of micrococcal nuclease. The methodology should be applicable to RNA and DNA and compatible with a variety of methods of hybridization. Sequence-dictated scission using Cibacron blue to tether the GST–MN fusion protein to the hybridizing oligonucleotide may provide a more robust method of cleaving the target than (1,10-phenanthroline)copper or any other chemical nuclease (Sigman et al., 1993a,b).

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