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Construction of a Z-DNA-specific restriction endonuclease

(chimeric restriction enzyme/conformational DNA cleavage/B-Z junction/cleavage mapping/protein engineering)

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ABSTRACT **Novel restriction enzymes can be created by fusing the nuclease domain of *Fok*I endonuclease with defined DNA binding domains. Recently, we have characterized a domain (Zα) from the N-terminal region of human double-stranded RNA adenosine deaminase (hADAR1), which binds the Z-conformation with high specificity. Here we report creation of a conformation-specific endonuclease, Zα nuclease, which is a chimera of Zα and *Fok*I nuclease. Purified Zα nuclease cleaves negatively supercoiled plasmids only when they contain a Z-DNA forming insert, such as (dC-dG)$_{13}$. The precise location of the cleavage sites was determined by primer extension. Cutting has been mapped to the edge of the B-Z junction, suggesting that Zα nuclease binds within the Z-DNA insert, but cleaves in the nearby B-DNA, by using a mechanism similar to type IIs restriction enzymes. These data show that Zα binds Z-DNA in an environment similar to that in a cell. Zα nuclease, a structure-specific restriction enzyme, may be a useful tool for further study of the biological role of Z-DNA.**

Z-DNA is a left-handed double-helical conformation distinct from right-handed B-DNA. Z-DNA is most easily formed in alternating purine-pyrimidine sequences. It is stabilized *in vitro* by a variety of environments, including negative supercoiling, high salt, divalent cations, and polyanions. (dC-dG)$_n$ sequences form Z-DNA more readily than other alternating sequences, with energetic penalties paid when dA-dT base pairs and bases out of alternation occur (1–3). Although a great deal is known about the biochemical and biophysical nature of Z-DNA, much less is known of its biological role. Z-DNA is believed to be formed *in vivo* as a result of changes in supercoiling caused by transcription (4), and Z-DNA has been detected in metabolically active transcribing mammalian nuclei (5, 6).

Ultimately, characterization of Z-DNA binding proteins will provide the most valuable information about the biological roles of Z-DNA. This laboratory has identified the chicken homologue of double-stranded RNA adenosine deaminase (ADAR1) as a specific Z-DNA binding protein (7). This enzyme is believed to be one of those involved in the editing of the glutamate receptor pre-mRNA, which is important in mammalian brain function (8). The high affinity of ADAR1 for Z-DNA has been shown to result from a region at the N terminus of the protein; a 77-aa peptide domain (Zα) that has been expressed in *Escherichia coli* and binds to Z-DNA by a variety of biophysical tests (9). The equilibrium binding constant of Zα for Z-DNA is 4 nM, as measured by the BIAcore instrument (Pharmacia), based on surface plasmon resonance. This measurement is comparable to 9 nM measured for the F_{ab} domain of the mAb against Z-DNA, Z_{22} (10). The finding of a Z-DNA binding domain, Zα, as part of an RNA editing enzyme suggests a possible role for Z-DNA in directing the recognition of RNA editing substrates.

*Fok*I is a type II class restriction enzyme, with two separable domains: a sequence-specific DNA binding domain at the N terminus, and a catalytic domain at C terminus that cleaves duplex DNA (11). This modular structure has made it possible to synthesize chimeric endonucleases with novel sequence specificities by replacing the DNA binding domain of *Fok*I with the DNA binding domain of another protein. Several new enzymes have been created in this manner, including Ubx-F_N and Zif-F_N (12–14) with F_N being the *Fok*I cleavage (nuclease) domain. These enzymes all exhibit the sequence specificity expected from their DNA binding domains. Here we describe the construction, purification, and characterization of a sequence-specific restriction enzyme containing the Zα Z-DNA binding domain. Specific cleavage by this enzyme is absolutely dependent on the presence of Z-DNA as demonstrated by the cleavage of a plasmid containing a (dC-dG)$_{13}$ insert only when it is supercoiled.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. All *E. coli* strains were K12 derivatives. RR1 was used for cloning of pET15b:Zα nuclease and BL21(DE3) is used for the expression of Zα nuclease fusion protein (12). pET15b:*Ubx-F*$_N$ and pACYA184:*lig* vectors were kindly provided by S. Chandrasegaran at The Johns Hopkins University, Baltimore. The plasmid pGEX-5X1-Zα was used to express the glutathione *S*-transferase-Zα fusion protein, which is described by Herbert *et al.* (9). pDPL6 and pDHg16 are used as substrates for Zα nuclease digestion (15).

The Construction of Zα Nuclease. The Zα domain coding 77 amino acids from amino acid residues 121 to 197 of the human ADAR1 gene was PCR-amplified. The Zα region from pGEX-5X1-Zα, a plasmid carrying the Zα gene, was amplified by using two primers, 5′-CCACGGCATATGAGAGGTGTT-GATTGCCTTTCCT-3′ flanked with the *Nde*I site (underlined) at 5′ end and 5′-TAGACTAGTCCCGGGAATTC-GATTTTCCAC-3′ flanked with the *Spe*I site (underlined) at the 3′ end. The amplified DNA was digested with *Nde*I and *Spe*I, and gel-purified. The fragment was then ligated to *Nde*I/*Spe*I cleaved pET15b:*Ubx-F*$_N$. The plasmid containing the Zα coding insert was verified with *Nde*I/*Spe*I double restriction enzyme digestion and further confirmed by Sanger dideoxy sequencing (16). The DNA coding for $(Gly_4Ser)_3$ linker was inserted at *Spe*I site by using the method of Kim *et al.* (13). The final construct of the expression vector, pET15b:*Zα nuclease*, contains the gene coding the N-terminal Zα domain (residues 121–197 of hADAR1) and the C-terminal nuclease domain of *Fok*I with the $(Gly_4Ser)_3$ linker between them.

Expression and Purification of Zα Nuclease. pET15b:*Zα nuclease* was transformed into *E. coli* BL21(DE3) carrying a ligase containing vector pACYC184:*lig*, which may help to alleviate toxic effects caused by Zα nuclease expression. It expresses *E. coli* ligase constitutively. The transformed cells

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Abbreviation: F_N, *Fok*I cleavage (nuclease) domain.

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were plated and grown overnight at 37°C on Luria–Bertani agar plates containing 100 μg/ml of ampicillin and 40 μg/ml of chloramphenicol. Cells were collected from plates and then transferred into fresh liquid: 2 × YT media (16 g of peptone, 10 g of yeast extract, 5 g of NaCl in 1 liter) containing 100 μg/ml of ampicillin and 40 μg/ml of chloramphenicol to a final OD_{600} of 0.05. The culture was grown at 37°C with vigorous shaking until the cell density reached $OD_{600} = 0.4$. The culture then was moved to 22°C and grown for another 30 min. The fusion protein was induced by 0.5 mM isopropyl β-D-thiogalactoside, and the cells were allowed to grow for an additional 6 hr at 22°C. The cells were harvested and resuspended in 1× bind buffer (5 mM imidazole/500 mM NaCl/20 mM Tris·Cl, pH 7.9) with 10% (vol/vol) glycerol and 0.1% Tween 20. The cells were disrupted by a French press and centrifuged at 20,000 × *g* for 2 hr at 4°C. The supernatant was filtered through a 0.45 μM filter unit before being loaded onto a 5-ml Hitrap chelation column (Pharmacia) charged with Ni^{2+} ion and preequilibriated with 1× bind buffer. The column then was washed with 6 vol of 1× bind buffer and 6 vol of 1× wash buffer (60 mM imidazole/500 mM NaCl/20 mM Tris·Cl, pH 7.9). The protein was eluted with 120 ml of a 60–600 mM linear imidazole concentration gradient, and 2.5-ml fractions were collected.

A nonspecific nuclease activity assay was used to detect the presence of the Zα nuclease fusion protein by incubating 0.5 μg of pUC19 plasmid with 0.5 μl of each fraction in the *Fok*I restriction enzyme digestion buffer (10 mM Tris·Cl, pH 7.5 at 37°C/10 mM $MgCl_2$/50 mM NaCl/1 mM dithioerythreitol) for > 1 hr at 22°C. In addition, a Western blot of the eluted fractions with anti-rabbit serum (a gift from S. Chandrasegaran) against the intact *Fok*I endonuclease was used to confirm the presence of Zα nuclease. The fractions containing Zα nuclease were pooled and diluted to 50 mM KCl in buffer A [1 mM EDTA/10% (vol/vol) glycerol/5 mM DTT/10 mM Tris phosphate, pH 8.0] by dialysis overnight at 4°C. It then was loaded onto a 1-ml Hitrap Heparin column pre-equilibriated with 50 mM KCl in buffer A. After washing the column with 10 vol of 50 mM KCl in buffer A, proteins were eluted by 50 ml of a 50–700 mM KCl linear salt gradient in buffer A. The eluates were collected in 1.2-ml fractions. A nonspecific nuclease activity assay and a Western blot again were used to check for the presence of Zα nuclease. Finally, the purity of Zα nuclease was determined by Commassie blue staining of a 12% SDS-polyacrylamide gel (17). The hybrid enzyme, Zα nuclease, was purified to near homogeneity with this procedure. The purified Zα nuclease was stored in 50% glycerol at −20°C for short-term storage or −80°C for long-term storage.

Zα Nuclease Digestion of Plasmids Containing Z-DNA. Two plasmids, pDPL6 and pDHg16, were isolated from *E. coli* by using alkaline lysis (15). pDHg16 contains a $(dC\text{-}dG)_{13}$ insert at the *Sma*I site of the parental plasmid, pDPL6. The plasmids were further purified with phenol extraction and precipitated with ethanol. The DNAs were dissolved with distilled H_2O, and the concentration of plasmids were determined at OD_{260}, assuming 1 OD is equivalent to 50 μg/ml. Quality of the plasmids was checked by 1% agarose gel electrophoresis. Sixty nanograms of purified Zα nuclease was preincubated with 300 ng of plasmid in 13.5 μl of reaction buffer at 22°C for 20 min. After that, 1.5 μl of 100 mM $MgCl_2$ was added to the reaction mixture. The final reaction contained 100 nM of Zα nuclease, 75 mM of KCl, 3 mM DTT, 5% glycerol, 200 μg/ml of *E. coli* tRNA, 10 mM $MgCl_2$, and 10 mM Tris·Cl (pH 8.0). Samples were further incubated for 4 hr at 22°C. The reactions were heat-inactivated by incubating at 50°C for 30 min. Twenty units of *Pst*I restriction enzyme was directly added to the reactions. The reactions were further incubated for 3 hr at 37°C. Five hundred nanograms of Proteinase K was added to the reaction mixture and incubated for 30 min at 50°C. The samples then were loaded on a 1% agarose gel for electrophoresis. DNA was visualized by ethidium bromide staining.

Primer Extension of DNA Fragments by Thermo Sequenase. The DNA fragments for primer extension were isolated by using a Qiaex II gel-extraction kit (Qiagen). The two primers for primer extension, *Eco*RI primer (catalog no. 1204) 5′-GTATCACGAGGCCCT-3′ and *Sal*I primer (catalog no. 1208) 5′-AGTCATGCCCCGCGC-3′, were purchased from New England Biolabs. The primers were end-labeled by T4 polynucleotide kinase and γ[^{32}P]ATP. The reaction contains 10–20 ng of DNA, 6.5 mM $MgCl_2$, 26 mM Tris·HCl at pH 9.5, 200 μM of each dNTP, 100,000–200,000 cpm of primer, and 1.5 units of Thermo Sequenase (Amersham) in 8 μl. One cycle of 4 min at 95°C, 1 min at 55°C, and 1 min 72°C was used for primer extension reaction with Thermo Sequenase. The reaction then was quenched by mixing with 4 μl of stop buffer (95% formamide/20 mM EDTA/0.05% bromophenol blue/0.05% xylene cyanol).

The Supercoiling of Circular Plasmids with Topoisomerase I. Ten micrograms of pDHg16 was preincubated in 50 μl of topoisomerase I buffer (50 mM Tris·HCl, pH 7.5/50 mM KCl/10 mM $MgCl_2$/0.1 mM EDTA/0.5 mM DTT/30 μg/ml BSA) at 22°C for 20 min with different concentrations of ethidium bromide. Twenty units of topoisomerase I (Life Technologies) was added to each reaction and incubated for 90 min at 37°C. Topoisomerase I and ethidium bromide then were removed by phenol and butanol extraction. DNA was precipitated with ethanol and dissolved in TE buffer (10 mM Tris·HCl, pH 8.0/1 mM EDTA) stored at −20°C until used.

RESULTS AND DISCUSSION

Construction and Purification of Zα Nuclease. The gene coding for the Zα domain was amplified by using two primers containing the *Nde*I and *Spe*I sites, respectively. The Zα gene fragment then was cloned into the pET15b:F_N plasmid encoding the nuclease domain of the *Fok*I endonuclease (12). The glycine-rich linker $(Gly_4Ser)_3$ then was inserted between the genes to provide flexibility between the two domains of the fusion protein. The resultant plasmid, pET15b:*Zα nuclease*, was transfected into an *E. coli* BL21(DE3) strain containing the pACYC184:*lig* plasmid. Induction of the fusion protein was carried out at 22°C to increase its solubility. The fusion protein contains an N-terminal $(His)_6$ tag for Ni^{2+} chelation affinity purification. While judging by Commassie blue staining, the protein was purified to homogeneity through two types of column chromatography, Ni^{2+} chelation and heparin agarose. The heparin column chromatography removes small degradation products (12) and eliminates the steps SP-Sepharose and gel-filtration used in the Zif-F_N nuclease preparation (13). Fractions were assayed for nonspecific nuclease activity, and the presence of *Fok*I nuclease was confirmed by Western blot by using anti-rabbit serum against the whole *Fok*I endonuclease protein. The size of the fusion protein is about 39 kDa in a 12% SDS/PAGE gel, consistent with the calculated molecular mass of the Zα nuclease. A gel-mobility shift assay with a radioactive Z-DNA probe and the purified Zα nuclease confirms that the Zα nuclease binds Z-DNA with a similar affinity to that of the Zα domain itself (data not shown).

Analysis of Cleavage by Zα Nuclease of Negatively Supercoiled Plasmids Containing Z-DNA. The activity of Zα nuclease was tested by using a plasmid substrate that contains a $(dC\text{-}dG)_{13}$ insert. This insert forms Z-DNA in negatively supercoiled plasmids at bacterial superhelical density ($\sigma \approx -0.055$) (15), but is in the B-DNA conformation when the plasmid is linearized or relaxed with topoisomerases. Cleavage efficiency was tested under a variety of conditions. The nuclease activity of the fusion protein was very temperature sensitive, and the enzyme was inactivated within 1 hr at temperatures above 30°C. This thermal lability has been

observed with other *Fok*I fusion endonucleases (12, 13). Digestion of plasmid was optimal at pH 8.0 and 75 mM NaCl. The cleavage was magnesium dependent, occurring more rapidly at 10 mM MgC_2 than at 2 mM. In contrast to other fusions made with the *Fok*I nuclease domains, cleavage by Zα nuclease remained specific at 10 mM $MgCl_2$. Under all conditions tested, a background of nonspecific nicking of supercoiled plasmids was observed, as has been reported for all the endonucleases produced by this method. It is likely that the nuclease domain is not as tightly regulated by the DNA-binding domain as is the case with *Fok*I. This background can be reduced by using tRNA as a nonspecific nucleic acid competitor. Other nucleic acids, such as poly(dl-dC)$_n$, had limited effects on reducing nonspecific cleavage. Under optimal conditions cleavage by Zα nuclease was complete within 4 hr.

The specific cleavage of substrates by Zα nuclease was mapped by using appropriate restriction enzymes and visualized by agarose gel electrophoresis (Fig. 1). The reaction mixtures were incubated with Zα nuclease for 4 hr at 22°C, and then moved to 50°C for 30 min to inactivate residual Zα nuclease activity. The second restriction enzyme, for example *Pst*I for pDHg16, was added directly to the reaction mixtures, and incubation continued for 3 hr at 37°C. If the Zα nuclease cuts substrates specifically at the (dC-dG)$_{13}$ insert, two DNA fragments (1.4 and 0.8 kb) would be present after the second digestion. Nicked substrates because of nonspecific nicking and partial cleavage, as well as undigested substrates, would be seen as a linearized DNA band in the same gel. As shown in Fig. 1*B*, the expected digestion products were produced by Zα nuclease. The amount of specific double-stranded cleavage was approximately 10–20%. In contrast, other *Fok*I fusion nucleases cleave up to 100% of the substrate. The result obtained with Zα nuclease would be expected if the conformation recognized by this enzyme was dependent on supercoiling. Any single-stranded nicking would result in loss of negative supercoiling essential for stabilizing the Z-DNA conformation, causing a loss of Z-DNA substrate and thus reducing the final yield of specifically cleaved product. In addition, the *Fok*I nuclease domain has only a single catalytic site (18). Cleavage of both strands may not always proceed to completion before the nuclease dissociates from the DNA, further reducing the digestion efficiency. Unlike wild-type *Fok*I, it is observed that relaxed cleavage specificity of the nuclease domain of the hybrid protein or mutants of *Fok*I sometimes causes single-strand cleavage (12, 13, 19).

To assess Z-DNA specificity, the parental plasmid pDPL6 also was digested under the same conditions. pDPL6 has no significant Z-DNA formation, even when it is highly negatively supercoiled (15). Additional control digestions were carried out on relaxed forms of both pDPL6 and pDHg16. The results are shown in Fig. 1*B*. The Zα nuclease specifically cut only negatively supercoiled pDHg16. Comparison of the fragments produced by *Bss*HII digestion (Fig. 1*B*, *Bss*HII), which cleaves at (dG-dC)$_3$ sequences, shows that the sizes of the two cleavage fragments are similar. Thus, the specific cleavage by Zα nuclease occurs near the Z-DNA or the (dC-dG)$_{13}$ sequences. When the substrate is relaxed with topoisomerase I so that the insert is in the B-DNA conformation, no specific doubled-stranded cleavage was observed. The results indicate that Zα nuclease recognizes only the (dC-dG)$_{13}$ sequence in Z-DNA form, stabilized by negative supercoiling, and not the B-form of the (dC-dG)$_{13}$ sequence. This result clearly indicates that Zα nuclease acts as a conformational specific restriction enzyme rather than recognizing (dC-dG)$_{13}$ in a sequence-specific manner.

Mapping Analysis of Zα Nuclease Cleavage Sites. Cleavage sites were mapped at single-nucleotide resolution, by using gel-purified DNA fragments produced from digestion of pDHg16 by Zα nuclease and *Pst*I. Thermo Sequenase was used

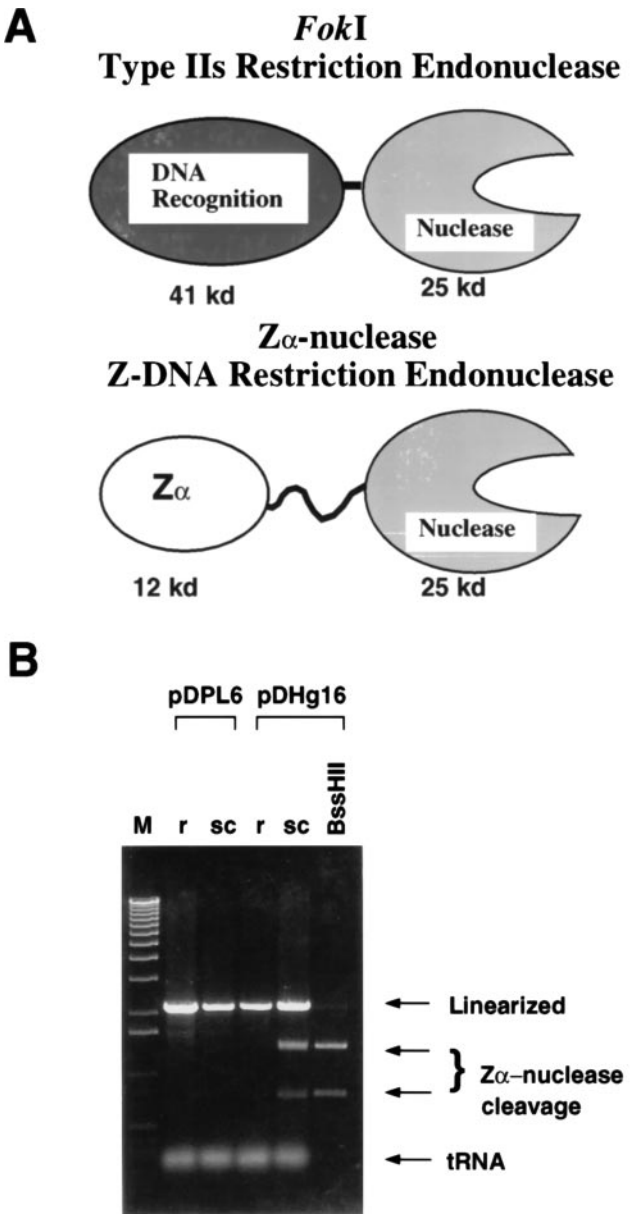

FIG. 1. Z-DNA cleavage by Zα nuclease. (*A*) *Fok*I has a modular structure with two separable domains, the DNA recognition domain and the nuclease cleavage domain. The Zα domain of hADAR1 replaces the DNA recognition domain of *Fok*I and is linked to the cleavage domain through a $(Gly_4Ser)_3$ linker (curved line). (*B*) Zα nuclease cleavage of the parental plasmid, pDPL6, and pDHg16 containing a (dC-dG)$_{13}$ insert in relaxed or negatively supercoiled form. This reaction is followed by *Pst*I digestion. The reactions were electrophoresed in a 1% agarose gel. Linear DNA (2.2 kb) and the Zα nuclease cleaved DNA products (1.4 and 0.8 kb) are indicated by arrows. The *Bss*HII restriction enzyme digestion of pDHG16 plasmid linearized with *Pst*I shows the boundaries of the (dC-dG)$_{13}$. This reaction yields similar-sized fragments to that of Zα nuclease digestion.

to extend end-labeled primers about 70 bp away from the (dC-dG)$_{13}$ insert. However, the insert causes severe compression in denaturing polyacrylamide gels when other thermolabile DNA polymerases or Klenow are used in primer extension, and Thermo Sequenase overcomes this problem. The cleavage sites were identified by using extensions of the end-labeled primers. The result is shown in Fig. 2. Most of the cuts by the Zα nuclease are detected in what are probably B-DNA regions within 16 bp of the (dC-dG)$_{13}$ insert. Almost no cleavage is detected in the Z-DNA region. The cleavage by

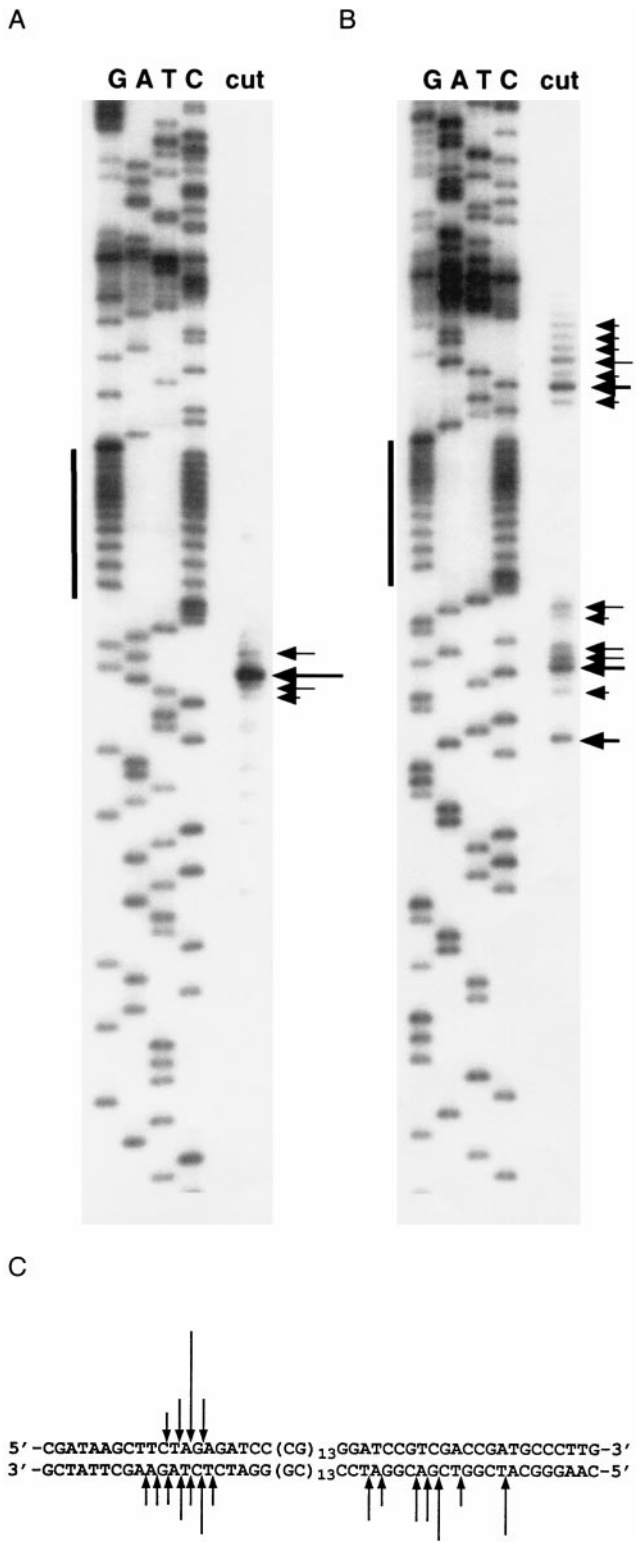

FIG. 2. Primer extension of the two cleavage product pDHg16 with Thermo Sequenase with either an *Eco*RI primer (*A*) or a *Sal*I primer (*B*). The reactions were electrophoresed in a 7% denaturing polyacrylamide gel. Sequencing reactions (G A T C) of pDHg16 with each primer are shown beside the corresponding primer extension reactions (cut). Arrows indicate the cleavage sites and intensities. The thick vertical line indicates the location of $(dC\text{-}dG)_{13}$ insert. (*C*) The position of the cleavage sites are mapped by vertical arrows.

Zα nuclease is therefore similar to those of type IIs class restriction enzymes and previously reported hybrid enzymes, which cleave DNA outside of their recognition sites (8). It is postulated that the Zα domain of the fusion protein recognizes Z-DNA and the nuclease domain cleaves the B-DNA region away from the Z-DNA recognition site.

The distance between the Z-DNA forming sequences and the cleavage sites is limited by the 15-aa linker, joining the Zα recognition domain to the nuclease domain. When fully extended, the linker could span somewhat over 50 Å or about 1.5 turns of the DNA duplex. The cleavage sites furthest away from the B-Z junctions at the ends of the $(dC\text{-}dG)_{13}$ insert are 13 and 16 bp distant. However, the flexibility of the linker residues also allows cleavage closer to the recognition site. Variations in the cleavage site also may arise because the $(dC\text{-}dG)_{13}$ insert is long and contains many different recognition sites for Zα. Indeed multiple cleavage sites can be seen (Fig. 2). Other fusion proteins and mutants of *Fok*I show a similar dispersion of DNA cleavage sites (12, 13, 19, 20).

It is unlikely that cleavage by Zα nuclease occurs within the Z-DNA region, as Z-DNA has proven refractory to cutting by other nucleases. For example, *Hha*I, which cleaves the B-DNA conformer of $(dC\text{-}dG)_n$, but not the Z-conformer, has been used to follow B-Z transitions (21). The nuclease domain from *Fok*I is likely to cleave B-DNA rather than any other forms of DNA or RNA. In the case of an RNA-DNA heteroduplex, the efficiency of cleavage of the DNA strand by a zinc finger-nuclease fusion protein, which can recognize DNA-RNA heteroduplxes, is reduced by about 50-fold (22).

The cleavage patterns on both sides of Z-DNA appear different and are asymmetric. There may be differences in the two B-Z junctions because of different flanking sequences, and the sequence preferences in the nuclease domain may contribute to this asymmetry. After the second restriction enzyme digestion, linearized DNA and total DNA from the Zα nuclease digestion also were purified and subjected to primer extension for comparison (data not shown). As expected, the cleavage patterns of the total DNA are roughly the sum of the specifically cleaved DNA and the linearized DNA. In addition, specific cleavage sites are observed in the primer extension experiment, which uses a linearized DNA template. These sites might be specific for Zα nuclease.

Plasmid Cleavage as a Function of Negative Superhelicity. Topoisomerase I relaxes supercoiled circular DNA. When a closed circular DNA is treated with topoisomerase I in the presence of intercalating reagents such as ethidium bromide, negative supercoiling of circular DNA can be introduced by changing the linking number once the intercalating reagent from the reaction is removed. The plasmid containing the $(dC\text{-}dG)_{13}$ insert thus was treated with topoisomerase I and with different concentrations of ethidium bromide to generate plasmids having different negative superhelicities. As the concentration of ethidium bromide increased, the degree of negative supercoiling in the plasmids also increased (Fig. 3*A*). The alternating purine-pyrimidine repeats of DNA requires negative supercoiling to stabilize the Z-DNA conformation as does the $(dC\text{-}dG)_{13}$ insert in plasmids. When the various negatively supercoiled plasmids were incubated with Zα nuclease, specific cleavage fragments by Zα nuclease appeared only after a certain degree of negative supercoiling, where there was enough to provide energy to convert the $(dC\text{-}dG)_{13}$ insert to Z-DNA (Fig. 3*B*). Thus the Zα nuclease cleaves substrate plasmids specifically only when the $(dC\text{-}dG)_{13}$ sequences adopts the Z-DNA conformation with sufficient negative supercoiling. This result confirms that the specific cleavage by Zα nuclease of plasmid substrates near the $(dC\text{-}dG)_{13}$ insert region is dependent on Z-DNA formation, rather than the sequence of the dC-dG repeat.

A Z-DNA Specific Restriction Enzyme. Our experiments clearly demonstrate that the Zα nuclease is specific for the Z-DNA conformation. We have not yet fully assessed the sequence-specificity of Zα. However, sequences with mixtures of dC-dG and dC-dA/dT-dG are recognized by Zα nuclease

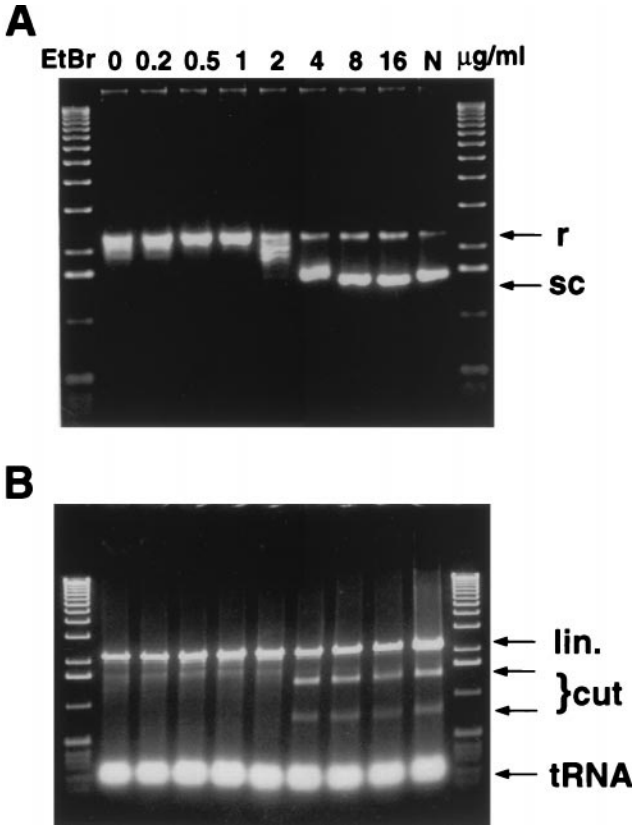

FIG. 3. The digestion of increasingly supercoiled pDHg16 by Zα nuclease. Plasmid pDHg16 with a $(dC\text{-}dG)_{13}$ insert was prepared at different superhelicities. Topoisomerase I was used in the presence of ethidium bromide and then electrophoresed in a 1% agarose gel (*A*). Plasmids isolated from *E. coli* at bacterial superhelicity is shown at N. Relaxed plasmids (r) and negatively supercoiled plasmids (sc) are indicated. The Zα nuclease digestion of plasmid substrates prepared in *A* is shown in *B* in the same column. DNA size markers (1 kb) are also shown (Life Technologies). Linearized plasmids (lin.), tRNA competitor, and two cleavage products (cut) are indicated by arrows.

in some cases. This result is of interest because many potential Z-DNA forming sequences identified from human or other organisms are the mixed repeats consisting of both dC-dG and dC-dA/dT-dG rather than only dC-dG. For instance, very few sites with dC-dG repeats longer than five are present in the yeast genome. To establish its wider application as a Z-DNA detection tool, the known Z-DNA forming sequences reported previously will be tested with the Zα nuclease. Schroth *et al.* (23) have developed a program that calculates the Z-DNA conversion preferences of DNA sequences. Sequences with a higher propensity to form Z-DNA are rated with a higher Z-score. They have used this program to search for sites of potential Z-DNA formation in known human genomic sequences. It will be of interest to see if there is a correlation between the Z-score of these sequences and the cleavage efficiency.

In addition, conditions may be found where the Zα nuclease has more specificity and stability and has a higher efficiency similar to that of naturally occurring restriction enzymes. Recently the structure of the *Fok*I restriction enzyme was solved by x-ray crystallography with a DNA oligomer substrate (18). Information from the structure of *Fok*I and the Zα domain may help in rational modifications of the Z-DNA specific restriction enzyme. The Zα nuclease may have important biological applications. It could provide a useful tool for directly identifying targets of the Zα domain of ADAR1 *in vivo* as well *in vitro*. Such experiments will provide valuable information about the function of the Zα domain of ADAR1. In addition, we plan to use the Zα nuclease to elucidate the relationship between negative supercoiling generated during transcription by RNA polymerase and Z-DNA formation. It is also known that Z-DNA forming sequences are not randomly distributed throughout the human genome. A previous study suggests that Z-DNA is located maximally near the transcription start sites (23). The nuclear material of transcriptionally active cells contains more Z-DNA than quiescent cells. Because Z-DNA is stabilized by negative supercoiling generated by transcription (4), this result suggests that Z-DNA may play a role in the regulation of transcription. Many questions are associated with Z-DNA formation. The Zα nuclease may provide an important tool to answer some of these questions.

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