Architectural Accommodation in the Complex of Four p53 DNA Binding Domain Peptides with the p21/waf1/cip1 DNA Response Element*

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High resolution chemical footprinting and cross-linking experiments have provided a basis for elucidating the overall architecture of the complex between the core DNA binding domain of p53 (p53DBD, amino acids 98–309) and the p21/waf1/cip1 DNA response element implicated in the G1/S phase cell cycle checkpoint. These studies complement both a crystal structure and earlier biophysical studies and provide the first direct experimental evidence that four subunits of p53DBD bind to the response element in a regular staggered array having pseudodyad symmetry. The invariant guanosines in the highly conserved C(A/T)|(T/A)G parts of the consensus half-sites are critical to the p53DBD-DNA binding. Molecular modeling of the complex using the observed peptide-DNA contacts shows that when four subunits of p53DBD bind the response element, the DNA has to bend approximately 20° to relieve steric clashes among different subunits, consistent with recent DNA cyclization studies. The overall lateral arrangement of the four p53 subunits with respect to the DNA loop comprises a novel nucleoprotein assembly that has not been reported previously in other complexes. We suggest that this kind of nucleoprotein superstructure may be important for p53 binding to response elements packed in chromatin and for subsequent transactivation of p53-mediated genes.

Wild type p53 is a widely distributed phosphoprotein that has become fundamental in cancer research (1–3). It functions as a tumor suppressor and is an essential component in the cell’s response to DNA damage (4). It acts as a transcriptional enhancer for a number of DNA damage and growth arrest genes including mdm2, gadd45, and p21/waf1/cip1, the last being involved in the G1/S phase checkpoint (5–8). When expressed at high levels, p53 suppresses transformation, arrests cells in G1 phase, and in some cases promotes apoptosis (9–11). Studies of tumorigenic mutants have shown that the majority are selectively located at sites that map directly to the specific DNA binding domain of p53 (12). This fact, supported by recent biochemical and molecular genetic evidence, has clearly demonstrated that much of the biological function of p53 is mediated by its DNA binding properties (13, 14).

Wild type p53 binds over 100 different naturally occurring response elements, of which approximately 60 show functional affinity. It has been estimated that the human genome contains approximately 200–300 such sites (15). Response elements differ in details of specific base sequence, but all contain two tandem decameric elements, each a pentamer inverted repeat. Most decamers follow the consensus sequence pattern RRRCA/A/T|(A/T)GYYY, where R and Y are purines and pyrimidines, respectively, and the vertical bar denotes the center of dyad symmetry (16). These decameric elements may be separated by as much as 21 bp without complete loss of p53 binding affinity, but functional sites, defined as the ability to transcriptionally activate a nearby reporter gene, evidently follow very closely the consensus decamer pattern with no or only very short intervening spacers (15). Wild type p53 binds response elements through a sequence-specific DNA binding domain extending from amino acid residue 96 to 308 (13). Studies of tumor-derived p53 mutants have shown that they are defective in sequence-specific DNA binding and consequently cannot activate transcription (17). These studies have demonstrated that sequence-specific DNA binding and transactivation are the key biochemical activities responsible for much of the biological function of p53.

A recent cocrystal structure of a p53DBD nucleoprotein complex has provided valuable insights into the binding specificity of p53 by identifying specific binding contacts (18). This important work has had a major impact on thinking about p53 structure-function relationships. However, both the complex size and the binding sequence were necessarily restricted in the cocrystal. Although the oligonucleotide used in the cocrystal contained a full 10-bp consensus half-site (Fig. 1B), only a single core domain peptide bound specifically with a consensus pentanucleotide, while a second bound nonspecifically 11 bp away and the third did not contact the DNA. Thus, many questions remain. These include the full origin of sequence specificity in DNA binding and the roles of specific p53 response elements, the multisubunit nature of the full p53 nucleoprotein complexes and the overall organization of p53 tetramers bound to the DNA recognition site, and the possible role of conformational changes in both the protein and the DNA as a consequence of complexation.

In the present study, we provide the first report of a struc-
The model is based on the results of several experiments sensitive to base-specific nucleoprotein contacts between the p53DBD peptides and response element DNA including hydroxyl radical footprinting, melting nucleoside analyses, and methylation and ethylation interference assays. Studies were conducted on a 65-bp oligonucleotide that includes the p21/waf1/cip1 response element; this response element contains both a consensus (Fig. 1A, boxes 1 and 2) and a nonconsensus (boxes 3 and 4) half-site. To facilitate comparison of the present work with the crystallographically determined contact sites (18), we also studied a 67-bp oligonucleotide containing the same 20-bp binding sequence used in the cocrystal structure, designated in this work as the Cho sequence (Fig. 1B). Our solution results generally agree with the crystallographic contacts for the Cho sequence, although we find evidence for a second binding site of reverse orientation within this sequence that was not observed in the cocrystal. Our results for the p21/waf1/cip1 site show unequivocally that four p53DBD peptides bind this response element in a staggared array and that each consensus pentanucleotide of the p21/waf1/cip1 site makes specific contacts with the p53DBD and its invariant guanosine nucleotide playing a critical role. Hydroxyl radical footprinting demonstrates structural microheterogeneity in the consensus binding sites, suggesting that sequence-dependent structural variability of response elements plays a critical role in the binding of p53 with DNA. Model building of the p53DBD-p21/waf1/cip1 nucleoprotein complex using the results of various chemical probes and footprinting shows that the four bound p53DBD peptides bend the response element by ~50° to relieve the steric clashes among the bound subunits. This model is in quantitative agreement with recent T4-DNA ligase-mediated cyclization studies (19) and with circular permutation gel retardation assays of the p53DBD-p21/waf1/cip1 complex (20).

MATERIALS AND METHODS

Oligonucleotide Synthesis and Labeling—Oligonucleotides used in the study (Fig. 1), were synthesized and were purified on a 15% denaturing polyacrylamide gel. Equimolar amounts of complementary single-stranded oligonucleotides were mixed and annealed by heating to 90 °C and slowly cooling down to room temperature. Oligonucleotide duplexes, designed with one base overhang, were uniquely labeled at different ends of both strands. The top strands were labeled at the 5′-end with [α-32P]dCTP using the Klenow fragment of DNA polymerase, and the bottom strand was labeled using [γ-32P]ATP and polynucleotide kinase using standard procedures (21). The labeled duplexes were purified once more on 10% native polyacrylamide gels to remove traces of labeled single-stranded DNA and free [γ-32P]ATP.

Preparation and Purification of the DNA Binding Domain of p53 (p53DBD)—A human p53 cDNA clone encoding amino acid residues 96–308 was amplified by polymerase chain reaction using p53-specific primers 5′-ATATGAGTCCTCAACAAGTCTCAGTTA-3′ and 5′-ATATGGATCCTCACAGTGCTCGCTTAGTGCTC-3′. The amplified product was cloned in the pET12a expression vector (Novagen), and the core DNA binding domain was overproduced in Escherichia coli BL21 (DE3). The cells were incubated at 37 °C until an A600 of 0.6–1.0 was attained, and 0.25 mM isopropyl β-D-thiogalactoside was added to induce the expression of the recombinant protein. Cells were harvested after 2 h by centrifugation, lysed in a French press, and sonicated for 2 min in 40 mM MES, pH 6.0, 100 mM NaCl, 5 mM dithiothreitol. The soluble fraction was loaded onto a Resource S column (Pharmacia Biotech Inc.) in 40 mM MES, pH 6.0, 5 mM dithiothreitol and was eluted in a 0–400 mM NaCl gradient. The pooled fractions were precipitated with ammonium sulfate addition to 80% saturation and purified further on a Sephadryl SR200 HR gel filtration column (Pharmacia) in 0.1 M Tris-propane-HCl, pH 6.8, 100 mM NaCl, 1 mM dithiothreitol. The purified p53DBD was checked on an SDS-polyacrylamide gel for purity.

Hydroxyl Radical Footprinting—Single end-labeled oligonucleotide duplexes (5 × 106 cpm, ~500 ng) were dissolved in a binding buffer containing 50 mM Tris-Cl, 10 mM dithiothreitol, and 50 mM NaCl (8 μl). Purified p53DBD (4 μg, ~105 cpm) at a molar ratio of 1:4 DNA:protein and the complex was allowed to form for 30 min on ice. Hydroxyl radical cleavage reactions were initiated by adding a mixture containing 8 mM FeSO4(NH4)2SO4,0.6M H2O, and 1 mM EDTA (20 μl), 0.3% H2O2 (10 μl), and 20 mM sodium ascorbate (2 μl). Reactions were carried out on ice for 2 min and were quenched by adding 0.1 mM thioeurea (5 μl). Each sample was mixed with 15% Ficoll (4 μl) and was loaded on a 4% native polyacrylamide gel containing 20 mM HEPES, pH 8.3, as a running buffer. The gels were run for about 1 h at 8 V/cm to separate bound DNA from traces of unbound oligonucleotides. The bound fraction was gel-eluted in 0.5 mM NH4Cl, 0.1 mM EDTA, extracted with phenol/chloroform, precipitated twice with ethanol, and run out on a sequencing gel. The control DNA was also cleaved under identical conditions.

Ethylation Interference Assay—Labeled oligonucleotides (1 × 106 cpm, ~1 μg), were mixed in 200 μl of 50 mM sodium cacodylate, pH 8.0, 1 mM EDTA containing sonicated salmon sperm DNA (1 μg) and tRNA (2 μg) and were incubated with 0.5 μl of dimethyl sulfate at room temperature for 2 min. The methylation reactions were terminated by adding 50 μl of 1.5 mM sodium acetate, 1 mM β-mercaptoethanol, 100 μg/ml tRNA and 750 μl of ethanol. The DNA was precipitated twice with ethanol, resuspended in 20 μl of 0.4 M NaCl and recompressed in 200 μl of 0.3 M sodium acetate containing 0.2 mM EDTA and was precipitated with 500 μl of ethanol. The pellet was washed with 70% ethanol, lyophilized, and dissolved in the binding buffer (18 μl). Purified p53DBD (5 μg) was added, and the complex was allowed to form for 30 min on ice. Each reaction was chased for 5 min with a 5 mM excess of cold oligonucleotide duplexes to titrate any nonspecific complex formation. The amount of cold oligonucleotides and chasing time was adjusted such that 90% of DNA remained bound. The bound and unbound fractions were separated on a mobility shift gel, as described above, eluted from the gel, precipitated with ethanol, washed with 70% ethanol, and loaded on a sequencing gel. Control DNA was also cleaved under identical conditions.

Hydration Interference Assay—The labeled oligonucleotides (1 × 106 cpm, ~1 μg), were mixed in 200 μl of 50 mM sodium cacodylate, pH 7.0, 1 mM EDTA containing sonicated salmon sperm DNA (1 μg) and tRNA (2 μg) and were incubated with 0.5 μl of dimethyl sulfate at room temperature for 2 min. The methylation reactions were terminated by adding 50 μl of 1.5 mM sodium acetate, 1 mM β-mercaptoethanol, 100 μg/ml tRNA and 750 μl of ethanol. The DNA was precipitated twice with ethanol, resuspended in 20 μl of 0.4 M NaCl and recompressed in 200 μl of 0.3 M sodium acetate containing 0.2 mM EDTA and was precipitated with 500 μl of ethanol. The pellet was washed with 70% ethanol, lyophilized, and dissolved in the binding buffer (18 μl). Purified p53DBD (4 μg) was added, and the complex was allowed to form for 30 min on ice. Samples were loaded on a mobility shift gel as described above, and the bound and unbound fractions were eluted from the gel, precipitated with ethanol, washed with 70% ethanol, and loaded on a sequencing gel. Control DNA was also treated with dimethyl sulfate under identical conditions and was analyzed on the gel.

Glutaraldehyde Cross-linking—Purified p53DBD (2 μg) was incubated after 2 h by centrifugation, lysed in a French press, and sonicated for 2 min in 40 mM MES, pH 6.0, 100 mM NaCl, 5 mM dithiothreitol. The solute fraction was loaded onto a Resource S column (Pharmacia Biotech Inc.) in 40 mM MES, pH 6.0, 5 mM dithiothreitol and was eluted in a 0–400 mM NaCl gradient. The pooled fractions were precipitated with ammonium sulfate addition to 80% saturation and purified further on a Sephadryl SR200 HR gel filtration column (Pharmacia) in 0.1 M Tris-propane-HCl, pH 6.8, 100 mM NaCl, 1 mM dithiothreitol. The purified p53DBD was checked on an SDS-polyacrylamide gel for purity.

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bated in the presence of labeled oligonucleotides containing the p21/waf1/cip1 and Cho sequences (Fig. 1, A and B) for 40 min on ice in 20 μl of DNA binding buffer. Increasing concentrations of freshly diluted glutaraldehyde were added for 15 min on ice. Partially cross-linked samples were dissociated by boiling in SDS and were loaded on an 8% SDS-polyacrylamide gel. The gel was run at 10 V/cm for 2 h and silver-stained to locate the cross-linked protein-DNA complexes. The same gel was dried and autoradiographed, and the DNA bands corresponding to the cross-linked species were located by superimposing the autoradiogram on the gel. p53DBD, used as a control in the experiment, was also treated under identical conditions. The protein molecular weight marker was heat-denatured in SDS and loaded as a control.

Electrophoretic Mobility Shift Assay—Labeled oligonucleotides of known concentration were mixed with poly(dI-dC) (200 ng) and incubated with varying amounts of p53DBD in DNA binding buffer at 4 °C. The complex was allowed to incubate for 40 min at 4 °C. An aliquot of the sample was incubated with 12 μg of RNAse A Cleavage, Gel Electrophoresis, and Densitometric Scan-ges singly end-labeled at the 5'-end of the bottom strand (3×10^6 cpn, ~200 ng) were incubated with 12 μg of p53DBD in DNA binding buffer. The complex was allowed to incubate for 40 min at 4 °C. An aliquot of the sample was checked on a nondenaturing polyacrylamide gel in 0.25× TBE at 4 °C. Identical unbound oligonucleotides were run in parallel. The bands were quantitated using a PhosphorImager (Bio-Rad), and the DNA binding affinities of the samples were determined as described by Carey et al. (22). The same gels were later used to determine the stoichiometry of p53DBD binding with the p21/waf1/cip1 and Cho sequences (19).

DNase I Footprinting—p21/waf1/cip1 and Cho duplexes singly end-labeled at the 5’-end of the bottom strand (3×10^6 cpn, ~200 ng) were incubated with 12 μg of p53DBD in DNA binding buffer. The complex was allowed to incubate for 40 min at 4 °C. An aliquot of the sample was checked on a nondenaturing polyacrylamide gel to ensure complete saturation of the DNA binding sites with p53DBD. The samples were electrophoresed on a 7% nondenaturing polyacrylamide gel in 0.25× TBE at 4 °C. Identical unbound oligonucleotides were run in parallel. The bands were quantitated using a PhosphorImager (Bio-Rad), and the DNA binding affinities of the samples were determined as described by Carey et al. (22). The same gels were later used to determine the stoichiometry of p53DBD binding with the p21/waf1/cip1 and Cho sequences (19).

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Hydroxyl Radical Footprinting of p53DBD with p21/waf1/cip1 and Cho Sequences—Hydroxyl radical cleavage (27) was used to footprint p53DBD complexes with the p21/waf1/cip1 and Cho sequences (Fig. 1, A and B). Fig. 2, A and B, shows hydroxyl radical footprinting data for the p53DBD-p21/waf1/cip1 and p53DBD-Cho complexes, respectively. Densitometric plots of the various lanes are shown in Fig. 2, E and F. The top strand of the naked p21/waf1/cip1 response element (as presented in Fig. 1A) shows reduced cleavage at CATG and TGTT base sequences within the 20-bp consensuses binding site, with clear minima at TG sequence elements (marked as arrows in

RESULTS

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Fig. 2. Hydroxyl radical cleavage (footprinting) (panels A, B, E, and F) and missing nucleoside analyses (panels C, D, G, and H). Lanes G and G + Â are Maxam-Gilbert guanosine and guanosine + adenosine sequencing reactions. Vertical brackets in panels A–D locate the p53 binding sites. Panels A and C, 65-mer oligonucleotide containing the p53DBD-p21/waf1/cip1 complex (Fig. 1A); panels B and D, 67-mer oligonucleotide containing the p53DBD-Cho complex (Fig. 1B). In panels A and B, lanes C and F refer to the absence (control) and presence of p53DBD, respectively. In panels C and D, lanes B and U refer to bound and unbound fractions, respectively. Panels E–H are densitometric plots corresponding to the gels in panels A–D, respectively. Plots a and b are cleavage patterns of the top strand in the absence and presence of p53DBD, respectively. Plots c and d are similar except for the bottom strand. The arrows indicate maxima or minima in the cleavage patterns (see “Results”). In panels E and F, solid brackets indicate bases showing reduced cleavage and dashed brackets higher cleavage frequency. In panels G and H, solid brackets indicate bases specifically discussed under “Results.”

The unique hydroxyl radical cleavage profiles suggest that the p21/waf1/cip1 response element has narrowed minor grooves at CATG and TGTT sequences and a relatively wider minor groove at the CCCAA sequence. p53DBD binding further narrows the minor grooves involving ATGT and TGTT bases in the two half-sites, compresses the major groove at the GTTGGG bases, and shields their sugar-phosphate backbone from the minor groove side, leading to their reduced cleavage. This may, in turn, further expose the sugar-phosphate backbone of the complementary CCCAA sequence from the minor groove side, making it more susceptible to hydroxyl radical cleavage. x-ray crystallography (28) and model building (25) on a variety of DNA sequences indicate that the minor groove width in the ACA/TGT sequence in the first half-site should be characterized by the distances between sugar moieties of G7 and G14 and the phosphate distances of G7–T3 and G14–T18 and by the similarly positioned nucleotides in the second half-site (Fig. 5A, b). Hydroxyl radical data indicate that p53DBD binding drastically reduces the cleavage frequency in the G7 and T18 in the first half-site and in G14 and T18 in the second half-site (Fig. 2E, a and b). Since hydroxyl radical cleavage is a diffusion-controlled process, the data clearly point to a relatively narrow minor groove in the CATG regions in the two half-sites, which is in accord with the crystal structure data (18).

The higher cleavage at the ACAT and CAT bases in the bottom strand as compared with the ATGT and TGTT sequence at the top strand probably indicates asymmetric distortion of the double helix in this region, leading to differential exposure of the sugar-phosphate backbone of the two strands to hydroxyl radicals. The relatively T-rich regions of the consensus binding site, which have narrow minor grooves, are spaced at integral helical periodicity and occur on the same face of the double
helix (Fig. 5A). It is also of interest that GGGC sequences, previously shown to have major groove-directed bending (29–31), occur in the central region of many functionally important p53 response elements. The helically phased CA:TG sequence elements in the highly conserved region of the consensus binding site have been shown to be kinked in the CAP nucleoprotein complex (32) and may be similarly kinked in other regulatory complexes (33). Thus, it is possible that intrinsic flexibility in these sequence elements may promote the formation of a more stable p53-DNA complex by facilitating specific protein-DNA and protein-protein interactions. A recent study from this laboratory has shown that p53DBD binds the p21/waf1/cip1 response element as a tetrapeptide with high cooperativity and that the DNA is bent by \(~50–60\)^° in solution (19). Thus, it is likely that a region of compressed major groove, located between two regions of narrow minor groove, as suggested by the present hydroxyl radical footprinting, may provide the structural basis for such a bending. We also observe that there is an inherent asymmetry in the footprinting patterns for the two half-sites, which otherwise might be expected to be identical.

We believe that such an inherent asymmetry in the complex may play a crucial physiological role in terms of bending directionality, as in the case of the TATA-binding protein-TATA complex (34).

The hydroxyl radical cleavage patterns for the Cho sequence (Fig. 1B) and its complex with p53DBD are shown in Fig. 2B, and densitometry plots are shown in Fig. 2F. The top strand of the unbound Cho sequence shows reduced cleavage at the TCT and the CA sequence elements within the consensus half-site, while a higher cleavage frequency occurs at the GGG bases (marked with dashed brackets). The cleavage frequency is also markedly reduced at a TT element outside this half-site (Fig. 2B, lane C; Fig. 2F, plot a). Binding with p53DBD further diminishes the cleavage frequency of the TCT and TT sequences, while it enhances the cleavage frequency in the GGG bases (Fig. 2B, lane F; Fig. 2F, plot b). However, cleavage in the bottom strand is enhanced in the complementary AGAC and AA sequences and reduced in the TTGC bases. Binding with p53DBD further reduces the cleavage pattern in the TTGC sequence. The data clearly point to a narrow minor groove in the TCT sequence and a relatively compressed major groove in the GGGC region. These footprinting data are in general agreement with the crystallographic results (18), in which a narrowed minor groove was observed in the TCTAG sequence due to high propeller twisting. However, the cleavage pattern in the GGGCA region demonstrates a relatively compressed and shielded major groove and indicates binding of p53DBD in this region, consistent with the crystallographic results (18).

**Missing Nucleoside Experiments**—Specific base contacts for both complexes were determined using missing nucleoside experiments (35). Fig. 2, C and D, show the missing nucleoside data for the p53DBD-p21/waf1/cip1 and p53DBD-Cho complexes respectively; corresponding densitometric plots are shown in Fig. 2, G and H. The bound fraction of DNA in the top strand of p53DBD-p21/waf1/cip1 complex (Fig. 1A) leads to weaker bands for bases in the two ATGT sequence elements in each half-site and intense bands for bases in the central CCCAAC sequence (Fig. 2C, lane B; Fig. 2G, plot a). The opposite pattern is observed in the unbound fraction, i.e. bands corresponding to bases in the central CCCA region are weak, whereas bands for bases ATGT in both of the half-sites are more intense (Fig. 2C, lane U; Fig. 2G, plot b). In the bottom strand, the protein-bound DNA shows weaker bands for bases in ATGT in both half-sites, whereas in the unbound fraction these bands show intense bands (Fig. 2C, lanes B and U; Fig. 2G, plots c and d). These data clearly show that ATGT sequence elements in both half-sites of the top and bottom strands make important contacts with the bound protein. The modification or absence of either of these base contacts greatly reduces the affinity of p53DBD with DNA. It is significant that these bases show reduced cleavage in direct hydroxyl radical footprinting (Fig. 2, A, B–E, and F), suggesting a narrow minor groove in these regions and further substantiating their direct involvement in the stability of the nucleoprotein complex.

Missing nucleoside data for the p53DBD-Cho complex are shown in Fig. 2D with corresponding densitometric plots in Fig. 2H. The top strand of the bound fraction shows relatively weak bands in the GGCA and CTA sequence elements (Fig. 2D, lane B; Fig. 2H, plot a). In the unbound fraction, strong bands are observed in this sequence element, indicating direct contact of these bases with the bound p53DBD (Fig. 2D, lane U; Fig. 2H, plot b). The bottom strand in the bound fraction shows weak bands in the TGCC element, while in the unbound fraction these bases exhibit much stronger bands (Fig. 2D, lanes B and U; Fig. 2H, plots c and d). Thus, the data clearly demonstrate that bases in the GGCA element in the top strand and in the TGCC element in the bottom strand make critical contacts with the bound peptide. Most of the contact sites probed by hydroxyl radical footprinting and missing nucleoside probing in the p53DBD-Cho complex are also observed in the crystal structure data (18).

**Methylation Interference Assays**—Methylation interference assays have been widely used to identify contacts between bound proteins and methylated guanosines in the major groove of the DNA (36). Fig. 3A shows the methylation interference results for the p53DBD-p21/waf1/cip1 complex. Corresponding densitometric plots of the different lanes are shown in Fig. 3E. The bound DNA fraction of the top strand (Fig. 1A) shows reduced cleavage (greater interference) at the guanosines in the two TG sequence elements (G^7^ and G^17^ in Fig. 1A) compared with more intense cleavage at these sites in the unbound fraction (Fig. 3A, lanes B and U; Fig. 3E, plots a and b). This suggests that these guanosines are in direct contact with p53DBD in the major groove. In the bottom strand, the bound DNA fraction shows reduced cleavage of guanosines at the two TG elements (G^4^ and G^14^) compared with much stronger bands in the unbound DNA fraction (Fig. 3A, lanes B and U; Fig. 3E, plots c and d). The residues in the central GGG region of the bottom strand show reduced cleavage in the bound DNA fraction as compared with the control DNA (lane C), but these guanosine signals are missing entirely from the unbound fraction.

These data clearly show that the central GGG guanosines in the bottom strand contact the p53DBD differently from those in the two TG elements. Methylation of the former does not affect p53DBD binding, whereas the guanosines in the TG doublets evidently make structurally important contacts with the protein, probably at the N-7 position, and when these sites are modified by methylation, p53DBD does not bind to DNA. It is important to note that TG guanosines are present in each pentamer quarter-site and constitute the invariant base in most of the high affinity p53 binding sites reported so far. All four guanosines in the TG base doublets (G^7^, G^17^, G^4^ and G^14^) are tandemly arrayed in the alternate major groove of the double helix, suggesting that all four subunits of p53DBD bind in the major groove.

The methylation interference data for the p53DBD-Cho complex are shown in Fig. 3B with corresponding densitometric plots in Fig. 3F. In the top strand, the p53DBD-bound fraction shows relatively weaker bands for the GGG (G^7^, G^9^, G^11^) sequence and G^13^ compared with the unbound fraction (Fig. 3B, lanes B and U; Fig. 3F, plots a and b). In the bottom strand,
both the bound and unbound fractions show relatively intense bands for guanosine G10, with the stronger band observed in the unbound DNA fraction. The data clearly indicate that the bases G7, G8, G9, and G13 in the top strand and G10 in the bottom strand make direct contact with the bound peptide, which is consistent with the cocrystal structure (18) in which Arg283 and Lys120 are in direct contact with G7 and G8, while Arg280 contacts G10. G13 (top strand) either makes direct contact with p53DBD at its N7 position or is in very close proximity with the peptide in the complex. This observation is also consistent with the cocrystal structure in which the sugar-phosphate backbone of G13 and T14 makes direct contact with Arg248. We do not observe contacts between p53DBD and bases in the GGA sequence element at the end of the top strand, i.e. G18, G19 and A20, as observed in the cocrystal structure.

Ethylation Interference Assays—Sugar-phosphate contacts were probed by ethylation interference (37). Results for the p53DBD-p21/waf1/cip1 complex are shown in Fig. 3 with corresponding densitometric plots of the bound and unbound fractions shown in Fig. 3G. For the top strand (Fig. 1A), the bound fraction shows weaker bands for bases in the ATGT sequence elements in the two half-sites with clear minima at the two TG base doublets (Fig. 3C, lane B; Fig. 3G, plot a), whereas in the unbound fraction, these two guanosine residues show significantly more intense bands (Fig. 3C, lane U; Fig. 3G, plot b). The AA sequence elements at the center of the binding site also appear as bands of medium intensity. For the bottom strand, the bound fraction (Fig. 3C, lane B; Fig. 3G, plot c) shows weaker bands in TGT sequence elements in both half-sites, with clear minima at the G residues (marked as arrows). On the other hand, bases in the TG elements appear as highly intense bands in the unbound fraction (Fig. 3C, lane U; Fig. 3G, plot d). These data clearly indicate that the DNA backbone at the ATGT sequences in the two half-sites is in contact with the p53DBD in the complex, which is consistent with the hydroxyl radical and missing nucleoside data. Particularly, the sugar-phosphate backbone at G7, G17, G4, and G19 makes very critical contacts with p53DBD, and ethylation of phosphates at these residues greatly reduces the binding affinity of the p53DBD-p21/waf1/cip1 complex.

The Binding of p53DBD to the p21/waf1/cip1 and Cho Binding Sites—Fig. 4 shows the results of several studies on the binding stoichiometry and positioning of the p53DBD peptide on the two DNA binding sites investigated. Glutaraldehyde cross-linking (38, 39) was used to determine the stoichiometry.
main, in agreement with gel band shift and ultracentrifugation results (19). With the p53DBD-Cho half-site, cross-linked species include trimers and tetramers in the SDS-polyacrylamide gel (Fig. 4A, lanes 3–5) but only monomers and dimers in the autoradiography (Fig. 4B, lanes 3–5). This indicates that p53DBD associates with the Cho sequence primarily as a monomer or dimer. The p53DBD peptide cross-linked in the absence of response elements can also weakly tetramerize, but the primary cross-linking products are monomers and dimers (Fig. 4A, lane 2). In Fig. 4, A and B, the DNA and protein bands move as doublets in the gel. An explanation for this commonly observed phenomenon may be that the faster band in each doublet arises from additional intrapeptide cross-linking and incomplete denaturation of the protein in SDS, leading to a more compact structure for the denatured complex and therefore slightly higher mobility (39).

A gel electrophoretic mobility shift assay was used to determine the binding affinity and stoichiometry of the p53DBD with the p21/waf1/cip1 and Cho sequences and is shown in Fig. 4C. It is evident that p53DBD binds the p21/waf1/cip1 response element only as a tetramer with high cooperativity. We assign the minor bands of slightly lower mobility than the tetramer complex to the nonspecific binding of p53DBD with the single-stranded duplex overlaps (40, 41). No intermediate bands, e.g. dimer or trimer, are evident. We estimate a mean equilibrium dissociation constant, $K_d$, of $4.2 \times 10^{-10}$ M for the p53DBD-p21/waf1/cip1 complex, which is in satisfactory agreement with the value ($8.3 \pm 1.4 \times 10^{-10}$ M obtained by analytical ultracentrifugation (19), since the $\sim 5$-fold difference most probably results from a lower dissociation rate of the complex in the gel (42). In the case of the Cho sequence, p53DBD binds as a dimer with an estimated mean equilibrium dissociation constant $K_d$ of $7.8 \times 10^{-7}$ M. This level of binding affinity is not much greater than that for many nonspecific nucleoprotein complexes. However, there is no evidence in Fig. 4, B, C, and E, for the association of a third p53DBD monomer as was observed in the cocrystal (18).

![Fig. 4. Glutaraldehyde cross-linking of p53DBD with oligonucleotides containing p21/waf1/cip1 and Cho sequences (Fig. 1, A and B). A, 8% SDS-polyacrylamide gel followed by silver staining. Lane 1, protein molecular weight markers. Lane 2, p53DBD cross-linked in the absence of DNA. Lanes 3, 4, and 5, different oligomeric states of p53DBD cross-linked to the Cho oligonucleotide in the presence of 0.01, 0.05, and 0.1% glutaraldehyde, respectively. Lanes 6, 7, and 8, different oligomeric states of p53DBD cross-linked to the p21/waf1/cip1 oligonucleotide in the presence of 0.01, 0.05, and 0.1% glutaraldehyde, respectively. B, autoradiograph of the gel in A showing cross-linked DNA species associated with lanes 3–8 of A. C, electrophoretic mobility shift assay of the p53DBD-Cho complex (Fig. 4, A and B). G, lanes 6, 7, and 8, and Cho sequences, respectively. D, autoradiograph of the gel in A showing cross-linked DNA species associated with lanes 3–8 of A. C, electrophoretic mobility shift assay of the p521/waf1/cip1 and Cho sequences. The molar ratios of p53DBD to DNA are given at the bottom of the gel. D and E, DNase I footprinting of the p21/waf1/cip1 and Cho sequences, respectively. The lanes marked G and G + A are the Maxam-Gilbert sequencing reactions; lanes C and F are control DNA and DNA with p53DBD, respectively.](Image)

FIG. 4. Glutaraldehyde cross-linking of p53DBD with oligonucleotides containing p21/waf1/cip1 and Cho sequences (Fig. 1, A and B). A, 8% SDS-polyacrylamide gel followed by silver staining. Lane 1, protein molecular weight markers. Lane 2, p53DBD cross-linked in the absence of DNA. Lanes 3, 4, and 5, different oligomeric states of p53DBD cross-linked to the Cho oligonucleotide in the presence of 0.01, 0.05, and 0.1% glutaraldehyde, respectively. Lanes 6, 7, and 8, different oligomeric states of p53DBD cross-linked to the p21/waf1/cip1 oligonucleotide in the presence of 0.01, 0.05, and 0.1% glutaraldehyde, respectively. B, autoradiograph of the gel in A showing cross-linked DNA species associated with lanes 3–8 of A. C, electrophoretic mobility shift assay of the p521/waf1/cip1 and Cho sequences. The molar ratios of p53DBD to DNA are given at the bottom of the gel. D and E, DNase I footprinting of the p21/waf1/cip1 and Cho sequences, respectively. The lanes marked G and G + A are the Maxam-Gilbert sequencing reactions; lanes C and F are control DNA and DNA with p53DBD, respectively.

of p53DBD bound to the p21/waf1/cip1 and Cho response elements. Fig. 4A shows an SDS-polyacrylamide gel electrophoresis analysis of the cross-linked species of the p53DBD-p21/waf1/cip1 complex (Fig. 4A, lanes 6, 7, and 8) and the p53DBD-Cho complex (lanes 3, 4, and 5) at three glutaraldehyde concentrations. The p53DBD-p21/waf1/cip1 complex shows four bands of 28, 46, 69, and 92 kDa, representing monomer, dimer, trimer, and tetramers, respectively. Direct autoradiography confirms that each band is associated with its corresponding DNA fragment (Fig. 4B, lanes 6, 7, and 8). This demonstrates clearly that the p53DBD peptide associates with the full p21/waf1/cip1 response element as a tetrapeptide, even in the absence of the wild type p53 oligomerization do-

A Comparison of the Footprinting Data for p53DBD Complexed with the p21/waf1/cip1 and Cho Sequences—We have summarized the above results on the p53DBD-p21/waf1/cip1 and p53DBD-Cho complexes in Figs. 5, panels A and B, respectively. For the p53DBD-p21/waf1/cip1 complex, certain structurally related characteristics become immediately apparent. (i) Most of the contact signals occur in the major groove of the DNA in a staggered array along the two helical turns of the response element in which each of the four bound p53DBD peptides occupies a single pentanucleotide quarter-site and faces outward, away from the DNA, in the same direction. The data indicate that each p53DBD binds to the major groove in agreement with the cocrystal structural study (18). (ii) Four

DISCUSSION
guanosine bases, G7 and G17 in the top strand and G4' and G14' in the bottom strand, which are part of an invariant sequence motif in each pentamer quarter-site, are identified as contacts in all of the four footprinting techniques used. These bases are in phase along the helix with a separation of 10 bp and are located in the major groove of the DNA. (iii) Most of the observed contact points are clustered in the major groove with relatively few contacts in the minor groove also in agreement with the specifically bound p53DBD in the crystal structure (18). (iv) The GTTG sequence of the bottom strand shows higher protection from hydroxyl radical cleavage than does the complementary CAAC sequence on the opposite strand, suggesting that it is shielded by bound peptide from the minor groove side, whereas the complementary CAAC sequence is exposed.

For the p53DBD-p21/waf1/cip1 nucleoprotein complex, the present results are most consistent with a model in which all four p53DBD peptides make their most important contacts between the major groove of the response element DNA with their loop sheet helix motif. The p53DBD peptides are bound to the major groove of each pentameric quarter-site in an alternating array so that, apart from DNA bending, the full complex possesses a quasi-C2 symmetry. The data also require that the conserved guanosines of each pentamer half-site play a crucial role in the binding. This model is not generally appropriate for the p53DBD-Cho complex, however. In this complex, the hydroxyl radical and the missing nucleoside contacts are symmetrical along the DNA, and the methylation and ethylation interference signals and some of the missing nucleoside contacts show a marked asymmetry. The footprinting contacts are arranged very differently from those in the p21/waf1/cip1 complex and suggest that there are two binding sites in the Cho sequence in opposite orientations along the DNA. One is to the GGCGA quarter-site element in the upper strand (Fig. 1B; Fig. 5B, c), and the second is to the TGCCC element in the lower strand. Contacts in the first of these are consistent with those observed in the cocrystal structure (Fig. 5B, a) (18), and since this is the only specific binding site observed crystallographically, it is likely that it is a site of higher binding affinity. A direct repeat of TGCTT, having close homology with the TGCCC element in the Cho sequence, has previously been shown to be a functionally important p53 binding site (44, 45).

The relatively lower quality of the footprinting data for the p53DBD-Cho complex compared with the p21/waf1/cip1 complex suggest that, in solution, a dynamic equilibrium may exist between complexes involving these two sites. In the p21/waf1/cip1 response element, all the four invariant guanosines occur in the TG sequence elements. By contrast, in the Cho sequence, only one guanosine occurs in the TG element, whereas the other occurs in an AG element. Since we observe intense contact signals for the guanosines in the p21/waf1/cip1 TG elements but not in the Cho half-site AG, this suggests that AG elements are very poor contact sites for p53 binding. Again, these results are consistent with earlier observations (18, 46).

**Stereochemical Model for the p53-DNA Complex**—To rationalize our footprinting and cross-linking data presented above, we have developed a computer-assisted structural model for the p53DBD-p21/waf1/cip1 complex. In this model, each of the four bound p53DBD peptides is attached to a single pentameric quarter-site as suggested by the present footprinting results.

The p53DBD coordinates are taken from the cocrystal structure; in that study, no changes in the p53DBD structure were observed upon DNA binding (18). Docking of the peptides to the DNA is determined by the specific contacts found here (Fig.
The model requires that, to avoid severe interpeptide steric clashes, the four p53DBD peptides must induce bending of the response element DNA by $\pm 50^\circ$ (Fig. 8), a result in close agreement with recent cyclization studies (19) and with circulatory permutation gel electrophoresis assays (20). Specifically, we find two sets of steric clashes in the complex between four p53 domains and "straight" DNA. The first of these occurs between the zinc-binding H1 helices (18) of peptides bound to two adjacent pentamers (Figs. 6A and 7A); these two p53 domains are bound to opposite sides of the duplex in opposite orientations and "embrace" the DNA. The second clash occurs between the two peptides in similar orientation and bound to the same side of the helix; they are separated by a single helical turn (Fig. 6C). It is important to note that these clashes were observed in all complexes involving unbent DNA that were analyzed (see "Materials and Methods").

The above steric clashes are relieved if the p21/waf1/cip1 DNA is bent by positive rolling through $15^\circ$ toward the major grooves in the CA:TG dimers indicated by chemical points: GAAC at positions 19 and 16 and GTCCCAAC at positions 33 and 30. Such axial bending does not interfere with the specific interaction of the p53DBD peptides with their cognate pentamers. The two adjacent rolls in the DNA that cause these changes are shown in Figs. 6B and 7B.

Fig. 6. Diagrams showing how molecular modeling determines steric clashes between the four p53DBD peptides bound to the p21/waf1/cip1 response element as directed by the chemical probe results and how these clashes are relieved by bending the DNA by a roll at the CA:TG sequence elements. Nomenclature for p53DBD structural elements is from Cho et al. (18). The zinc-binding H1 helices are shown as ellipses. The H2 recognition helices are shown as sharply pointed regions buried in the major grooves. The four pentamer response element quarter-sites are numbered 1–4. Pentamers 1 and 3 and pentamers 2 and 4 are in a parallel orientation, while pentamers 1 and 2 and pentamers 3 and 4 are antiparallel. A, in unbent DNA, binding of two p53DBD peptides to pentamers 1 and 2 (antiparallel orientation) is accompanied by steric hindrance between the H1 helices (overlapped ellipses; see legend to Fig. 7A for details). B, this clash is relieved by bending the DNA toward the major groove along the dyad axis by a positive roll at the TT:CA sequence elements as discussed under "Discussion" (see legend to Fig. 7B for details). C, in unbent DNA, binding of two p53DBD peptides to pentamers 1 and 3 (parallel orientation) is accompanied by steric hindrance. The darkened overlap area represents steric clash between residues 99, 167, 170, and 210 from one peptide and residues 224, 140, 199, and 201 from the other; a steric clash is presumed if the distance between two heavy atoms in a residue pair is less than 2.4 Å. D, this clash is relieved by bending the DNA toward the major grooves along the dyad axes at two points separated by 10 bp. This bending is the same as in B and is affected by a positive roll at the TT:CA sequence elements as discussed under "Discussion."

Fig. 7. Interactions between the H1 helices and L3 loops in the p53-DNA complexes for unbent DNA (18) (A) and bent DNA (B). A, the H2 helix from the left domain (green) interacts specifically with the pentamer GGGCA (also shown in green) (18). The right domain is located symmetrically, so that its H2 helix (blue) interacts with the pentamer AAGAT shown in blue (for details, see "Materials and Methods"). Inset, the H1 helices interdigitate, producing unacceptable steric clashes (the heavy atoms in the protein are shown as spheres of 50% van der Waals radii). The zinc atoms are shown as large red spheres. The clashes specifically involve Arg$^{181}$ and Pro$^{177}$. Similar clashes occur using other unbent DNA models including canonical B-form DNA (see "Materials and Methods"). B, bending the DNA toward the major groove at the junction between the green and blue pentamers relieves the H1-H1 clash (the bend is directed away from the viewer). The important spatial complementarity between the two H1-L3 moieties is shown in the inset; in this view, the protruding residues are Arg$^{181}$ (in the H1 helix) and Met$^{243}$ (in the L3 loop).
CA: TG dimers induce a bend toward the major groove as indicated in Fig. 6, B and D. It is important to note that the same DNA bends relieve both types of clashes: antiparallel, as in Figs. 6 A and 7 A, and parallel, as in Fig. 6 C. The putative rolls in the CA: TG steps are consistent with the well known flexibility of these dinucleotides (33), demonstrated in numerous x-ray structures of pure B-DNA and protein-DNA complexes (26, 32, 47), as well as by gel electrophoresis (48). According to energy calculations, these dinucleotides can also bend anisotropically toward the major groove (25).

This direction of DNA bending is entirely consistent with the consensus p53-binding sequence. All four possible tetramers allowed by the consensus, i.e., CA: TG, CT: TG, CA: AG, and CT: AG, contain dinucleotide elements that can flex toward the major groove. In addition to CA: TG, the CT: AG element also prefers bending toward the major groove (26, 49, 50). AA: TT or AT: AT dinucleotides are the central elements in three of the tetranucleotides. Both dinucleotides prefer a negative or zero roll angle (26); thus, the minor groove is expected to be relatively narrow, which is desirable for interactions with the highly conserved Arg248 (18). The CT: AG tetranucleotide contains the TA: TA element, which can assume roll angles between −6.4° in free DNA (51) to +12° in the trp repressor complex (52) and appears, in addition, to possess unusual torsional flexibility (31, 32). However, the CT: AG element is reported to be extremely rare in functional genomic p53 response elements (15).

In the detailed model presented in Fig. 8, the roll angles for CA: TG are taken as 15°. This leads to an overall bend of −50°, which is consistent both with the present experimental results and with the previous cyclization studies (19). CA: TG roll angles between 15 and 20° are sufficient to relieve all peptide-peptide clashes; 15° seems preferable, however, since this leads to a structure in which the p53DBD peptides are in close proximity to one another (Figs. 7 B and 8) and hence can interact to produce the observed binding cooperativity (19).

According to this model, shown in Fig. 8, the C-terminal residues of p53 are located on the inside of the DNA curve. The
C-terminal domains are involved in oligomerization, protein-protein interactions, and the modulation of DNA binding (53). This arrangement may be advantageous for the stabilization of the entire superstructure in the binding of the wild type protein. On the other hand, the N-terminal region, which contains the transactivation domain, is located on the outside of the loop, where it may be more accessible to other proteins. The model presented in Fig. 8 suggests that the intrinsic bendability of p53 response elements favors the same direction of DNA bending as in the p53 nucleoprotein complex. Thus, p53 response elements may be wrapped in chromatin in such a way as to facilitate the approach and binding of the protein. In other words, p53 may prefer to bend response element DNA from the side that is normally exposed in chromatin structures. This is consistent with a transcriptional role for p53 that may be mediated by the architectural organization of chromatin (54, 55) and the looping of DNA (56).

Conclusions—The p21/waf1/cip1 site is one of the most important functional sites for p53 binding presently known (57). The full site consists of a consensus (16) and a nonconsensus half-site, making it generally representative of a broad class of p53 response elements (15). The data presented here provide a more complete picture than previously available for the overall spatial arrangement of the peptides on the p21/waf1/cip1 response element DNA along with critical p53DBD-DNA contacts and important information on the conformation of the binding site DNA. The footprinting and contact data show that a p53DBD is bound in the major groove on each of the four pentameric quarter-sites of the p21/waf1/cip1 response element with the peptides oriented in the same direction. Molecular modeling shows that relief of steric clashes in the bound peptides requires the DNA to bend at flexible TG:CA sequence elements, resulting in an overall bend of ~50° over the full response element away from the peptide complex. Such a bending motif differs from previously observed motifs in that major groove-binding proteins usually wrap the DNA (32, 47), whereas minor groove binders bend the DNA in the direction observed here (58).

The present model is realistic in terms of p53 function, however, since it suggests that the C-terminal domains of the full p53 protein lie on the concave side of the bend and the N-terminal transactivation domain on the convex side (Fig. 8); this would be a convenient arrangement both in terms of chromatin structure and of possible allosteric regulation in the intact protein (59). In addition, wild type p53 interacts with a variety of cellular and viral oncoproteins through its acidic transactivation domain (60). The proposed arrangement of this domain coupled with inherent flexibility in the response element may facilitate the formation of such multiprotein supercomplexes. This view of the p53DBD-p21/waf1/cip1 complex also underscores the symbiosis between the absolute structural and the chemical probes/molecular modeling approaches. Although no high resolution co-crystal structure is yet available for the p55DBD-p21/waf1/cip1 complex, the results from the existing structural study (18) complement the chemical data presented here, and the two permit the development of a reasonable model for the full tetrapeptide complex.

It is known that TG:CA sequence elements are ubiquitous in genomic regulatory sites and particularly so in those in which DNA bending may occur in the regulatory nucleoprotein complexes (33). Various studies have shown that these elements are flexible, with potential for abrupt kinking (25, 48, 61, 62), and such kinking has been observed structurally in a limited number of nucleoprotein complexes (32, 47). Hence, it is quite likely that helically phased TG:CA or other flexible sequence elements play a critical role in the ability of p53 response elements to bend and accommodate the protein in the p53 complex, thus enhancing the stability of the complex through more precise, and perhaps more extensive, protein-DNA contacts. Most functional p53 binding sites contain helically phased TG:CA sequence elements in approximately the same relative positions (16). This requirement may also provide a structural explanation for the fact that p53 response elements that contain spacers between the palindromic half-sites have generally been observed to be highly unstable and nonfunctional (15).

The present results suggest that sequence-dependent structural and dynamical properties of the response element DNA may modulate both the stability and structure of p53 nucleoprotein complexes. This is consistent with earlier studies that demonstrated substantial DNA bending in several p53DBD-DNA complexes (19) and suggests a possible role for the variation observed in the sequences of known naturally occurring functional p53 response elements (15). The present findings support the concept that both specificity and stability of p53-DNA nucleoprotein complexes are controlled by a complex interplay of specific sequence contacts as well as inherent structural features of the DNA. In addition to deformation of the DNA and the sequence-directed requirement of the two narrow minor grooves and a compressed major groove as found in this study, these effects must include a (possibly variable) set of specific protein-DNA and intra-p53 protein-protein contacts and may also include specific or nonspecific inter-p53DBD interactions.

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