Distinct Phosphate Backbone Contacts Revealed by Some Mutant Peptides of Zinc Finger Protein Sp1: Effect of Protein-Induced Bending on DNA Recognition†

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ABSTRACT: By using some mutant peptides of transcription factor Sp1, phosphate backbone contacts with the DNA binding protein containing three zinc fingers have been investigated by alkylation interference, circular permutation, DNase I footprinting, and methylation protection methods. The ethylation interference analyses of Sp1(R565S) and Sp1(K595S) mutants demonstrate that arginine at 565 position and lysine at 595 position interact with the phosphate between G(3) and G(4) and with the phosphate between G(9) and G(10) in GC-box DNA, respectively. On the basis of the experimental results for Sp1(K535G), Sp1(537−623), and Sp1(530−623), lysine and glutamine at 535 and 536 positions have been clarified to be in contacts with phosphate between G(7) and G(8) and with phosphate outside GC-box, respectively. In particular, glutamine at the N-terminal side of zinc finger 1 is a key amino acid residue to induce DNA bending and also participates in total base specificity of Sp1. The present study strongly indicates that (1) each zinc finger is not independent for the DNA interaction with Sp1 and (2) DNA base recognition of the zinc finger protein is influenced by local conformational change of DNA induced by the protein binding.

Zinc finger of C_2H_2-type is a ubiquitous DNA-binding motif found in a variety of eukaryotic regulartory proteins and has 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}. In a C_2H_2-type zinc finger, the metal-dependent domain consists of a first β-strand, a turn, a second β-strand, a turn, and a helix. Invariant Cys and His residues are involved in chelating a zinc ion tetrahedrally and form a globular domain with a hydrophobic core made by three well-conserved hydrophobic amino acid residues (Frankel et al., 1987; Berg, 1988, Párraga et al., 1988; Lee et al., 1989a,b, 1991; Michael et al., 1992). This tetrahedral coordination structure can also be produced by the binding of cobalt(II), cadmium(II), ferrous(II), manganese(II), and nickel(II) ions, and such a metal substitution affects the sequence specificity of DNA by zinc finger protein (Frankel et al., 1987; Krizek & Berg, 1992; Thiesen & Bach, 1991a; Nagaoka et al., 1993). Amino acid residues at four key positions in or near the helix seem to play an important role in specific base contacts (Berg, 1992; Pavletich & Pabo, 1991, 1993; Fairall et al., 1993). The observations suggest that the mutations of these key amino acid residues in zinc finger are valuable for design of new proteins to recognize any DNA sequences. Several mutagenic experiments to achieve this aim have been carried out (Thiesen & Bach, 1991b; Nardelli et al., 1991, 1992; Desjarlais & Berg, 1992, 1993; Thukral et al., 1992; Choo & Klug, 1994a,b; Jamieson et al., 1994; Rebar & Pabo, 1994; Taylor et al., 1995). Zinc finger motif is very attractive for *de novo* design of a new DNA binding protein, because it recognizes an asymmetric DNA sequence. Indeed, zinc finger is versatile for the constructions of artificial restriction enzyme and transcription repressor (Nagaoka et al., 1994; Choo et al., 1994).

DNA binding protein specifically binds to DNA through the recognitions of its bases, sugar−phosphate backbone, and helical structure (Pabo & Sauer, 1992). Although the recognition of DNA bases is well-investigated, phosphate backbone contacts and local conformation of DNA are also important for protein−DNA interaction. In particular, the backbone contacts are significant because they govern the geometry between protein and DNA and thereby enhance the sequence-specific binding of protein (Pabo & Sauer, 1992). Here, we investigated DNA backbone contacts by three zinc fingers from human transcription factor Sp1 that binds to the decanucleotide region, termed GC-box, in the upstream sequence of several human or viral promoters. The deletion and/or change of specific amino acid residues was conducted in the linker and front regions of the first zinc finger. The results of methylation interference for these mutants revealed that (1) the amino acid residues at linker region have little effect on base specificity of the zinc finger and (2) glutamine of the position 536, which locates at N-terminal side of the first zinc finger, significantly influences the base specificity. In addition, CPA,[1] DNase I footprinting analysis, and methylation protection analysis clarified that the structural alteration of DNA is clearly induced by the interaction between backbone phosphate of DNA and glutamine (536 position) of the zinc finger protein.

MATERIALS AND METHODS

Materials. All enzymes were purchased from New England Biolabs (Beverly, MA), except for restriction enzymes *Xho*I, *Bgl*II, and *Sal*I obtained from Takara Shuzo (Kyoto, Japan). Labeled compound [γ-^{32}P]ATP was supplied with DuPont. Dimethyl sulfate and *N*-nitroso-*N*-ethylurea

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[1] Abbreviations: bp, base pair; G-strand, guanine-rich strand of GC-box sequence in pCPA4; C-strand, cytosine-rich strand of GC-box sequence in pCPA4; CPA, circular permutation assay; DNase I, deoxyribonuclease I.

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were obtained from Aldrich (Milwaukee, WI) and Sigma (St. Louis, MO), respectively. All other chemicals were of commercial reagent grade.

Preparation of Zinc Finger Fragment from Sp1. The plasmid pBS-Sp1-fl was kindly provided by Dr. R. Tijan. The *Bam*HI−*Hin*dIII fragment (1062 bp) of pBS-Sp1-fl containing zinc finger domain (530−696 residues) was inserted into pUC118 (Takara Shuzo, Kyoto) to prepare a single-stranded DNA. This construct was designated pUCSp1F. For all mutagenesis, the method using uracil-containing DNA was employed (Kunkel et al., 1991). An *Eco*RI site was made immediately after the stop codon on pUCSp1F, and its *Bam*HI−*Eco*RI fragment (503 bp) was cut out and inserted into pUC119. This plasmid was renamed as pUCSp1(530−696). In order to delete the C-terminal 73 amino acid residues (624−696 residues), a nonsense codon was made at glycine of the position 624 on pUCSp1(530−696). From this plasmid renamed as pUCSp1-(530−623), Sp1(530−623), its deletion mutant, and its single-point mutants were prepared. The resultant single-point mutants were termed according to the following rule; the mutant replaced by glycine of lysine at position 535 was named as Sp1(K535G). Regarding the construction of a deletion mutant, Sp1(537−623), glutamine (536 position) was altered to methionine with new *Nde*I site. In the cases of single-point mutants, Sp1(K535G), Sp1(R565S), Sp1-(R565K), and Sp1(K595S), the target amino acid residues were changed into the desired amino acid residues. In order to overexpress these peptides in *Escherichia coli*, the *Bam*HI−*Eco*RI fragment (530−623) of Sp1, the single-points mutants, or the *Nde*I−*Eco*RI fragment (537−623) of Sp1 was cut out from pUCSp1(530−623) or their respective derivatives and then inserted into similarly digested plasmid pEV3b, namely, a pET3b (Novagen, Madison, WI) derivative without "N-terminal T7•Tag sequence", to construct pEVSp1 or its derivatives. All sequences of the mutated or inserted regions were confirmed by DNA sequence analysis using *Bca*BEST dideoxy sequencing kit (Takara Shuzo, Kyoto). The methods for the overexpression, the purification, and the holding of zinc finger fragment were the same as the previous method (Kuwahara & Coleman, 1990).

Preparation of Plasmid for Circular Permutation Assay. We constructed a new plasmid "pCPA5" for CPA. This plasmid is based on pIBI24 (International Biotechnologies Inc., New Haven, CT). Eight oligonucleotides that are analogous to sequences of the multicloning site of pIBI24 were synthesized on an Applied Biosystems 391 DNA synthesizer and annealed to make four DNA cassettes. The first cassette was inserted between a unique *Eco*RI site and a *Hin*dIII site of pIBI24 (pCPA1), and this plasmid was cleaved by *Hin*dIII. The second cassette was inserted in (pCPA2) and then digested at a single unique *Hin*dIII site. The third cassette was ligated to (pCPA3). Repeating this step once again, we constructed pCPA4 which contains single GC-box and is a precursor of the plasmid (pCPA5) for circular permutation assay. The smaller fragment of *Bln*I−*Nhe*I of pCPA4 was inserted into pCPA4 at the *Nhe*I site to obtain pCPA5. The insert sequence was ascertained by using *Bca*BEST dideoxy sequencing kit (Takara Shuzo, Kyoto).

Circular Permutation Assay. CPA was carried out under two conditions according to the modified method of Crothers et al. (1991). Under the condition that the peptide/GC-box DNA molar ratio is very high, binding reaction mixture (4 μL) was prepared as follows: various restricted fragments from pCPA5 were dephosphorylated by calf intestinal alkaline phosphatase, purified by a 5% nondenaturing polyacrylamide gel, and ^{32}P-labeled at 5′-end with T4 polynucleotide kinase. The Sp1 mutant (5 pmol) was added to the DNA solution containing 2.5 mM Tris and 0.4 μg of sonicated calf thymus DNA. In the experiment with excess of GC-box DNA, the binding reaction mixture (4 μL) contained 2.5 mM Tris, 5 pmol of peptide, and 3.0 μg of restriction fragment that the 3 kb vector fragment for competitor DNA was not removed. No action was taken to remove vector fragment which serves as competitor DNA. After incubation at 20 °C for 30 min, loading dye (1 μL) was added to the mixture immediately before electrophoresis on a 5% nondenaturing polyacrylamide gel (acrylamide/bisacrylamide = 29:1). After the electrophoresis at 150 V for 1 h in 22.5 mM Tris-borate buffer, the bands were visualized by autoradiography or ethidium fluorescence.

Manipulation of Singly End-Labeled DNA Fragment. The end-labeled DNA fragments for footprinting and interference experiments were prepared from pCPA4. To gererate the fragment labeled at the 5′-end of guanine-rich strand (G-strand), a *Bgl*II-digested pCPA4 restriction fragment was dephosphorylated, ^{32}P-labeled, and recut with *Mlu*I. The produced singly end-labeled fragment (90 bp) was purified with a 5% nondenaturing polyacrylamide gel. For the opposite strand (C-strand), the labeling procedure is the same as for G-strand, except for usages of *Mlu*I and *Bgl*II at the first digestion and the cutting after labeling, respectively.

Ethylation Interference Analysis. Ethylation interference experiment was carried out according to the method of Wissmann and Hillen (1991). In 200 μL of a cacodylate buffer containing 1 μg of sonicated calf thymus DNA, the 5′-end-labeled *Bgl*II−*Mlu*I fragment of pCPA4 was partially ethylated by 50% *N*-nitroso-*N*-ethylurea at 50 °C for 1 h. After the termination of ethylation with ethanol precipitation, the precipitated DNA was lyophilized. Binding reaction mixtures (50 μL) contained the ethylated restriction fragment (0.6 pmol), the Sp1 mutant (0.6−1.2 pmol), and 10 mM Tris buffer (pH 8.0). After incubation at 20 °C for 30 min, the peptide-bound and free DNAs were separated on a 5% nondenaturing polyacrylamide gel and eluted from the gel with a standard elution buffer. The recovered DNAs were reacted in 35 μL of 10 mM phosphate buffer (pH 7.0) containing 1 mM EDTA and 143 mM NaOH at 90 °C for 30 min and then lyophilized. The cleavage products were analyzed on a 15% polyacrylamide/7 M urea sequencing gel. The bands were visualized by autoradiography, and the densitometric analysis was performed with a Pharmacia LKB 2222 Ultra Scan laser densitometer.

Methylation Interference and Protection Analyses. Methylation interference and protection analyses were investigated as described previously (Kuwahara et al., 1993). The DNA used in these studies was the 5′-end-labeled *Bgl*II−*Mlu*I fragment of pCPA4. To investigate 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 one to two (5−10% bound).

DNase I Footprinting Analysis. DNase I footprinting analysis was carried out according to the method of Brenowitz et al. (1986). Each reaction mixture (final volume 20 μL) contained 20 mM Tris buffer (pH 8.0), 22.5 mM

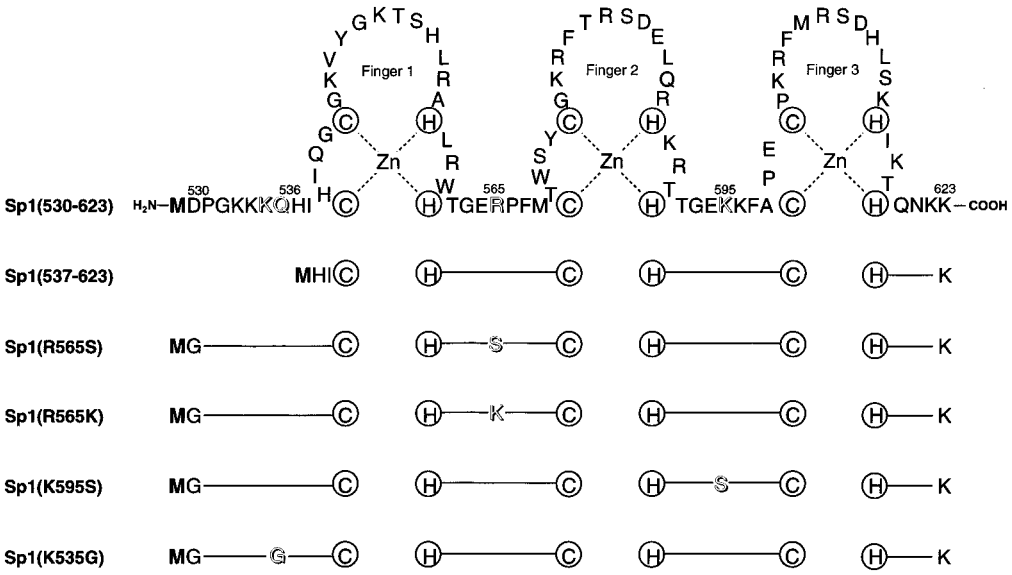

FIGURE 1: Amino acid sequences of various peptide mutants of Sp1 written by one-letter codes. Invariant ligands, cysteines, and histidines are circled. The N-terminal methionines and the mutated residues are bolded and shaded, respectively. The numbers on some residues are the numbers of amino acid sequences in native Sp1. Lines depict homology with the wild type, and the nomenclature is explained in the text.

NaCl, 5 mM $CaCl_2$, 10 mM $MgCl_2$, 0−30 pmol of the Sp1 mutant, 0.4 μg of sonicated calf thymus DNA, and the 5′-end-labeled *Bgl*II−*Mlu*I fragment of pCPA4. After incubation at 20 °C for 30 min, the sample was digested with DNase I (final concentration 0.7 unit/mL) at 20 °C for 2 min. In order to stop the reaction, 20 μL of DNase I stop solution (0.1 M EDTA and 0.6 M sodium acetate) and 100 μL of ethanol were added to the sample solution. The cleavage products were lyophilized and then analyzed on a 15% polyacrylamide/7 M urea sequencing gel. The bands were visualized by autoradiography.

RESULTS

Ethylation and Methylation Interference Analyses. Figure 1 summarizes the three zinc finger domain of Sp1 and its some mutants employed in our experiment. Figure 2 shows the experimental result of ethylation interference analysis, suggesting the interaction between phosphate backbone of DNA and amino acid residues of Sp1 peptides. The contact points obtained from the densitometric estimation of Figure 2B are given in Figure 3. The three zinc finger domain of Sp1 revealed clear contacts with the phosphates in both the DNA strands, in particular overlap contacts at the positions from five to eight in the GC-box. The results for mutants Sp1(R565S) and Sp1(K595S) demonstrated that arginine (position 565) and lysine (position 595) interact with the phosphates between G(3) and G(4) and between G(9) and G(10), respectively. The result for Sp1(R565K) was the same as that for Sp1(530−623) (data not shown). By comparison of the results for Sp1(K535G), Sp1(537−623), and Sp1(530−623), the backbone contacts of lysine (position 535) and glutamine (position 536) were detected. Namely, K535 and Q536 interact with the phosphate between G(7) and G(8) and with the phosphate outside GC-box, respectively. In addition to K535, Sp1 possesses other lysine residues, K533 and K534. In order to clarify roles of these lysine residues, we prepared two mutants, Sp1(K533G) and Sp1(K534G). The results of ethylation interference for Sp1(K533G) and Sp1(K534G) were identical to that for Sp1(530−623) (data not shown).

Figure 4 presents the results of the methylation interference analyses for diverse Sp1-peptide mutants. To obtain the information for not only strong contacts but also weak contacts, we performed the experiments under the condition that 5−10% of DNA are bound by the peptide (Clemens et al., 1992). In the C-strand (Figure 4, panel, bottom), Sp1(530−623) and our all mutants indicated contacts with G(5′), G(10′), and G(11′). However, these three contacts were clearly weakened in Sp1(537−623). In the G-strand (Figure 4, panel, top), Sp1(530−623), Sp1(K595S), and Sp1(K535G) showed the almost same results. In these cases, the strong contacts at G(2), G(3), G(4), and G(6) and the weak contacts at G(1), G(8), and G(9) were determined. In Sp1(R565S) and Sp1(R565K), on the other hand, the contacts at G(1), G(8), and G(9) were stronger, and a weak contact at G(7) was newly observed. Very interestingly, Sp1(537−623) produced no strong contacts with the bases of GC-box DNA. The weak contacts at G(2), G(3), and G(4) and also the considerably weak contacts at G(1) and G(6) were detected. To bear out lowering of the base specificity of Sp1(537−623), competition assays with poly(dI-dC) or poly(dA-dT) were carried out. The DNA binding of Sp1(530−623) was not inhibited remarkably by these competitors, whereas that of Sp1(537−623) was strongly prevented. Nevertheless, the dissociation constants of these mutants were evaluated to be approximately 4 nM, and the value was parallel to that of Sp1(530−623).

Circular Permutation Assay. Figure 5 shows the DNA constructs and the result of CPA. Several restriction sites exist on both the sides of GC-box at regular intervals in pCPA5 (Figure 5B). By using various restriction fragments prepared by the digestion, we performed CPA. Figure 5C-(a) gives the electrophoretic results for DNA only, Sp1(530−623), and Sp1(537−623) in the reaction with excess of peptide. All the restriction fragments of DNA exhibited the mobility same to the control DNA. In Sp1(530−623), by contrast, the fragment containing the GC-box situated near the molecular center moved at a minimum distance. The difference of mobility distance among the respective lanes was not large, but clear and reproducible. On the other hand,

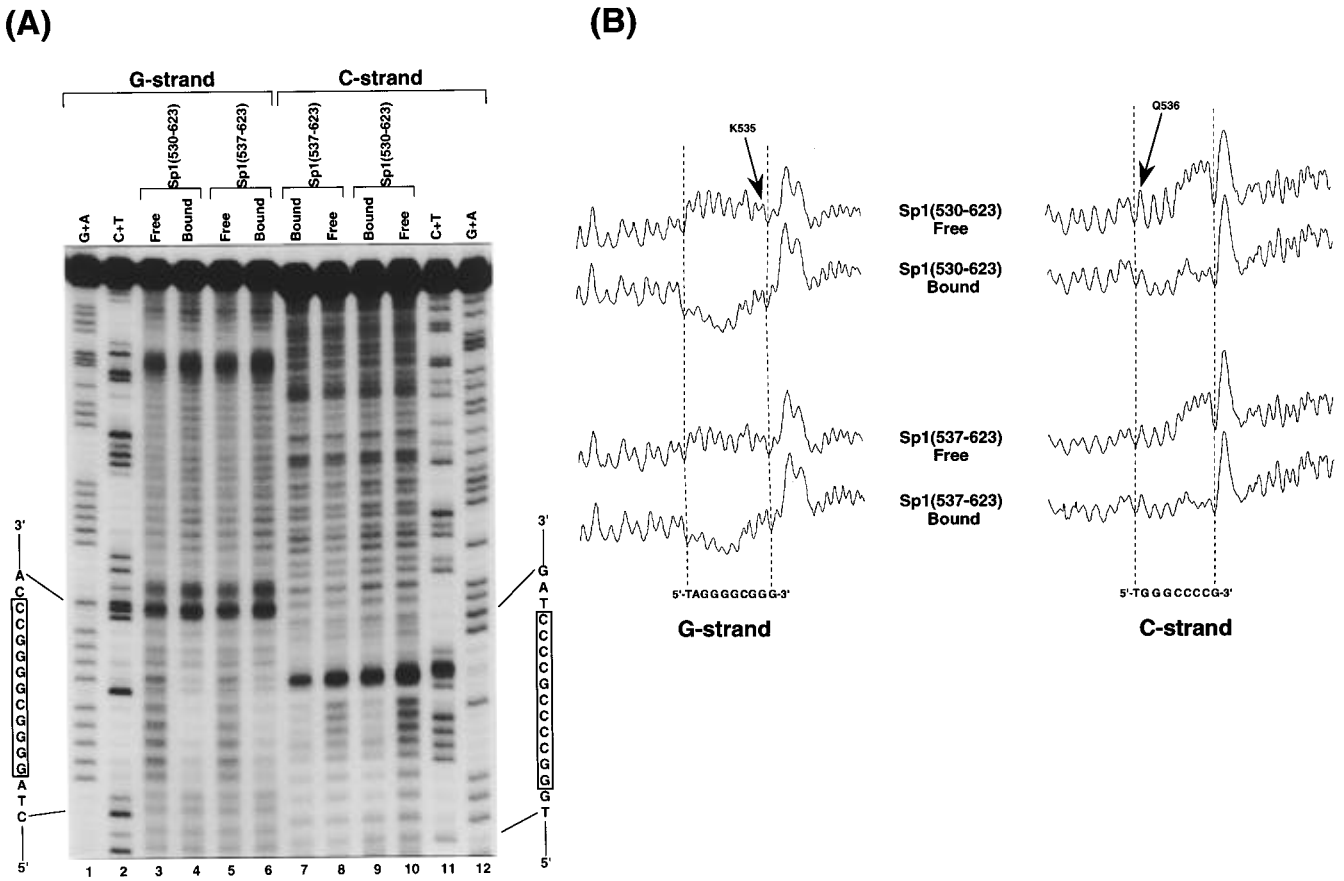

FIGURE 2: Ethylation interference analyses for Sp1(530−623) and Sp1(537−623). (Panel A) Autoradiograms for G-strand (lanes 1−6) and C-strand (lanes 7−12) on a denaturing polyacrylamide gel. Lanes 1 and 12 represent the purine-specific ladders, and lanes 2 and 11 represent pyrimidine-specific ladders, respectively. Lanes 3−10 show free and the peptide-bound DNA samples. The experimental conditions are described in Materials and Methods. (Panel B) Densitometric analyses of the autoradiograms for G-strand (left) and C-strand (right). The scans for free and the peptide-bound DNAs were adjusted to match at the edge. The interfered regions are divided by dotted lines, and the contacts points of K535 and Q536 are indicated by arrows.

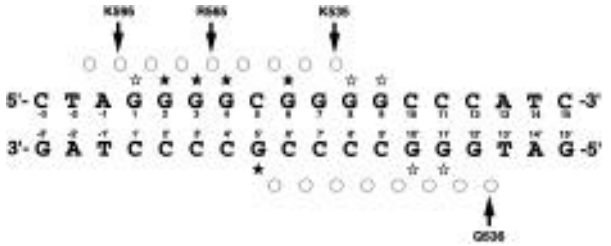

FIGURE 3: Summary of interactions between Sp1(530−623) and GC-box DNA. The number indicates the base position used in Results and Discussion. Open circles, filled stars, and open stars exhibit the contacted phosphates, the strongly-recognized bases, and the weakly-recognized bases, respectively. Among them, the phosphates revealed by mutational experiments are pointed to by an arrow with the residue number.

all the DNA fragments bound with Sp1(537−623) moved down at the almost same distance. Under the condition that excessive of DNA was used, a similar result was also obtained (Figure 5C(b)). Except for Sp1(537−623), all other mutants demonstrated the same result as Sp1(530−623) (data not shown).

DNase I Footprinting Analysis. Figure 6 presents the results of DNase I footprinting analyses for Sp1(530−623) and Sp1(537−623). In the G-strand (Figure 6, left panel), the binding of Sp1 peptides protected all the residues in GC-box and 3−4 residues of 5′-side out of GC-box. At the 3′-side of GC-box, hypersensitive cleavages were observed. In the C-strand (Figure 6, right panel), the almost same results as in the G-strand were obtained. The phosphate between T(13′) and A(14′) residues in Sp1(530−623) was not attacked by DNase I, but clear cleavage at this site was detected in Sp1(537−623). In both DNA strands, the cleavage of GC-box was perfectly inhibited by 20 pmol of Sp1(537−623). On the other hand, 20 pmol of Sp1(530−623) did not absolutely protect the GC-box from the digestion of DNase I.

Methylation Protection Analysis. Figure 7 indicates the results of methylation protection analyses for Sp1(530−623) and Sp1(537−623) (G-strand only). The binding of Sp1-(530−623) protected all the guanine residues except for G(7) in GC-box from the attacking of dimethyl sulfate. The hypersensitive cleavage was observed only at G(7) site. In the case of Sp1(537−623), all the guanine residues in GC-box were equally protected from the methylation. The hypermethylation was not detected at G(7), but at C(12) outside GC-box in the G-strand.

DISCUSSION

Specific Interaction between Sp1 Peptides and GC-Box DNA. In zinc finger motif, basic amino acid residues such as lysine and arginine are highly conserved at N-terminal side of β-sheet region or at C-terminal side of helix region. A similar situation is also observed in linker and start/end regions of zinc finger domain. The X-ray crystallographic evidence of Zif268 and GLI reveals that these basic amino acid residues are often in contact with backbone phosphate

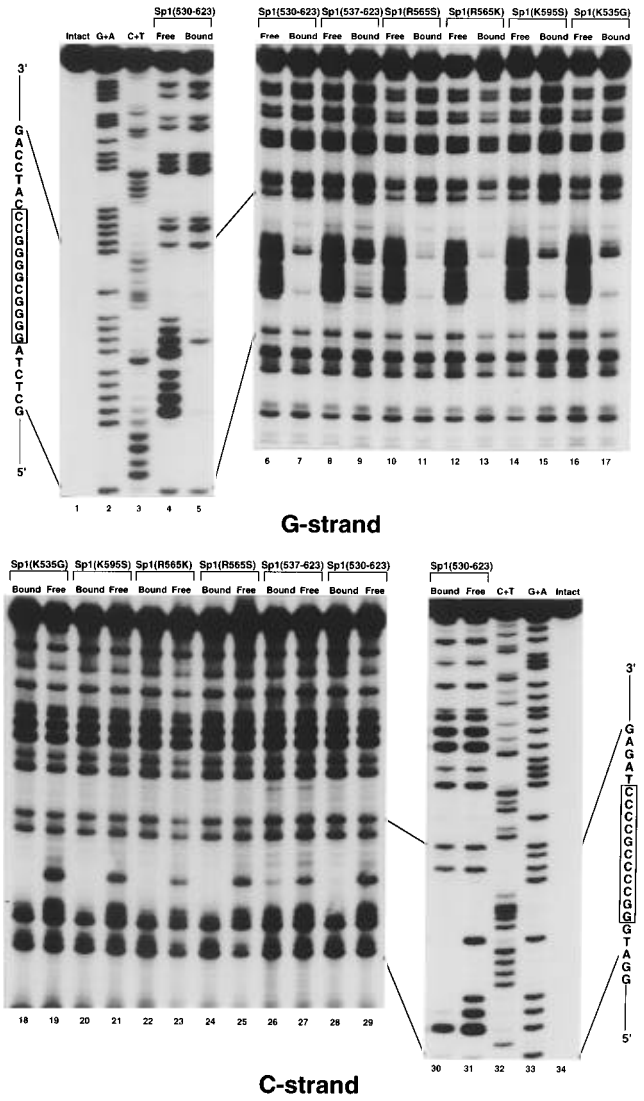

FIGURE 4: Methylation interference analyses for diverse Sp1-peptide mutants. The peptide-bound and free DNAs were isolated, cleaved, and analyzed on a denaturing polyacrylamide gel. The peptide-bound sample (20 μL) contained the methylated restriction fragment (0.6 pmol), sonicated calf thymus DNA (1 μg), the Sp1 mutant (0.6−1.2 pmol), and 10 mM Tris buffer (pH 8.0). The peptide-free sample was the same to the peptide-bound sample, except for the absence of the Sp1 mutant. Lanes 1 and 34 are the lanes of intact DNA. Lanes 2 and 33 represent the purine-specific ladders, and lanes 3 and 32 represent pyrimidine-specific ladders, respectively. Lanes 4−31 show free and the peptide-bound DNA samples. The experimental procedure was performed according to our previous paper (Kuwahara et al., 1993).

of DNA duplex (Pavletich & Pabo, 1991, 1993). As shown in Figure 3, all the amino acid residues at the mutated positions interact with the backbone phosphate. The same result of ethylation interference between Sp1(R565K) and Sp1(530−623) indicates that a lysine residue is available at the position 565 instead of arginine residue. No specific contacts with DNA backbone phosphate were observed in Sp1(K533G) and Sp1(K534G) in spite of increasing flexibility by the change of lysine to glycine. Therefore, K535 clearly interacts with the backbone phosphate, but K533 and K534 seem to have no specific contacts. Of special interest is the fact that glutamine residue at the position 536 (Q536) unequivocally recognizes the phosphate outside GC-box in C-strand. In most of zinc finger proteins, the position equivalent to Q536 is proline residue which does not interact with DNA backbone phosphate. Evidently, the backbone contact mode seen in Sp1(530−623) is distinct from that in Zif268, GLI, TTK, and TFIIIA (Sakonju & Brown, 1982; Pavletich & Pabo 1991, 1993; Fairall et al., 1993). In the latter cases, the recognized phosphates are restricted almost in one strand of duplex DNA. On the contrary, Sp1(530−623) interacts with the phosphates in both the DNA strands and also an overlapped contact is observed. On the basis of our previous report, the zinc fingers 1 and 2 recognize the first (positions 1−3 in G-strand) and second triplets (positions 4−6 in G-strand), respectively (Kuwahara et al., 1993). This information suggests that the geometry of finger 1 in major groove of GC-box DNA may be different from those of fingers 2 and 3. Indeed, the zinc finger 1 interacts with the bases in both the DNA strands with almost equal affinity, whereas the zinc fingers 2 and 3 recognize only the bases in G-strand (Figure 4). Suzuki et al. (1994) classified the C_2H_2-type zinc finger into two subtypes based on the mode of backbone phosphate contact by β-sheet region. However, the zinc finger 1 of Sp1 is proposed to be a hybrid type.

The results of methylation interference for Sp1(K595S) and Sp1(K535G) were the same as that for Sp1(530−623), strongly suggesting that lysine residues at 595 and 535 positions do not play a difinitive role for the DNA binding of Sp1. On the other hand, Sp1(R565S) and Sp1(R565K) were in stronger contact with GC-box DNA than Sp1(530−623). In the case of TFIIIA, the mutant of lysine→serine change at the linker region demonstrated weaker DNA binding than wild type (Choo & Klug, 1993). This observation is not true for all zinc finger proteins, because the dissociation constants for Sp1(530−623) and its mutants were estimated to be approximately 4 nM. In addition, the lysine residue at the linker region of ADR1 gave no serious effects on its DNA interaction (Thukral et al., 1991). In our opinion, the zinc finger 1 of Sp1(530−623) has an orientation toward DNA different from that of zinc fingers 2 and 3 of Sp1 and the three zinc fingers of Zif268. In distinction to the linker having K595, the linker containing R565 connects two zinc fingers of different type. Concerning the position of R565, serine which is short to interact with phosphate backbone and lysine which is in a Drosophila *Krüppel*-type linker, may be better for Sp1. The arginine residue is presumably a weak inhibitory factor for DNA binding of Sp1 because of its long side chain. The *Krüppel*-type linker, TGEKP, is conserved in many zinc finger proteins. Of course, the best linker sequence is different among the types of zinc fingers. We must consider the linker sequence together with the finger sequence in the evaluation about DNA recognition by zinc finger. On the other hand, the weak interaction of Sp1(537−623) to GC-box DNA suggests that the mutation at the zinc finger 1 influences the base specificity of the zinc fingers 2 and 3, that is, each zinc finger is not independent.

Detection of DNA Structural Change by Circular Permutation Assay, DNase I Footprinting Analysis, and Methylation Protection Analysis. Why is the base specificity diminished in Sp1(537−623)? We performed CPA to obtain information about the structural alteration of DNA, in particular about DNA bending. As clearly shown in Sp1(530−623) of Figure 5, fragment D moved most slowly. The result suggests that the bending center locates at the center of GC-box, though the degree of bending is not large. This is distinct from the result of Ikeda et al. (1993) that substantial bending occurs

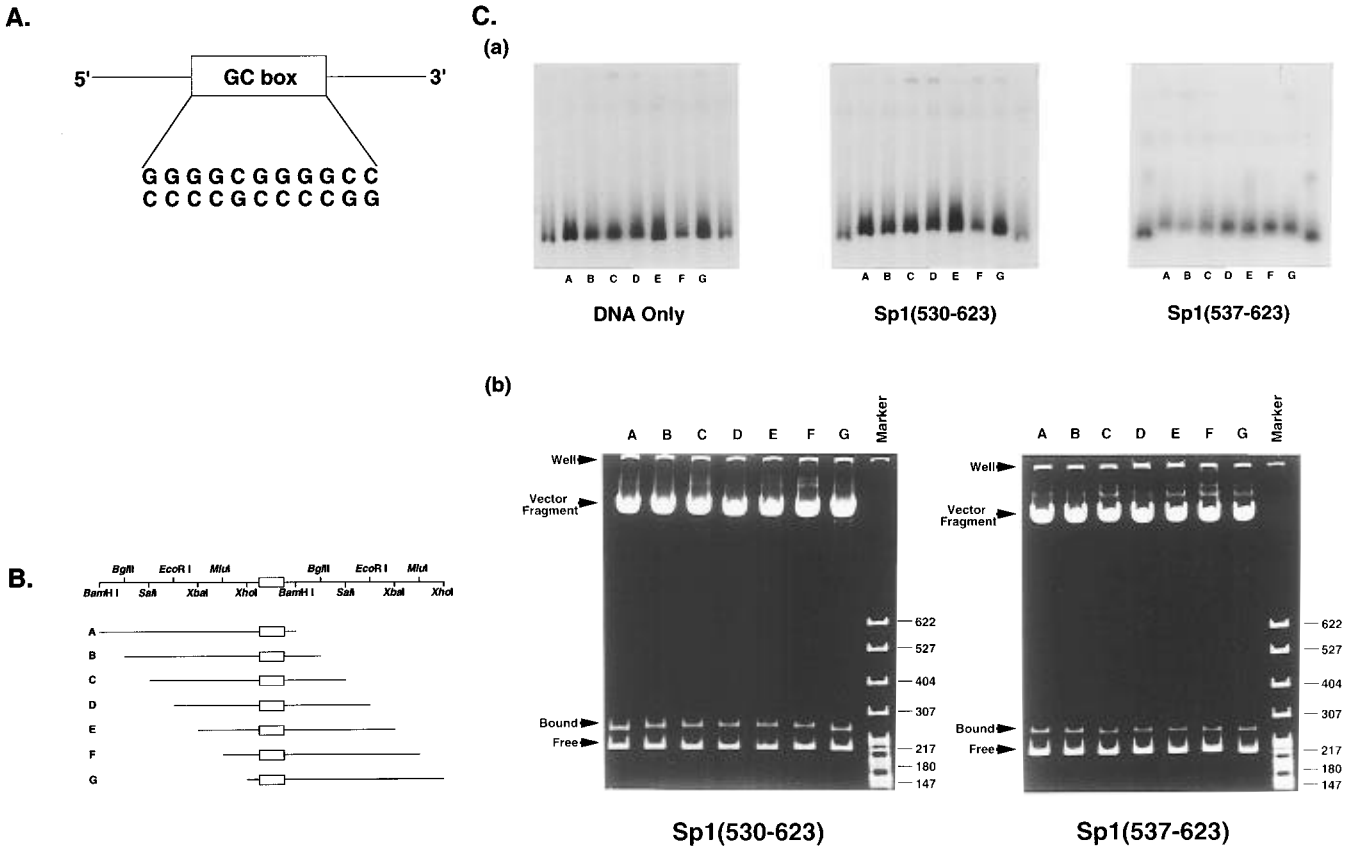

FIGURE 5: Structure of DNA constructs and results of circular permutation assay. (Panel A) GC-box cloned in the circularly permutated DNA fragments. In this experiment, the base sequence recognized by Sp1(530−623) is defined as the GC-box. (Panel B) Circularly permutated fragments generated by cutting with seven restriction enzymes. The relative position of the GC-box represented by white box is shown for each fragment. (Panel C) Gel mobility shift assays by using of circularly permutated fragments. (a) and (b) show the assays using a large excess of peptide and GC-box DNA, respectively. In lanes A−G, the DNA fragments displayed in panel B are used, respectively. The both end lanes in (a) present peptide-free control lanes that the fragment E is used. The right lanes in (b) are the lanes of marker DNA from a *Msp*I digest of pBR322. The experimental conditions are described in Materials and Methods.

at 5′-side of GC-box. In their study, native Sp1 was used, and hence the region outside zinc finger domain may have some interaction with DNA. The basic amino acid residues at N-terminal and linker regions appears to be not involved in the DNA bending. On the other hand, all the DNA fragments bound with Sp1(537−623) moved with almost same mobility (Figure 5C). The present data indicate that Q536 is one key residue to bend DNA and that the local structural change of DNA affects the base-specific recognition of zinc finger protein.

The DNase I footprinting analysis also supports the loss of DNA bending by the binding of Sp1(537−623). Figure 6 revealed that the suppression of DNA digestion with DNase I is considerably different between Sp1(530−623) and Sp1-(537−623). Although the DNA cleavage for GC-box was strongly inhibited by the binding of 20 pmol of Sp1(537−623), the GC-box DNA was still digested under the condition of 30 pmol of Sp1(530−623). This result suggests that DNA duplex is bent toward major groove and is cleaved from minor groove by the Sp1(530−623) binding. The data suggest that Q536 takes part in DNA bending by Sp1(530−623) binding.

The difference of DNA binding mode between Sp1(530−623) and Sp1(537−623) is elucidated from the methylation protection analysis. Our previous methylation protection analysis for the three zinc fingers of Sp1 showed that hypersensitive cleavage is seen at G(7) in the G-strand, and that this phenomenon is due to the change of the helical twist angle at G(6)−G(7) step and also is caused by C-terminal two fingers (Kuwahara et al., 1993, unpublished results). All the guanine residues in GC-box were equally protected from methylation by the binding of Sp1(537−623). Hypermethylation was not detected at G(7), but at C(12) outside GC-box in the G-strand. Thus, zinc fingers 2 and 3 of Sp1(537−623) have a distinct binding mode from that of Sp1(530−623). These results suggest that the bending of GC-box DNA is not caused by the binding of Sp1(537−623) and that DNA binding mode of Sp1(530−623) differs from that of Sp1(537−623).

Relationship between Structural Change of DNA and Base Recognition by Zinc Finger. Of interest is the result of methylation interference for Sp1(537−623), in which glutamine 536 is changed to methionine and also lysine at N-terminus is deleted. In Sp1(537−623), the recognition ability of bases in GC-box lowered in both the strands of DNA duplex. However, Sp1(K535G) did not show such the decrease of base specificity. Both the dissociation constants of Sp1(537−623) and Sp1(K535G) were approximately 4 nM. These results are reminiscent of DNA binding of single zinc finger peptide, suggesting that the base specificity is not parallel to the DNA binding strength in zinc finger protein (Frankel et al., 1987). Therefore, this phenomenon is probably due to the mutation at Q536. Zif268 possesses proline residue at a position equivalent to Q536 in Sp1. This proline residue, which is highly conserved among many zinc finger proteins, has van der Waals contacts with the side

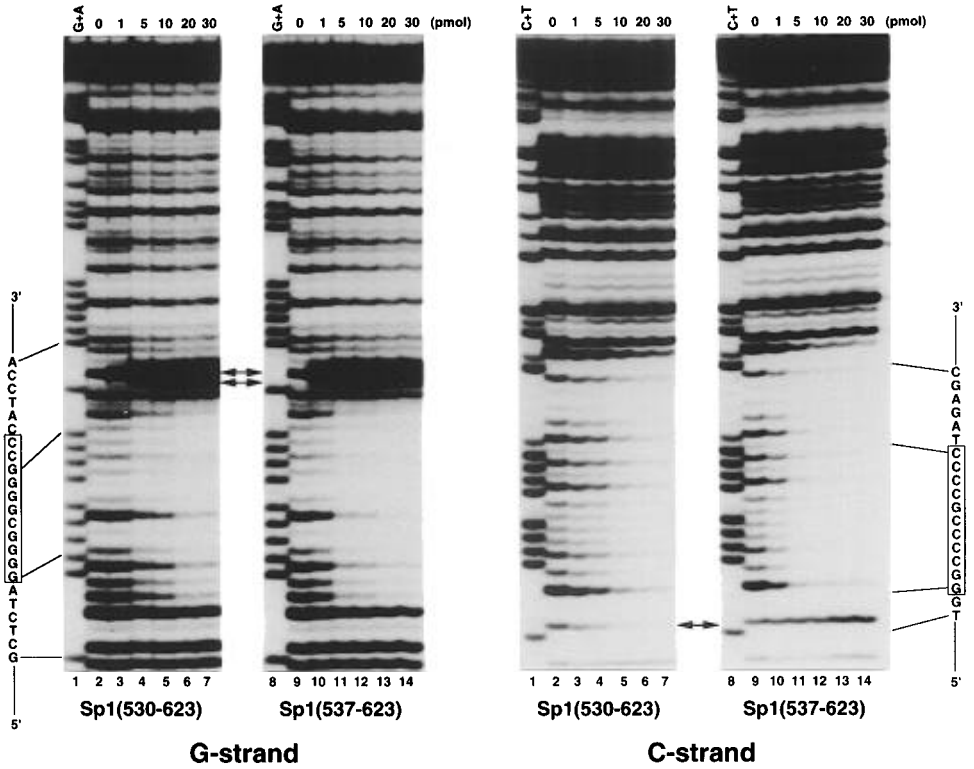

FIGURE 6: DNase I footprinting analyses for Sp1(530−623) and Sp1(537−623). The left and right panels show the results for G- and C-strands, respectively. Lanes 1 and 8 in G-strand represent the purine-specific ladders, and lanes 1 and 8 in C-strand represent the pyrimidine-specific ladders, respectively. The samples contained the following amounts of peptide: none (lanes 2 and 9), 1 pmol (lanes 3 and 10), 5 pmol (lanes 4 and 11), 10 pmol (lanes 5 and 12), 20 pmol (lanes 6 and 13), and 30 pmol (lanes 7 and 14). Arrows in G- and C-strands indicate the hypersensitive cleavage sites and the recovered cleavage site, respectively.

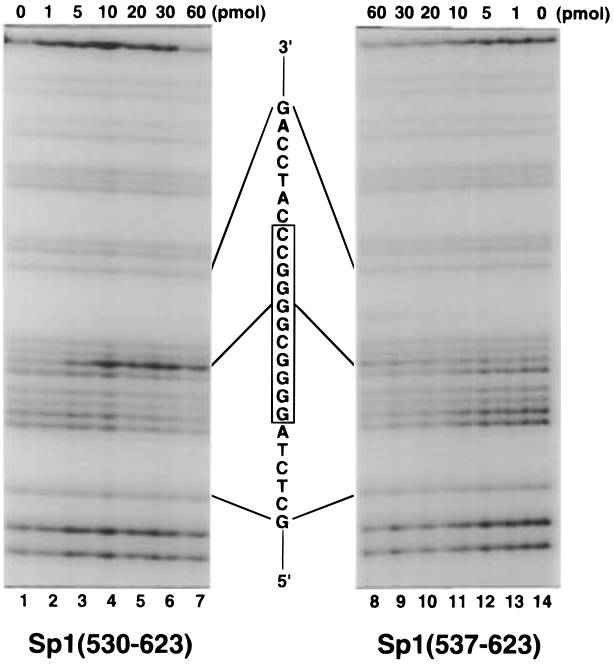

FIGURE 7: Methylation protection analyses for Sp1(530−623) and Sp1(537−623) (G-strand only). The samples contained the following amounts of peptide: none (lanes 1 and 14), 1 pmol (lanes 2 and 13), 5 pmol (lanes 3 and 12), 10 pmol (lanes 4 and 11), 20 pmol (lanes 5 and 10), 30 pmol (lanes 6 and 9), and 60 pmol (lanes 7 and 8).

chain of aromatic amino acid residue at C-terminal side and plays an important role in forming zinc finger (Pavletich & Pabo, 1991). The roles of glutamine 536 and the conformation of finger 1 in Sp1(530−623) have never been clarified. Sp1(537−623) contains three zinc(II) ions as demonstrated by the titration of *p*-hydroxymercuryphenylsulfonic acid and also has a folded conformation as supported by circular dichroism feature (data not shown). It is important that the zinc finger 1 influences the base recognition of the zinc fingers 2 and 3, namely, each zinc finger is not independent. The zinc finger 1 of Sp1(537−623) appears to act like a "*inhibition finger*" that hinders the specific binding of adjacent fingers to DNA. Presumably, this is due to two conformational alteration of DNA. From the results of CPA and DNase I footprinting, the bending of DNA is not induced by the binding of Sp1(537−623), whereas Sp1(530−623) gives rise to the DNA bending. Typical examples, such as *su(HW)* protein that significantly affects the DNA binding of zinc finger, have been reported (Spana & Corces, 1990). In this protein, the flexibility of AT-rich sequence outside the binding site is predicted to influence DNA bending and DNA binding of zinc finger. Our bending model is distinct from the above-mentioned model, because the present DNA is not bent in the absence of protein. Another conformational alteration of Sp1(530−623) is the helical twist angle at G(6)−G(7) step revealed by the methylation protection analysis. Although such an alteration was not observed in Sp1(537−623), the GC-box of DNA was explicitly protected from the methylation (Figure 7). The results suggest that the DNA binding mode of the zinc fingers 2 and 3 in Sp1(537−623) changes from that in Sp1(530−623). Indeed, the DNA conformational alteration caused by specific binding of the zinc fingers 2 and 3 seems to be essential for the binding of Sp1 (Kuwahara et al., unpublished results). Therefore, phosphate backbone contact of glutamine 536 probably contributes to appropriate DNA binding of the zinc finger 1, inducing DNA bending and change of G(6)−G(7) helical twist. In zinc finger−DNA interaction, the local

structural change of DNA induced by the backbone phosphate contacts plays an important role for accurate base recognition and DNA binding by zinc finger protein.

In conclusion, the present study used some mutant peptides demonstrates that (i) the best linker sequence is different by the types of connecting zinc fingers, (ii) each zinc finger is not independent in the DNA recognition by zinc finger protein, and (iii) backbone phosphate contact of Sp1 contributes to the local structural changes of DNA by Sp1 binding and affects the total base specificity of Sp1. These results provide good information for the elucidation of the DNA recognition by zinc finger protein and the design of new zinc finger proteins.

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