Cooperative Bending of the 21-Base-Pair Repeats of the SV40 Viral Early Promoter by Human Sp1†

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ABSTRACT: The overall structural features of the multimeric complex between Sp1 and the 21-base-pair repeat of the early promoter region of SV40 DNA have been determined using hydroxyl-radical footprinting; (+)-CC-1065, a sequence-specific minor groove bending probe; and circularization experiments. The results show that the 21-base-pair repeat region has an intrinsically in-phase bent structure that is stabilized upon saturation Sp1 binding by protein–DNA and protein–protein interactions to produce a looping structure. The direction of the Sp1-stabilized bending of DNA occurs into the minor groove and is localized between each of the Sp1 binding sites. These results are used as the basis to propose a looping structure for the multimeric Sp1 21-base-pair repeat region of SV40 DNA. Last, these results provide a rationale for the recently observed inhibition of basal transcriptional levels by site-specific triple-helical DNA complexes.

The regulation of eukaryotic gene transcription is operated primarily through the interaction of various trans-acting transcription factors (e.g., basal transcriptional factors, activators, and repressors) with cis-acting DNA promoter elements (Johnson & McKnight, 1989; Mitchell & Tjian, 1989; Müller & Schaffner, 1990; Ptashne, 1988). The binding sites for transcriptional activators are generally located proximal to the transcriptional initiation site. Furthermore, both *in vivo* and *in vitro* transcriptional factors bound to distal regulatory sequences are also believed to interact with RNA polymerase II and/or other proximal transcriptional factors in order to facilitate the activation of transcription (Müller & Schaffner, 1990; Müller et al., 1989; Hai et al., 1988). It also appears that certain trans-acting activators can work synergistically in combination with other trans-acting activators to modulate gene expression (Herschlag & Johnson, 1993; Lin et al., 1990; Li et al., 1991).

Sp1 is a sequence-specific transcription factor that binds to G- and C-rich elements via three zinc fingers and activates transcription via glutamine-rich domains (Dynan & Tjian, 1983; Gidoni et al., 1984; Kadonaga et al., 1986, 1987, 1988; Courey & Tjian, 1988; Pugh & Tjian, 1990). Transcriptional activation by Sp1 has been studied most extensively with the SV40 early promoter, although Sp1-responsive promoters have been found in a wide variety of viral and cellular promoters (Dynan & Tjian, 1983; Gidoni et al., 1984, 1985; Janson & Pettersson, 1990; Dynan et al., 1985). The region upstream of the SV40 promoter contains six Sp1 binding sites (GC boxes) within the three 21-bp[1] repeats. Two additional tandem repeats of 72 bp are positioned toward the late promoter side of the SV40 control region. Complete deletion of three 21-bp repeats, as well as both 72-bp repeats, severely limits early promoter function as well as virus viability (Banerji et al., 1981; Everett et al., 1983). Although Sp1 is generally considered to be a protein that interacts with promoter-proximal elements, Sp1 also possesses the ability to mediate the long-range activation of transcription via protein–protein interactions between DNA-bound Sp1 molecules and other transcriptional factors (Courey et al., 1989).

Recently, it has been shown that site-specific triple-helical complexes reduce the level of basal transcription even in the absence of Sp1 (Maher et al., 1992). This suggests that the intrinsic DNA structure of the Sp1 binding region is an important factor in transcriptional regulation. Multiple binding sites appear to be one general feature of promoters that are Sp1 responsive, as exemplified in the SV40 early promoter region (Gidoni et al., 1984; Kadonaga et al., 1986). However, it is not clear why Sp1 binding sites are arranged in the manner that Sp1 binds once every 10–12 bp, which roughly corresponds to one complete turn of the DNA helix. This situation is similar to the TFIIIA DNA complex, where zinc fingers bind in successive major grooves of DNA in a periodic manner (Churchill et al., 1990). In this study, we have addressed the question of the significance of the periodicity of Sp1 binding alongside the significance of sequences located between Sp1 binding sites.

(+)-CC-1065 is a very potent antitumor antibiotic that was isolated from the culture broth of *Streptomyces zelensis* in the late 1970s (Hanka et al., 1978). The biological activity of (+)-CC-1065 stems from its ability to alkylate N3 of adenine on duplex DNA in a sequence-selective manner and modify the structure and local conformation of DNA (Hurley & Draves, 1993). Previous studies from this laboratory, in collaboration with scientists from The Upjohn Company, have shown that (+)-CC-1065 lies in the minor groove of duplex DNA covering a 4–5-bp region to the 5′ side of modified adenine (Hurley et al., 1984; Reynolds et al., 1985; Scahill et al., 1990). As a consequence of formation of the (+)-CC-1065–(N3)-adenine adduct, (+)-CC-1065 induces bending, winding, and stiffening of the DNA (Lee et al., 1991; Sun & Hurley, 1992). Significantly, the bending produced by (+)-CC-1065 has characteristics similar to those of an A-tract (Lin et al., 1991; Sun et al., 1993a; Koo & Crothers, 1988). Armed with this insight, we have recently demonstrated the utility of (+)-CC-1065 in probing the bending sites produced by the A-protein of Mu transposase in the *att* L3 sequence (Ding et al., 1993).

To enhance our understanding of the structural details of the complex between Sp1 and 21-bp repeats, we have used

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[1] Abbreviations: bp, base pair(s); DDW, double-distilled water.

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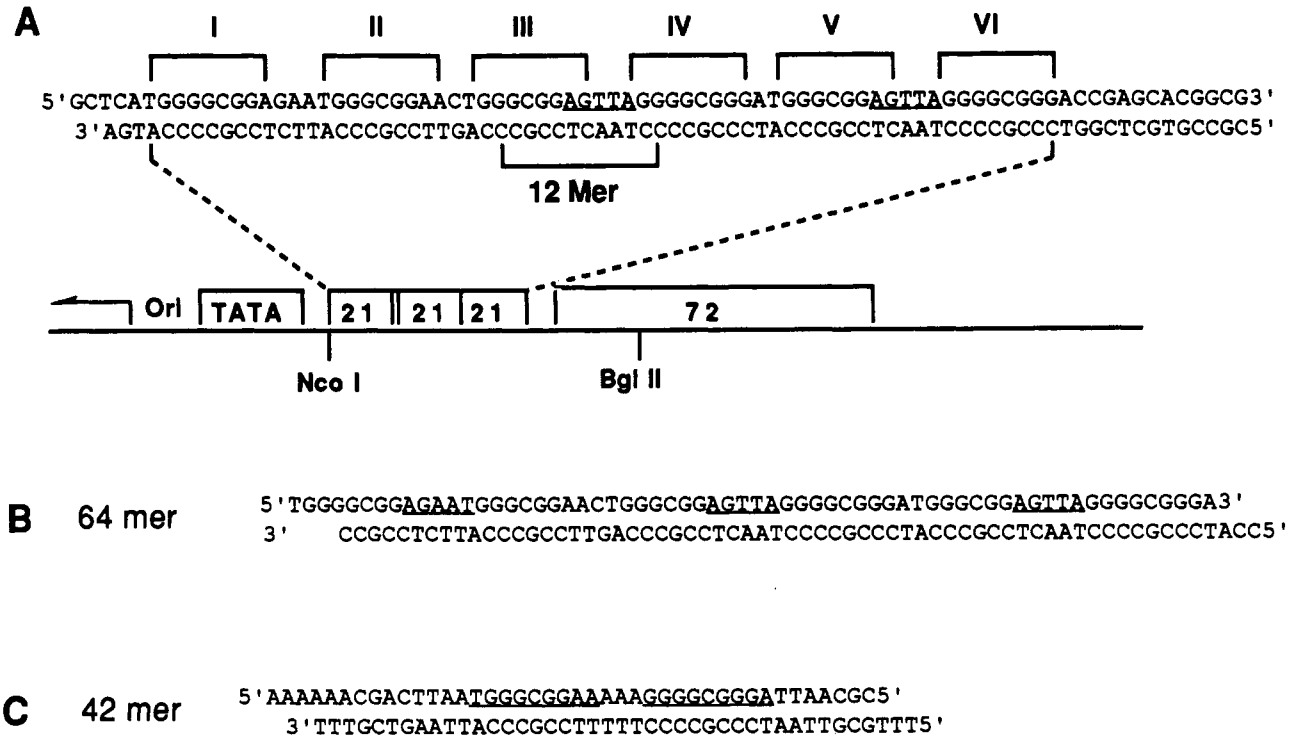

FIGURE 1: (A) Early promoter region of SV40 DNA containing the 21-bp repeat region. The 80-mer sequence containing the six GC boxes (I–VI) is shown as an inset. The two restriction enzyme sites used in construction of the substrates for footprinting and circularization experiments are shown in the lower region. In the inset, the two (+)-CC-1065 bonding sites (5′-AGTTA) are underlined, and in the lower bracket is the 12-mer sequence (5′-GGCGGAGTTAGG) used in the high-field NMR study (Lin et al., 1992). (B) Sequence of the 64-mer used in experiments to determine the intrinsic bending due to the intervening sequences between the GC boxes (underlined). A similar sequence in which the underlined sequences were replaced by 5′-CGCCG was used as a control. (C) Sequence of the 42-mer used to determine the direction of bending due to binding of Sp1 to the GC boxes.

hydroxyl-radical footprinting, competition experiments between Sp1 and (+)-CC-1065, and circularization experiments. Our results provide excellent evidence for the general conclusion that, at saturation binding of Sp1 to DNA, the six Sp1 molecules each make similar contacts with their six GC boxes by interaction through the major groove to induce an overall curvature of the 21-bp repeats toward the minor groove. In addition, when the 21-bp repeat region is saturated with six Sp1 molecules, the two (+)-CC-1065 bonding sites are still accessible and reactive toward (+)-CC-1065. Last, we note the previously undocumented observation that the "transient kink" at the 5′-GT/AC step of the sequence 5′-AGTTA is an important locus for the initial bending of DNA upon binding of Sp1 (Lin et al., 1992).

EXPERIMENTAL PROCEDURES

Materials. (+)-CC-1065 was a gift from The Upjohn Company (Kalamazoo, MI). Electrophoretic reagents were purchased from Bio-Rad. Other chemicals for chemical DNA sequencing and hydroxyl-radical footprinting were from Aldrich Chemical Company; [γ-^{32}P]ATP and [α-^{32}P]dGTP were from ICN; and X-ray film, intensifying screens, and developing chemicals were from Eastman Kodak. Human Sp1 protein and DNase I were from Promega. The T4-polynucleotide kinase and DNA polymerase I (Klenow fragment) were from United States Biochemical. T4 DNA ligase and other restriction endonucleases were from New England Biolabs. We are grateful to Robert Tjian for supplies of Sp1 used in the initial stages of this investigation.

Oligonucleotide Synthesis. The oligonucleotides were synthesized on an automated DNA synthesizer (Applied Biosystems 381A) by the phosphoramidite method (Gait, 1984). The oligomers were then deprotected by heating at 55 °C overnight with saturated ammonium hydroxide, dried under vacuum, and redissolved in DDW.

Preparation and End-Labeling of DNA. The 80-mer oligomer DNA molecules containing the 21-bp repeats of the SV40 viral early promoter region (Figure 1A) were constructed by the ligation of two 40-mer duplex DNA molecules and labeled with [α-^{32}P]dGTP by standard methods at the 3′ end of the DNA. This oligomer DNA was used in the construction of the (+)-CC-1065 site-directed adduct and in the footprinting experiments with the hydroxyl-radical and DNase I. For DNase I and hydroxyl-radical footprinting of the coding strand, the plasmid pCAT (Promega) that contains the SV40 viral early promoter region was digested with NcoI and dephosphorylated with calf thymus intestinal alkaline phosphatase (see Figure 1A). Dephosphorylated restriction fragments were labeled with [γ-^{32}P]ATP and cleaved a second time with *Bgl*II to remove the radioactive label on the noncoding strand. Labeled DNA molecules (100 bp) were separated by electrophoresis on an 8% nondenaturing polyacrylamide gel and recovered by crushing the gel fragment, extracting the DNA with DDW, and lyophilizing the DNA solution. Lyophilized DNA was redissolved in DDW and further purified by phenol/chloroform extraction and ethanol precipitation.

Footprinting with DNase I. Purified human Sp1 (Promega) was combined with radiolabeled DNA in 40 μL of binding buffer consisting of 10 mM Tris·HCl (pH 7.6), 0.01% Nonidet P-40 (NP-40), 10 mM $MgCl_2$, and 5% glycerol and incubated at 30 °C for 10 min. Enzymatic cleavage was initiated by adding 0.1 unit of DNase I, and incubation was carried out for 2 min. The reaction was stopped by the addition of the same volume of alkaline sequencing dye (80% formamide and 10 mM NaOH).

Hydroxyl-Radical Footprinting. Purified human Sp1 was combined with radiolabeled DNA in 40 μL of the same drug

binding buffer, except for a different glycerol concentration (final 2.5%), and incubated at 30 °C for 10 min. Cleavage was initiated by adding 160 μL of a solution containing 10 mM Tris·HCl (pH 7.6), 0.01% NP-40, 10 mM $MgCl_2$, 20 μM Fe(II), 40 μM ethylenediaminetetraacetic acid, 0.3% H_2O_2, and 1 mM sodium ascorbate and was stopped by the addition of 20 μL of 3 M sodium acetate, 10 μg of yeast tRNA, and 3 vol of ethanol. The mixture was incubated in a −70 °C freezer for 2 h, and the DNA was precipitated by centrifugation at 4 °C. The dried DNA pellet was redissolved in DDW, extracted with phenol/chloroform, and precipitated with ethanol.

Circularization Experiment. Plasmid pCAT (Promega) was digested with *BglII* and dephosporylated with calf thymus intestinal phosphatase (see Figure 1A). Restriction fragments were digested with *NcoI* and ligated after the inactivation of restriction endonuclease. Ligated DNA was phosphorylated with [γ-^{32}P]ATP and electrophoresed through a 6% polyacrylamide gel to isolate a 200-bp fragment containing 12 Sp1 binding sites at the center of the fragment. This 200-bp restriction fragment was incubated at room temperature for 10 min with different concentrations of human Sp1 protein in 20 μL of a ligase buffer containing 10 mM Tris·HCl (pH 7.8), 10 mM $MgCl_2$, and 1 mM ATP. Ligation was initiated by the addition of 2000 units of T4 DNA ligase (New England Biolabs), and the mixture was incubated for 12 h. The ligated reaction products were applied to a 6% polyacrylamide gel to separate each species of DNA.

For the determination of the contribution of 5′-AGTTA and 5′-AGAAT to the circularization efficiency, a 64-mer (Figure 1B) containing three 21-bp repeats was synthesized and phosphorylated with [γ-^{32}P]ATP and polynucleotide kinase. As a "nonbent" control DNA, a new 64-mer DNA was synthesized, in which the sequences 5′-AGTTA and 5′-AGAAT were replaced with the sequence 5′-CGCCG. For the determination of the direction of Sp1-induced DNA bending, a 42-mer oligomer DNA containing GC boxes III and IV (Figure 1C) with an A_5-tract in place of the 5′-AGTTA sequence was phosphorylated with [γ-^{32}P]ATP and polynucleotide kinase. Phosphorylated 42-mer oligomer DNA (2 ng) was incubated for 10 min with a 4 molar excess of human Sp1 protein in 20 μL of a ligase buffer, and the ligation reaction was initiated by the addition of 2000 units of T4 DNA ligase and incubated for 12 h. Ligated reaction products were applied to 6% polyacrylamide gel electrophoresis to separate each species of DNA molecules.

RESULTS

High-Resolution Hydroxyl-Radical Footprinting of Sp1 on the 21-bp Repeats of the SV40 Viral Early Promoter Region Suggests a Repetitive Pattern of Sp1 Binding to This Region. We have employed hydroxyl-radical footprinting to study in detail the structure of the complex of six Sp1 proteins with the 21-bp repeat region (Figure 1). The hydroxyl-radical footprinting method provides a valuable means to examine the interaction of a DNA binding protein with the backbone of DNA, since hydroxyl radicals are small enough to cause strand breakage in DNA, even when complexed with DNA binding proteins or small DNA interactive ligands (Shafer et al., 1989; Burkhoff & Tullius, 1988). However, a direct interaction of the protein with DNA molecules through a minor groove site or severe changes in the DNA backbone conformation, such as bending in toward the minor groove, are also expected to cause significant reductions in the magnitude of hydroxyl-radical cleavage (Churchill et al., 1990; Yang & Nash, 1989).

Hydroxyl-radical footprinting patterns of both the noncoding [(−) strand] and coding [(+) strand] strands of the 21-bp repeat region of the SV40 early promoter region after formation of the complex with Sp1 molecules are shown in Figure 2A,B, respectively. These hydroxyl-radical data are also presented in the form of three densitometric tracings of the (−) strand (Figure 2C) taken at the three different levels of saturation of Sp1 binding, corresponding to lanes 1–3 in Figure 2A. It is evident from both the densitometric tracings (Figure 2C) and the footprinting gels (Figure 2A,B) that, at the highest concentrations of Sp1, there is an enhancement of hydroxyl-radical cleavage at the *central part* of each GC box region, while at this same concentration of Sp1, the cleavage efficiency *between each* GC box is diminished (lane 3 in Figure 2A,B and trace c in Figure 2C). This regular undulating pattern at the highest concentration of Sp1 is common to both the coding and noncoding strands, but there is about a 3-bp offset toward the 3′ side of the coding strand (Figure 2D), which is consistent with minor groove cleavage by hydroxyl radical. This repeating enhancement/diminution pattern is evident within the region covering GC boxes II–VI, but is particularly obvious in regions IV–VI. The enhancement of hydroxyl-radical cleavage within the GC regions in which Sp1 makes contacts in the major groove suggests that some widening of the minor groove occurs on the opposite side. Conversely, the diminished hydroxyl-radical cleavage of regions between each GC box indicates that the Sp1 molecules might make direct contacts with those regions through the minor groove or that Sp1 binding in the major groove causes a structural change in the region between each GC box. We favor the latter explanation, since no adenine residues are protected from modification with dimethyl sulfate (Gidoni et al., 1984). This footprinting pattern is compatible with the current model for the Sp1–DNA complex, in which three zinc fingers bind in the major groove of the GC box and partially wrap around the double helix (Pavletich & Pabo, 1991). This result also provides strong evidence that, at saturation binding of protein, each Sp1 molecule has a *similar relationship* to each GC box within the 21-bp repeats, since the footprinting patterns of each unit GC box complexed with Sp1 molecules are quite similar to each other. It is important to note that even in the *absence* of Sp1 (lane 1 in Figure 2A,B and trace a in Figure 2C), there is already a distinct trend toward this undulating pattern, particularly for GC boxes III–VI.

Saturation of Sp1 Protein Binding to the 21-bp Repeat Region Does Not Inhibit (+)-CC-1065 Bonding to the 5′-AGTTA Sequence.* (+)-CC-1065 shows high reactivity for covalent bonding to the sequence 5′-AGTTA* (* indicates the covalent bonding site for (+)-CC-1065), which is located between GC boxes III and IV and V and VI of the 21-bp repeat region (Figure 2D) (Reynolds et al., 1985). The thermal strand breakage assay (Reynolds et al., 1985) can be used to detect both the sequence location and the extent of covalent reaction of (+)-CC-1065 in the 21-bp repeats. Using this assay, we have determined how preincubation of DNA with Sp1 affects (+)-CC-1065 bonding to the sequence 5′-AGTTA*. In Figure 3A, the results of three sets of different but parallel incubations with the 21-bp repeat regions are shown. In lanes 1–4, the 21-bp region was treated with incrementally increasing concentrations of Sp1 and subjected to DNase I footprinting to determine the amount of Sp1 required to attain saturation binding (lanes 2–4). In lanes 5–8, the same 21-bp region was incubated with increasing concentrations of (+)-CC-1065. At the highest concentration of (+)-CC-1065 (lane 8), strong reaction at the two 5′-AGTTA sequences is attained. Finally, in lanes 9–11, the same

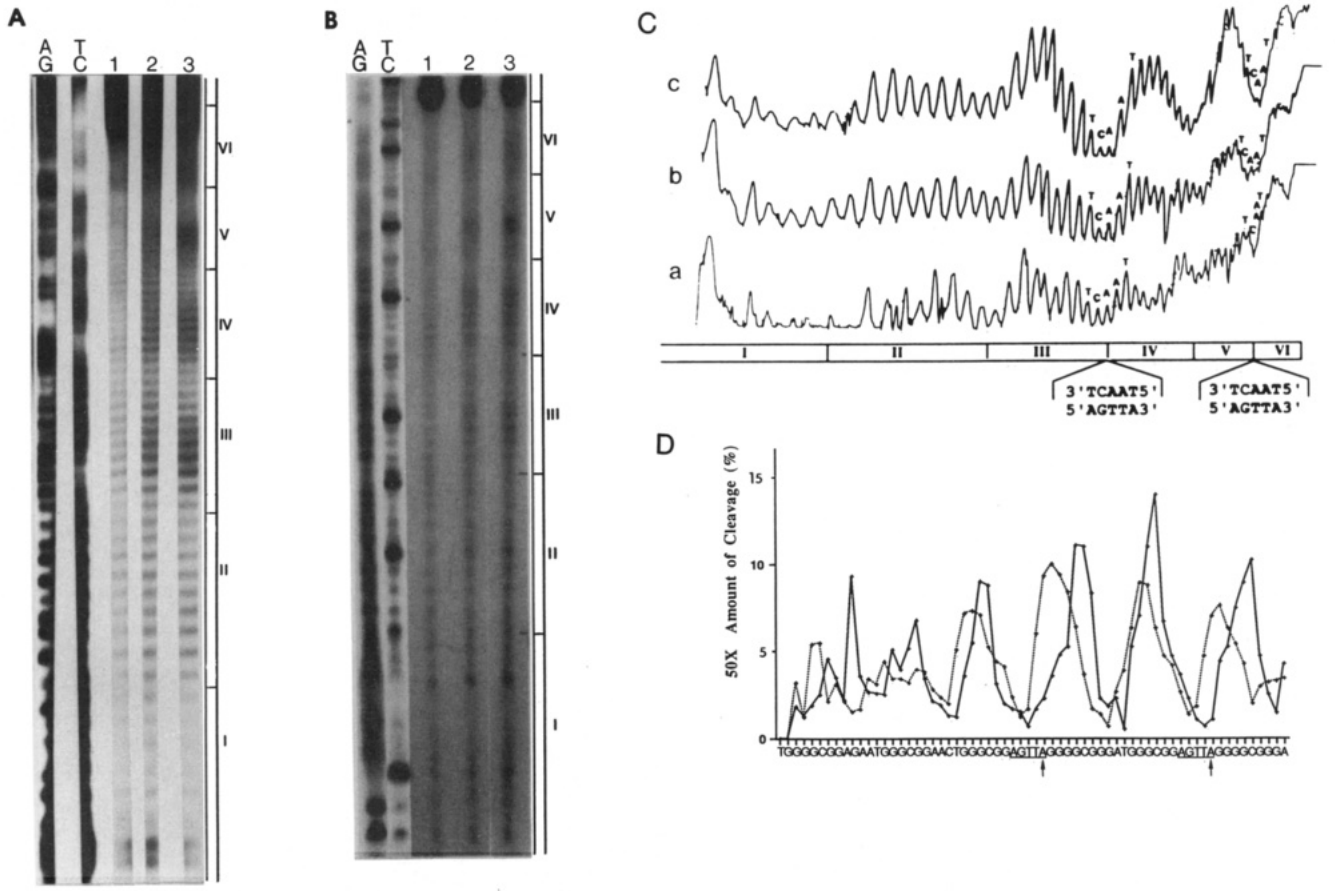

FIGURE 2: Hydroxyl-radical footprinting of Sp1 on the 80-mer and the 100-bp *Nco*I–*Bgl*II restriction fragment containing the 21-bp repeat region. Shown in A and B are the gel electrophoresis patterns of the hydroxyl-radical cleavage products on the noncoding and coding strands, respectively. AG and TC represent purine- and pyrimidine-specific cleavage reactions. Lanes 1–3 contain 0, 30, and 60 ng of Sp1, respectively, with about 50 fmol of DNA. The GC boxes (I–VI) are shown to the right side of each gel. (C) Letters a, b, and c refer to the densitometric scans of lanes 1–3, respectively, from A. (D) Comparison of the hydroxyl-radical cleavage pattern of the upper (—) and lower (···) strands (Figure 1) in the presence of saturating concentrations of Sp1 molecules (i.e, from lane 3 in Figure 2A,B).

concentration of (+)-CC-1065 as that used in lane 8 was added to samples that also contained amounts of Sp1 equivalent to those used in the samples in lanes 2–4. In parallel time course experiments, incubation of the 21-bp repeat with (+)-CC-1065, both in the absence and presence of saturating amounts of Sp1, was carried out (Figure 3B). The results of both experiments reveal that preincubation of the 5′-end-labeled restriction fragment, which contains the 21-bp repeats with saturating levels of Sp1, *does not* significantly decrease the extent of reaction of (+)-CC-1065 at the sequence 5′-AGTTA* (see lanes 9–11 in Figure 3A,B). This observation is particularly significant because hydroxyl-radical footprinting had revealed diminished cleavage within the sequence 5′-AGTTA in the presence of Sp1 (Figure 2D).

Taken together, the results of the hydroxyl-radical footprinting and competition experiments between Sp1 and (+)-CC-1065 strongly suggest that it is not steric occlusion by Sp1 that causes the diminution of hydroxyl-radical cleavage, since the minor groove is still open to modification by (+)-CC-1065 in the presence of Sp1. Since (+)-CC-1065 did not show an increase in reactivity in the presence of saturating amounts of Sp1, conditions that might be argued to provide an enhanced site of reactivity of (+)-CC-1065, we propose that either the rate-limited step is not associated with minor groove compression or, more likely, the actual protein-induced shape compression is not optimal for drug reaction. Since it could also be argued that (+)-CC-1065 was still able to react with its bonding sites on the 21-bp repeat due to the rapid exchange between bound and unbound Sp1 species, transiently exposing the (+)-CC-1065 bonding sites, a control experiment was carried out using pluramycin, a second alkylating agent that reacts in the major groove within the GC region occupied by Sp1. Pluramycin is an antitumor antibiotic that covalently modifies N7 of guanine in DNA via an intercalation-threading mechanism (Sun et al., 1993b). An experiment essentially similar to that described above with pluramycin in place of (+)-CC-1065 was carried out. In contrast to the results with (+)-CC-1065 (Figures 3A,B), Sp1 is able to almost completely protect GC boxes II–VI from alkylation by pluramycin (D. Sun and L. H. Hurley, unpublished results).

Saturation Binding of Sp1 to the GC Boxes in the 21-bp Repeat Region of SV40 Preferentially Facilitates Formation of an Intramolecular Circularization Product of a 200-mer and Inhibits Formation of the Intermolecular Ligation Product. Direct measurement of protein-induced DNA bending can be achieved by monitoring the formation of circular DNA produced by the ligation of linear DNA-containing protein binding sites upon the addition of protein (Kahn & Crothers, 1992; Lyubschenko et al., 1991). Therefore, to investigate Sp1-induced or -stabilized bending of DNA, a 200-bp DNA fragment was constructed to contain *six* 21-bp repeats (12 GC boxes) of the SV40 early promoter in the central region (see Figure 1A). Upon ligation of this fragment in the presence of *subsaturating* concentrations of Sp1 molecules, linear multimers (400-mer, 600-mer, and 800-mer) of the 200-bp DNA fragment were generated in significant amounts, constituting 33% and 34% of the total ligation product (lanes 3 and 4, respectively, in Figure 4A). At subsaturating levels of Sp1, the overall cyclization efficiency is reduced, presumably due to protein-induced stiffening of DNA, and there is also a preferential inhibition of intramolecular over intermolecular ligation of 200-mers. The preferential inhibi-

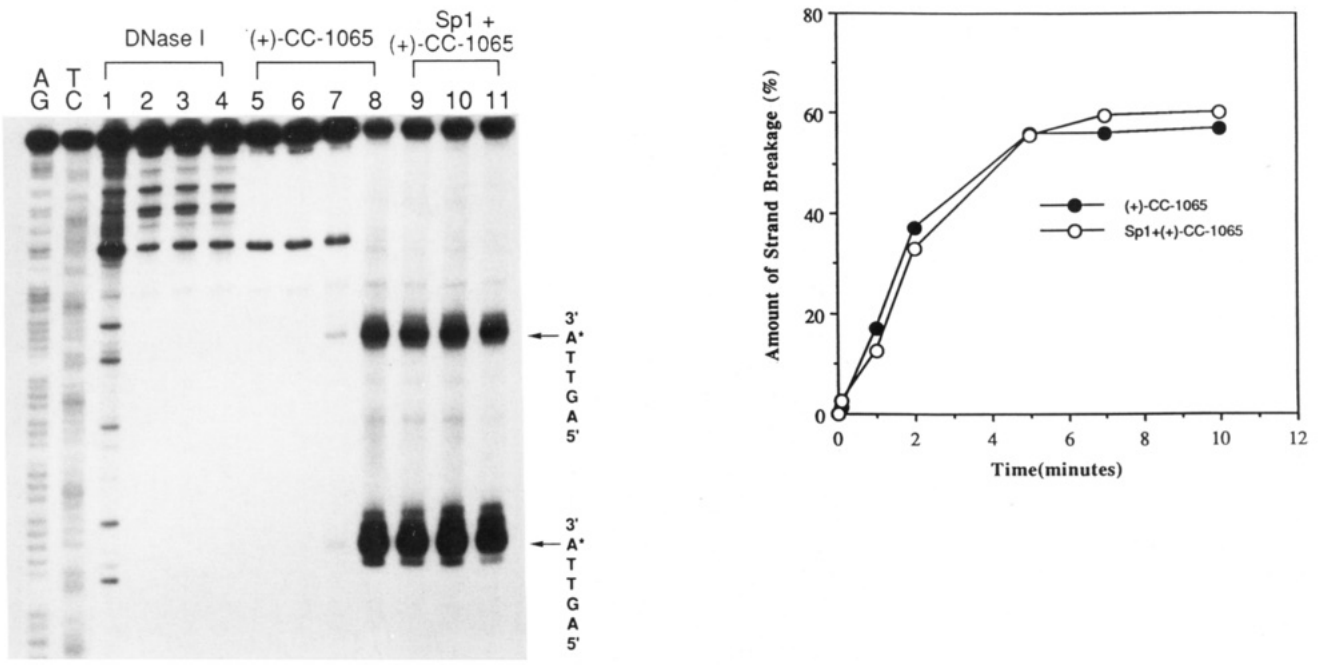

FIGURE 3: Effect of prebinding of Sp1 to the 21-bp repeats on covalent modification of 5′-AGTTA* with (+)-CC-1065. (A, left) Gel electrophoretic analysis of the 5′-^{32}P single-end-labeled 100-bp *Nco*I–*Bgl*II restriction fragment after treatment with Sp1 (lanes 1–4), (+)-CC-1065 (lanes 5–8), or Sp1 and (+)-CC-1065 (lanes 9–11). Samples containing 0, 15, 30, and 60 ng of Sp1 (lanes 1–4, respectively), with about 20 fmol of DNA, were digested with DNase I. Lanes 5–8 contained 0, 0.6, 3, and 15 pmol of (+)-CC-1065 with about 20 fmol of DNA. In lanes 9–11, DNA (20 fmol) was incubated at 30 °C for 10 min with 15, 30, and 60 ng of Sp1, respectively, and then reacted with 15 pmol of (+)-CC-1065 for 10 min. For lanes 5–11, reactions were stopped by adding 80 μL of solution containing 10 μg of calf thymus DNA and heating the sample at 95 °C for 10 min to induce strand breakage at the drug modification sites. Thermally treated samples were phenol/chloroform extracted and ethanol precipitated. DNA pellets were dried and redissolved in sequencing dye (10 mM NaOH and 80% formamide) prior to electrophoresis. (B, right) Time course of the modification of the 5′-AGTTA* sequence between GC boxes III and IV with (+)-CC-1065 in the presence of 60 ng of Sp1 (○) or in the absence of Sp1 (●). The amounts of strand breakage (%) at different times were determined by laser scanning of the autoradiogram.

tion of intramolecular ligation is most likely attributable to the binding of Sp1 molecules to GC boxes separated by an unoccupied Sp1 site (i.e., binding at sites III and V in the 21-bp repeat region). This dimeric protein–DNA complex would not lead to the protein–protein interactions necessary to stabilize the bent DNA structure, which are required for efficient intramolecular cyclization. On the other hand, intermolecular ligation would not be adversely affected by this phenomenon. However, at *saturating* levels of Sp1 molecules (lanes 5 and 6 in Figure 4A), the linear multimeric form of the 200-bp DNA was significantly diminished (9.8% and 2.5%, respectively, of the total ligation product), and multimer linear forms higher than 400-bp DNA were not detectable in lane 6. In contrast, the product of *intra*molecular cyclization increased from 67% of total ligation at subsaturating levels to 97.5% at saturating levels of Sp1. In accord with the results of the hydroxyl-radical footprinting experiments in the absence of Sp1, significant amounts of intramolecular cyclization products were produced in the absence of Sp1 (see lane 2, Figure 4A,B). Histogram representation of the ligation experiment is shown in Figure 4B. These results suggest that saturation binding levels of Sp1 molecules convert the linear 200-bp DNA molecules into the nonligated circular 200-bp structure, which is then an unfavorable substrate for further ligation to form linear multimers. It is significant that the circular form of DNA was formed even in the absence of Sp1, implying that these linear DNA molecules have a strong intrinsic tendency to form circular DNA molecules (see following).

Intervening Sequences between the GC Boxes Are an Important Determinant of the Intrinsic Bending of the 21-bp Repeat Region of SV40. The significant amount of monomer circular DNA found even in the absence of Sp1 (see Figure 4A,B) suggests that the 21-bp repeat has some phased intrinsic bending property. The hydroxyl-radical footprinting pattern in the absence of Sp1 of this same region reveals compaction of the minor groove at the 5′-AGTTA sequences and also at sequences separated from these positions by one helical turn (see Figure 2C, scan a). These observations suggested that the intervening sequences between the GC boxes might contribute to the overall intrinsic bending of the 21-bp repeat region. In order to test this possibility, a 64-mer sequence in which the intervening sequences, 5′-AGTTA and 5′-AGAAT, were replaced by 5′-CGCCG was prepared and compared to the natural sequence for intrinsic bending by a circularization efficiency assay. The results in Figure 5A show a comparison of the T4-catalyzed ligation ladders of the natural 64-mer and its GC-substituted oligomer. In the oligomer (64-mer in Figure 1B), in which the 5′-AGTTA and 5′-AGAAT sequences have been substituted by 5′-CGCCG sequences, the amount of circular DNA (C3, C4) is significantly reduced relative to the natural sequence DNA (compare lanes 1 and 2 in Figure 5A), and the optimal circularization size is increased from C3 to above C5 (Figure 5B). Last, a comparison of R_L values[2] for the natural vs the GC-substituted oligomer (Figure 5C) shows that the natural DNA has an intrinsic bending that is decreased upon substitution of the intervening sequences with a GC-rich region. Taken together, these results strongly suggest that the 5′-AGTTA sequence and its helically phased AT-rich regions are responsible for the intrinsic bending of the 21-bp repeat region of SV40 DNA.

Sp1-Induced Bending of DNA Occurs in a Direction toward the Minor Groove of DNA. The *direction* of Sp1-induced DNA bending was determined using a 42-mer oligomer (Figure 1C), which contains one of the 21-bp repeats of the

[2] R_L is defined as the ratio of apparent size to true size for each of the ligation products.

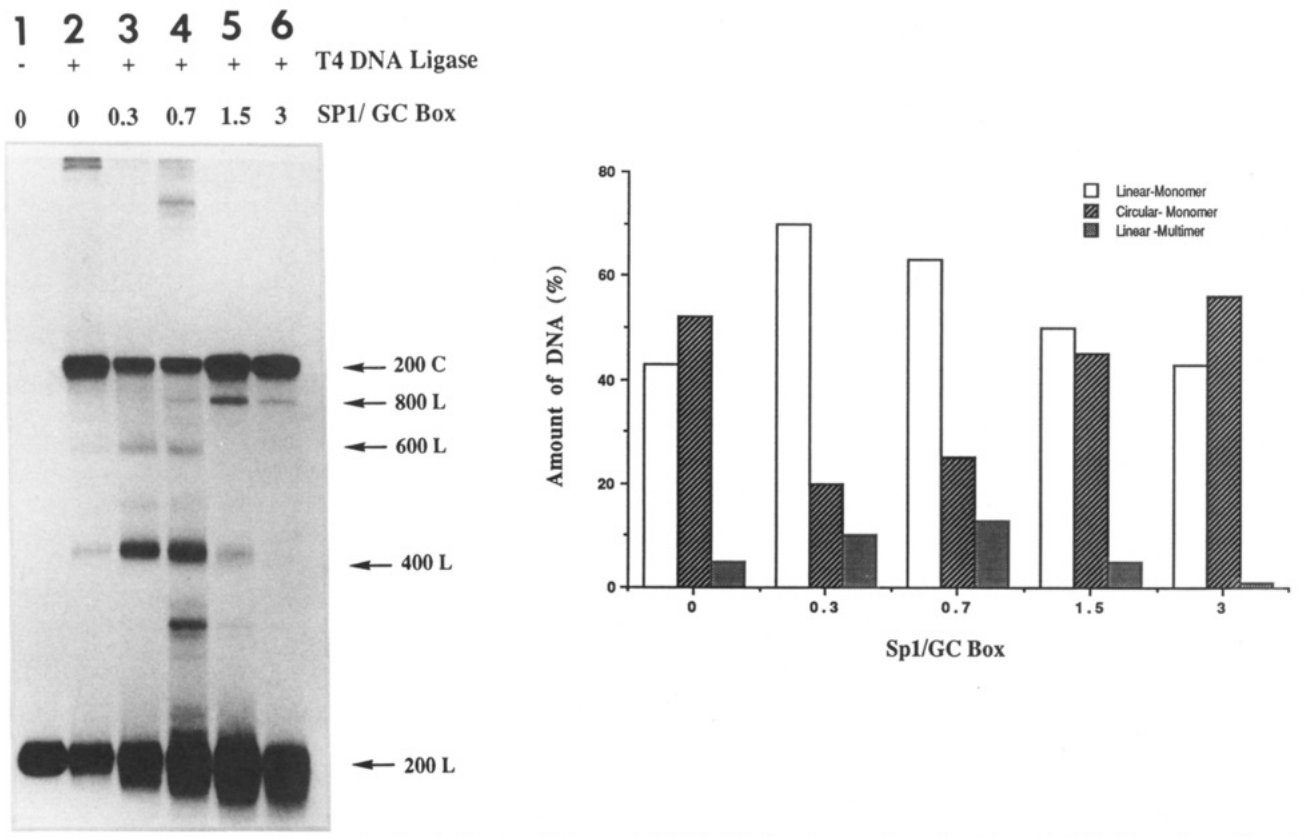

FIGURE 4: (A, left) Effect of Sp1 on the circularization efficiency of 200-bp DNA substrate. Lane headings (1–6) indicate the molar ratio (0–3) of Sp1 molecules to GC boxes of DNA substrate. Reactions containing 50 fmol of DNA with 2000 units of T4 DNA ligase in the presence of the indicated amounts of Sp1 were incubated at room temperature for 12 h. (B, right) Histogram representation of the autoradiogram described in A.

SV40 early promoter in the central region, except that the 5′-AGTTA sequence is replaced with an A_5-tract, which is expected to amplify the overall circularization process. Upon ligation of this oligomer, an A_6-tract is also generated exactly two helical turns away from the center of the A_5-tract sequence. If Sp1 molecules induce DNA bending toward the major groove of DNA, it would be expected that *out-of-phase* bent DNA would be produced in the presence of Sp1 molecules, resulting in a significant *decrease* in the amount of circular DNA. As shown in Figure 6, in the presence of Sp1 the formation of five and six *linear* multimers (L5 and L6) was significantly decreased relative to a control experiment carried out in the absence of Sp1, while the amount of *circular* DNA of six and eight multimers (C6 and C8) was *increased*.[3] This result strongly suggests that Sp1 molecules induce DNA bending toward the minor groove upon binding to GC boxes, which is consistent with the hydroxyl-radical footprinting results shown in Figure 2 and the competition drug bonding experiments carried out in the presence of Sp1 (Figure 3).

DISCUSSION

Using a combination of hydroxyl-radical footprinting, circularization experiments, and competition experiments between Sp1 and (+)-CC-1065, we have gained considerable insight into the structural features of the complex between Sp1 and the 21-bp repeat region of the SV40 viral early promoter region. The main point is the bending of the 21-bp repeats by cooperative binding of six Sp1 molecules. In addition, the hitherto undocumented importance of the 5′-AGTTA sequence located between GC boxes III and IV and V and VI in providing a locus for bending, resulting in the clustering of Sp1 molecules, is implicated. Last, the relationship between the high reactivity of this sequence to (+)-CC-1065 and its importance in Sp1 binding to the 21-bp repeat region is revealed. This work has important implications in understanding the mechanisms for transcriptional regulation by Sp1 and how drugs such as (+)-CC-1065, which can mimic a bent DNA structure upon reaction with DNA, may serve as useful probes for determining protein-induced bends in DNA.

Importance of the Propensity of the 5′-AGTTA Sequence To Form a Bent DNA Structure in the Molecular Mechanisms for Recognition of the 21-bp Repeat Region by Sp1 and (+)-CC-1065. (+)-CC-1065 is a potent antitumor agent that reacts covalently with DNA in a sequence-specific manner to form an N3 adenine adduct within the minor groove of DNA (Hurley & Draves, 1993; Hurley et al., 1984; Reynolds et al., 1985). As a consequence of covalent reaction with DNA, the minor groove is narrowed and the DNA assumes a bent structure, which has structural features (direction and magnitude) that are similar to those associated with the intrinsic bending of A-tracts (Lin et al., 1991; Sun et al., 1993a). We have recently proposed a "truncated junction bend" model for this bent DNA structure, which is analogous to Crothers's junction bend model for A-tracts (Koo & Crothers, 1988), except that the bend is more sharply focused (Sun et al., 1993a). Our model was based upon the combined results of high-field NMR studies, hydroxyl-radical footprinting, and gel electrophoresis of a (+)-CC-1065-modified 12-mer containing the highly reactive 5′-AGTTA* sequence and flanking bases from the 21-bp repeat region (see Figure 1). Particularly intriguing to us was the very high reactivity of this sequence to (+)-CC-1065 and its juxtaposition in the

[3] An anonymous referee pointed out that the odd-numbered circles are weaker in intensity than the even-numbered circles and that a possible explanation for this end alignment or writhe effect is that the 42-mers produce in- and out-of-phase ligamers.

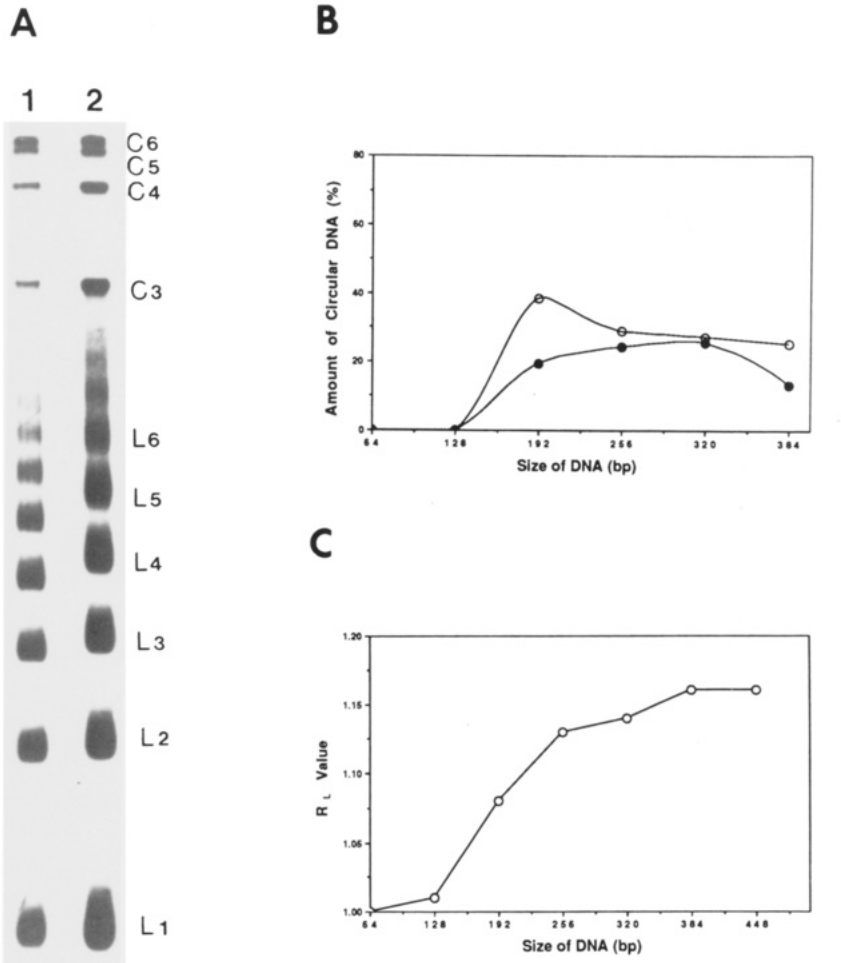

FIGURE 5: (A) Autoradiogram of the first-dimensional gel electrophoresis of the ligation reactions of the 64-mer oligomer containing the 21-bp repeat region (Figure 1B) and the equivalent 64-mer oligomer containing a 5′-CGCCG sequence in place of the 5′-AGTTA and 5′-AGAAT sequences (lanes 2 and 1, respectively). The upper family of bands (C3–C6) is circular 3–6-mers of the 64-bp precursor. The lower family of bands (L1–L6) is linear 1–6-mers. Both types of species of DNA were further separated in the second dimension by electrophoresis in a 6% polyacrylamide gel containing 50 μg/mL chloroquine phosphate (D. Sun and L. H. Hurley, unpublished results). Each band was then extracted from the gel and loaded onto a 4% denaturing polyacrylamide gel to determine the size of the DNA, using the methods described previously (Ulanovsky et al., 1986; Husain et al., 1988). (B) Graphical representation of the circularization efficiency for each given size of DNA. Each band was isolated, and the radioactivity was measured using scintillation counting to determine the ratio of the amount of circular species of DNA to the total amount of DNA at each given DNA size (from lane 1 (●) and lane 2 (○) in part A). (C) Plot of the R_L values of the native 64-mer vs the total length of ligated multimers of the 64-mer containing the 21-bp repeat region calculated from the comparative gel migration distances determined for the two samples shown in panel A.

21-bp repeat region with the two *highest* affinity Sp1 binding sites (III and V in Figure 1A).

A structural study (Lin et al., 1992) using high-field NMR, molecular modeling, and hydroxyl-radical footprinting on the 12-mer *duplex* containing this 5′-AGTTA* sequence provided some important observations. First, although this 12-mer duplex does not show intrinsic bending, the minor groove is narrowed and highly propeller twisted within the 5′-TT/AA region. There is rapid local internal motion at one of the adenines across from the covalent modification site (underlined in 5′-TAAC•T) and distortion, both at the cytosine (C•) and on the backbone on either side of this site. This results in a "transient kink" at the 5′-GT/AC step. Upon adduct formation with (+)-CC-1065, these features, except for the internal motion, are entrapped and in some cases exaggerated, and in addition, a discontinuity at the 5′-TA*/AT step (covalent modification site A*) is induced. The junctions in the model are positioned on either side of the 5′-TT/AA sequence, and the locus of bending is located between the two thymines. A summary of these structural features in the 12-mer duplex and the (+)-CC-1065 12-mer duplex adduct is shown in Figure 7.

In the present study, we have used hydroxyl-radical footprinting to probe the structural changes in DNA upon Sp1 binding to this region, gel electrophoresis to examine Sp1-induced bending of DNA, and (+)-CC-1065 to probe for overall groove structure and reactivity after Sp1 binding to the 21-bp repeats. The diminished cleavage of the region between each GC box (see Figure 2) reveals an important structural aspect of the Sp1 21-bp repeat complex. Generally, diminished hydroxyl-radical cleavage of the DNA backbone can be attributed to either close contacts between the sugar phosphate backbone and DNA binding ligand (e.g., small molecules or DNA binding proteins) or a structural change in minor groove geometry (Burkhoff & Tullius, 1988; Churchill et al., 1990). DNA bending, which results in diminished hydroxyl-radical cleavage as a consequence of compression of the minor groove (Shafer et al., 1989; Burkhoff & Tullius, 1988; Churchill et al., 1990), is the most well-characterized structural change in minor groove geometry. Suspicion that the correct explanation involves *bending* of DNA rather than steric occlusion for the diminished hydroxyl-radical cleavage stems from the observation that both 5′-AGTTA sequences located between GC boxes III and IV and V and VI show relatively diminished cleavage compared to the other regions (see panel a in Figure 2C), even in the *absence* of Sp1. Perhaps more persuasive is the comparison of the intrinsic bending of the 64-mer containing the 5′-AGTTA vs the straight control

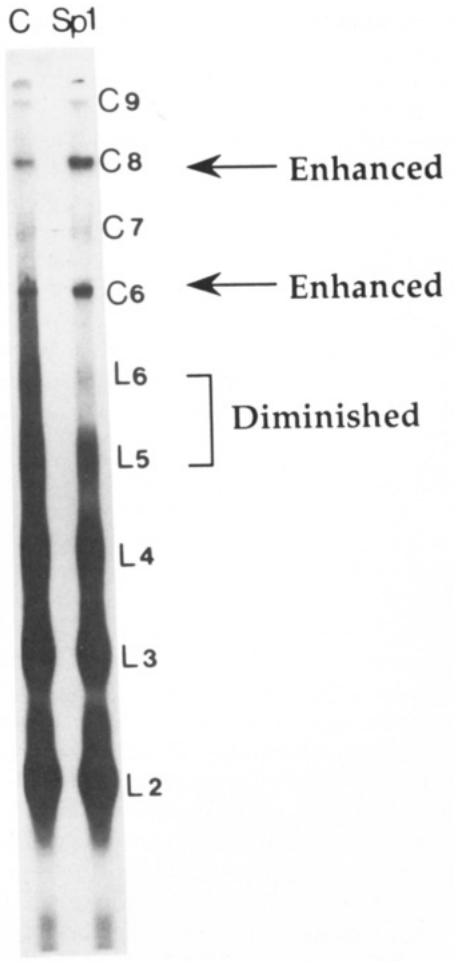

FIGURE 6: Autoradiogram of the first-dimensional gel electrophoresis of the ligation reaction of oligomer 42A without Sp1 (C) and with Sp1 (Sp1). The bands of the upper family (C6–C9) are circular 6–9-mers of the 42-bp precursor. The bands of the lower family (L2–L6) are noncircular 2–6-mers. The size of the DNA was determined as described in the legend for Figure 5A.

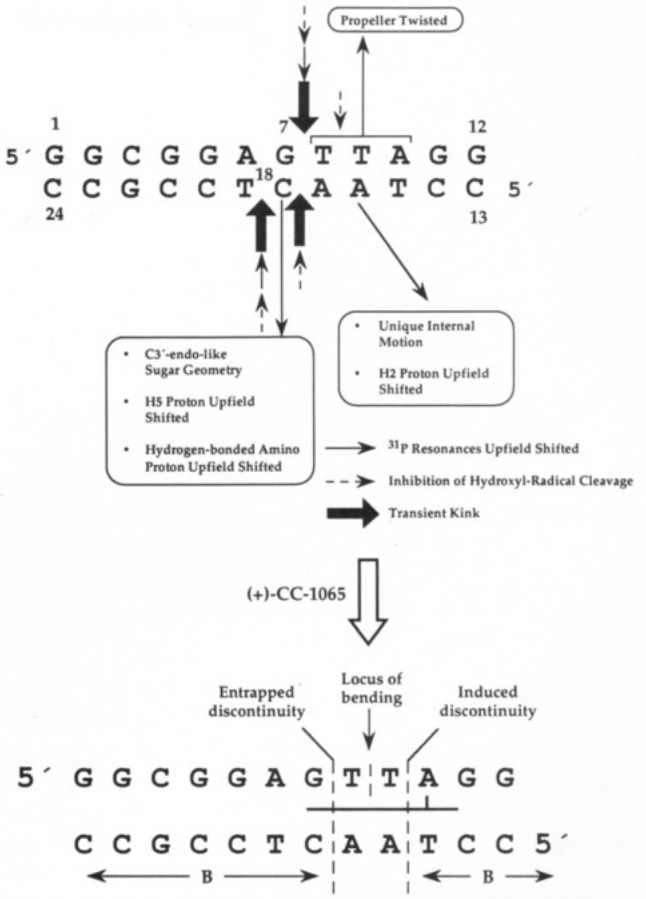

FIGURE 7: Summary of unusual structural features of the 12-mer duplex (Lin et al., 1992) and the (+)-CC-1065-modified 12-mer duplex adduct (Sun et al., 1993a).

DNA in which this sequence has been replaced by a GC sequence that eliminates this intrinsically bent DNA structure. These data demonstrate that this sequence has a tendency to form a bent DNA structure.

Furthermore, we propose that the binding of Sp1 molecules to the 21-bp repeat takes advantage of the preexisting tendency of this 5′-AGTTA sequence in order to form a bent DNA structure to create a boundary between consecutive Sp1 binding sites. Our results show that upon Sp1 binding to the 21-bp repeat region, the minor groove of the 5′-AGTTA sequence is further narrowed (compare a and c in Figure 2C) and, significantly, (+)-CC-1065 is still able to react at this sequence in an apparently normal way (Figure 3). That Sp1 binding to GC boxes III and IV or V and VI does not create *more* reactive sites for (+)-CC-1065 by freezing the 5′-AGTTA* sequences into a bent DNA structure can be rationalized if it is assumed that the Sp1-induced bent DNA structure is not optimal for drug reaction with DNA or that this is not the rate-limited step for the reaction. We propose that, just like (+)-CC-1065, Sp1 is able to take advantage of the transient kink at the 5′-GT/AC step and the associated propensity to form a bent DNA structure, and this is why Sp1 has the highest affinity for the GC boxes at the III and V sites. Furthermore, upon saturation binding of the 21-bp repeats with Sp1, we propose that the Sp1 molecules bound at GC boxes III and IV and V and VI stabilize a bent DNA structure, with the locus of bending around the center of the 5′-AGTTA sequence. Last, the apparently normal reactivity of (+)-CC-1065 at this sequence, even in the presence of saturation levels of Sp1, provides compelling evidence for Sp1-induced bending rather than steric occlusion being responsible for the inhibition of hydroxyl-radical cleavage.

The evidence for Sp1-induced bending of the 21-bp repeats is strongly supported by the results of the circularization experiments (Figures 4–6). In the first experiment, a 200-bp DNA fragment was constructed to contain inverted repeats of the two sets of six Sp1 binding sites of the SV40 viral early promoter region. Even in the absence of Sp1 binding, this 200-bp DNA produced significant amounts of the circular form of the 200-bp DNA, as well as linear multimers, implying that the transient kink in the 5′-AGTTA sequence might also have an important role in the circularization of 200-bp DNA. Moreover, in the presence of a subsaturating level of Sp1, the circular form of the 200-bp fragment was decreased and linear multimers were increased. We attribute this preference for intermolecular over intramolecular ligation to the lack of protein–protein interactions in protein–DNA complexes in which Sp1 binding occurs at sites (III and V) that are separated by one GC box not occupied by Sp1. These dimeric protein–DNA complexes would not be expected to stabilize the bent DNA structure, which would favor intramolecular ligation, and thus intermolecular ligation becomes more prevalent. However, upon saturation of the 200-bp DNA with Sp1, the formation of linear multimers from the 200-bp DNA was *severly inhibited*, while the formation of 200-bp circular DNA was *increased*. These results suggest that the 200-bp DNA complexed with 12 Sp1 molecules attains a nearly circular form. The decrease in formation of linear multimers from monomer 200-bp DNA also suggests that Sp1 molecules, through protein–protein interactions, can stabilize DNA molecules to form a rigid circular form and make this DNA

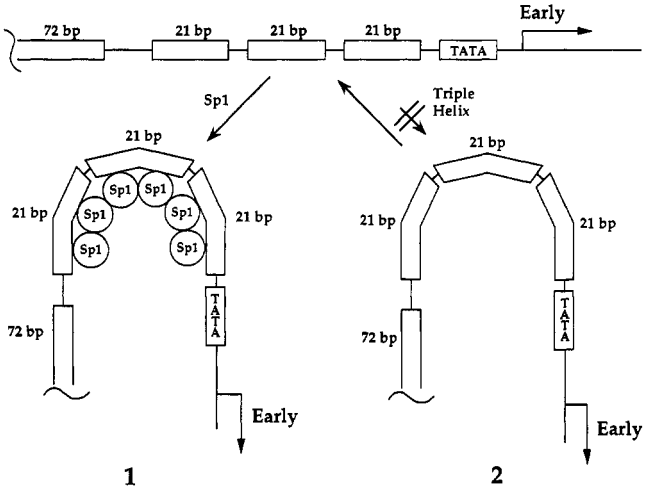

FIGURE 8: Proposed scheme for the formation of a looping structure (**1**) facilitated by interaction of Sp1 with the 21-bp repeats. While the linear structure predominates in the absence of saturating amounts of Sp1, small amounts of the looping structure (**2**) can be spontaneously produced in the absence of Sp1, but this conversion is blocked by triple-helical complexes (Maher et al., 1992).

an unfavorable substrate for further multimerization. In the second experiment, a 42-bp oligomer was used to provide further evidence for the Sp1-induced DNA bending and to determine the *direction* of bending by Sp1, which was shown to be in toward the minor groove. In the SV40 virus, the early promoter region contains six Sp1 binding sites in three 21-bp repeats between the TATA box and the 72-bp enhancers. Taking all of these results together, we propose that the six clustered Sp1 molecules binding within this region interact together to form a multiprotein–DNA complex having the looping structure shown in Figure 8. Presumably, the looping structure is stabilized to protein–protein interactions as well as protein–DNA interactions.

Transcriptional Implications of the Proposed Model for the Complex between Sp1 and the 21-bp Repeat Region. Electron microscopy studies have shown that Sp1-bound distal elements in the eukaryotic promoter can interact directly with those bound to the proximal element (Mastrangelo et al., 1991; Su et al., 1991). Upon the basis of the results of the hydroxyl-radical cleavage and circularization experiments described here, together with the ability of (+)-CC-1065 to still react with DNA in the presence of saturating amounts of Sp1, we propose that the multiple binding of Sp1 to the 21-bp repeats produces an overall bending of about 150–180° within the 21-bp repeat region (six Sp1 molecules, each contributing about 30°; see **1** in Figure 8). Since the Sp1 binding region that consists of three 21-bp repeats spanning 64 bp is located upstream adjacent to the TATA box for early transcription of SV40 and downstream of the 72-bp enhancer regions (Dynan & Tjian, 1983; Gidoni et al., 1984; Janson & Pettersson, 1990), we propose that the stabilization of a 180° bend in the 21-bp repeats produced by the cooperative binding of six Sp1 molecules to the six GC boxes might facilitate the interaction between the transcriptional factors complexed with the 72-bp enhancer region and basal transcriptional factors (e.g., TFIID, RNA polymerase) to enhance the transcriptional rate at the early promoter region. The looping model proposed here also explains the apparent paradox, identified by Gidoni and co-workers (1984), that the 21-bp repeat, which is an *asymmetric* sequence, can produce a *symmetrical* effect, since upon inversion of the repeats the promoter remains competent. The Sp1-induced curvature of the 21-bp repeat region would, of course, be insensitive to inversion.

Our present conclusion, based on the results of hydroxyl-radical cleavage and the circularization experiments, that the 21-bp repeat region has an inherent propensity to form a looping DNA structure (see **2** in Figure 8) in the absence of Sp1, provides an explanation for the observed degree of basal repression produced by triple-helix complexation (Maher et al., 1992), even in the *absence* of Sp1. The proposal that triple-helix-induced repression involves changes in DNA flexibility is in accord with the ideas presented here, namely, that DNA flexibility and bending are important components of the transcriptional regulation in the early promoter region of SV40 DNA. Last, the model proposed here has probable implications for other multi-zinc-finger binding proteins, such as TFIIIA (Churchill et al., 1990), in their protein–DNA complexes.

Structural and Biological Implications of the Modification of the 21-bp Repeat Region by (+)-CC-1065. DNA molecules have long been considered to be the molecular target for the selective action of a number of antiviral and antitumor compounds (Shea & Milligan, 1992). As a refinement of this postulate, we have proposed that it is more accurate to consider the real drug receptor as the DNA complexed with DNA binding proteins rather than the naked DNA since, *in vivo*, most DNA molecules are coated with DNA binding proteins, such as transcriptional factors and histone molecules (Hurley, 1989). In most cases, it is to be expected that the formation of drug–DNA complexes will be significantly inhibited by the addition of increasing amounts of proteins prior to the addition of drug molecules (Broggini et al., 1989; Reeves & Nissen, 1990; Dorn et al., 1992). However, in the case of (+)-CC-1065 and related analogous compounds, prebinding of 21-bp repeats with Sp1 molecules *did not* inhibit the binding of (+)-CC-1065 to the sequence 5′-AGTTA* (see Figure 3), while in control experiments Sp1 completely inhibited the binding of pluramycin to the sequence 5′-AG*TTAG* (* indicates the pluramycin binding sites). The previous arguments put forth in this paper rationalize the high reactivity of (+)-CC-1065 with the 21-bp repeats, even in the presence of Sp1.

In a parallel study (Sun & Hurley, 1994), we have determined the effect of (+)-CC-1065 on the binding of Sp1 to the 21-bp repeats of SV40 DNA. As a consequence of alkylation of the two 5′-AGTTA* sequences, which reside between GC boxes III and IV and boxes V and VI, protein binding to the 3′ sites is completely abolished, and there is a significant decrease in Sp1 binding to the other regions. The effect of substituting A_5-tracts for the (+)-CC-1065 bonding sequence was intermediate between the unmodified 5′-AGTTA* and the drug-modified sequences. It is proposed that a structural distortion of DNA associated with stiffening of the helix induced by the drug adduct formation is primarily responsible for the inhibition of binding of Sp1 molecules to 21-bp repeats, rather than steric hindrance due to the occupancy by drug molecules of the minor groove within that region.

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