Artificial Nine Zinc-Finger Peptide with 30 Base Pair Binding Sites†

Tatsuya Kamiuchi, Emiko Abe, Miki Imanishi, Tamaki Kaji, Makoto Nagaoka, and Yukio Sugiura*

Institute for Chemical Research, Kyoto University, Uji, Kyoto 611-0011, Japan

Received May 13, 1998; Revised Manuscript Received July 6, 1998

ABSTRACT: It is well-known that DNA binding of native nine zinc-finger protein TEIIIA is dominated by interaction of select few fingers. Newly designed zinc-finger peptide Sp1ZF9 containing nine Cys_2−His_2 type motifs has been manipulated. The DNA-binding property of Sp1ZF9 was compared with those of native three zinc-finger Sp1(530−623) and artificial six zinc-finger Sp1ZF6 peptides. Although the equilibrium time was less than 0.5 h for Sp1(530−623)−DNA complex, Sp1ZF6 and Sp1ZF9 required approximately 48 and 72 h, respectively, for full complex formation. Evidently, the footprinting analysis demonstrated that Sp1ZF9 and Sp1ZF6 bind at least 27 and 18 contiguous base pairs of DNA sequence, respectively. Sp1ZF9 showed two step bindings to DNA, namely first the recognition of GC (5′-GGG-GCG-GGGCC-3′) sequence by the N-terminal Sp1 domain and next the recognition of the corresponding target sequences by the middle and C-terminal Sp1 domains. In contrast with unimolecular binding of Sp1ZF9 and Sp1ZF6, two Sp1(530−623) molecules bind to one GCIII (5′-GGG-GCG-GGG-GGG-GCG-GGG-GGG-GCG-GGGCC-3′) site region. Semispecific complex formed at the beginning of Sp1ZF9−DNA interaction has also been characterized by kinetic analysis using surface plasmon resonance. Interestingly, the association rate constants for GC and GCIII complexes of Sp1ZF9 are smaller than those of the corresponding Sp1(530−623) complexes. Of special interest is the fact that new nine zinc-finger peptide Sp1ZF9 can bind to DNA sequence of approximately 30 base pairs. Such multi zinc-finger peptides may be useful as genome-specific transcriptional switches in future.

DNA-binding proteins selectively bind to specific DNA sequence, and play an important role in biological systems (*1*). Zinc-finger domain of Cys_2−His_2 type is a typical class of DNA-binding protein and contains the sequence of (Tyr, Phe)-X-Cys-X_{2-4}−Cys-X_3−Phe-X_5-Leu-X_2-His-X_{3-5}−His, usually in tandem arrays (*2*−*5*). The X-ray crystal structures of the Zif268− and GLI−DNA complexes revealed the characteristic DNA-binding mode of zinc-finger proteins as follows: (1) recognition of 3 bases/one finger motif, (2) structure of tandemly repeated finger domain, and (3) binding to the sequence of asymmetric base pairs (*6*−*9*). Transcription factor Sp1 involves three Cys_2−His_2 type zinc-finger motifs at the C-terminus of protein (*10*) and is closely related to Zif268 (*11*, *12*). Indeed, Sp1 strongly binds to GGG-GCG-GGG sequence. On the basis of the nature of Cys_2−His_2 type zinc-finger motif and recognition bases of Sp1, we designed novel nine zinc-finger peptide Sp1ZF9 and also its DNA-binding properties were compared with those of three finger Sp1(530−623) and six finger Sp1ZF6 peptides. DNA binding of nine zinc-finger protein TFIIIA is well-known to be dominated by interaction of select few fingers (*2*, *8*, *13*−*15*). Therefore, it is of special interest to create a new nine zinc-finger peptide that can bind to DNA sequence over an extended region of 30 base pairs. Such multi zinc-finger peptides may be hopeful in future gene therapy strategies. Certainly, molecules with high DNA-binding affinity and long sequence specificity in the human genome are useful tools in molecular biology and potentially in human medicine (*16*−*18*).

MATERIALS AND METHODS

Chemicals. T4 polynucleotide kinase and restriction enzymes were purchased from New England Biolabs, except for *Eco*47III and *Xho*I obtained from Takara Shuzo (Kyoto, Japan) and *Age*I obtained from Nippon Gene (Tokyo, Japan). Labeled compound [γ-^{32}P]ATP was supplied by DuPont. The plasmid pBS−Sp1-fl was kindly provided by Dr. R. Tjian. All other chemicals were of commercial reagent grade.

Construction of Genes and Peptide Expression. The pUC−Sp1(530−623), which codes the three zinc-finger region, was constructed as described previously (*19*). Sp1ZF6 and Sp1ZF9 were constructed by linking two and three Sp1's zinc-finger domains with the TGEKP linker peptide. *Bam*HI−*Sty*I fragment (84 bp) containing the *Krüppel-type* linker (TGEKP) and gene was synthesized on an Applied Biosystems 391 DNA synthesizer. This fragment was inserted into pUC−Sp1(530−662). The *Eco*47III fragment (264 bp) was cut out and inserted into similarly digested pUC−Sp1(530−623) (*19*). The plasmid was renamed as pUC−Sp1ZF6. The middle Sp1 gene fragments for Sp1ZF9 were created by PCR with primer set of pUC−Sp1(530−623) as template, and the middle Sp1 for Sp1ZF9 was flanked by *Age*I sites at the 5′- and 3′-ends. The primer pair was the *Age*I site primers, namely 5′-ACCGGTGAAAAACCGCATATTTGCCACATC-

† This study was supported in part by a Grant-in-Aid for Priority Project "Biometals" (08249103) and Scientific Research (B) (09557200 and 10470493) from the Ministry of Education, Science, Sport, and Culture, Japan. We also thank for the grant from the Houansha Foundation.

* Corresponding author. Phone: 81-774-38-3210. Fax: 81-774-32-3038. E-mail: sugiura@scl.kyoto-u.ac.jp.

S0006-2960(98)01111-8 CCC: $15.00
Published on Web 09/05/1998

3′ for cording strand and 5′-CGGTTTTTCACCGGT-GTGGGTCTTGATATG-3′ for noncording one. This fragment modified with *Age*I sites was constructed by ligation into *Age*I site of pUC−Sp1ZF6. The *Age*I enzyme site between two and three Sp1 fragments encodes amino acid TG, part of the TGEKP linker peptide. All sequences were confirmed by DNA sequence analysis using BcaBEST dideoxy sequencing kit (Takara Shuzo, Kyoto). The DNA fragments of Sp1(530−623), Sp1ZF6, and Sp1ZF9 were cut out and inserted into the similar digested plasmid pEV-3b (*19*). These zinc-finger peptides were overexpressed in *Escherichia coli* strain BL21(DE3)pLysS as described previously (*20*). Purification procedures were performed at 4 °C. *E. coli* cells in which Sp1 peptides were overexpressed, were resuspended and lysed in phosphate buffer (10 mM phosphate buffer, pH 7.0, 130 mM NaCl, and 2.7 mM KCl). After centrifugation, the supernatant containing the soluble form of zinc-finger peptides was purified by a cation-exchange chromatography (HighS, Bio-Rad and MiniS, Pharmacia) with phosphate buffer (pH 8.0 or 7.0) using NaCl gradient (0.13 to 1 M), except that the purification of Sp1ZF9 was performed by HighS only. The isolated peptides were dialyzed with 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, and 1 mM β-mercaptoethanol, and then stored at 4 °C. To estimate purity of the isolated peptides, we demonstrated by Coomassie Blue-stained SDS−PAGE gels. Peptide concentrations and zinc analyses were determined by amino acid analysis and atomic absorption spectrophotometry respectively, and then Sp1(530−623), Sp1ZF6, and Sp1ZF9 contained approximately 3, 6, and 9 zinc atoms/mol of the peptides, respectively.

Preparations of Plasmid and DNA Fragments. The GC-box oligonucleotides were synthesized by using an Applied DNA synthesizer 391. These oligonucleotides contain the target binding site predicted from the transcription factor Sp1: GC, 5′-GGG GCG GGGCC-3′; GCII; 5′-GGG GCG GGG GGG GCG GGGCC-3′; and GCIII, 5′-GGG GCG GGG GGG GCG GGG GGG GCG GGGCC-3′. The synthesized oligonucleotides were annealed and inserted in pBluescript II KS+ (Toyobo, Osaka). The *Sac*I−*Xho*I fragment was cut out for the experiments, except for use of the *Hin*dIII−*Xba*I fragment for gel mobility shift assay.

Gel Mobility Shift Assays. Gel mobility shift assays were carried out under the following condition. Each reaction mixture contained 10 mM Tris buffer (pH 8.0), 50 mM NaCl, 0.5 mM β-mercaptoethanol, 0.1 mM $ZnCl_2$, 0.05% Nonidet P-40, 25 ng/μL poly(dI-dC) (Pharmacia), 5% glycerol, the 5′-end-labeled *Hin*dIII−*Xba*I fragment (∼100pM), and 0−1000 nM of zinc-finger peptide. After incubation at 20 °C for 0.5−72 h, the sample was run on a 12% polyacrylamide sequencing gel with 1×TB buffer at 20 °C. The bands were visualized by autoradiography. Apparent dissociation constants were determined by using Kaleida Graph program (Abelbeck Software).

DNase I Footprinting Analyses. DNase I footprinting experiments were performed according to the method of Brenowitz et al (*21*). Binding reaction mixture contained 10 mM Tris buffer (pH 8.0), 50 mM NaCl, 5 mM $CaCl_2$, 10 mM $MgCl_2$, 20 ng/μL of sonicated calf thymus DNA, the 5′-end labeled *Sac*I−*Xho*I fragment (approximately 15 000 cpm), and 0−1 μM peptide. After incubation at 20 °C for 30 min, the sample was digested with DNase I (20 milliunits) at 20 °C for 2 min. After the reaction, 30 μL of DNaseI stop solution (0.1 M EDTA and 0.6 M sodium acetate) and 50 μL of phenol/chroloform were added to sample solution. After ethanol precipitation, the cleavage products were analyzed on an 8% polyacrylamide/7 M urea sequencing gel. The bands were visualized by autoradiography.

Methylation Interference Analyses. Methylation interference assays were investigated as described previously (*22*), except for reaction time (0.5 or 72 h). The binding reaction mixture contained 10 mM Tris buffer (pH 8.0), 50 mM NaCl, 50 ng/μL of sonicated calf thymus DNA, 5% glycerol, the 5′-end labeled methylated *Sac*I−*Xho*I fragment (approximately 200K cpm), and 20−50 nM peptides. To examine both the strong and weak base contacts in the experiment of methylation interference, we selected the experimental condition that the peptide/DNA molar ratio in binding reaction is about 10−20% bound.

Kinetic Analysis Using Surface Plasmon Resonance. The principle of operation of the BIAcore biosensor has been described before. 5′-Biotinylated oligonucleotides were annealed with the complementary strand in Tris-HCl buffer (pH 8.0) containing 50 mM NaCl and 2 mM EDTA. The duplex solution was injected over a streptavidin-coated sensor chip (SA5, Pharmacia Biosensor) until a suitable level (∼1000 RU) was achieved. Tris-HCl buffer (50 mM Tris-HCl, 250 mM KCl, 10 mM $MgCl_2$, 0.005% Tween20, and 0.1 mM $ZnCl_2$, pH 7.5) was used both as flow buffer and sample preparation buffer. Bound protein was eluted from the DNA by three repeats of a short pulse (5 μL) of 0.05% SDS. This regeneration procedure did not alter to any measurable extent the ability of the immobilized DNA to bind protein in subsequent cycles at 25 °C. The association was followed for 7 min and the dissociation for 10 min at a flow rate of 30 μL/min. Analysis of the data was performed using the evaluation software supplied with the instrument (BIAevaluation version 3.0). To eliminate small bulk refractive change differences at the beginning and end of each injection, a control sensorgram obtained over a surface modified with biotin was subtracted for each peptide injection. For obtaining the association and dissociation rate constants, the following equations were used, respectively.

$$R = R_{eq}[1 - e^{-(k_a C + k_d)(t - t_0)}] \quad (1)$$

$$R = R_0 e^{-k_d(t - t_0)} \quad (2)$$

R, R_0, and R_{eq} are the response at time t, t_0, and at equilibrium, respectively; C is the concentration of protein; k_a is the association rate constant; k_d is the dissociation rate constant; and t_0 is the start time of the dissociation or association.

RESULTS

Design of Multiple Zinc-Finger Proteins. Novel multiple zinc-finger peptides, Sp1ZF6 and Sp1ZF9, were newly created from zinc-finger motif of transcription factor Sp1 (Figure 1). These peptides were constructed by connecting C-terminal Sp1 molecule to N-terminal of a following one. The *Krüppel-type* linker (Thr-Gly-Glu-Lys-Pro), which is conserved in many zinc-finger proteins, was selected for connection of Sp1 finger domains. This linker plays in

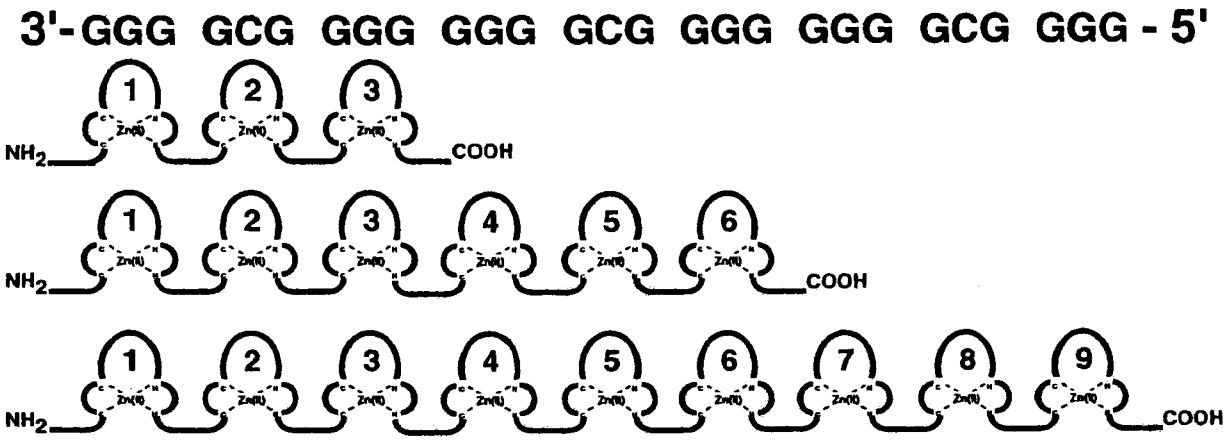

FIGURE 1: Sp1(530−623), Sp1ZF6, and Sp1ZF9.

controlling the orientation and spacing of adjacent finger and also is involved in nonspecific interaction with phosphate backbone of DNA (*6*, *8*, *19*, *23*, *24*).

Binding Specificity of Sp1(530−623), Sp1ZF6, and Sp1ZF9. In gel mobility shift assays, the binding sites were predicted from Sp1 recognition site (G**GG GCG G**GG) (*10*). The equilibrium time was less than 0.5 h for Sp1(530−623)−DNA complex. By contrast, Sp1ZF6 and Sp1ZF9 required approximately 48 and 72 h, respectively. To determine binding specificity of the multiple zinc-finger peptides, we performed the gel mobility shift assays with two DNA fragments (Figure 2). The binding affinity of Sp1(530−623) was not significantly different in three DNA fragments. Sp1ZF6 showed about 20-fold preferential binding to GCII compared with GC. Sp1ZF9 gave approximately 30-fold higher affinity with GCIII than GC. The results reveal that the length of the binding DNA sequence is dependent on the number of these zinc-finger motifs. On the other hand, the binding affinities for GCIII complexes of Sp1ZF6 and Sp1ZF9 were considerbly close. Probably, this is because GCIII sequence contains both GC and GCII sequences. Figure 3 demonstrates that two Sp1(530−623) molecules bind to one GCIII fragment but Sp1ZF9 (or Sp1ZF6) does with unimolecule.

DNA Binding of Multiple Zinc-Finger Peptides. To examine the DNA-binding site of Sp1ZF6 and Sp1ZF9 on GCIII, DNase I footprinting assays were performed (Figure 4). Under lower peptide concentration, Sp1(530−623) bound the GC-box of 3′-portion. With increasing the peptide concentration, the GC-box of 5′-portion was protected and also the hypersensitive breakages were detected at C(14) and G(15) within the middle GC-box. Clearly, two Sp1(530−623) peptides bound to GCIII. On the other hand, Sp1ZF6 and Sp1ZF9 exhibited different binding features from Sp1-(530−623). Sp1ZF6 bound to longer sites than 18 bp of 3′-end in the GCIII. Sp1ZF9 protected slightly longer binding sites than the 27 bp target site.

Finger-Base Interaction of Sp1ZF9 in Comparison with Sp1ZF6 and Sp1(530−623). When Sp1ZF9, Sp1ZF6, and Sp1(530−623) bound to the GCIII, methylation interference patterns are shown in Figure 5. Two molecules of Sp1(530−623) comes in contact with one DNA GCIII fragment in increasing peptide concentration. Therefore, we carried out the experiment under the peptide concentration that two bound bands were detected. In the case of one Sp1(530−623) binding, the guanine contacts were observed at G(20), G(21), G(22), and G(24) of the 3′-end. In the bindings of two molecules, base contacts with G(1), G(2), G(3), G(4), G(6), G(7), G(8), G(9), G(10), G(20), G(21), G(22), G(24), and G(26) were detected. Clearly, the contacts with guanine bases of middle GC-box were not demonstrated. On the other hand, Sp1ZF6 presented the contacts with the guanine residues at 3′-portion of GCIII, namely G(11), G(12), G(13), G(15), G(16), G(17), G(18), G(19), G(20), G(21), and G(22). In the case of Sp1ZF9-DNA interaction, the experiments were carried out during an incubation period of 0.5 and 72 h. The lower mobility band of two bound bands at 0.5 h gave the contacts with G(18), G(19), G(20), G(21), G(22), G(24), G(25), G(26), and G(27) on the 3′-portion. By contrast, the upper band in this condition presented the contacts with G(2), G(3), G(4), G(6), G(7), G(8), G(9), G(10), G(11), G(12), G(20), G(21), and G(22). Under reaction conditions at 72 h where only one bound band was detected, G(1), G(13), G(17), G(18), and G(24) evidently added to their contact bases, and also all contacts were stronger than those in the 0.5 h reaction. Figure 6 summarizes these experimental results.

Binding Affinity of Sp1ZF9 to GCIII. To estimate accurately the binding affinity, the active peptide concentration should be calculated on the basis of only the preparation fraction which is active to bind to DNA. However, the larger peptide may be expected to be less likely to fold and more likely to be oxidized in a long equilibrium period. By using the peptide prepared freshly, therefore, we determined the approximate DNA-binding affinity of Sp1ZF9. Figure 7 compares representative gel mobility shifts for GCIII-binding of Sp1ZF9 at 0.5 and 72 h. In the case of 72 h, the apparent equilibrium dissociation constant (K_d) was 1.2 ± 0.3 nM. Recently, we determined that the dissociation constant (K_d) of three zinc-finger Sp1(530−623) peptides for GC-box DNA is 3.5 ± 0.5 nM (*25*).

DISCUSSION

TFIIIA and GLI proteins containing nine and five zinc-fingers, respectively, use only few select fingers for their DNA bindings (*2*, *8*, *12*−*14*). Multiple zinc-finger proteins wraping around DNA major grooves by all zinc-fingers and their binding to such a long sequence are unknown. Our nine zinc-finger peptide Sp1ZF9 evidently binds a 27 bp continuous sequence with high affinity and specificity. The

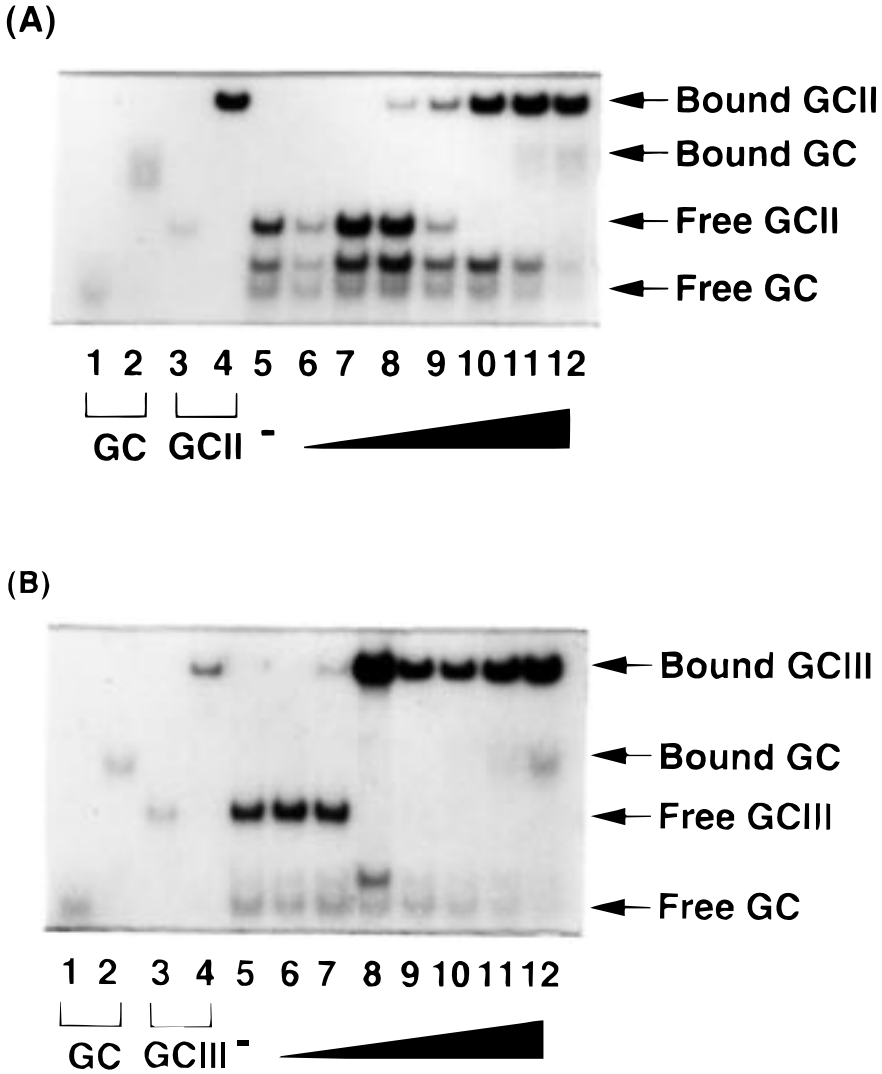

FIGURE 2: Gel mobility shift assays for binding specificity. (A) Sp1ZF6 bindings to GC and GCII: lanes 1 and 2, GC only; lanes 3 and 4 GCII only; and lanes 5−12, 0, 0.1, 0.3, 1, 3, 10, 30, and 100 nM of peptide. (B) Sp1ZF9 bindings to GC and GCIII. lanes 1 and 2, GC only: lanes 3 and 4, GCIII only; and lanes 5−12, 0, 0.1, 0.3, 1, 3, 10, 30, and 100 nM of peptide.

DNA binding characteristics of the nine finger peptide were clarified in comparison with those of Sp1(530−623) and Sp1ZF6 containing three and six zinc-fingers, respectively.

When the time to reach equilibrium of DNA complex was measured, Sp1ZF6 and Sp1ZF9 required approximately 48 and 72 h, respectively. That of Sp1(530−623) was less than 0.5 h. Natural nine finger protein TFIIIA is known to reach equilibrium for 1 h (*26*). These differences may arise from the binding mode that new peptides slowly wrap around DNA duplexes with all fingers. In the case of TFIIIA, the three finger domains of C- and N-terminals, respectively, interact with the one turn of duplex DNA and this protein binds from one side of the DNA (*14*, *15*).

From the results of gel shift assays, Sp1ZF6 has higher affinity for GCII than GC, and Sp1ZF9 for GCIII than GC. On the other hand, these peptides exhibit the binding selectivity within one GCIII fragment. Sp1ZF6 binds preferentially to the 3′-end GC-box of GCIII. The evidence demonstrates that Sp1ZF6 has high specificity for a sequence containing 5′-GGG GCG GGG**C**-3′. Primarily, Sp1ZF9 also interacts selectively with this sequence. In addition, Sp1-(530−623) showed the higher affinity to this binding site than 5′-GGG GCG GGG**G**-3′ site (*10*, *27*, *28*). The methylation interference analyses for Sp1ZF6 and Sp1ZF9 also reveal that the N-side domain rather than the C-side in all finger domains is preferential in the sequence-specific DNA binding of these multiple finger peptides. The N-terminal Sp1 domain in six or nine zinc-fingers of Sp1ZF6 or Sp1ZF9 is the most similar to Sp1(530−623). These results demonstrate that our peptides have sequence specificity and its favorable sequence depends on both the number of motif and character of Sp1(530−623).

The result of gel mobility shift assays for Sp1(530−623) bound to GCIII indicates that two Sp1(530−623) molecules interact with GCIII. Presumably, the DNase I footprinting evidence suggests that, first, Sp1(530−623) binds specifically to 3′-portion GC sequence and the second molecule is forced to bind to the distant site from the first binding site because of steric hindrance. Two molecules of Sp1(530−623) could not bind in close proximity to a continuous DNA sequence. On the other hand, the bound band of Sp1ZF6 or Sp1ZF9 to GCIII is finally only one and these binding sites are at least 18 and 27 bp continuous sequences. The facts suggest that the multiple zinc-finger peptide−DNA interaction forms s 1:1 complex. Very recently, Liu et al. also showed similar results that six zinc-finger proteins designed from structure-

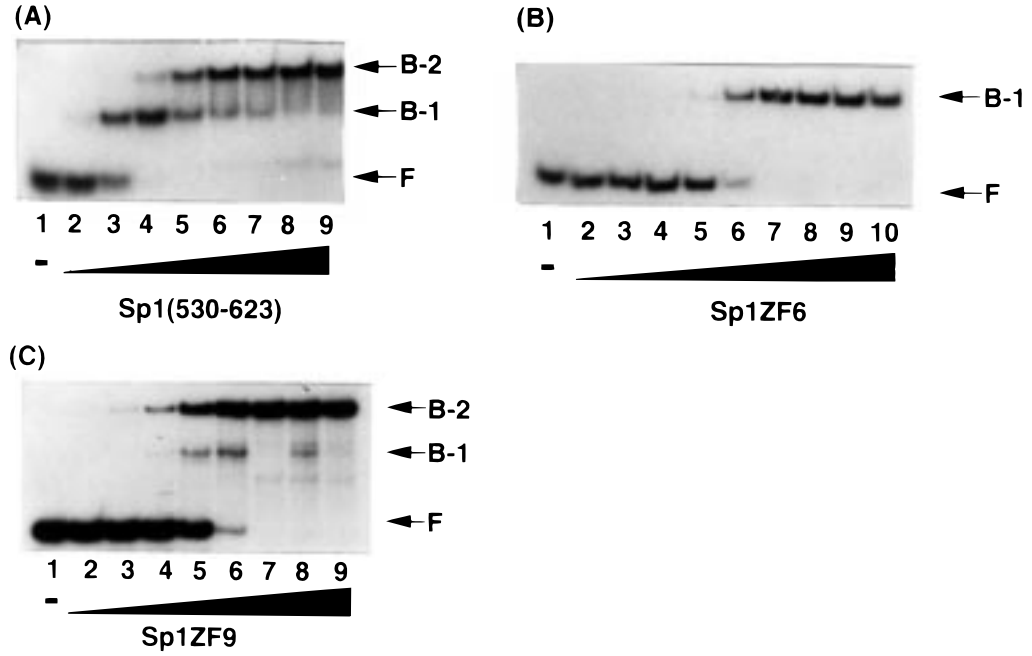

FIGURE 3: Gel mobility shift assays for Sp1(530−623), Sp1ZF6, and Sp1ZF9 bindings to GCIII-box DNA at 0.5 h. (A) Sp1(530−623)−DNA complex: B-1 and B-2 are one and two peptide-bound DNAs. F is peptide-free DNA. Lanes 1−9, 0, 1, 10, 30, 100, 200, 300, 600, and 1000 nM of peptide. (B) Sp1ZF6−DNA complex: B-1 and F are peptide-bound and -free DNA bands. Lanes 1−10, 0, 3.125, 6.25, 12.5, 25, 50, 100, 200, 400, and 800 nM of peptide. (C) Sp1ZF9−DNA complex: B-1 and B-2 show disappeared and newly appeared peptide-bound DNAs after 72 h. F is peptide-free DNA. Lanes 1−9, 0, 6.25, 12.5, 25, 50, 100, 200, 400, and 800 nM of peptide.

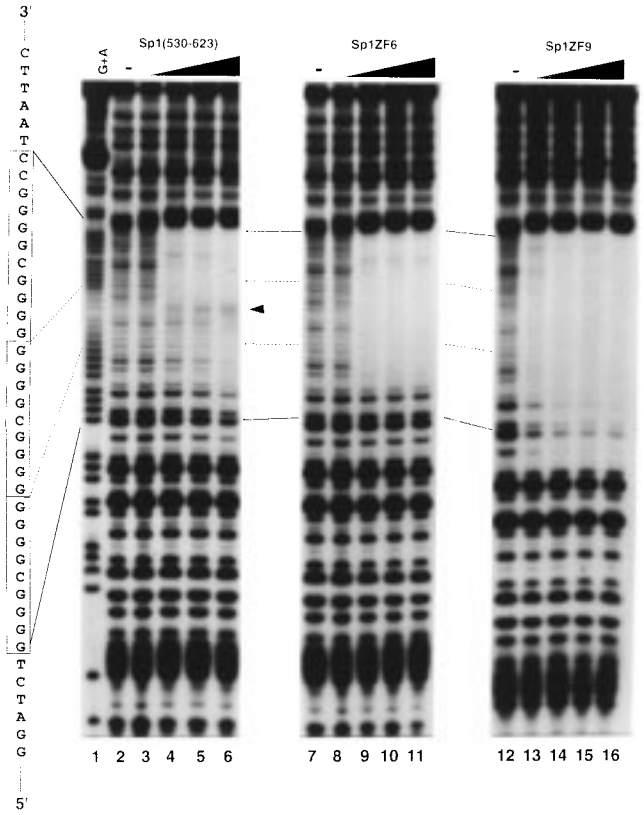

FIGURE 4: DNaseI footprinting analyses for Sp1(530−623), Sp1ZF6, and Sp1ZF9 bindings to GCIII-box DNA fragment (108 bp). The closed triangle represents enhance site of cleavage. Lane 1, G+A (Maxam−Gilbert reaction products); lanes 2, 7, and 12, 20 μg/mL BSA; lanes 3−6, Sp1(530−623); lanes 8 and 9, Sp1ZF6; and lanes 13−16, Sp1ZF9. Peptide concentration is increased as follows: 0, 125, 250, 500, and 1000 nM.

based modeling of Zif268 bind a long 18 bp DNA sequence in a sequence-specific fashion (*17*).

DNase I molecule is larger than organic chemical reagents, and hence, its DNA footprinting technique is able to detect an extent of DNA site covered with the peptide. Methylation interference analysis demonstrates detailed contacts of amino acid−nucleotide base. These methods were performed at the reaction times of 0.5 and 72 h. Sp1ZF6 bound to GCIII did not show significant difference of the band concentration between peptide-free and -bound at 0.5 h, except that Sp1ZF6 likely binds to two GC-boxes of the 3′-end of GCIII. This binding tendency was remarkably clear at 72 h. Namely, Sp1ZF6 strongly contacts only with two GC-boxes of the 3′-end of GCIII. At the reaction time of 0.5 h in Sp1ZF9−GCIII DNA interaction, the lower bound band presented contacts with some guanine bases of the 3′-end of GCIII. On the other hand, the upper bound band at the same reaction time indicated contacts with some guanine bases of the opposite (5′) end of GCIII. At 72 h, the lower band disappeared and the upper band was only detected. Interestingly, Sp1ZF9 exhibited strong contacts with some guanine bases in two GC boxes of the 5′-end and weak contacts with some guanine bases of the 3′-end of GCIII. Sp1ZF9 first recognizes a sequence GGG GCG GGGCC by N-terminal Sp1 domain, namely the intermediate of Sp1ZF9−DNA complex. Next, the middle and C-terminal Sp1 domains bind to the 5′-side of the sequence site, and finally the selected fingers of Sp1ZF9 interact with their best contact bases. The strong interaction with Sp1ZF9 results from the 5′-side sequence of GCIII. Although all fingers of these multiple finger peptides engage with duplex DNA, the DNA recognition ability of each finger is different.

Results from gel mobility shift and DNase I footprinting experiments have revealed that Sp1ZF9 (9 finger peptide) efficiently forms a specific binding complex with the expected 27 bp DNA sequence after a long equilibrium period. At the beginning of binding reaction, a mobility-

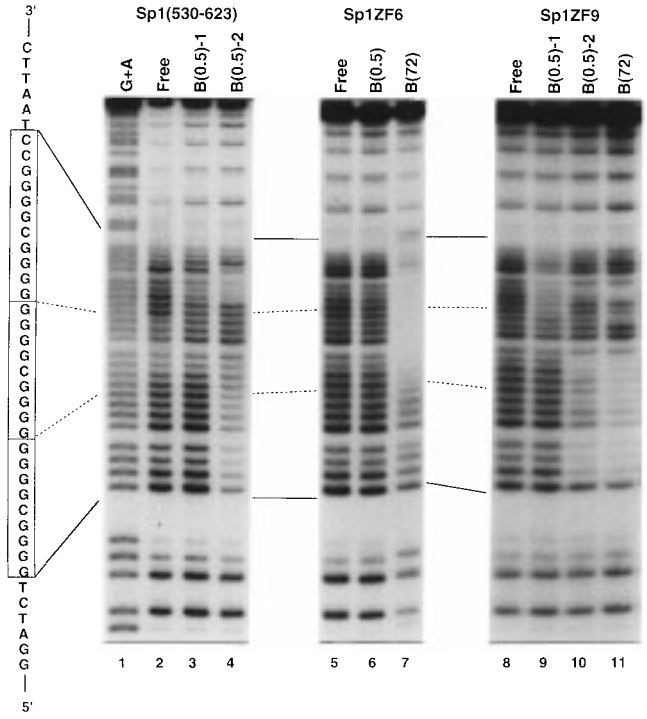

FIGURE 5: Methylation interference analyses for Sp1(530−623), Sp1ZF6, and Sp1ZF9 bindings to GCIII-box. Lane 1, G+A (Maxam−Gilbert reaction products); lanes 2−4, Sp1(530−623); lanes 5−7, Sp1ZF6; lanes 8−11, Sp1ZF9. F is peptide-free DNA. The parenthesis shows reaction time (h). B-1 and B-2 present the lower and upper DNA bands, respectively.

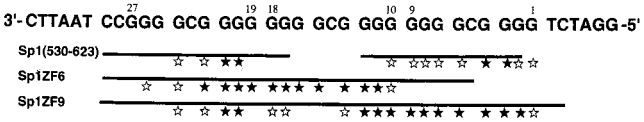

FIGURE 6: Summary of Sp1(530−623), Sp1ZF6, and Sp1ZF9 bindings to GCIII-box. Rod-line represents DNA sequence sites protected by peptide from DNaseI-digestion. Filled and open stars exhibit strongly- and weakly contacted bases respectively, revealed by methylation interference analysis.

shifted band that migrates faster than the final shifted band was observed. Such a time-dependent intermediate band was never observed in the cases of Sp1(530−623) (3 finger peptide). The faster migrating band disappears with increasing the incubation time. Mobilities of the faster migrating bands observed in 9 finger peptide/GC and /GCIII binding mixtures are almost the same, indicating that net charge, shape, and flexibility of 9 finger/GC and 9 finger/GCIII intermediate complexes are very similar. We postulated that the faster migrating band observed at the beginning of equilibrium represents a semispecific peptide−DNA complex. With this mechanism, the 9 finger peptide first forms a semispecific complex with only 3 finger matches with a 9 bp sequence. The intermediate complex then rearranges to the specific complex, and it is this process that takes a very long equilibrium period. To characterize the binding complex that observed at the beginning of the reaction, kinetic aspects of the binding reaction were analyzed by BIAcore instrument with GC and GCIII DNAs immobilized on the surface. A typical sensorgram is shown in Figure 8. The association and dissociation phases for the 3 finger/GC and 3 finger/GCIII complexes are quite similar at the peptide concentration of 50 nM, while a slightly larger amount of 3 finger peptide binds to GCIII. This is expected because GCIII contains three tandem GC box sequences, and gel

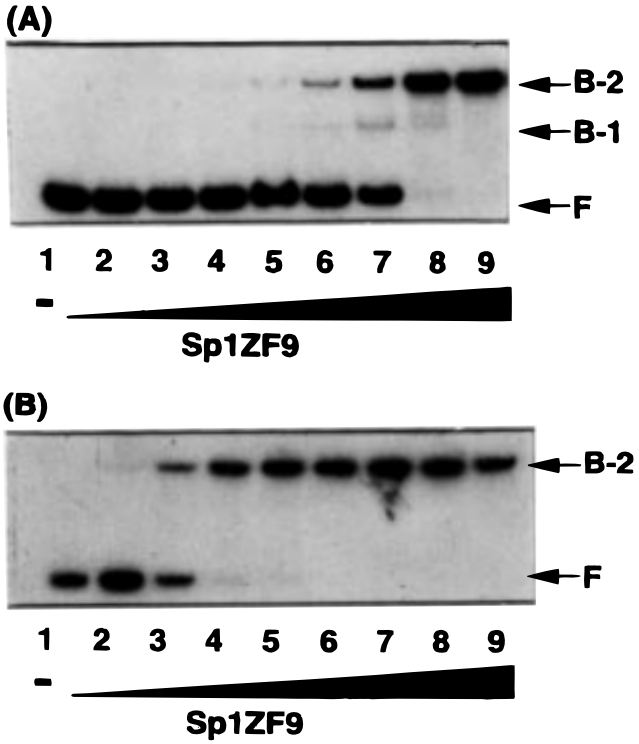

FIGURE 7: Gel mobility shift assay for Sp1ZF9 to GCIII-box DNA. (A) The incubation time is 0.5 h. Lanes 1−9, 0, 0.5, 1, 2, 5, 10, 20, 50, and 100 nM. (B) The incubation time is 72 h. Lanes 1−9, 0, 0.5, 1, 2, 4, 8, 16, 32, and 64 nM. F is protein-free DNA. B-1 and B-2 show protein-bound DNAs at 0.5 h, but after 72 only B-2 is detectable.

mobility shift experiments showed that two 3 finger peptides bind in a noncooperative manner to GCIII sequence. The association reactions for the 9 finger/GC and 9 finger/GCIII complexes closely resemble each other. This is also the case

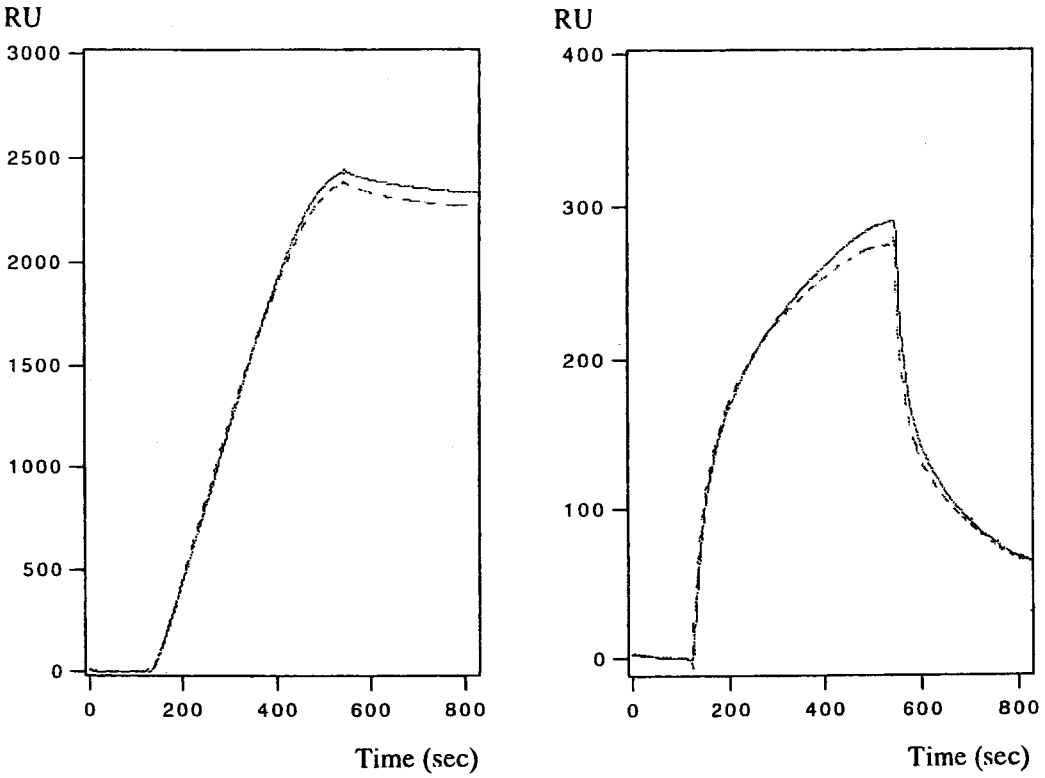

FIGURE 8: Sensorgrams for binding reaction of Sp1ZF9(left) and Sp1(530−623) (right) to GCIII(—) and GC(---) DNAs.

Table 1: Association and Dissociation Rate Constants for Sp1ZF9 and Sp1(530−623) to DNAs

peptide	DNA	k_a (M^{-1} s^{-1})	k_d (M^{-1})	K_a
Sp1ZF9	GC	$(4.95 \pm 1.25) \times 10^4$	$(1.45 \pm 0.35) \times 10^{-4}$	$(3.72 \pm 1.66) \times 10^8$
	GCIII	$(5.47 \pm 1.91) \times 10^4$	$(1.04 \pm 0.61) \times 10^{-4}$	$(9.66 \pm 7.50) \times 10^8$
	SS	$(1.25 \pm 0.07) \times 10^5$	$(1.67 \pm 0.50) \times 10^{-4}$	$(8.37 \pm 0.30) \times 10^8$
Sp1(530−623)	GC	$(2.70 \pm 0.36) \times 10^5$	$(3.49 \pm 0.97) \times 10^{-3}$	$(1.73 \pm 0.52) \times 10^8$
	GCIII	$(1.75 \pm 0.60) \times 10^5$	$(2.36 \pm 0.53) \times 10^{-3}$	$(0.84 \pm 0.44) \times 10^8$
	SS	$(0.83 \pm 0.16) \times 10^5$	$(2.46 \pm 0.33) \times 10^{-3}$	$(3.54 \pm 1.12) \times 10^7$

for the dissociation reaction. Thus, the 9 finger peptide binds both GC and GCIII DNAs in a similar manner. Association and dissociation rate constants for the binding reactions of Sp1ZF9 and Sp1(530−623) peptides were obtained by monitoring the binding reactions with a variety of peptide concentrations (Table 1). Comparison of the kinetic parameters listed in Table 1 clearly indicates that kinetic aspects for the formation of 9 finger/GC and 9 finger/GCIII are essentially identical while the dissociation rate constant for 9 finger/GC complex is slightly larger than that of the 9 finger/GCIII complex. Because the time scale of BIAcore experiments is much shorter than that of gel shift experiments, the binding reaction of 9 finger peptide monitored here unlikely represents the formation of specific complex. Rather, it mainly describes the formation of an intermediate complex that has been observed as the faster migrating band in the gel shift experiments. The 9 finger peptide shows an order of magnitude smaller dissociation rate constants than that for the specific 3 finger peptide even though the 9 finger complex is thought to be a semispecific complex. In the cases for the 3 finger peptide, kinetic parameters for the formation of GC and NON complexes are clearly different in the dissociation rate constants. Even if such a semispecific complex forms at the beginning of the binding reaction, such complex is thermodynamically too unstable to be observed for 3 finger peptide. Interestingly, the association rate constants for the 9 finger/GC and /GCIII complexes are smaller than those observed for the 3 finger peptide. It is possible that the 9 finger peptide has some folded structure which slows the association reaction of the 9 finger peptide. In fact, the association rate constant for the 9 finger peptide/ssDNA complex is similar to that for the specific 3 finger/GC complex. Because the ssDNA has more flexible structure than the dsDNA, this observation supports the idea that the folded structure slows the association reaction of the 9 finger peptide to dsDNA. If this is the case, the linker region likely plays its role not only in the thermodynamic aspects but also in the kinetic aspects of the DNA binding of Sp1ZF9. We must determine folding and DNA interaction of Sp1ZF9 and Sp1(530−623) by circular dichroism and NMR methods.

In conclusion, a newly designed nine zinc-finger peptide Sp1ZF9 binds a contiguous 27 bp DNA. The multiple zinc-finger peptide has two steps of the sequence recognition and binding for peptide−DNA complex formation. Recently, zinc-finger motifs contacting with various sequences were selected by the technique of phage display (*29*−*32*). The present results would provide good information for design of new DNA-binding proteins to recognize long DNA sequence. Indeed, human Y-box binding protein gene promoter (*33*) and human immune activation (Act-2) gene (*34*) include GCIII-like long GC sequences. In future gene

therapy, such multi zinc-finger proteins may be useful as genome-specific transcriptional switches.

ACKNOWLEDGMENT

We are grateful to Dr. T. Morii for pertinent advice on our experiments.

REFERENCES

1. Pabo, C. O., and Sauer, R. T. (1984) *Annu. Rev. Biochem. 53*, 293−321.
2. Miller, J., Mclachan, A. D., and Klug, A. (1985) *EMBO J. 4*, 1609−1614.
3. Klug, A., and Rhodes, D. (1987) *Trends. Biochem. Sci. 12*, 464−470.
4. Evans, R. M., and Hollenberg, S. M. (1988) *Cell 52*, 1−3.
5. Krizek, B. A., Amann, B. T., Kilfoil, V. J., Merkled. L., and Berg, J. M. (1991) *J. Am. Chem. Soc. 113*, 4518−4523.
6. Pavletich, N. P., and Pabo, C. O. (1993) *Science 252*, 809−817.
7. Elrod-Erickson, M., Rould, M. A., Nekludova, L., and Pabo, C. O. (1996) *Structure 4*, 1171−1180.
8. Pavletich, N. P., and Pabo, C. O. (1993) *Science 261*, 1701−1707.
9. Berg, J. M., and Shi, Y., (1996) *Science 271*, 1081−1085.
10. Kadonaga, J. T., Carner, K. R., Masiarz, F. R., and Tjian, R. (1987) *Cell 51*, 1079−1090.
11. Christy, B. A., Lau, L. F., and Nathans, D. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 7857−7861.
12. Berg, J. M., (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 11109−11110.
13. Zang, W. Q., Veldhoen, N., and Romaniuk, P. J. (1995) *Biochemistry 34*, 15545−15552.
14. Clemens, K. R., Lion, X., Wolf, V., Wright, P. E., and Gottesfeld, J. M. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 10822−10826.
15. Harpes, J. J., and Tullius, T. D. (1992) *J. Mol. Biol. 227*, 407−417.
16. Kim, J. S., Kim, J., Cepek, K. L., Sharp, P. A., and Pabo, C. O. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 3616−3620.
17. Liu, Q., Segal, D. I., Ghiara, J. B., and Barbas, C. F., III. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 5525−5530.
18. Gottesfeld, J. M. Nealy, L., Trauger, J. M., Baird, E. E., and Dervan, P. B. (1997) *Nature (London) 387*, 202−205.
19. Nagaoka, M., and Sugiura, Y. (1996) *Biochemistry 35*, 8761−8768.
20. Kuwahara, J., and Coleman, J. E. (1990) *Biochemistry 29*, 8627−8631.
21. Brenowitz, M., Senear, D. F., Shea, M. A., and Ackers, G. K. (1986) *Methods Enzymol. 130*, 132−181.
22. Kuwahara, J., Yonezawa, A., Futamura, M., and Sugiura, Y. (1993) *Biochemistry 32*, 5994−6001.
23. Smith, J. F., Hawkins, J., Leonard, R. E., and Hanas, J. S., (1991) *Nucleic Acids Res. 19*, 6871−6876.
24. Choo, Y., and Klug, A., (1993) *Nucleic Acids Res. 21*, 3341−3346.
25. Yokono, M., Saegusa, N., Matsushita, K., and Sugiura, Y. (1998) *Biochemistry 37*, 6824−6832.
26. Romaniuk, P. J., (1990) *J. Biol. Chem. 265*, 17593−17600.
27. Kadonaga, J. T., Jones, K. A., and Tjian, R. (1986) *Trends Biochem. Sci. 11*, 20−23.
28. Kriwacki, R. W., Schults, S. C., Steitz, T.A., and Caradonna, J. P. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 9759−9763.
29. Choo, Y., and Klug, A., (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 11163−11167.
30. Choo, Y., and Klug, A., (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 11168−11172.
31. Jamieson, A. C., Wang, H., and Kim, S., (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 12834−12839.
32. Greisman, H. A., and Pabo, C. O. (1997) *Science 275*, 657−661.
33. Makino, Y., Ohga, T., Toh, S., Koike, K., Okumura, K., Wada, M., Kuwano. M., and Kohno, K. (1996) *Nucleic Acids Res. 24*, 1873−1878.
34. Lipes, M. A., Napolitana, M., Jeang, K.-T., Chang, N. T., and Leonard, W. J. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 9704−9708.

BI9811112