Finger-Positional Change in Three Zinc Finger Protein Sp1: Influence of Terminal Finger in DNA Recognition†

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Received October 10, 2000; Revised Manuscript Received December 1, 2000

ABSTRACT: The connection of functional modules is effective for the design of DNA binding molecules with the desired sequence specificity. C_2H_2-type zinc finger proteins have a tandemly repeated array structure consisting of independent finger modules and are expected to recognize any DNA sequences by permutation, multi-connection, and the substitution of various sets of zinc fingers. To investigate the effects of the replacement of the terminal finger on the DNA recognition by other fingers, we have constructed the three zinc finger peptides with finger substitution at the N- or C-terminus, Sp1(zf223), Sp1(zf323), and Sp1(zf321). From the results of gel mobility shift assays, each mutant peptide binds preferentially to the target sequence that is predicted if the fingers act in a modular fashion. The methylation interference analyses demonstrate that in the cases of the N-terminal finger substitution mutants, Sp1(zf223) and Sp1-(zf323), the N-terminal finger recognizes bases to different extents from that of the wild-type peptide, Sp1(zf123). Of special interest is the fact that the N-terminal finger of the C-terminal finger substitution mutant, Sp1(zf321), shows a distinct base recognition from those of Sp1(zf123) and Sp1(zf323). DNase I footprinting analyses indicate that the C-terminal finger (*active finger*) induces a conformational change in the DNA in the region for the binding of the N-terminal finger (*passive finger*). The present results strongly suggest that the extent of base recognition of the N-terminal finger is dominated by the binding of the C-terminal finger. This information provides an important clue for the creation of a zinc finger peptide with the desired specificity, which is applicable to the design of novel drugs and biological tools.

The design of DNA targeting proteins with desired functions is one of the most significant problems in the *post-genome* era and may lead to the creation of new drugs and biological tools. To achieve this goal, it is necessary to establish the DNA recognition rule by the DNA binding molecules with a well-defined modular structure such as the C_2H_2-type zinc finger and pyrrole−imidazole polyamide (*1, 2*). The C_2H_2-type zinc finger motif has a tandemly repeated structure consisting of independent modules with the consensus sequence: (Tyr,Phe)-X-Cys-$X_{2,4}$-Cys-X_3-Phe-X_5-Leu-X_2-His-X_{3-5}-His-X_{2-6}. Each finger domain is mostly connected by a well-conserved linker and has a compact globular $\beta\beta\alpha$ structure due to tetrahedral binding of a zinc ion with invariant cysteines and histidines. The first crystal structural analysis of the Zif268−DNA complex has provided useful information about the DNA recognition by the C_2H_2-type zinc finger motif (*3, 4*). In the complex, each zinc finger makes direct base contacts by using amino acids at positions −1, 2, 3, and 6 in the α-helix that recognizes overlapping four-base-pair subsites mainly on the guanine-rich strands of the binding site. However, two unique features opposed to the modularity of the zinc finger have been reported in the DNA recognition by the zinc finger protein. One is the unbalanced influence of these fingers on the DNA binding observed in the DNA bindings of Zif268 (*5*), the C-terminal three fingers of TFIIIA (*6*), and WT1 (*7*). In them, the N-terminal fingers make particularly smaller contributions to the high-affinity DNA binding than the other fingers. The other influence is the relative finger position on the DNA binding of the finger. The order change of zinc fingers in an array and sequential selection via the phage display give rise to the unexpected effects on the DNA base recognition by zinc fingers (*8, 9*).

Transcription factor Sp1 is a sequence-specific DNA binding protein derived from the HeLa cell (*10, 11*). Sp1 has three contiguous repeats of a C_2H_2-type zinc finger motif at the C-terminus and activates transcription in various viral and cellular genes by binding to the GC box, which has the decanucleotide consensus sequence 5′-(G/T)GGGCGG(G/A)(G/A)(C/T)-3′ (*11*−*15*). Together with the crystallographic evidence of the Zif268−DNA complex, some previous studies about the Sp1−DNA interaction revealed that the three zinc fingers of Sp1 also make a different contribution to GC-box DNA binding (*16*−*19*). Namely, the contribution is reduced in the order: C-terminal finger > central finger > N-terminal finger. Moreover, the N-terminal zinc finger of Sp1 uniquely binds to DNA, whereas the central and C-terminal zinc fingers typically bind to DNA (*19*). The finger does not utilize His(3), but instead utilizes Lys(−1) for the recognition of the guanine bases at the center and the 3′-end of the 5′-GGG-3′ subsite despite prevalent

† This study was supported in part by a Grant-in-Aid for COE Project "Element Science" (12CE2005), Priority Project "Biometals" (08249103), and Scientific Research (10470493·12470505) from the Ministry of Education, Science, Sports, and Culture, Japan.

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10.1021/bi0023596 CCC: $20.00
Published on Web 01/17/2001

recognition of guanine at the center of the 5′-GGG-3′ subsite by His(3).

In this study, we prepared three mutant zinc finger peptides, Sp1(zf223), Sp1(zf323), and Sp1(zf321), and evaluated the effect of the terminal finger on the base recognition of the other fingers in the DNA binding of three zinc finger peptide. The wild-type and mutant peptides preferably bind to their predicted binding sequences. Methylation interference and DNase I footprinting analyses suggest that the different degrees of base recognition of the N-terminal finger of the mutant peptides from that of wild type is due to the DNA conformational change induced by the binding of the C-terminal finger.

MATERIALS AND METHODS

Materials. All enzymes were purchased from New England Biolabs (Beverly, MA), except for the restriction enzyme *Age*I obtained from Nippon Gene (Tokyo, Japan). The synthesized oligonucleotides for construction of the genes and substrate DNAs were acquired from Amersham Pharmacia Biotech. 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.

Preparation of Zinc Finger Peptides from Sp1. The primary structures of all the zinc finger peptides used in this study are summarized in Figure 1A. Sp1(zf123), which is the alias for Sp1(530−623), is coded on plasmid pEVSp1-(530−623) as previously described (*20*). For the creation of Sp1(zf223) and Sp1(zf323), the finger 2, 3, and 2−3 gene fragments were amplified by PCR with the primer set of pEVSp1(530−623) as a template. The amplified single-finger fragments were designed to be flanked by the N-terminal region with the *Bam*HI site at the 5′-end and the *Age*I site at the 3′-end. On the other hand, the double-finger fragments were amplified as they were designed to be flanked by the *Age*I site and the C-terminal region with the *Eco*RI site at the 5′- and 3′-ends, respectively. The *Age*I enzyme site in the linker region encodes amino acids Thr-Gly, part of the linker peptide between fingers 1 and 2. By digesting one set of single- and double-finger fragments with enzymes and ligating them into the similarly digested pEV3b, we constructed the plasmids pEVSp1(zf223) and pEVSp1(zf323), which code Sp1(zf223) and Sp1(zf323), respectively. The plasmid pEVSp1(zf321) coding Sp1(zf321) was created by ligating the N-terminal double-finger gene fragment from pEVSp1(zf323) and the finger 1 gene fragment from pEVSp1(530−623) in the same manner as described above. All sequences were confirmed by the *Bca*BEST Dideoxy Sequencing Kit (Takara Shuzo, Kyoto, Japan). These zinc finger peptides were overexpressed in *Escherichia coli* strain BL21-(DE3)pLysS and purified as previously described (*19*), except for the use of 1 mM dithiothreitol as the reductant.

Preparation of Substrate DNA Fragments. The substrate oligonucleotides contain the target binding site predicted from the binding mode of the transcription factor Sp1: GC(123), 5′-GGG GCG GGGCC-3′; GC(223), 5′-GGG GCG GCGGC-3′; GC(323), 5′-GGG GCG GGGGC-3′; GC(321)-1, 5′-GGGC GCG GGGGC-3′; and GC(321)-2, 5′-GGGCC GCG GGGGC-3′. The synthesized oligonucleotides were annealed and inserted in pBluescript II SK+ (Stratagene, La Jolla, CA). The *Hin*dIII−*Xba*I fragment was cut out and labeled at the 5′-end by ^{32}P for the experiments.

Circular Dichroism (CD) Measurements. The CD spectra of the wild-type and mutant zinc finger peptides of Sp1 were recorded on a Jasco J-720 spectropolarimeter in 10 mM Tris-HCl (pH 8.0), 50 mM NaCl, 1 mM dithiothreitol, and 5 μM zinc finger peptide at 20 °C.

Gel Mobility Shift Assays. Gel mobility shift assays were carried out under the following conditions. Each reaction mixture contained 10 mM Tris-HCl (pH 8.0), 50 mM NaCl, 1 mM dithiothreitol, 10 μM $ZnCl_2$, 25 ng/μL poly(dI-dC), 0.05% Nonidet P-40, 5% glycerol, 40 mg/μL BSA, the ^{32}P-end-labeled substrate DNA fragment (∼50 pM), and 0−4 μM zinc finger peptide. After incubation at 20 °C for 30 min, the sample was run on a 12% polyacrylamide gel with Tris−borate buffer at 20 °C. The bands were visualized by autoradiography and quantified with NIH image software (version 1.62). The dissociation constants (K_d) of the Sp1 peptide−DNA fragment complexes were estimated based on a previously reported procedure (*19*).

Methylation Interference Analyses. Methylation interference assays were investigated as previously described (*18*, *20*). The binding reaction mixture contained 10 mM Tris-HCl (pH 8.0), 50 mM NaCl, 1 mM dithiothreitol, 10 μM $ZnCl_2$, 20 or 25 ng/μL competitor DNA, 0.05% Nonidet P-40, 5% glycerol, the ^{32}P-end-labeled methylated DNA fragment (∼40 nM, 400 Kcpm), and 10−500 nM zinc finger peptides. As competitor DNAs, 20 ng/μL sonicated calf thymus DNA and 25 ng/μL poly(dI-dC) were used for the experiments shown in Figures 3 and 4, respectively. To examine both the strong and weak base contacts in the methylation interference experiment, we selected the experimental conditions such that the peptide/DNA molar ratio in the binding reaction was about 10−20% bound. Densitometric measurements were obtained with NIH image software (version 1.62). The extent of interference was estimated as previously described (*19*).

DNase I Footprinting Analyses. DNase I footprinting experiments were performed according to the method of Brenowitz et al. (*21*). The binding reaction mixture contained 20 mM Tris-HCl (pH 8.0), 15 mM NaCl, 5 mM $CaCl_2$, 10 mM $MgCl_2$, 20 ng/μL sonicated calf thymus DNA, the 5′-end-labeled substrate DNA fragment (∼8 nM, 20 000 cpm), and 0−10 μM peptide. After incubation at 20 °C for 30 min, the sample was digested with DNase I (0.75 milliunit/μL) at 20 °C for 2 min. The reaction was stopped by the addition of 20 μL of DNase I stop solution (0.1 M EDTA and 0.6 M sodium acetate) and 100 μL of ethanol. After ethanol precipitation, the cleavage products were analyzed on a 10% polyacrylamide/7 M urea sequencing gel. The bands were visualized by autoradiography.

RESULTS

Design of Mutant Zinc Finger Peptides, Sp1(zf223), Sp1-(zf323), and Sp1(zf321) and Their Predicted Binding Sequences. To study the effect of the terminal finger of the three finger peptide on DNA binding of the peptide, we prepared three mutant zinc finger peptides, Sp1(zf223), Sp1-(zf323), and Sp1(zf321) (Figure 1A, left panel). Sp1(zf223) and Sp1(zf323), in which the N-terminal finger 1 of Sp1-(zf123) is replaced with fingers 2 and 3, respectively, are

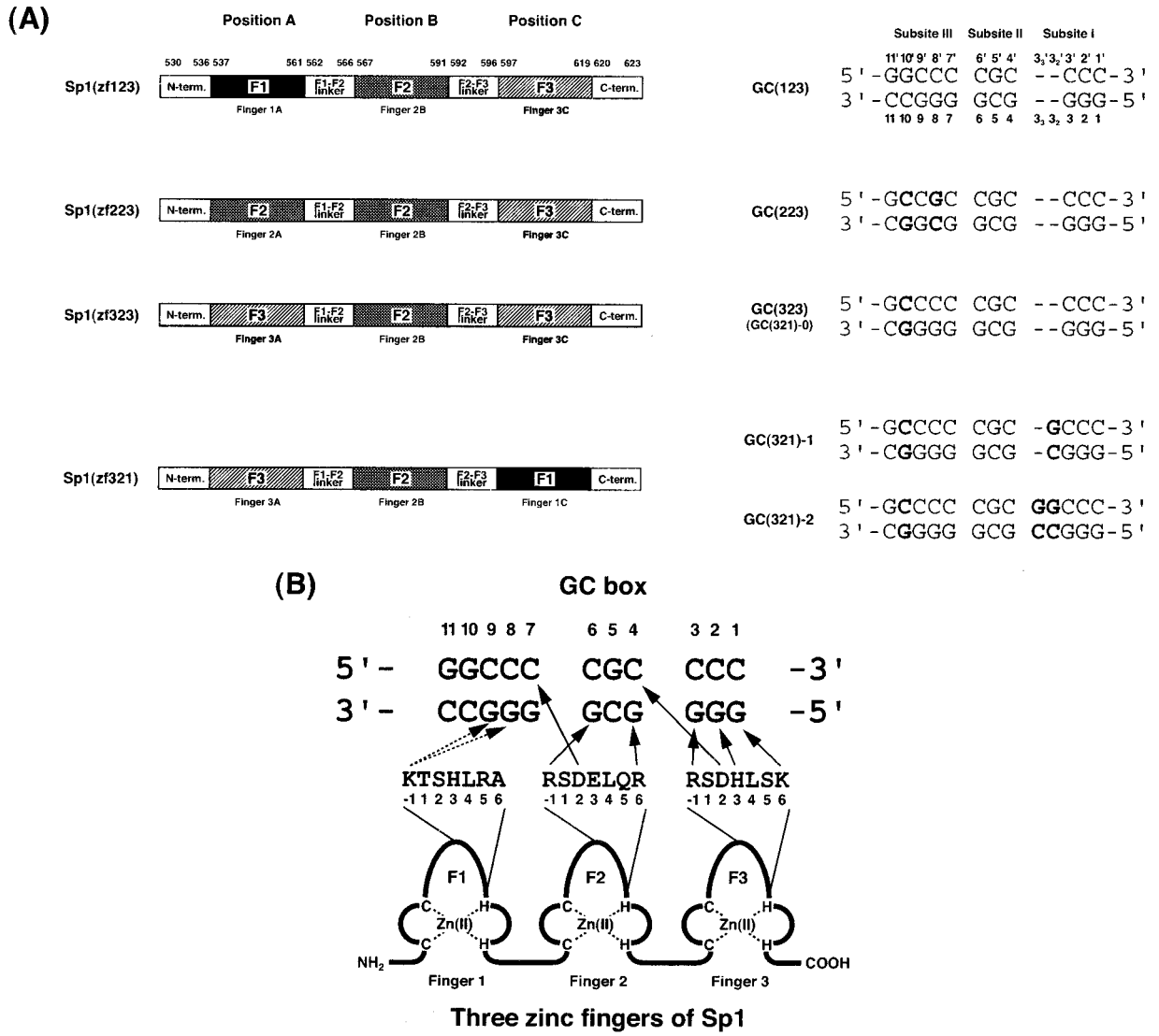

FIGURE 1: (A) Primary structures of wild-type and mutant zinc finger peptides of Sp1 (left) and their predicted binding sequences (right). The designation of each zinc finger is shown by the original name (fingers 1−3) with an alphabetical letter indicating the absolute position (positions A−C). Substituted or inserted nucleotides in the mutated GC-box sequences are depicted in boldface type. The base numbers in the wild-type and mutant GC boxes are also shown. (B) Mode of putative interaction of Sp1 with GC-box DNA. Amino acid residues at the N-terminus of the α-helix in each finger are depicted by their one-letter codes with the number of helical positions below. Solid arrows show the amino acid−base contacts assumed by the DNA binding mode of Zif268, and dotted arrows depict the contacts indicated by our previous report.

the mutants for the evaluation of the N-terminal finger properties. On the other hand, the effect of replacing the C-terminal finger on the DNA binding mode is estimated by the preparation of a mutant peptide, Sp1(zf321), which has three zinc fingers arrayed inversely in comparison with Sp1(zf123). Our previous study demonstrated that the finger 3-deleted mutant, Sp1(zf12), formed no stable complex with the GC-box DNA under our experimental conditions because of the small contribution of finger 1 to the DNA binding (*19*). Therefore, we did not design Sp1(zf121) but Sp1-(zf321), considering the deficiency of the DNA binding ability of Sp1(zf121).

The predicted binding sequences, which are designed from the putative DNA binding mode of Sp1 (Figure 1B; *19*), are shown in Figure 1A (right panel). The wild-type GC-box sequence, GC(123), is derived from the mouse dihydrofolate reductase promoter (I and III) (*12, 13*). GC(223) and GC-(323) are the target sequences for Sp1(zf223) and Sp1(zf323), respectively. From the results of the previous interference experiment for Sp1(zf123), finger 1A of Sp1(zf123) recognizes uniquely the five-base-pair subsite (*19*). On the contrary, the zinc finger proteins such as Zif268 exhibit the typical recognition of the three-base-pair subsite by each finger domain (*3*). To clarify whether the base recognition mode of finger 1C of Sp1(zf321) is typical or unique, we prepared two sequences, GC(321)-1 and -2, in addition to GC(321)-0 which is identical to the target sequence for Sp1-(zf323).

Examination of the Folding Property of Wild-Type and Mutant Zinc Finger Peptides of Sp1. To examine the change in the folding property by finger substitution, we analyzed the secondary structures of the peptides based on measurements of the CD spectra. Figure 2 shows the CD measurement results for the peptides at 20 °C. The spectrum for Sp1(zf123) was similar to those of the single- and three-finger peptides of Sp1 previously described (*22*−*24*). Negative Cotton effects in the far-UV region with a minimum at 206 nm and a shoulder around 222 nm suggest that Sp1-

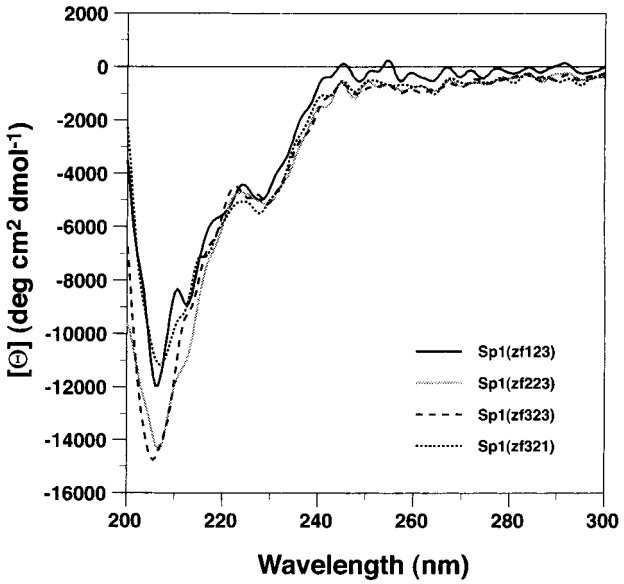

FIGURE 2: CD spectra of wild-type and mutant zinc finger peptides of Sp1 at 20 °C.

Table 1: Dissociation Constants (K_d) for Spl(zf123), Sp1(223), Sp1(zf323), and Sp1(zf321) Binding to Wild-Type (GC) and Mutant GC Boxes

	K_d (nM)[a]			
binding site[b]	Sp1(zf123)	Sp1(zf223)	Sp1(zf323)	Sp1(zf321)
GC(123)	5.3 ± 0.4	41 ± 2.2	24 ± 1.1	994 ± 45
GC(223)	57 ± 2.6	3.9 ± 0.2	60 ± 2.3	286 ± 29
GC(323)	22 ± 1.6	21 ± 1.0	6.6 ± 0.2	314 ± 15
GC(321)-1	714 ± 74	>4000	512 ± 57	>4000
GC(321)-2	>4000	>4000	>4000	ND[c]

[a] Apparent dissociation constants were determined by titration using a gel mobility shift assay as described under Materials and Methods. Values are averages of three or more independent determinations with standard deviations. [b] The nomenclature is described in the text (see Figure 1). [c] ND, not determined.

(zf123) has an ordered secondary structure. The spectrum for Sp1(zf321) was quite similar to that of Sp1(zf123). On the other hand, Sp1(zf223) and Sp1(zf323) exhibited spectra somewhat different from those of Sp1(zf123) and Sp1(zf321). As for the ellipticities at 206 nm, the values of Sp1(zf123) and Sp1(zf321) ($[\theta]_{206} = -11\,936$, $-10\,984$, respectively) were distinct from those of Sp1(zf223) and Sp1(zf323) ($[\theta]_{206} = -14\,205$, $-14\,673$, respectively). This is probably due to the difference in the composition of the fingers. These results indicate that the conformation of the finger domain in each peptide is not identical but comparable with that of each other. We obtained the same results at 4 °C (data not shown).

Evaluation of the Binding Affinity of Mutant Zinc Finger Peptides to Wild-Type and Mutant GC Boxes. By using gel mobility shift assays, we obtained the dissociation constants (K_d) of these peptide−DNA complexes as summarized in Table 1. Sp1(zf123) binds to GC(123) with a 5.3 nM dissociation constant, which is comparable to the previously reported value, whereas GC(223) and GC(323) dissociation constants were 57 and 22 nM, respectively. The K_d values for the Sp1(zf223)−GC(123), −GC(223), and −GC(323) complexes were 41, 3.9, and 21 nM, respectively. On the other hand, the K_d values for Sp1(zf323) binding to GC(123), GC(223), and GC(323) were 24, 60, and 6.6 nM, respectively. These results suggest that each mutant mentioned above binds to the predicted binding sequence with the highest affinity under our experimental condition.

On the contrary, Sp1(zf321) binds to the wild-type and mutant GC boxes with a 70−250-fold lower affinity than the other peptides. The dissociation constants of Sp1(zf321) for GC(123), GC(223), and GC(323) are 994, 286, and 314 nM, respectively, indicating that GC(223) and GC(323) are preferable to GC(123) for the binding of Sp1(zf321) to DNA. All peptides used in this study showed no effective binding to GC(321)-2. GC(321)-1 was bound only by Sp1(zf123) and Sp1(zf323) with 714 and 512 nM dissociation constants, respectively.

Specific Base Recognition Mode of Mutant Zinc Finger Peptides Revealed by Methylation Interference Analysis. Figure 3A shows the methylation interference patterns of Sp1(zf123), Sp1(zf223), and Sp1(zf323) for GC(123), GC(223), and GC(323), respectively. The extent of the interference based on a densitometric analysis is shown by histograms (Figure 3B). The interference patterns at subsites I and II were the same among these peptides. In contrast, distinct interference patterns were observed at subsite III. Namely, the recognition of G7 in Sp1(zf223) and Sp1(zf323) was 5-fold stronger than in Sp1(zf123), and the recognitions of G10 in Sp1(zf223) and Sp1(zf323) and G11′ in Sp1(zf223) were almost lost. In Figure 3C, we compared the extent of the interference of fingers 2 and 3 on the basis of the difference in the relative position, suggesting that the difference has a significant effect on the base recognition of the cognate fingers.

Figure 4 shows the results of the methylation interference assay for Sp1(zf321). From the results of the evaluation of K_d, Sp1(zf321) binds to GC(223) and GC(323) with a higher affinity than the other DNAs. Therefore, we carried out an experiment using such peptide−DNA combinations in which Sp1(zf323) was employed as the control. Panels A and B depict the electrophoretic results and the densitometric analysis for Sp1(zf321), respectively. There is no obvious difference in the extent of interference between the Sp1(zf321)− and Sp1(zf323)−GC(223) complexes. In the binding to GC(323), Sp1(zf321) shows an interference pattern distinct from that of Sp1(zf323). The bases at subsite III were not strongly recognized by Sp1(zf321), as is distinct from the case with Sp1(zf323).

Analysis of Conformational Changes of DNA Induced by Binding of Mutant Zinc Finger Peptides. Figure 5 shows the DNase I footprinting patterns of Sp1(zf123), Sp1(zf223), Sp1(zf323), and Sp1(zf321) for their high-affinity binding sequences. All mutant peptides protected the wild-type or mutant GC box from cleavage by DNase I in both strands. Hypersensitive cleavage was observed at the 5′-AA-3′ step outside the GC box in the guanine-rich strand (G-strand), whereas no cleavage induction by protein binding was detected in the cytosine-rich strand (C-strand). In addition, hypersensitive cleavage was also induced in the G-strand by the binding of Sp1(zf321) between G8 and G9 in the GC box. The same footprinting patterns were also obtained in the case of the wild-type and mutant Sp1 peptide−GC(123) complexes (data not shown).

DISCUSSION

Effects of Replacement of Finger 1 in Sp1(zf123) on DNA Binding Affinity and Specificity. Based on our mutational

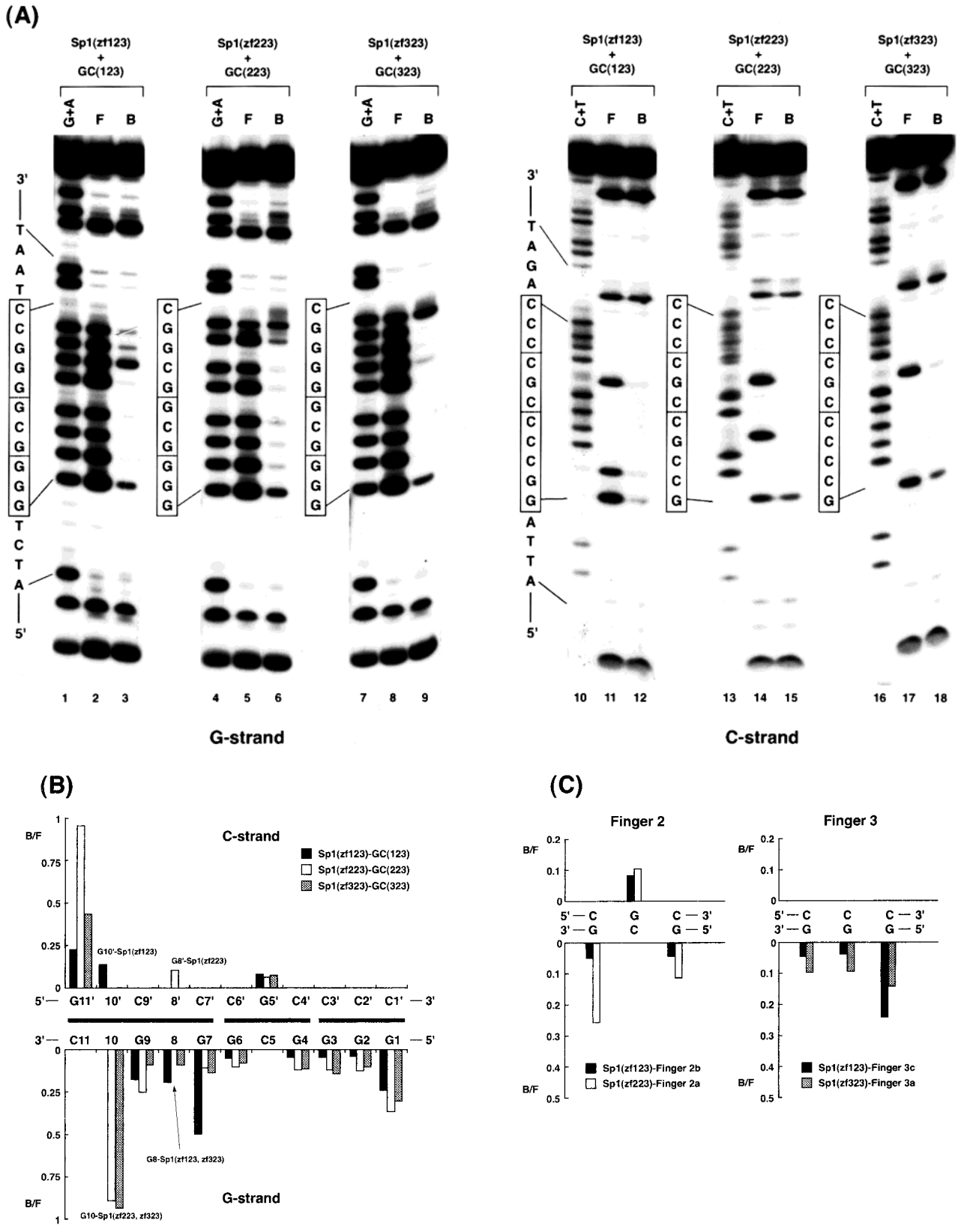

FIGURE 3: (A) Methylation interference analyses for Sp1(zf123), Sp1(zf223), and Sp1(zf323) binding to GC(123), GC(223), and GC(323), respectively. The left (lanes 1−9) and right (lanes 10−18) panels show the results for the G- and C-strands, respectively. Lanes 1, 4, and 7 and lanes 10, 13, and 16 contain G+A and C+T of the Maxam−Gilbert sequencing reactions, respectively. The remaining lanes represent free (F) and peptide-bound (B) DNA samples. In these experiments, calf thymus DNA was used as a competitor (see Materials and Methods for details). (B) A histogram showing the extent of methylation interference by Sp1(zf123), Sp1(zf223), and Sp1(zf323). Three or more autoradiograms of the gels were scanned with a densitometer, and the average extent of interference was calculated as the ratio of the cutting probabilities for the two bands (B/F). (C) Direct comparisons of the extent of interference between finger 2B of Sp1(zf123) and finger 2A of Sp1(zf223) (left) and between finger 3C of Sp1(zf123) and finger 3A of Sp1(zf323) (right).

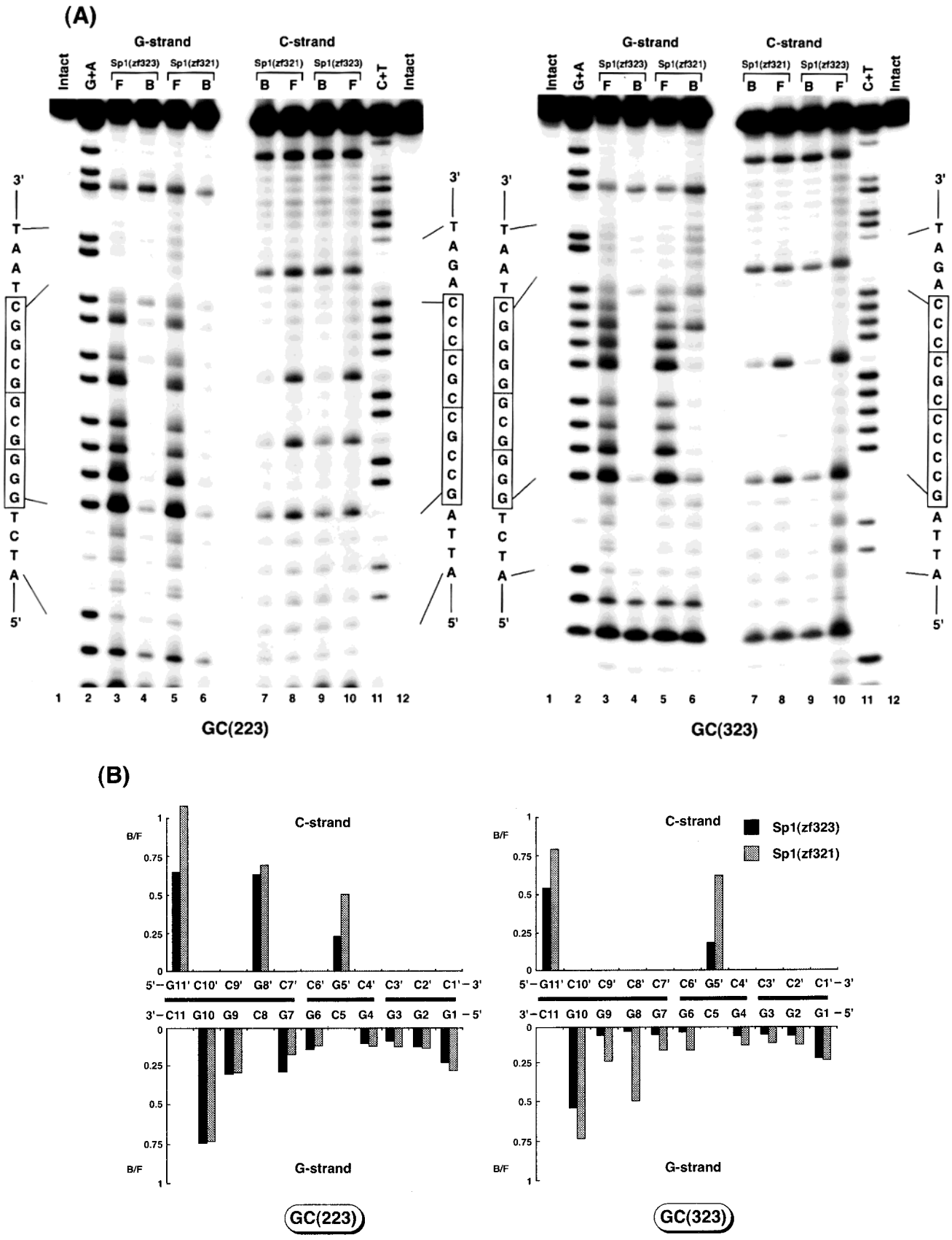

FIGURE 4: (A) Methylation interference analyses for Sp1(zf323) and Sp1(zf321) binding to GC(223) and GC(323). The left and right panels show the results for GC(223) and GC(323), respectively. Lanes 3−10 represent free (F) and bound (B) DNA samples. Lanes 2 and 11 contain G+A and C+T of the Maxam−Gilbert sequencing reactions, respectively. Lanes 1 and 12 show intact DNA. In these experiments, poly(dI-dC) was used as a competitor (see Materials and Methods for details). (B) Histograms showing the extent of methylation interference by Sp1(zf323) and Sp1(zf321) for GC(223) (left) and GC(323) (right). Two or more autoradiograms of the gels were scanned with a densitometer, and the average extent of interference was calculated as the ratio of the cutting probabilities for the two bands (B/F).

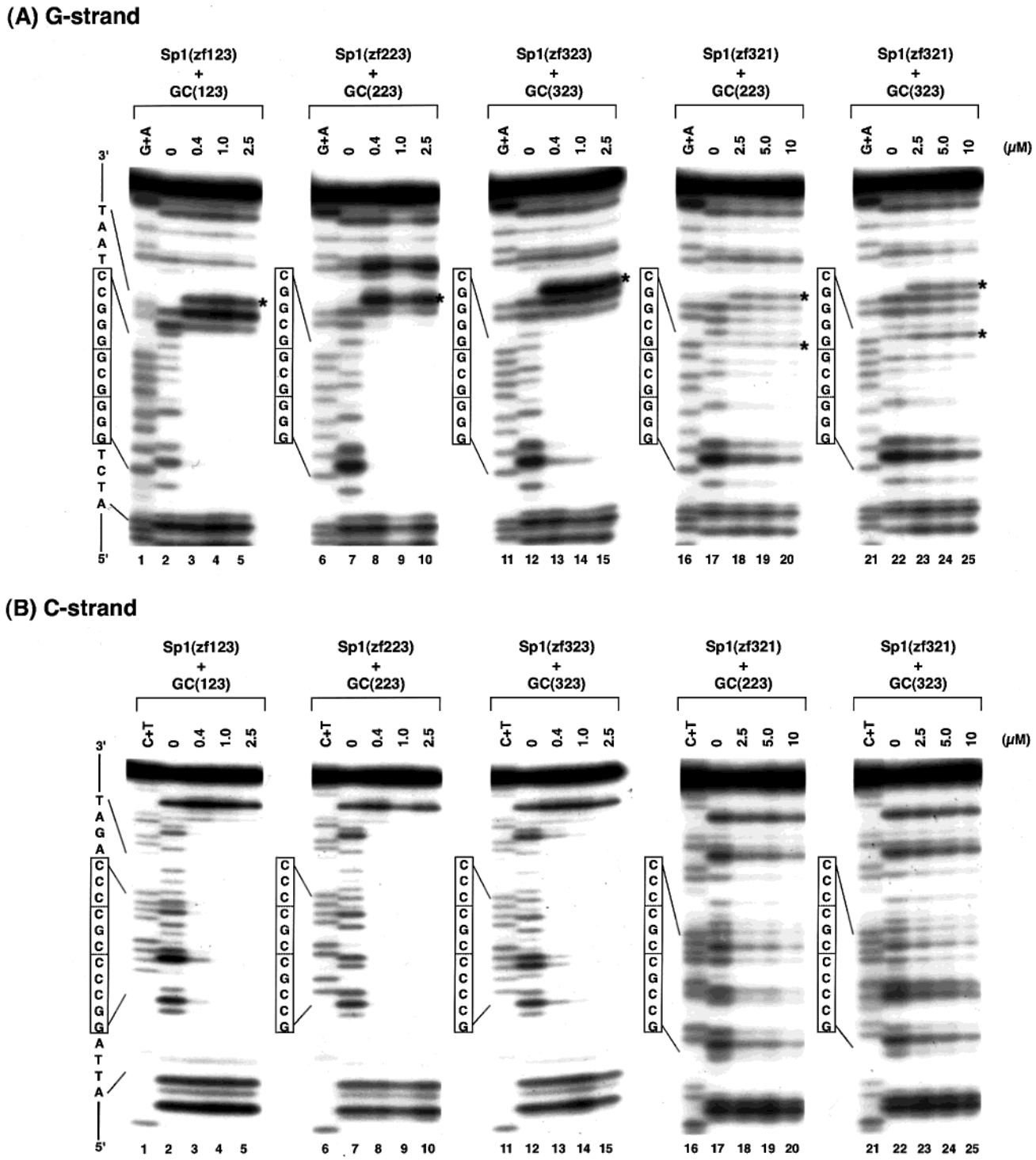

FIGURE 5: DNaseI footprinting analyses for Sp1(zf123), Sp1(zf223), Sp1(zf323), and Sp1(zf321) binding to their high-affinity binding sequence. Panels A and B show the results for the G- and C-strands, respectively. The asterisks represent the enhance sites of cleavage. Lanes 1, 6, 11, 16, and 21 in panel A, G+A (Maxam−Gilbert reaction products); lanes 1, 6, 11, 16, and 21 in panel B, C+T (Maxam−Gilbert reaction products). Peptide concentrations and the combinations of peptide and substrate DNA are noted in the figure.

analyses of Sp1(zf123) and the GC boxes previously reported (*19*), the relative contribution of the three fingers of Sp1(zf123) to the DNA binding affinity was shown to be reduced in the order of finger 3 > finger 2 > finger 1. It is expected that the replacement of finger 1 with finger 2 or 3 in Sp1(zf123) leads to an increase in the DNA binding affinity of Sp1(zf123). The results presented here show that both Sp1(zf223) and Sp1(zf323) preferentially bind to their predicted binding sequences with a dissociation constant comparable to that of Sp1(zf123). In contrast, the deletion of finger 1 from Sp1(zf123) results in an 89-fold reduction in the binding affinity to GC(123) (*19*). Together with this evidence, the results indicate that these mutants evidently make use of all three fingers in DNA binding and that the N-terminal fingers, fingers 2A and 3A of Sp1(zf223) and Sp1(zf323), respectively, make an equivalent contribution to the DNA binding of finger 1A of Sp1(zf123).

The extent of base recognition of Sp1(zf223) and Sp1(zf323) appears to be distinct in part from that of Sp1(zf123). From the results of the methylation interference analyses,

the two fingers at positions B and C of the mutant peptides maintain the same base recognition mode as that of Sp1-(zf123). With respect to their fingers at position A, the strong interference patterns of Sp1(zf223) and Sp1(zf323) at G7 in the G-strand suggest that they recognize bases at subsite I like fingers 1 and 2 of Zif268, respectively, in contrast to finger 1A of Sp1(zf123), which recognizes G8 and G9 by Lys(−1) and exhibits a weak interference pattern at G7. The direct comparison of the extent of base recognition by them with that in their native positions demonstrates the difference in the extent of recognition (Figure 3C). In Sp1(zf323), G7 is recognized 1.5-fold stronger than G1. The extent of the recognition is also stronger than that of G7 in Sp1(zf123) by a factor of 3.7. In the DNA binding of the zinc finger protein, the helicity of the recognition helix increases by the C-cap formation in the linkers connecting adjacent fingers at the C-terminal side (*25*). The increase in the recognition of G7 in the Sp1(zf323)−GC(323) complex may be elucidated by the increment of helicity induced by new C-cap formation in finger 3A of Sp1(zf323). However, the mutation of Ala(6) to arginine in finger 1A of Sp1(zf123), which was carried out for the increase in the recognition of G7 in the Sp1(zf123)−GC(123) complex considering the typical base recognition mode of the zinc finger peptide, had no drastic effect on the recognition of G7 in GC(123) despite the plausibility of the original C-cap formation in finger 1A of Sp1(zf123) (Matsushita and Sugiura, unpublished data). This evidence suggests that the extent of recognition of G7 by the finger at position A of these Sp1 peptides is undetermined only by the C-cap formation in the finger.

In contrast, the recognition of G9 by finger 2A of Sp1-(zf223) is 5-fold weaker than the recognition of G6 by finger 2B. A rationale for the disruption of the base recognition by a terminal zinc finger is reported as an end effect (*5*). In this theory, the base recognition by the amino acids such as Arg(18) and Arg(80), which are situated at positions −1 and 6 in the α-helices of fingers 1 and 3 of Zif268, respectively, is less sensitive to the mutation to glycine than the other critical amino acid residues. This is not applicable to our system for the following reason: no decrease in the base recognition by finger 3A of Sp1(zf323) was observed, whereas a similar decrease was detected in the Sp1(zf223)−GC(223) complex.

Effects of Replacement of Finger 3 in Sp1(zf123) on DNA Binding Affinity and Specificity. Our previous report revealed that finger 1A of Sp1(zf123) recognizes the five-base-pair subsite (*19*). On the basis of several X-ray crystallographic analyses (*3, 4, 26*−*29*), fingers 1C, 2B, and 3A of Sp1(zf321) are expected to bind to subsites I, II, and III, respectively, and the predicted binding sequence for finger 1C is not the five- but the three-base-pair site, 5′-NGG-3′, in the G-strand. The actual DNA binding affinity of Sp1(zf321) for various GC boxes estimated by the calculation of the K_d values suggests that a pertinent interaction between finger 1C and subsite III does not occur in GC(321)-1 or -2, but in GC-(123), GC(223), and GC(323). The inability of Sp1(zf321) binding to GC(321)-1 and -2 also demonstrates that Sp1-(zf321) did not bind to the DNA by the N-terminal two-finger domain in contrast to the ability of the two-finger peptide, Sp1(zf23), to bind to GC(123) (*19*). This evidence indicates that the constitution and order of the finger is important for DNA binding of the zinc finger protein with a high affinity and specificity despite the sufficiency of the two-finger domain for DNA binding. For the DNA binding of the two-finger peptide composed of fingers 2 and 3, fingers 2 and 3 need to be aligned in the direction from amino- to carboxyl-termini.

As evidenced by the comparison of the results of the methylation interference analyses and estimation of the K_d values of Sp1(zf321) with those of other mutants, the base recognition mode of finger 1C of Sp1(zf321) is distinct from that of finger 1A of Sp1(zf123). Finger 1C of Sp1(zf321) appears to have the typical base recognition mode of the zinc finger protein. That is to say, Lys(−1) and His(3) recognize G3 and G2 in subsite I. G1 might be recognized by Arg(5). The base recognition by Arg(5) was also discovered in the GLI−DNA complex, in which Arg(5) in finger 5 recognizes guanine at position 4′ (*26*). In addition, it is of special interest that the base recognition mode of finger 3A of Sp1(zf321) is clearly different from that of finger 3A of Sp1(zf323). Replacement of finger 3C by finger 1 in Sp1(zf323) induced a 15-fold reduction in the extent of recognition of G8 by finger 3A. As mentioned earlier, the extent of base recognition by the finger at position A is distinct from that of the finger at the native position in the cases of Sp1(zf223) and Sp1(zf323). Therefore, the zinc fingers at positions A and C are *passive* and *active* fingers, respectively.

Effects of Conformational Change of DNA Induced by the Binding of Active Finger on DNA Binding of Passive Finger. Several conformational changes of DNA induced by the binding of the zinc finger protein, such as bending (*20, 27, 29, 30*), local distortion (*18*), and unwinding (*31*), have been reported. DNA bending is induced by the bindings of Tramtrack, Sp1, and TFIIIA. Based on our CD data for the wild-type and mutant peptides, no drastic conformational change in each finger domain appears to occur, suggesting that the conformational change of DNA has effects on the DNA binding of the peptides. For the investigation of the existence of DNA bending, DNase I footprinting analyses are available, since hypersensitive cleavage of DNA by DNase I induced by the binding of protein is generally attributed to a conformational change in the DNA, in particular bending (*32*). In fact, such a hypersensitive cleavage has also been observed in the 3′-region outside the GC-box in the G-strand for the binding of the zinc finger of Sp1 to the GC-box DNA, and this is consistent with the evidence of DNA bending by circular permutation analyses (*19, 20, 30*). Therefore, we applied DNase I footprinting analyses to the examination of the DNA bending by the Sp1 mutants. Our footprinting results clearly show that Sp1-(zf321) produces bending of the GC-box DNA at subsite III. The hypersensitive cleavage at the G8−G9 step does not occur in the binding of Sp1(zf323), indicating that finger substitution at position C in Sp1(zf323) induces a structural change. The other conformational change of DNA by Sp1 binding is local distortion of the GC-box DNA in the 3′-region of the G-strand revealed by our previous footprinting analyses (*18*). In the binding of Sp1(zf123) to GC(123), finger 1A recognizes the bases of the region in a unique manner. Together with the result that finger 1 shows the typical base recognition mode by transferring from positions A to C, it is suggested that the conformational distortion has an effect on the base recognition mode and/or extent of the

zinc finger at position A. Moreover, the distortion is induced by the binding of the C-terminal two fingers of Sp1(zf123) (Sogo et al., unpublished data), indicating that Sp1(zf223) and Sp1(zf323) also cause a distortion in subsite III by the C-terminal two fingers upon binding to DNA and fingers 2A and 3A may show different extents of base recognition than that in the native positions. In the mechanism described above, the N- and C-terminal fingers in the three-finger-peptide are *passive* and *active* fingers for base recognition of DNA, respectively.

In this paper, we report "the active and passive fingers mechanism in DNA recognition by three-zinc-finger peptide" which is based on the conformational change in DNA induced by the C-terminal finger. This concept is not always applicable to the DNA binding of other zinc finger proteins. In fact, all zinc fingers equivalently bind to DNA without any induction of a conformational change in DNA during the DNA binding of Zif268 and YY1 (*3*, *4*, *28*). In the N-terminal six zinc fingers of the TFIIIA−DNA complex, however, DNA is bent in the binding regions of fingers 1 and 2 (*29*). Recently, we showed that the three-zinc-finger domain binds to DNA as one unit during the DNA binding of the zinc finger protein by multiconnection of identical zinc fingers (Nagaoka et al., unpublished data). Therefore, in the design of zinc finger proteins with a novel sequence preference, selection of the third finger (*passive finger*) at the N-terminus of a two-finger domain containing an *active finger* is desirable. Thus far, the DNA recognition code of the zinc finger has been analyzed by a phage display method (*33*−*36*). While most of sequences can be recognized by zinc finger proteins, unrecognized sequences remain. We may overcome this problem by considering the presented information in the design of novel zinc finger peptides. Additionally, such peptides give promise of the application of the zinc finger peptide to the design of novel drugs and biological tools.

SUPPORTING INFORMATION AVAILABLE

One figure showing DNase I footprinting analyses of wild-type and mutant peptides for GC(123) (1 page). This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Wolfe, S. A., Nekludova, L., and Pabo, C. O. (1999) *Annu. Rev. Biophys. Biomol. Struct. 29*, 183−212.
2. Dervan, P. B., and Bürli, R. W. (1999) *Curr. Opin. Chem. Biol. 3*, 688−693.
3. Pavletich, N. P., and Pabo, C. O. (1991) *Science 252*, 809−817.
4. Elrod-Erickson, M., Rould, M. A., Nekludova, L., and Pabo, C. O. (1996) *Structure 4*, 1171−1180.
5. Choo, Y. (1998) *Nucleic Acids Res. 26*, 554−557.
6. Zang, W.-Q., Veldhoen, N., and Romaniuk, P. J. (1995) *Biochemistry 34*, 15545−15552.
7. Nakagama, H., Heinrich, G., Pelletier, J., and Housman, D. E. (1995) *Mol. Cell. Biol. 15*, 1489−1498.
8. Desjarlais, J. R., and Berg, J. M. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 2256−2260.
9. Wolfe, S. A., Greisman, H. A., Ramm, E. I., and Pabo, C. O. (1999) *J. Mol. Biol. 285*, 1917−1934.
10. Dynan, W. S., and Tjian, R. (1983) *Cell 32*, 669−680.
11. Kadonaga, J. T., Carner, K. R., Masiarz, F. R., and Tjian, R. (1987) *Cell 51*, 1079−1090.
12. Kadonaga, J. T., Jones, K. A., and Tjian, R. (1986) *Trends Biochem. Sci. 11*, 20−23.
13. Bucher, P. (1990) *J. Mol. Biol. 212*, 563−578.
14. Miller, J., McLachlan, A. D., and Klug, A. (1985) *EMBO J. 4*, 1609−1614.
15. Brown, R. S., Sander, C., and Argos, P. (1985) *FEBS Lett. 186*, 271−274.
16. Kriwacki, R. W., Schultz, S. C. Steitz, T. A., and Caradonna, J. P. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 9759−9763.
17. Berg, J. M. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 11109−11110.
18. Kuwahara, J., Yonezawa, A., Futamura, M., and Sugiura, Y. (1993) *Biochemistry 32*, 5994−6001.
19. Yokono, M., Saegusa, N., Matsushita, K., and Sugiura, Y. (1998) *Biochemistry 37*, 6824−6832.
20. Nagaoka, M., and Sugiura, Y. (1996) *Biochemistry 35*, 8761−8768.
21. Brenowitz, M., Senear, D. F., Shea, M. A., and Ackers, G. K. (1986) *Methods Enzymol. 130*, 132−181.
22. Kuwahara, J., and Coleman, J. E. (1990) *Biochemistry 29*, 8627−8631.
23. Frankel, A. D., Berg, J. M., and Pabo, C. O. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 4841−4845.
24. Hori, Y., Suzuki, K., Okuno, Y., Nagaoka, M., Futaki, S., and Sugiura, Y. (2000) *J. Am. Chem. Soc. 122*, 7648−7653.
25. Laity, J. H., Dyson, J., and Wright, P. E. (2000) *J. Mol. Biol. 295*, 719−727.
26. Pavletich, N. P., and Pabo, C. O. (1993) *Science 261*, 1701−1707.
27. Fairall, L., Schwabe, J. W. R., Chapman, L., Finch, J. T., and Rhodes, D. (1993) *Nature 366*, 483−487.
28. Houbaviy, H. B., Usheva, A., Shenk, T., and Burley, S. K. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 13577−13582.
29. Nolte, R. T., Conlin, R. M., Harrison, S. C., and Brown, R. S. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 2938−2943.
30. Sjøttem, E., Andersen, C., and Johansen, T. (1997) *J. Mol. Biol. 267*, 490−504.
31. Shi, Y., and Berg, J. M. (1996) *Biochemistry 35*, 3845−3848.
32. Suck, D., Lahm, A., and Oefner, C. (1988) *Nature 332*, 464−468.
33. Reber, E. J., and Pabo, C. O. (1994) *Science 263*, 671−673.
34. Choo, Y., and Klug, A. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 11163−11167.
35. Choo, Y., and Klug, A. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 11168−11172.
36. Jamieson, A. C., Kim, S.-H., and Wells, J. A. (1994) *Biochemistry 33*, 5689−5695.

BI0023596