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Hybrid restriction enzymes: Zinc finger fusions to *Fok* I cleavage domain

(*Flavobacterium okeanokoites*/chimeric restriction endonuclease/protein engineering/recognition and cleavage domains)

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ABSTRACT **A long-term goal in the field of restriction-modification enzymes has been to generate restriction endonucleases with novel sequence specificities by mutating or engineering existing enzymes. This will avoid the increasingly arduous task of extensive screening of bacteria and other microorganisms for new enzymes. Here, we report the deliberate creation of novel site-specific endonucleases by linking two different zinc finger proteins to the cleavage domain of *Fok* I endonuclease. Both fusion proteins are active and under optimal conditions cleave DNA in a sequence-specific manner. Thus, the modular structure of *Fok* I endonuclease and the zinc finger motifs makes it possible to create "artificial" nucleases that will cut DNA near a predetermined site. This opens the way to generate many new enzymes with tailor-made sequence specificities desirable for various applications.**

Since their discovery nearly 25 years ago (1), type II restriction enzymes have played a crucial role in the development of the recombinant DNA technology and the field of molecular biology. The type II restriction endonucleases and modification methylases are relatively simple bacterial enzymes that recognize specific sequences in duplex DNA. While the former cleave DNA, the latter methylate adenine or cytosine residues within the recognition site so as to protect the host genome against cleavage. So far, over 2500 restriction-modification (R-M) enzymes have been identified, and these are found in widely diverse organisms (2). These enzymes fall into numerous "isoschizomer" (identically cleaving) groups with about 200 sequence specificities. Discovery of new enzymes involves tedious and time-consuming effort that requires extensive screening of bacteria and other microorganisms (3). Even when one finds a new enzyme, more often than not, it falls into the already discovered isoschizomer groups. Furthermore, most naturally occurring restriction enzymes recognize sequences that are 4–6 bp long. Although these enzymes are very useful in manipulating recombinant DNA, they are not suitable for producing large DNA segments. For example, restriction enzymes that recognize DNA sequences 6 bp long result in cuts as often as every 4096 bases. In many instances, it is preferable to have fewer but longer DNA strands, especially during genome mapping. Rare cutters that recognize 8-bp-long sequences cut human DNA (which contains about 3 billion bp) every 65,536 bases on average. So far, only a few restriction endonucleases with recognition sequences longer than 6 bp (rare cutters) have been identified (New England Biolabs catalog). R-M systems appear to have a single biological function—namely, to protect cells from infection by foreign DNA that would otherwise destroy them. The phage genomes are usually small. It stands to reason, then, that bacteria select for R-M systems with small recognition sites (4–6 bp) because these sites occur more frequently in the phages. Therefore, a long-term goal in the field of R-M enzymes has been to generate restriction endonucleases with longer recognition sites by mutating or engineering existing enzymes (3).

Our studies on proteolytic fragments of *Fok* I endonuclease (from *Flavobacterium okeanokoites*; belonging to the type IIS class) have revealed an N-terminal DNA-binding domain and a C-terminal domain with nonspecific DNA-cleavage activity (4–7). The modular structure of *Fok* I endonuclease suggested that it might be feasible to construct hybrid endonucleases with novel sequence specificities by linking other DNA-binding proteins to the cleavage domain. Recently, we reported the construction of the first "chimeric" restriction endonuclease gene by linking the *Drosophila Ubx* homeodomain to the cleavage domain of *Fok* I (8).

Unlike the homeodomains, the zinc finger proteins, because of their modular structure, offer an attractive framework for designing chimeric restriction enzymes with tailor-made sequence specificities. The Cys_2His_2 zinc finger proteins are a class of DNA-binding proteins that contain sequences of the form (Tyr, Phe)-Xaa-Cys-Xaa_{2-4}-Cys-Xaa_3-Phe-Xaa_5-Leu-Xaa_2-His-Xaa_{3-5}-His, usually in tandem arrays (9). Each of these sequences binds a zinc(II) ion to form the structural domain termed a zinc finger. These proteins, like many sequence-specific DNA-binding proteins, bind to the DNA by inserting an α-helix into the major groove of the double helix (10). The crystallographic structure of the three zinc finger domains of Zif268 (a mouse immediate early protein) bound to a cognate oligonucleotide reveals that each finger interacts with a triplet within the DNA substrate. Each finger, because of variations of certain key amino acids from one zinc finger to the next, makes its own unique contribution to DNA-binding affinity and specificity. The zinc fingers, because they appear to bind as independent modules, can be linked together in a peptide designed to bind a predetermined DNA site. Although more recent studies suggest that the zinc finger–DNA recognition is more complex than originally perceived (11, 12), it still appears that zinc finger motifs will provide an excellent framework for designing DNA-binding proteins with a variety of new sequence specificities. In theory, one can design a zinc finger for each of the 64 possible triplet codons, and, using a combination of these fingers, one could design a protein for sequence-specific recognition of any segment of DNA. Studies to understand the rules relating to zinc finger sequences/DNA-binding preferences and redesigning of DNA-binding specificities of zinc finger proteins are well underway (13–15). An alternative approach to the design of zinc finger proteins with new specificities involves the selection of desirable mutants from a library of randomized fingers displayed on phage (16–20). The ability to design or select zinc fingers with desired specificity implies that DNA-binding proteins containing zinc fingers will be made to order. There-

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Abbreviations: R-M, restriction-modification; Zif-F_N, zinc finger–*Fok* I cleavage domain fusion protein.

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fore, we reasoned that one could design "artificial" nucleases that will cut DNA at any preferred site by making fusions of zinc finger proteins to the cleavage domain of *Fok* I endonuclease (Zif-F_N). Here, we report the deliberate creation of two zinc finger hybrid restriction enzymes and the characterization of their DNA cleavage properties.

MATERIALS AND METHODS

The complete nucleotide sequence of the *Fok* I R-M system has been published (21, 22). Experimental protocols for PCR have been described elsewhere (4). The purification of proteins using His·Bind resin (23) is as outlined in the Novagen pET system manual. The protocol for SDS/PAGE is as described by Laemmli (24). The nucleotide sequences of the zinc finger fusion constructs were confirmed by Sanger's dideoxynucleotide sequencing method (25). Supplementary material describing the procedures for the construction of the clones producing Zif-F_N and the purification of the Zif-F_N is available upon request.

RESULTS AND DISCUSSION

Construction of Overproducer Clones of Zif-F_N Using PCR. Two plasmids containing three consensus zinc fingers each (CP-QDR and Sp1-QNR) were kindly provided by Jeremy Berg (Department of Biophysics and Biophysical Chemistry, The Johns Hopkins School of Medicine). They have been shown to preferentially bind to 5′-G(G/A)G G(C/T/A)G GC(T/A)-3′ and 5′-G(G/A)G GA(T/A) GG(G/T)-3′ sequences, respectively, in double-stranded DNA (13–15). We used the PCR technique to link the zinc finger proteins to the cleavage domains (F_N) of *Fok* I endonuclease (Fig. 1*A*). The hybrid gene, *Zif-F_N*, was cloned as a *Xho* I/*Nde* I fragment into the pET-15b vector (26), which contains a T7 promoter for expression of the hybrid protein. We also inserted a glycine linker $(Gly_4Ser)_3$ between the domains of the fusion protein to confer added flexibility to the linker region (Fig. 1*B*). This construct links the zinc finger proteins through the glycine linker to the C-terminal 196 amino acids of *Fok* I that constitute the *Fok* I cleavage domain (8). This construct also tags the hybrid protein with six consecutive histidine residues at the N terminus. These residues serve as the affinity tag for the purification of the hybrid proteins by metal-chelation chromatography (23) with Novagen's His·Bind resin. This histidine tag, if necessary, can be subsequently removed by thrombin. The hybrid endonucleases with a histidine tag were used in all experiments described below.

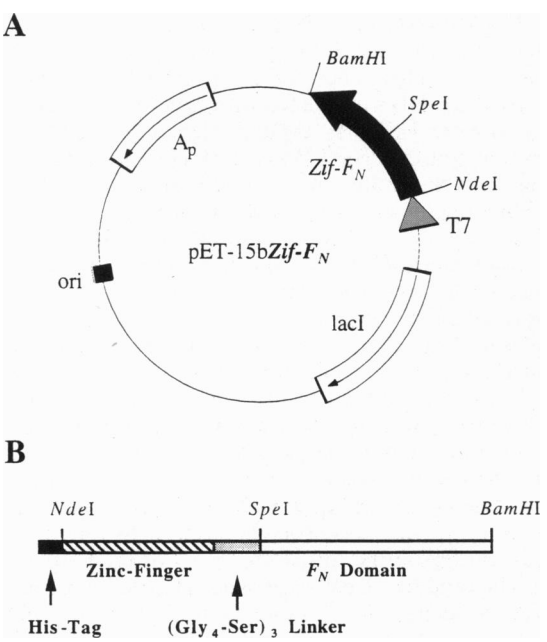

FIG. 1. Construction of expression vector of hybrid enzyme Zif-F_N. (*A*) Structure of plasmid pET-15b*Zif-F_N*. In this construct the *Zif-F_N* gene is placed downstream of the T7 promoter. (*B*) Map of the *Zif-F_N* gene. PCR was used to construct expression vectors of the zinc finger fusions.

The clones carrying the hybrid genes may not be viable, since there is no methylase available to protect the host genome from cleavage by the hybrid endonuclease. We have circumvented this problem as follows: (*i*) The hybrid genes were cloned into a tightly controlled expression system (26) to avoid any deleterious effect to the cell. (*ii*) In addition, we increased the level of DNA ligase within the cell by placing the *Escherichia coli lig* gene on a compatible plasmid, pACYC184, downstream of the chloramphenicol promoter. This vector expresses DNA ligase constitutively. BL21 (DE3) served as the host for these experiments. It contains a chromosomal copy of the T7 RNA polymerase gene under *lacUV5* control, the expression of which is induced by the addition of isopropyl β-D-thiogalactoside. After induction of the recombinant cells with 0.7 mM isopropyl β-D-thiogalactoside, the hybrid proteins were purified to homogeneity using His·Bind resin, a SP-Sepharose column, and gel-filtration chromatography. The SDS/PAGE (24) profiles of the purified hybrid enzymes are shown in Fig. 2 *A* and *B*. Their size is ≈38 kDa and agrees well with that predicted for the fusion proteins. Identities of the hybrid proteins were further confirmed by probing the immunoblot with rabbit antiserum raised against *Fok* I endonuclease (Fig. 2*C*).

Analysis of the Cleavage Activity of the Zif-F_N Hybrid Enzymes. To determine whether the zinc finger fusion proteins cleave DNA, we used 48.5-kb λ DNA as the substrate. The Sp1-QNR fusion protein cleaves λ DNA into ≈9.5-kb and ≈39-kb fragments (Fig. 3, lane 4). The cleavage is highly specific, and the reaction proceeds almost to completion. The CP-QDR fusion protein cleaves λ DNA primarily into ≈5.5-kb and ≈43-kb fragments (Fig. 3, lane 3). This appears to be the major site of cleavage. There are two other minor sites within the λ genome for this fusion protein. Addition of yeast tRNA to the reaction mixture reduces cleavage at the minor site(s). Under these reaction conditions, there was no detectable random nonspecific cleavage as seen from the nonsmearing of the agarose gels.

The cleavage is sensitive to buffer conditions, pH, and the purity of the DNA substrate. The kinetics of the cleavage of the λ DNA substrate using CP-QDR and Sp1-QNR fusions are shown in the Fig. 4. The cleavage occurs mainly at the major DNA-binding site within the λ genome at short incubation time. The cleavage at the secondary sites becomes more pronounced with longer incubation times in the case of CP-QDR fusion (Fig. 4*A*). The cleavage occurs predominantly at the major DNA-binding site in the case of the Sp1-QNR fusion. Only a few weaker bands appear even after long incubation times, suggesting that there is only one major DNA-binding site for Sp1-QNR in the λ DNA substrate (Fig. 4*B*). The reactions appear to proceed almost to completion (>95% cleavage) within 4 hr. The kinetics of the cleavage of the λ DNA substrate by wild-type *Fok* I is shown in Fig. 4*C*. The cleavage reaction by *Fok* I endonuclease proceeds to completion within 15 min. The rate and efficiency of cleavage by the hybrid endonucleases are much lower compared to wild-type *Fok* I.

We have also studied the effect of temperature and salt concentrations (KCl and $MgCl_2$) on Sp1-QNR fusion protein cleavage activity using λ DNA as a substrate. The results of these experiments are shown in Fig. 5. The cleavage efficiency by Sp1-QNR appears to decrease with increasing temperatures

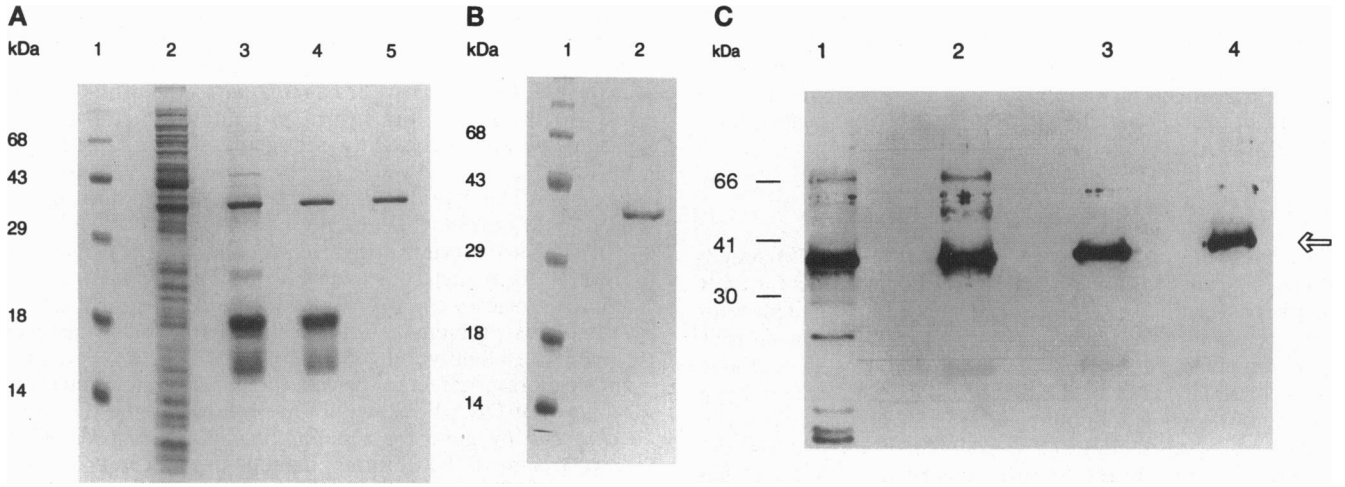

FIG. 2. Purification of Zif-F_N hybrid enzymes. (*A*) SDS/PAGE profiles at each step in the purification of the Sp1-QNR hybrid enzyme. Lanes: 1, protein standards; 2, crude extract from induced cells; 3, His·Bind resin column; 4, SP-Sepharose column; 5, gel-filtration column. (*B*) SDS/PAGE profile of CP-QDR fusion protein. Lanes: 1, protein standards; 2, purified CP-QDR fusion protein. (*C*) Western blot profile at each step of purification of the Sp1-QNR hybrid enzyme using antisera raised against *Fok* I endonuclease. Lanes: 1, crude extract from induced cells; 2, His·Bind resin column; 3, SP-Sepharose column; 4, gel-filtration column. The arrow indicates the intact fusion protein.

(Fig. 5*A*). Room temperature (22°C) appears to be the optimal temperature for the cleavage reaction. This may indicate decreased binding of the Sp1-QNR fusion protein to the λ DNA substrate at higher temperatures. The optimal salt concentration for cleavage appears to be 75 mM KCl. Under these conditions, the reaction proceeds to completion (Fig. 5*B*). The cleavage efficiency appears to drop off with increasing KCl concentration. This can be attributed to the instability of the protein–DNA complex at higher salt concentrations. The effect of increasing the $MgCl_2$ (cofactor) concentration on the cleavage reaction is shown in Fig. 5*C*. The efficiency of cleavage increases with $MgCl_2$ concentration, and the reactions proceed to completion. However, with increasing $MgCl_2$ the nonspecific cleavage by the *Fok* I nuclease domain becomes more pronounced. The optimal $MgCl_2$ concentration for the cleavage reaction appears to be between 2 and 3 mM.

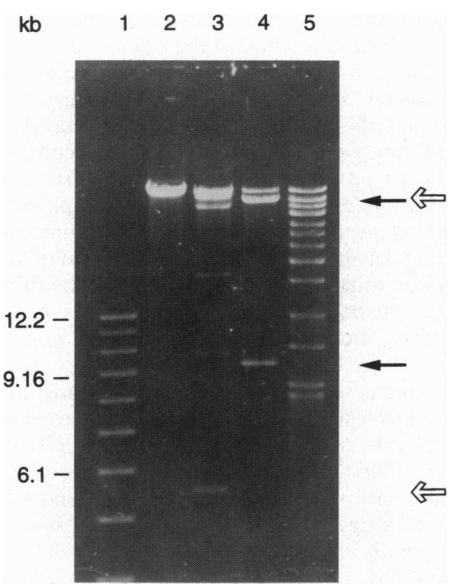

FIG. 3. Cleavage of λ DNA (48.5 kb) substrate by the hybrid protein Zif-F_N. The DNA (30 μg/ml; ≈1 nM) was incubated with the enzymes (≈10 nM) in 35 mM Tris·HCl, pH 8.5/75 mM KCl/100 μM $ZnCl_2$/3 mM dithiothreitol containing 5% (vol/vol) glycerol, yeast tRNA at 25 μg/ml, and bovine serum albumin at 50 μg/ml for 20 min at room temperature in a total volume of 25 μl. $MgCl_2$ was then added to a final concentration of 2 mM and the mixture incubated at room temperature for 4 more hr. The reaction products were analyzed by 0.5% agarose gel electrophoresis. Lanes: 1, kilobase ladder; 2, λ DNA; 3, λ DNA digested with CP-QDR [the substrate cleaves into ≈5.5-kb and 43-kb fragments (open arrows)]; 4, λ DNA digested with Sp1-QNR [the substrate cleaves into ≈9.5-kb and ≈39-kb fragments (closed arrows)]; 5, high molecular weight markers from BRL (from top to bottom: 48.5, 38.4, 33.5, 29.9, 24.8, 22.6, 19.4, 17.0, 15.0, 12.2, 10.1, 8.6, and 8.3 kb, respectively). Weaker bands result from cleavage at the minor DNA-binding sites. tRNA of the reaction mixture runs outside the region shown.

These experiments demonstrate that the cleavage activity of the CP-QDR and Sp1-QNR fusions are quite reproducible. Furthermore, they also show that the reaction conditions can be optimized for site-specific cleavage as well as for the complete cleavage of the substrate.

These results are consistent with what is known about zinc finger–DNA interactions. The zinc finger–DNA recognition appears to be by virtue of only two base contacts of the triplet per zinc finger (10). Therefore, zinc fingers may recognize more than one DNA sequence differing by one base in the central triplets. This may explain why the CP-QDR hybrid enzyme recognizes several DNA sites with different affinities and then cuts these sites with different efficiencies. Thus, the subsite bindings of relatively moderate affinity may contribute to the degeneracy of cleavage. On the other hand, the Sp1-QNR fusion suggests that a hybrid restriction enzyme with a high sequence specificity can be engineered by using the appropriate zinc finger motifs in the fusion constructs.

Analysis of the DNA Sequence Preference of the Zif-F_N Hybrid Enzymes. Determination of the major DNA-binding sites of CP-QDR and Sp1-QNR fusion proteins was done in two steps. First, by using a series of known restriction enzyme digests of the λ DNA followed by cleavage with the fusion protein, the site was localized within a 1- to 2-kb region of the genome. Second, a 300-bp λ DNA fragment containing the major cleavage site was isolated. This substrate was end-labeled with ^{32}P on the top DNA strand or the bottom DNA strand. The products of cleavage of each labeled substrate were analyzed by denaturing polyacrylamide gel electrophoresis (25) followed by autoradiography.

The maps of the primary recognition and cleavage site(s) of the CP-QDR and Sp1-QNR fusion proteins found in the λ genome are shown in Fig. 6. The CP-QDR fusion protein preferentially binds to 5′-GAG GAG GCT-3′, which is one of the four predicted consensus sites that occur in the λ genome.

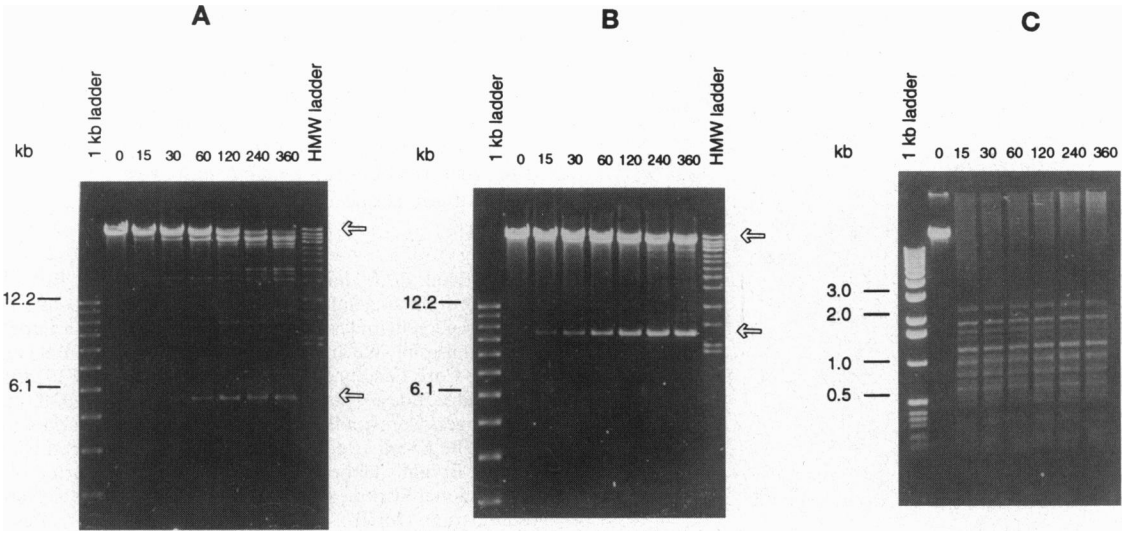

FIG. 4. Kinetics of cleavage of the λ DNA substrate by CP-QDR (*A*) and Sp1-QNR (*B*) hybrid enzymes and wild-type *Fok* I (*C*). The reaction conditions are described in Fig. 3 legend. Aliquots (12 μl) were removed from a 90-μl reaction mixture at 0, 15, 30, 60, 120, 240, and 360 min. The products were analyzed by agarose gel electrophoresis. The arrows indicate the major cleavage products of the λ DNA substrate. The λ DNA was digested with 18 units of wild-type *Fok* I in a volume of 90 μl using New England Biolabs buffer. HMW, high molecular weight.

The Sp1-QNR fusion does not bind to any of the four predicted consensus sites that are present in the λ genome. It preferentially binds to the 5′-GAG GGA TGT-3′ site that occurs only once in the λ genome. The two bases that are different from the reported consensus recognition site of Sp1-QNR are underlined. The reported consensus DNA-binding sites of the zinc finger proteins were determined by affinity-based screening (13–15). This method utilizes a library of DNA-binding sites. Underrepresentation of any of the possible sites within this library may lead to the identification of a subsite as the optimal DNA-binding site. Alternatively, the fusion of the zinc finger proteins to the *Fok* I cleavage domain may alter the DNA sequence specificity. This is unlikely because the binding sites for the previously reported Ubx-F_N and one of the two Zif-F_N fusions described here agree with the reported consensus DNA sites. As many more zinc finger fusions are engineered and characterized, this apparent discrepancy may be resolved. If the sequence specificity of the hybrids is indeed altered, then we need to develop a fast and efficient screening method to identify or select the DNA-binding sites of the hybrid restriction enzymes.

The specificity of the two hybrid restriction enzymes described here is different. More than likely, the specificity of these enzymes is determined solely by the DNA-binding properties of the zinc finger motifs. It appears that the hybrid endonucleases do turn over—that is, the fusion proteins come off the substrate after cleavage. Both enzymes cleave the top strand near the binding site; they cut the bottom strand at two distinct locations. Both fusions show multiple cuts on both strands of the DNA substrate (Fig. 6). One possibility is that the cleavage domain is not optimally positioned for cutting. Naturally occurring type IIS enzymes with multiple cut sites have been reported in the literature (27). The variations in the cleavage pattern of the two hybrid enzymes can be attributed

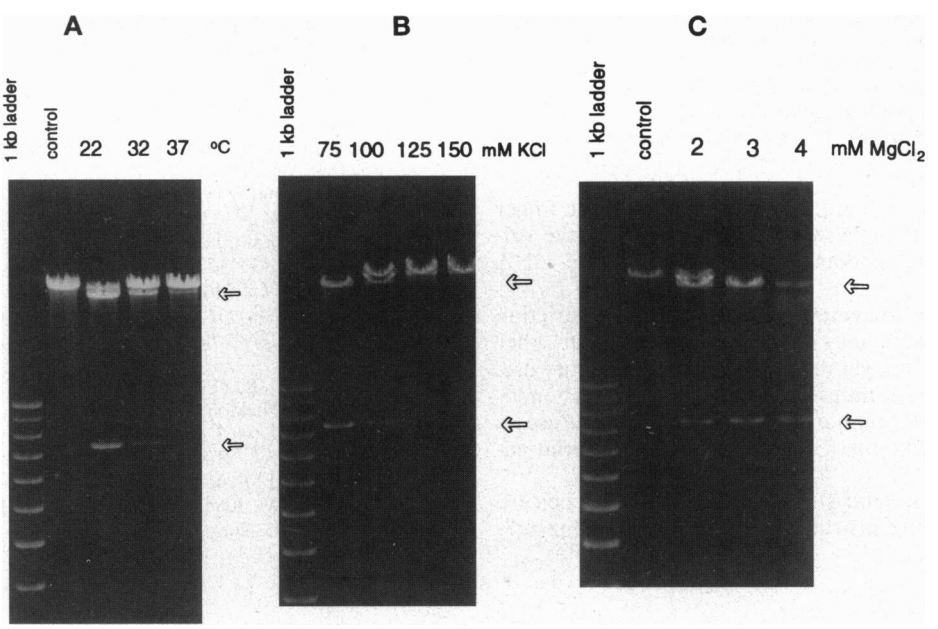

FIG. 5. The effect of temperature (*A*), KCl (*B*), and $MgCl_2$ (*C*) on the cleavage activity of the hybrid enzyme Sp1-QNR-F_N. The reaction conditions are the same as described in Fig. 3 legend except for the variables, which are shown on the top of the figures. Arrows show the major cleavage products from the λ DNA substrate.

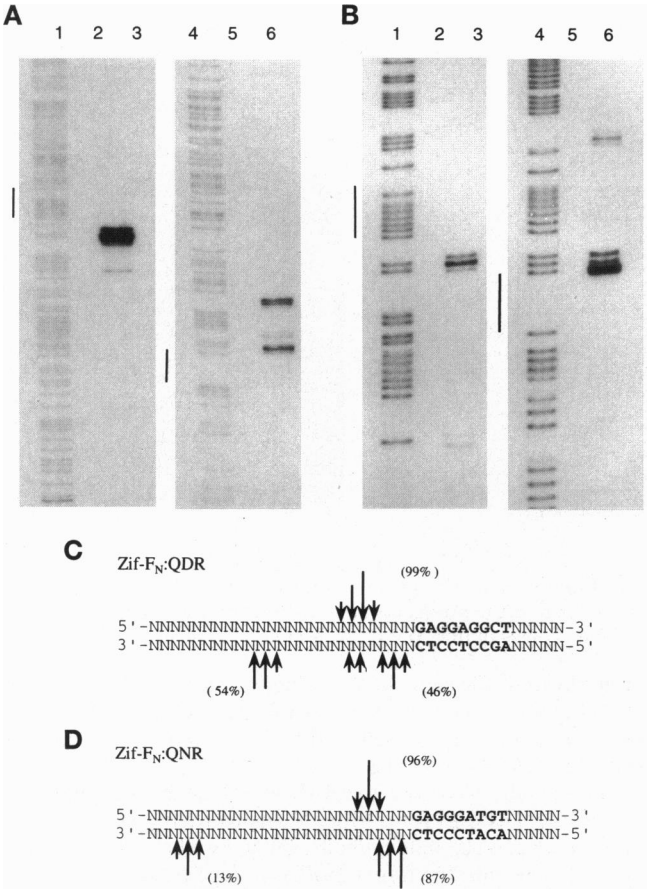

FIG. 6. Analysis of the distance of cleavage from the recognition site by Zif-F_N. Cleavage products of the ^{32}P-labeled DNA substrate containing a single binding site by Zif-F_N along with G+A sequencing reactions were separated by electrophoresis on a 8% polyacrylamide gel containing 6 M urea; the gel was dried and exposed to an x-ray film for 6 hr. (*A* and *B*) Cleavage products from the substrates by CP-QDR (*A*) and Sp1-QNR (*B*). Lanes: 1 and 4, G+A sequencing reaction; 2, substrates containing ^{32}P-label on top strand, 5′-GAG GAG GCT-3′ and 5′-GAG GGA TGT-3′, respectively; 3, Zif-F_N digestion products; 5, substrates containing ^{32}P-labeled on bottom strand, 5′-AGC CTC CTC-3′ and 5′-ACA TCC CTC-3′, respectively; and 6, Zif-F_N digestion products. The locations of the DNA-binding sites for the hybrid enzymes are indicated by vertical lines. (*C* and *D*) Map of the major recognition and cleavage site(s) of Zif-F_N hybrid enzymes. The recognition site is indicated by boldfaced type, and the site(s) of cleavage are indicated by arrows. The percent cleavage at each location is shown in brackets.

to the differences in the mode of binding of the zinc finger motifs to their respective DNA-binding sites and to the orientation of the nuclease domain within the enzyme–DNA complex.

Hybrid Restriction Enzymes. While the hybrid restriction enzymes described here may be suitable for several other applications, they are not yet ready for routine laboratory use. Further experiments are necessary to refine the hybrid endonucleases into enzymes that are efficient for practical use as laboratory reagents like the naturally occurring bacterial enzymes.

In summary, the modular structure of *Fok* I endonuclease has made it possible to construct chimeric restriction enzymes by linking other DNA-binding proteins to the cleavage domain of *Fok* I. The modular structure of zinc finger proteins enables one to design or select peptides that will bind DNA at any predetermined site. The convergence of these two areas of research makes it possible to create artificial nucleases that will cut DNA near a predetermined site. Thus, this approach opens the way to generate many new restriction enzymes with tailor-made sequence specificities desirable for various applications.

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