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Discrimination of DNA Binding Sites by Mutant p53 Proteins

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Critical determinants of DNA recognition by p53 have been identified by a molecular genetic approach. The wild-type human p53 fragment containing amino acids 71 to 330 ($p53_{71-330}$) was used for in vitro DNA binding assays, and full-length human p53 was used for transactivation assays with *Saccharomyces cerevisiae*. First, we defined the DNA binding specificity of the wild-type p53 fragment by using systematically altered forms of a known consensus DNA site. This refinement indicates that p53 binds with high affinity to two repeats of PuGPuCA.TGPyCPy, a further refinement of an earlier defined consensus half site PuPuPuC(A/T).(T/A)GPyPyPy. These results were further confirmed by transactivation assays of yeast by using full-length human p53 and systematically altered DNA sites. Dimers of the pentamer AGGCA oriented either head-to-head or tail-to-tail bound efficiently, but transactivation was facilitated only through head-to-head dimers. To determine the origins of specificity in DNA binding by p53, we identified mutations that lead to altered specificities of DNA binding. Single-amino-acid substitutions were made at several positions within the DNA binding domain of p53, and this set of p53 point mutants were tested with DNA site variants for DNA binding. DNA binding analyses showed that the mutants Lys-120 to Asn, Cys-277 to Gln or Arg, and Arg-283 to Gln bind to sites with noncanonical base pair changes at positions 2, 3, and 1 in the pentamer (PuGPuCA), respectively. Thus, we implicate these residues in amino acid-base pair contacts. Interestingly, mutant Cys-277 to Gln bound a consensus site as two and four monomers, as opposed to the wild-type p53 fragment, which invariably binds this site as four monomers.

The gene for the tumor suppressor protein p53 is commonly mutated in human tumorigenesis (15, 22, 27, 35). Wild-type p53 negatively regulates cell growth and division, whereas p53 mutants do not suppress cell growth and in some cases can promote the growth of tumor cells (5, 10, 14, 20). The physiological pathway of p53 in the control of cell growth is not clearly understood. However, there is considerable evidence for regulation of transcription as a mechanism of p53 action. p53 protein is capable of specific DNA binding and transactivation, which are necessary for suppression of cellular transformation (1, 8, 9, 13, 19, 29, 34). Most of the natural mutations associated with tumorigenesis occur in the central domain of p53, which is essential for interaction with DNA (2, 13, 28, 33, 37), T antigen (23, 30), and cellular proteins p53BP1 and p53BP2 (16). Natural mutations occurring in this domain result in the loss of DNA binding and transactivation (19, 25, 29, 34). Several DNA binding sites of wild-type p53 have previously been described; these include repeats of the pentamer TGCCT (1, 8), two copies of the 10-bp motif PuPuPuC(A/T)(T/A)GPyPyPy (6, 11), and variations of this latter motif in some genes (7, 18, 26, 38–40).

The crystal structure of the DNA binding domain has provided some insight into the origin of specificity in p53-DNA interactions (3). Recently, we identified several p53 residues that were implicated in specific DNA binding by alanine mutagenesis (33). In this paper, we identify amino acid-base pair contacts in the p53-consensus DNA complex by a genetic approach. First, systematic alterations of the known p53 consensus DNA site were made, and the binding specificity of wild-type p53 was determined. We then made several single-amino-acid substitutions by in vitro mutagenesis of the DNA binding domain of p53. This set of p53 point mutants and DNA site variants was used to identify contacts in the p53-DNA complex between individual amino acids of p53 and individual base pairs of the DNA site.

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MATERIALS AND METHODS

Mutagenesis and expression of p53. The phagemid pRSET A(L) was used for mutagenesis and expression in *Escherichia coli* of p53 with a histidine tag at its N terminus. Cloning of the wild-type p53 fragment, $p53_{71-330}$, encoding amino acids 71 to 330 into pRSET A(L) has been described previously (33). For in vitro mutagenesis of *p53*, a uracil-containing template, pRSET A(L)-*p53*, was obtained by coinfecting this phagemid with M13K07 helper phage into *E. coli* CJ236. Synthetic oligonucleotides bearing the appropriate mismatches were used to synthesize a second strand from the uracil-containing template. Double-stranded DNA sequencing was performed to screen for correct mutants. The conditions for induction and purification of proteins have been described previously (33). Briefly, total protein extracts made from IPTG (isopropyl-β-D-thiogalactopyranoside)-induced cells were clarified by centrifugation and passed through a Ni nitrilotriacetic acid column (Qiagen), and proteins were step eluted with native imidazole elution buffer.

Plasmids for *p53* transactivation assays. Plasmid pY10-*p53* was used for expression of the wild-type human p53 gene in *Saccharomyces cerevisiae* (33). This plasmid contains 2μm sequences, the *TRP1* gene for selection in yeast, the *Bla* gene for selection in *E. coli*, and the p53 gene under the control of the yeast *ADH1* promoter. The reporter plasmid pY28, which is derived from pLGK669Z, is a *URA3*, 2μm-based plasmid containing the *CyC1-lacZ* gene (12, 33). Double-stranded 43-bp oligonucleotides containing 20-bp canonical and noncanonical p53 binding sites and flanked by identical sequences were ligated into *Kpn*I-*Xho*I-digested pY28. For transactivation assays, yeast CM1 cells were cotransformed with plasmid pY10-*p53* and reporter plasmid pY28 containing one copy of the consensus binding site or a variant binding site.

DNA binding assays. DNA binding assays were performed with a purified p53 fragment containing amino acids 71 to 330 and expressed in *E. coli*. The DNA sites used in binding assays are listed in the appropriate figures. The flanking sequences of consensus and altered double-stranded DNA sites were identical; the upper strand from 5′ to 3′ was CAA ACG CGT AGC C (binding site) TCG CGA CAG GTC C. p53 protein was incubated in 25 μl of binding buffer (25 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], 50 mM KCl, 20% glycerol, 0.5 mM dithiothreitol, 1 mg of bovine serum albumin per ml) at room temperature for 20 min in the presence of 100 ng of poly(dI-dC) and 1 ng

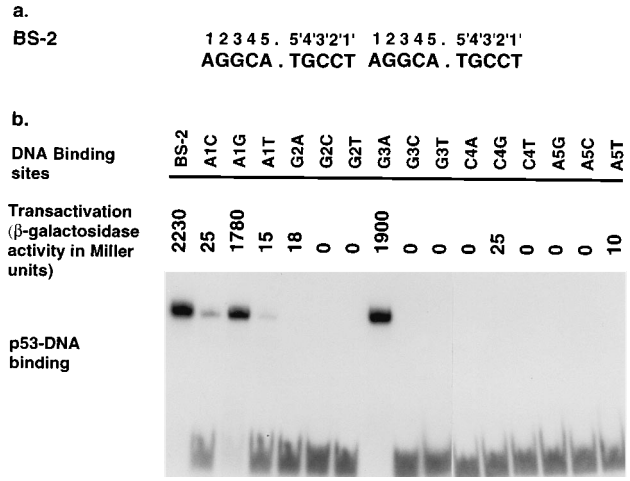


FIG. 1. DNA-binding specificity of wild-type p53. (a) DNA sequence of the consensus site used in p53 binding studies. The upper-strand DNA sequence of the bases flanking this site was 5′ CAA ACG CGT AGC C (binding site) TCG CGA CAG GTC C 3′, which is unrelated to the consensus site. (b) Oligonucleotides containing symmetric changes in BS-2 were chemically synthesized with 13-base flanking sequences on each end as described in Materials and Methods. A1C represents a change at the first position of the pentamer AGGCA from the AT base pair to the CG base pair in both dyads, as well as the symmetric changes at position 1′ shown in panel a. One nanogram of the indicated end-labeled DNA site and 50 ng of the *E. coli*-expressed and purified p53 fragment containing amino acids 71 to 330 were incubated in DNA binding buffer. Protein-DNA complexes were resolved on 6% acrylamide gels (0.5× Tris-borate-EDTA). For transactivation assays, CM1 cells were cotransformed with plasmid pY10-*p53*, encoding full-length human p53, and pY28, a minimal *CYC1-lacZ* reporter plasmid containing one copy of BS-2 or the indicated variant binding site. Five-milliliter cultures of strain CM1 were grown in synthetic medium containing 2% glucose and lacking uracil and tryptophan for plasmid selection to an optical density of 1.0. Cells were prepared, and β-galactosidase enzyme activities were determined as previously described (24). Enzyme activities are reported in Miller units (24) and are averages of three independent assays.

of end-labeled binding site (25,000 cpm) with or without the unlabeled competitor DNA. Protein-DNA complexes were resolved on 6% acrylamide gels (0.5× Tris-borate-EDTA), and the gels were dried and autoradiographed. For competition assays, binding reactions were mixed with increasing amounts of unlabeled DNA. The amounts of free and bound DNA were quantitated by using a Molecular Dynamics PhosphorImager.

Cross-linking of p53 in p53-DNA complexes. The stoichiometries of p53-DNA complexes of wild-type p53 and p53 mutant C-277→Q (C277Q) were determined by protein cross-linking with glutaraldehyde. DNA binding assays were performed with an end-labeled radioactive DNA probe containing the 20-bp p53 binding site A1G (see Fig. 1). p53 complexes were excised from a preparative scale mobility shift gel and incubated for 15 min at room temperature with increasing concentrations of glutaraldehyde. Complexes were eluted, concentrated with a microcentricon-100 (Amicon), boiled in sodium dodecyl sulfate (SDS)-sample buffer, run on an SDS–10% polyacrylamide gel, and blotted to nitrocellulose, and p53 protein was detected with pAb 240 antibody and an enhanced chemiluminescence procedure using an ECL kit (Amersham).

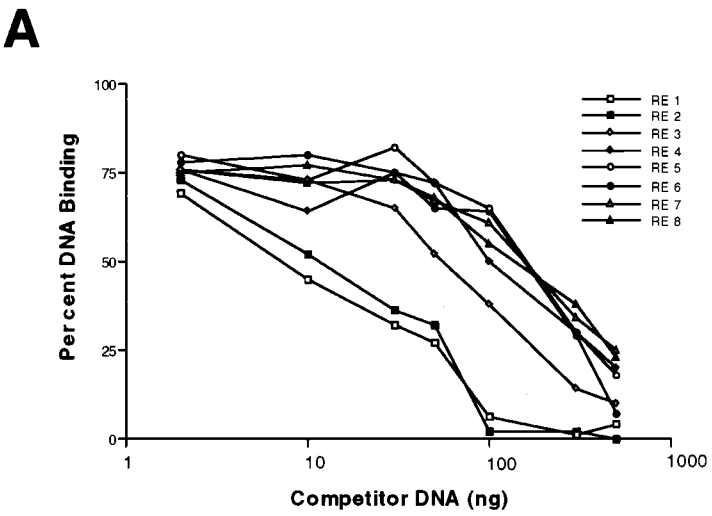
Yeast strain, growth conditions, and β-galactosidase assays. *S. cerevisiae* CM1 (*MATα pep4-3 trp1Δ ura3*) was used in this study. Yeast cells were grown in yeast extract-peptone-dextrose or appropriate selective minimal media. β-Galactosidase assays were done in liquid cultures. Five-milliliter yeast cultures were grown in the appropriate selective media to an optical density at 600 nm of between 0.8 to 1.0. Cells were then prepared and permeabilized as previously described (24). Activities are reported in units as defined by Miller (24).

RESULTS

DNA binding specificity of p53. To evaluate the DNA binding specificities of wild-type p53, all 15 possible symmetrical mutations in both copies of the 10-bp palindromic sequence were made (Fig. 1a). According to the nomenclature used in Fig. 1, A1C represents symmetric changes at positions 1 and 1′ of AGGCA.TGCCT from AT to CG and from TA to GC, respectively, in both repeats. Mutant binding sites were evaluated for DNA binding by wild-type p53 and for the ability to transactivate in yeast. A compilation of data is presented in Fig. 1b. Mobility shift assays with the wild-type p53 segment containing amino acids 71 to 330 and variant DNA sites showed that changing the guanine at position 2, cytosine at position 4, or adenine at position 5 resulted in the loss of all detectable binding. At positions 1 and 3, either purine was acceptable. Wild-type p53 exhibits strong specificity for an AT or GC base pair at position 1 of the DNA site; however, binding was adversely affected by a TA or CG base pair substitution at this position. At position 3, AT and GC base pairs were equally acceptable, but TA and CG substitutions resulted in the complete loss of DNA binding. In vitro DNA binding was corroborated by yeast-based transactivation assays with mutant DNA sites in a minimal *CYC1-lacZ* promoter and expression of wild-type full-length p53 (Fig. 1b). Thus, on the basis of these data, the deduced consensus binding site is two copies of the 10-bp motif PuGPuCA.TGPyCPy. This sequence is a further refinement of the previously reported consensus sequence PuPuPuC(A/T).(T/A)GPyPyPy (6, 11). The G at position 2 and A at position 5 of the pentamer PuPuPuC(A/T) appear to be critical for high-affinity DNA binding activity and transactivation.

Orientation requirement of quarter DNA site repeats for high-affinity DNA binding by p53. We further investigated the orientation requirement of the pentamer quarter sites for high-affinity DNA binding and transactivation by p53. Consensus p53 binding consists of two repeats of head-to-head (h-h) dimers of pentamer quarter sites (RE1) (Fig. 2). DNAs containing two repeats, either h-h (RE1) or tail-to-tail (RE2), performed equally well in competition with BS-2. Surprisingly, p53 did not transactivate through RE2, indicating that the h-h orientation of pentamer repeats is important for transactivation. Efficient competition by RE2 of the p53-DNA complex might have resulted from the presence of three pentamer repeats, each in an orientation similar to that in RE1. Since this orientation is shifted by 5 bp relative to that in RE1, the p53 complex is rotated half a turn on the DNA, possibly removing important interactions with p53 subunits or other transcriptional factors and thus resulting in the loss of transactivation. Binding sites with a single dyad in h-h orientation (RE3), two or three pentamer repeats that are 0 or 5 bp apart (RE3 to RE7), or four direct repeats (RE8) were poor competitors of the p53-consensus DNA complex and did not transactivate. RE9, which contains five repeats of the pentamer quarter site 5 bp apart, was able to compete and transactivate to a limited degree.

Identification of amino acid-base pair contacts. Residues K-120, R-248, R-249, R-273, C-277, R-280, D-281, R-282, and R-283 were chosen for further mutagenesis on the basis of alanine scanning mutagenesis (33) and the p53 crystal structure (3). Several amino acid changes were made at each position. By in vitro mutagenesis, we replaced K-120 with Arg, Gln, Asn, Thr, Glu, Asp, and His; C-277 with Arg, Lys, Gln, Asn, Thr, Glu, Asp, and His; D-281 with Arg, Lys, His, Thr, Gln, Asn, and Glu; and R-248, R-249, R-273, R-280, R-282, and R-283 with Lys, His, Thr, Gln, Asn, Asp, and Glu. Mutant p53 proteins were expressed as p53 fragments containing amino acids 71 to 330 in *E. coli* and were purified to approximately 80% homogeneity by using a His tag. All proteins were soluble and expressed in bacteria at levels of 5 to 7% of total cell protein (steady-state levels). All subsequent p53 mutant designations are based on the single-letter amino acid code (e.g., R248K is Arg-248 changed to Lys). Mutant proteins were used for DNA binding assays with labeled BS-2 and symmetrically altered binding sites. Only 7 of 65 p53 mutants containing



B

	β-Galactosidase Activity (Miller Units)	ng DNA for 50% Competition
RE1	2320	16
RE2	18	20
RE3	2	90
RE4	0	145
RE5	10	210
RE6	2	225
RE7	5	280
RE8	0	180
RE9	260	50

FIG. 2. Competition experiments that show affinities to pentamer quarter sites in different orientations. (A) Binding reactions similar to those in Fig. 1A that contained $p53_{71-330}$ and labeled BS-2 were challenged with increasing amounts (0, 2, 5, 10, 30, 50, 100, 300, and 500 ng) of the indicated unlabeled DNA. Gels were dried, and the amounts of free and bound labeled DNA was quantitated with a Molecular Dynamics PhosphorImager. The percent DNA bound was plotted against the $\log_{10}$ of the amount of competitor used in each experiment. (B) The amount of each competitor required to reduce DNA binding by 50% is indicated. For transactivation assays, reporter plasmid pY28 containing the indicated DNA sites and pY10-*p53* were introduced into yeast strain CM1. β-Galactosidase activities are reported in Miller units (24) and were determined as described in the legend to Fig. 1. ←, the pentamer repeat, TGCCT.

single-amino-acid substitutions had detectable binding. Four of these seven mutants had altered DNA binding specificities. The activities of all mutants that resulted in any detectable binding are summarized in Table 1. Mobility shift assays of mutants with new binding specificities or a loss-of-contact phenotype are shown in Fig. 3.

No mutants of R-249, R-280, D-281, or R-282 showed any detectable DNA binding with canonical or noncanonical sites, emphasizing their critical structural roles in p53-DNA interactions. Mutants R283Q, K120N, and both C277Q and C277R bound to variant sites containing base pair changes at positions 1, 2, and 3, respectively, in the consensus pentamer to which the wild type p53 did not bind (Fig. 3 and Table 1). The binding specificities of these mutant p53 proteins were similar to that of wild-type p53 at all other positions (Fig. 1 and 3). R283Q bound to A1T, to which the wild-type protein bound very poorly. Mutant K120N preferentially recognized altered base pairs CG and TA at position 2, to which wild-type p53 is unable to bind. Mutants C277Q and C277R displayed new specificity phenotypes at position 3 by binding to variant site G3T much better than it bound to BS-2. C277Q also displayed detectable binding activity to mutant site G3C. On the basis of these data,

TABLE 1. DNA binding specificities of the wild type and point mutants of $p53_{71-330}$[a]

p53 protein	DNA binding activity[b]															
	BS-2	A1C	A1G	A1T	G2A	G2C	G2T	G3A	G3C	G3T	C4A	C4G	C4T	A5G	A5C	A5T
Wild type	+++	±	+++	±	−	−	−	+++	−	−	−	−	−	−	−	−
R273K	±	−	+++	−	−	−	−	++	−	−	−	−	−	−	−	−
K120N	+	−	+++	−	−	++	+++	+++	−	−	−	−	−	−	−	−
K120R	±	−	++	−	−	−	−	++	−	−	−	−	−	−	−	−
C277Q	+	−	++	−	−	−	−	+++	±	+++	−	−	−	−	−	−
C277R	+	−	++	−	−	−	−	+++	−	+++	−	−	−	−	−	−
R248K	±	−	++	−	−	−	−	−	−	−	−	−	−	−	−	−
R283Q	±	−	+++	++	−	−	−	+++	−	−	−	−	−	−	−	−

[a] The fragment of p53 containing amino acids 71 to 330.

[b] Data are taken from Fig. 1 and 3 and similar experiments. A minus sign (−) indicates no detectable binding with 100 ng of protein. Increasing levels of binding are indicated by ±, +, ++, and +++.

R-283, K-120, and C-277 are implicated in interactions with bases at positions 1, 2, and 3, respectively, of the pentamer repeat PuGPuCA. None of the R-248 and R-273 mutations resulted in binding to additional sites. However, analysis of mutant R248K showed that it was unable to bind the consensus site or any variant at position 3, in contrast to wild-type p53. It retained wild-type specificity at position 1 by binding to variant A1G efficiently. This loss of R248K binding with respect to base pair position 3 suggests that the R-248 side chain is critical for recognition of Pu at position 3.

Stoichiometry of p53 binding. In gel mobility shift assays of mutant C277Q, an additional faster-migrating complex (C-II) was observed (Fig. 3 and 4A). The wild-type p53 fragment containing amino acids 71 to 330 ($p53_{71-330}$) but lacking the oligomerization domain (amino acids 325 to 365) invariably shows one complex (C-I) with a consensus binding site (Fig. 1 and 4A). At higher protein concentrations, C-II could also be driven into the slower mobility complex, C-I (Fig. 4A). To investigate the oligomeric states of p53 in C-I and C-II, glutaraldehyde cross-linking was performed at various concentrations of the cross-linker to achieve partial cross-linking between p53 molecules. Cross-linked p53 molecules were fractionated on a denaturing gel. Protein was detected by a Western blot (immunoblot) with anti-p53 antibodies. Under

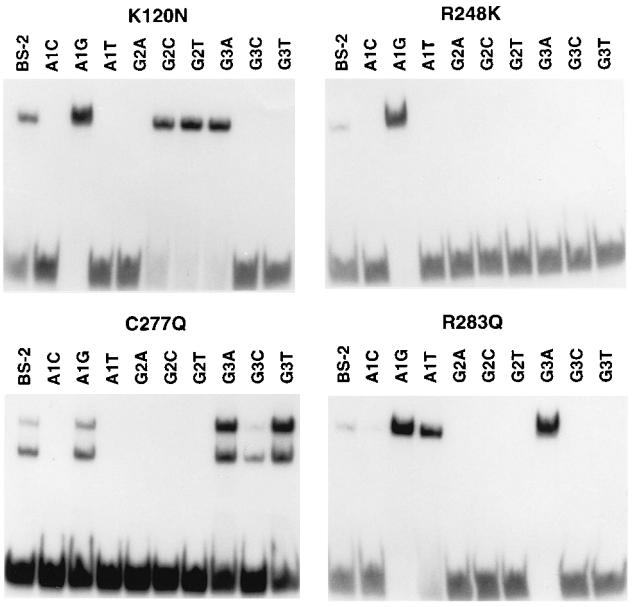


FIG. 3. DNA binding activities of p53 mutants. *E. coli*-expressed and purified p53 fragments with the indicated amino acid substitutions were analyzed by mobility shift assays with end-labeled DNA probes containing symmetric changes corresponding to positions 1, 2, and 3 of pentamer quarter site AGGCA as described in the legend to Fig. 1. Fifty nanograms of mutant protein and 1 ng of labeled BS-2 or mutant variant DNA site were used in all DNA binding reactions.

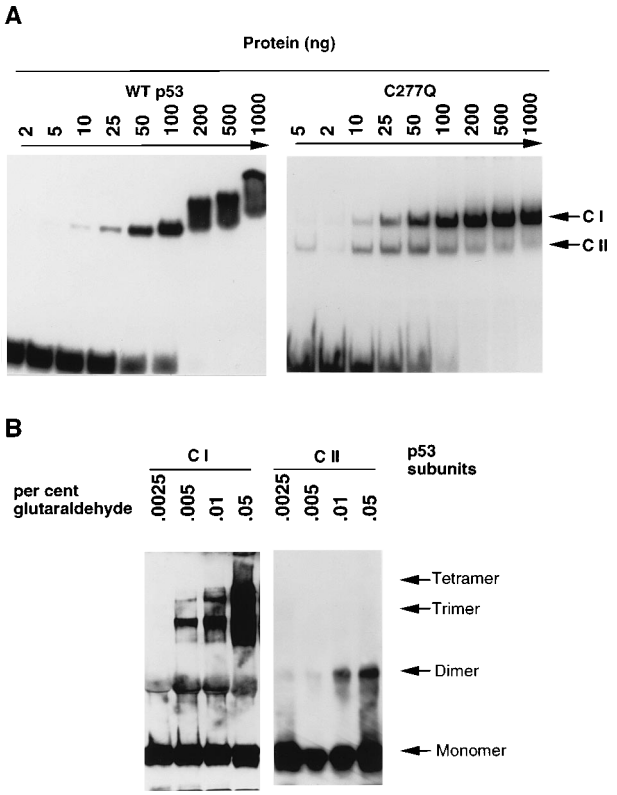


FIG. 4. Stoichiometries of p53-DNA complexes of wild-type $p53_{71-330}$ and the C277Q mutant. (A) The conditions for DNA binding assays were the same as those described in the legend to Fig. 1. The indicated amounts of protein and 1 ng of radioactive probe containing the 20-bp p53 binding site A1G (Fig. 1) were used in DNA binding assays. (B) C-I and C-II were excised from preparative scale mobility shift gel assays of C277Q and wild-type p53 with A1G. p53 protein (250 ng) was used in 5× DNA binding reaction mixture for each assay. Excised complexes were incubated for 15 min at room temperature with the indicated concentrations of glutaraldehyde. p53 complexes were eluted, concentrated with a microcentricon-100, boiled in SDS-sample buffer, and run on an SDS–10% polyacrylamide gel. Proteins were immunoblotted with pAb 240 antibody and detected by an enhanced chemiluminescence procedure using an ECL kit from Amersham.

conditions of partial cross-linking, C-I migrated as four bands, resolving at R_fs of a tetramer, trimer, dimer, and monomer of p53. At higher glutaraldehyde concentrations, C-I resulted in increased intensity of oligomeric p53. C-II resolved into two bands, corresponding to a dimer and monomer, under partial cross-linking conditions, and shifted to more dimer p53 at higher cross-linker concentrations (Fig. 4B). These results suggest that four monomers of wild-type $p53_{71-330}$ bind to the site containing two repeats of the h-h site. This observation, together with the absence of any faster-migrating mobility complex, demonstrates that binding by the wild-type p53 fragment which lacks the oligomerization domain is highly cooperative. In the case of C277Q, the appearance of C-II may result from reduced interaction between p53 molecules on DNA.

DISCUSSION

Two repeats of the 10-bp motif PuPuPuC(A/T)·(T/A)GPyPyPy were determined to be high-affinity binding sites for p53 on the basis of a binding site selection-amplification assay (6). Further investigation of this binding site revealed that the wild-type p53 fragment binds with high affinity to two repeats of motif PuGPuCA·TGPyCPy. The G and A at positions 2 and 5, respectively, in the pentameric quarter site are critical determinants of p53 DNA binding. A G and an A were also present at these positions in the high-affinity p53 binding site reported by Funk et al., which had been obtained from a mixture of random sequences synthesized in vitro (11). In the crystal structure of the p53-DNA complex, only one monomer bound to the dimer site GGGCAAGTCT (3). This may have been the result of the presence of an unfavorable adenine at the underlined position. A T at this position seems critical for high-affinity binding by p53.

High-affinity DNA binding and transactivation through four pentameric quarter sites arranged in an orientation of two h-h dimers indicate that proper positioning of p53 molecules on DNA is important for transactivation. RE2, which also contains four quarter sites but as two tail-to-tail dimers, was effective in the DNA binding assay but did not result in transactivation. In the tail-to-tail DNA site, the orientation of p53 subunits on DNA is different, which perhaps affects the intermolecular interactions between p53 subunits and the other proteins involved in transcription. Alternatively, the difference in DNA binding activity and transactivation may be due to the difference in sizes of p53 used in the two experiments. p53 also bound and transactivated through RE9, which contains pentamer repeats 5 bp apart. Transcriptional activity through this site was 10-fold lower than that through the consensus RE1 site. A naturally occurring arrangement of pentamers, similar to that in RE9, through which p53 transactivates has been reported previously (8). In addition, model building studies of p53 and mass spectroscopic study of the p53-DNA complex have indicated that the linker between the oligomerization and DNA binding domains is flexible, allowing some variability in the spacing of individual DNA binding domains on the DNA, in general agreement with the observations made here (17, 21, 31).

Amino acid-base pair interactions between p53 and its consensus DNA site were inferred from DNA binding specificity mutations in the DNA binding domain of p53. The conclusions based on the behavior of these mutants support and extend observed interactions in the p53-DNA crystal structure (3). No mutants of R-249, R-280, D-281, or R-282 showed any detectable DNA binding with canonical or noncanonical sites, emphasizing their structural roles. In the crystal structure, the guanidinium group of R-280 donates two hydrogen bonds to N-6 and O-6 of the invariant guanine in the CG base pair at position 4. It is also involved in salt bridge formation with the carboxylate of D-281. Even a conservative change to lysine was ineffective, emphasizing the importance of arginine at this position. In conjunction with the results for DNA site variants, these data strongly suggest the critical roles of the cytosine at position 4 and the R-280 side chain in governing the DNA binding specificity of wild-type p53. The side chains of R-249, D-281, and R-282 do not make any direct contacts with DNA but are involved in forming a network of interactions that stabilize the DNA binding region of p53.

Interactions between the original and substituted amino acids of altered-specificity mutants of p53 with their cognate DNA sites seem chemically feasible. K120N, C277Q, C277R, and R283Q bound to variant sites to which wild-type p53 did not bind. R283Q bound efficiently to A1T, to which the wild-type protein did not bind, and also to A1G. These variant bases provide hydrogen bond acceptors in their O-4 and O-6 positions, respectively, together with hydrogen bond donor groups from the complementary base pair. This is reversed in the original base pair, AT, and variant A1C, which may account for the origin of this observation. Mutant K120N preferentially recognized base pairs TA and CG at position 2, to which wild-type p53 did not bind. The reduction in side chain length with the change from K to N may result in the observed change in preference from purine to pyrimidine at this position. In the crystal structure, the side chain of K-120 donates hydrogen bonds to N-7 and O-6 of G at position 2 in the pentamer quarter site. The amide side chain of the substituted amino acid in K120N may hydrogen bond with variant base pairs at position 2. Substitutions C277Q and C277R also bound variant DNA sites that wild-type p53 did not recognize. In wild-type p53, C277 is suggested to have two potential modes of interaction with target DNA, either accepting a hydrogen bond from N-4 of cytosine in the GC base pair at position 3 or donating a hydrogen bond to O-4 of thymidine in the AT at position 3. Thus, C277Q and C277R may switch between these two interactions, both of which are stronger than the wild type. Alternatively, the longer side chain of C277R may permit it to form interactions with the phosphate backbone as well as the base pairs. Genetic analysis has also identified a direct role for the R-248 side chain in DNA binding. This residue is one of the most frequently mutated in various human cancers. R248K binds efficiently to GGGCA (A1G [Table 1]). It has lost contact with G3A, to which wild-type p53 binds strongly. Our data indicate the importance of the guanidinium group of arginine at this position in discriminating base pair changes at position 3. On the basis of these results, both C-277 and R-248 contribute to recognition of the base pair at position 3. In the crystal structure study, the role of the R-248 side chain in governing DNA binding specificity was not clear. This residue was close to the minor groove of DNA. However, the DNA site used in the X-ray crystal study was different from the one used in this analysis; perhaps this resulted in the differences between the two observations. Analysis of various substitutions at R-273 showed that only R273K was able to partially replace the binding specificity of R-273, indicating the importance of positive charge and the guanidinium group. Consistent with this result, in the p53-DNA crystal structure, the guanidinium group of R-273 makes DNA backbone contact with the base pair at position 5 and is also involved in salt bridge formation with D-281.

Interestingly, in gel mobility shift assays of mutant C277Q, an additional faster-migrating complex was observed. The wild-type p53 fragment containing amino acids 71 to 330 but lacking the oligomerization domain (4, 17, 21, 32) invariably

shows one complex with a consensus binding site. Analysis of more slowly and faster-migrating mobility bands showed that these contain four and two monomers of p53, respectively. Earlier studies have indicated that the p53 fragment which lacks the oligomerization domain, though predominantly monomers in solution, still has a tendency to associate into oligomers (36). Thus, this tendency of the wild-type p53 fragment lacking the oligomerization domain to associate in solution may result in highly cooperative DNA binding. Structural studies of the oligomerization domain have indicated that monomer-monomer interactions in the formation of a dimer are more extensive and stronger than interactions between dimers to form tetramers (17, 21). Furthermore, model building studies of the p53 DNA binding domain (3) suggested that protein-protein contacts between dimers, possibly by the H1 helices of the DNA binding domain, result in further oligomerization. Perhaps these interactions between p53 subunits contribute to highly cooperative DNA binding by the p53 fragment lacking the oligomerization domain. C-277 mutants may affect this activity.

With the stoichiometry of one monomer per pentamer, four monomers bind to four pentameric quarter sites in the h-h orientation. High-affinity DNA binding and transactivation through quarter sites arranged in this orientation suggest that proper positioning of p53 molecules on DNA is important for transactivation. Since each pentamer repeat in an h-h dimer represents half a turn in the DNA helix, monomeric p53 proteins are likely to be present on the same surface of DNA, as has been proposed by Cho et al. (3).

While this paper was under review, Wang et al. published a study of the interaction of p53 with its consensus DNA site (37a). That study indicated that the p53 fragment lacking the tetramerization domain also binds the consensus DNA site with a high degree of cooperative interactions. Furthermore, by altering the spacing within the half and full consensus sites, they suggest that p53 binds DNA with rotational specificity.

Molecular genetic analysis of the p53-DNA complex, together with the crystal study, provides a general framework for p53-DNA interactions. However, the natural response elements identified so far usually differ at two to three critical base pair positions from the consensus site or only contain half a consensus site (7, 18, 26, 38, 40). In vitro binding studies show that wild-type p53 binds poorly to a single h-h sequence and does not transactivate in yeast-based assays. These results argue that wild-type p53 might bind to native DNA sites with different kinetics and/or require another protein(s) for efficient binding and regulation of genes. Additionally, the characterizations of p53 mutants in this study that possess altered DNA binding specificities also suggest that natural mutants bind as yet unidentified DNA sites that are suboptimal for wild-type p53. Though this is speculation at present, it is indeed plausible, given the existence of natural p53 mutants which result in gains of function (5).

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