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# Specific Interaction of p53 with Target Binding Sites Is Determined by DNA Conformation and Is Regulated by the C-terminal Domain*

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**Transcriptional activation of p53-regulated genes is initiated by sequence-specific DNA binding of p53 to target binding sites. Regulation of sequence-specific DNA binding is complex and occurs at various levels. We demonstrate that DNA topology is an important parameter for regulating the selective and highly specific interaction of p53 with its target binding sites. Specific binding of wild-type p53 is greatly enhanced when cognate binding sites are present in a non-linear stem-loop conformation. The C-terminal domain plays a key role in regulating the specific interactions of p53 with target binding sites in a DNA conformation-dependent manner. The C-terminal domain is required for binding to target sites in a non-linear DNA conformation in contrast to the strong inhibitory effects of the C terminus on p53 interaction with linear DNA. We propose that selective binding of p53 to various promoters may be determined by the DNA conformation within p53 cognate sites.**

A major function of the tumor suppressor p53 is that of a pleiotropic transcription factor controlling the expression of target genes by either activating or inhibiting the activity of p53-responsive promoters. p53 has several DNA binding activities of which sequence-specific DNA binding (SSDB)[1] (1) is the most important because it is indispensable for the transcriptional activity of the protein. The importance of SSDB is highlighted by the fact that the most frequent p53 mutations found in human cancers are located within the DNA binding domain encoding region (2, 3) and abrogate the SSDB of p53. The specific interaction of p53 with target DNA is complex and tightly regulated. Promoter recognition by p53 is determined by the presence of p53-response elements (PREs), which share homology with the consensus sequence 5′-(PuPuPuC(A/T)(T/A)GPyPyPy)$_n$-3′ (1). C-terminally unmodified p53 seems to be inactive in SSDB; however, modifications at the C terminus, like binding of the C-terminal-specific antibody PAb421, phosphorylation, or deletion of the 30 C-terminal amino acids, strongly enhance SSDB under certain *in vitro* conditions (4). The data indicate that the C terminus regulates binding of p53 to target DNA, although the underlying mechanisms are not entirely understood. Several models have been proposed to explain the influence of the C-terminal domain on SSDB by p53. The *conformation model* postulates that the p53 protein exists in two conformationally distinct forms termed *latent* (for DNA-binding inactive p53) and *activated* (for DNA-binding active p53). According to this model, the C-terminal domain in latent p53 interacts directly with the core domain and thereby inhibits SSDB of p53 (5). A conformational switch was proposed as the mechanism that relieves the allosteric inhibition by the C terminus and converts p53 from latent into an activated form. However, such a hypothesis is not supported by the results of structural studies that recently demonstrated that the conformation of DNA binding-inactive and DNA binding-active p53 forms is largely identical (6). These findings indicate that the C terminus may regulate SSDB by other mechanisms. The *competition model* explains the inhibitory effect of the C-terminal domain by its ability to bind DNA unspecifically (7). According to this model, unspecific DNA binding of the C terminus interferes with the sequence-specific interaction with DNA mediated by the core domain. Modifications of the C terminus, such as phosphorylation or binding of PAb421, may displace unspecific bound DNA and thereby facilitate SSDB (7).

Both models suggest that SSDB is inhibited by the C terminus directly (conformation model) or indirectly (competition model), thereby necessitating the activation of p53 for SSDB. However, an alternative view is that SSDB of p53 is not inhibited by the C terminus and therefore does not need activation under more physiological conditions. Espinosa and Emerson (8) observed that the p53 C terminus inhibited SSDB to the *p21* promoter only in short naked DNA, but not when the *p21* promoter was assembled into chromatin *in vitro* (8). Moreover, deletion of the C terminus decreased SSDB and, consequently, transactivation of the chromatin-assembled *p21* promoter (8). The findings indicate that SSDB of p53 requires a certain level of structural DNA organization, which may be missing in short linear DNA.

Commonly, two identical repeats of the 5′-(PuPuPuC(A/T)(T/A)GPyPyPy)$_n$-3′ sequence are used in DNA binding assays as a p53 target binding site. However, the comparison of such "optimized" binding sites with functional PREs identified in various p53-regulated promoters reveals that "natural" p53 binding sites differ significantly from the optimized version. Notably, known PREs are never composed of identical decam-

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[1] The abbreviations used are: SSDB, sequence-specific DNA binding; CON, control; CREB, cAMP-response element-binding protein; DBD, DNA binding domain; dec, decamer; EGF-R, epidermal growth factor receptor; EMSA, electromobility shift assay; MUT, mutated; ODN, oligonucleotide; PRE, p53-response element; TGF-α, transforming growth factor-α; wt, wild-type.

TABLE I
*p53 response elements (PREs) identified in p53-regulated genes*

Decamers that perfectly conform to the p53 consensus are shown in italic capital letters and are underlined. Bases violating the consensus are shown in small letters. Boldface and underlined sequences correspond to decamers that perfectly conform to the p53 consensus.

| p53 target | PREs |
|---|---|
| *p21* (52) | ***GAACATGTCC*** cAACATGTT |
| *pcna* (53) | ***GAACAAGTCC*** GGGCATaTgT |
| *cyclin G* (54) | AGACcTGCCC ***GGGCAAGCCT*** |
| *msh2* (55) | ***AGGCTAGTTT*** AAGtTtcCTT |
| *gml* (56) | AtGCTTGCCC ***AGGCATGTCC*** |
| *mdm2* (57) | GGtCAAGTTg GGACAcGTCC–$N_{17}$ GAGCTAaGTCC tGACATGTCT |
| *egf-r* (58) | AGACTAGgCC ***GAGCTAGCCC*** GAGCgAGCTC |
| *p2xm* (59) | GAACAAGggc ***GAGCTTGTCT*** |
| *pα26* (60) | GGACAAGTCT ccACAAGTTC |
| c-*fos* (61) | ***GGACTTGTCT*** GAGCgcGTgC AcACTTGTCa |
| *bax1* (62) | tcACAAGTTa ***AGACAAGCCT*** GGGCgTGggC |
| *puma*-BS2 (63) | ctGCAAGTCC tGACTTGTCC |
| *mcg10*-PRE1 (64) | GGtCTTGgCCC AGACTTaGCaG |
| *mcg10*-PRE2 (64) | GAACTTaagaCC GAGGCTCt GGACAAGTTg |
| *fas/apo1* (65) | cAACATGgTg-$n_3$ AGACT–GTTT–$n_8$-GGGCATGTac AAACATGTCa |
| c-HA-*ras* (66) | AtACTTGTC ggag GcACcAGCgCcgcg GGGCcTGCa ggctg GcACTAGCCT gccc GGGCAcGCCgt |
| *mck* (67) | tGGCgTGTgC tccc tGGCAAGCCTatGACATGgCCg GGGCcTGCCT |
| *pig3* (68) | cAGCTTGCCC AccCATGCTC |
| *tgf-α* (69) | GGGCAgGCCC tGcCTAGTCT AGcCAAGTCT tGGCAAGCgg |
| *ibp-3* (70) | AAACAAGCCac cAACATGCTT |
| *p202* (71) | cTACATGaCTT ctAccCATGCTT cAGCTAGTTT tAAcAcCTTGaTC |

ers (Table I). Furthermore, because of the high frequency of bases deviating from the consensus, PREs exhibit significant sequence diversity (Table I). Typically, only one decamer (half-site) stringently adheres to the consensus, whereas the other decamer(s) deviate from the consensus to a varying extent. In PREs of some p53-regulated genes, all decamers contain bases that violate the consensus (*e.g.* c-Ha-*ras*, *pig3*, *fas/apo1*, *tgf-α*). Contente *et al.* (11) have recently reported that the p53-dependent transcription of the *pig3* gene is critically dependent on the presence of a $(TGYCC)_n$ pentanucleotide repeat and not on the p53-consensus sequence identified previously (9). The $(TGYCC)_n$ pentanucleotide repeat is part of a microsatellite sequence and bears only very limited sequence homology to the p53 consensus. Nevertheless, this sequence mediates sequence-specific transactivation of *pig3* by p53, whereas the consensus-like binding site from the *pig3* promoter appears to be dispensable (9). The sequence variance among PREs is puzzling because it remains unclear how the sequence heterogeneity of PREs is compatible with the required high specificity for their interaction with p53. As an explanation, we and others have proposed that the sequence variance of PREs may be important to provide DNA with structural flexibility, which appears to be an important parameter determining p53 interactions with DNA (8, 10, 11). p53 binding to unusual DNA structures has been demonstrated in studies investigating the involvement of p53 in repair and recombination pathways (12–14). So far, the two modes of p53 interaction with DNA, sequence-specific and DNA structure-dependent, have been studied independently from each other. With regard to SSDB, only the presence of the specific cognate motif has been considered. However, it is likely that the presence of the specific sequence motif is not the only parameter determining specific binding of p53 to its cognate sites, which may also be influenced by structural features of DNA (8, 10, 15).

In this study we analyzed the impact of DNA structure on the sequence-specific interaction of p53 with PREs derived from known p53 regulated promoters and with target binding sites containing variations of the consensus motif. We demonstrate that the presentation of specific binding sites in a non-linear stem-loop conformation determines strong binding of p53 which does not require C-terminal modification by PAb421. Our data demonstrate that the C-terminal domain exerts opposite effects on the interaction of p53 with target sites depending on the conformation of the DNA; while inhibiting the specific interaction of p53 with linear DNA, the C terminus is required for the specific binding to stem-loop DNA. Our data may therefore provide a base for understanding the complex regulation of p53 interactions with DNA. With regard to transcriptional activation, selective promoter recognition might be determined by both binding of other proteins at the C terminus of p53 and by structural organization of the DNA within the p53 target binding sites.

## EXPERIMENTAL PROCEDURES

*Preparation of p53 Binding Sites Presented in Linear or Non-linear DNA Conformation*—All DNA substrates were prepared from synthetic oligonucleotides (ODNs) listed in Table II. To obtain the PRE-p21 in a linear form, the PRE-p21(lin/s) DNA was end labeled with T4 polynucleotide DNA kinase and [$\gamma$-$^{32}$P]ATP and annealed with unlabeled complementary PRE-p21(lin/as) DNA at a molar ratio of 1:1. Non-linear DNA substrates containing short stem-loop structures on one strand were obtained by annealing of two oligonucleotides from which only the shorter, T3-1 oligonucleotide was radiolabeled at the 5′-end. The longer oligonucleotides contained 13 bases at the 5′-end which were complementary to 13 bases at the 3′-end of T3-1 DNA, and 12 bases at the 3′-end complementary to 12 bases at the 5′-end of T3-1. The central parts of the longer oligonucleotides were comprised of self-complementary sequences either containing or lacking p53-specific cognate motifs (Table II). Annealing of the T3-1 ODN with a stem-loop-forming oligonucleotide allowed the formation of a three-way junction structure that contained a stem-loop on one strand. Such DNA structures can be easily distinguished from single-stranded or linear double-stranded DNA molecules in 8% native polyacrylamide gels (shown in Fig. 2*A*) allowing their purification. Eluted DNA was recovered by ethanol precipitation, resuspended in buffer containing 10 mM Tris (pH 7.8) and 60 mM NaCl, and used in electromobility shift assay (EMSA) or DNase I protection experiments.

*Protein Purification*—Recombinant p53 proteins expressed in insect cells were isolated as described by Bessard *et al.* (16) and purified by ion exchange chromatography (FPLC, Amersham Biosciences).

*EMSA*—DNA binding experiments were performed using 50 ng of recombinant p53 proteins in a reaction mixture containing 5 ng of poly(dI·dC) (Amersham Biosciences) and 2 $\mu$g of bovine serum albumin in 50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 1 mM dithiothreitol, 20% glycerol, and 50 mM NaCl. After a 20-min preincubation at room temperature, 20,000 cpm of the labeled DNA probe was added, and the incubation was continued for another 25 min. Samples were loaded onto a 4% native polyacrylamide gel and separated by electrophoresis in 10 mM Tris-HCl (pH 7.8), 0.2 mM EDTA, 1.25 mM NaOAc, and 8 mM acetic

TABLE II

*Oligonucleotides used for the preparation of DNA substrates in linear or in stem-loop DNA conformation*

p53 cognate motives are shown in bold characters. Underlined sequences are complementary to a 12 bp at the 5′-end and 13 bp at the 3′-end of the T3–1 oligonucleotide.

| | ODNs for linear dsDNA[a] |
|---|---|
| PRE-p21(lin/s) | 5′- gctctgcc **GAACATGTCC cAACATGTTg** ccgctctg-3′ |
| PRE-p21(lin/as) | 5′- cagagcgg **cAACATGTTg GGACATGTTC** ggcagage-3′ |
| | ODNs for short stem-loop DNAs |
| T3–1 | 5′-ccgcggtaccat tacctaaggcgtc-3′ |
| p21-dec1 | 5′-gacgccttaggta cctggcctgcct **GAACATGTCC** ggcctgccgtgg atggtaccgcgg-3′ |
| p21-dec2 | 5′-gacgccttaggta cctggcctgcct **cAACATGTTg** ggcctgccgtgg atggtaccgcgg-3′ |
| p21-dec1+2 | 5′-gacgccttaggta cctgcc**GAACATGTCCcAACATGTT**gggcctgatggtaccgcgg-3′ |
| EGF-R | 5′-gacgccttaggta **AGACTAGgCC GAGCTAGCCC GAGCgAGCTC** agactaggcc atggtaccgcgg-3′ |
| TGF-α-dec3 | 5′-gacgccttaggta cctggcctgcct **AGcCAAGTCT** gggcctgcctgg atggtaccgcgg-3′ |
| CON-loop | 5′-gacgccttaggta cctggcctgcct **GGACTTGCCT** ggcctgcctgg atggtaccgcgg-3′ |
| MUT-loop | 5′-gacgccttaggta cctggcctgcct **GGAgTTcCCT** ggcctgcctgg atggtaccgcgg-3′ |
| CON-paired | 5′-gacgccttaggta cctggcctgcca **GGACTTGCCT** ggcaggccagg atggtaccgcgg-3′ |
| SCR-loop | 5′-gacgccttaggta ccagcctccgcacgctcaccgagcgctggcagg atggtaccgcgg-3′ |
| SCR-paired | 5′-gacgccttaggta cctggcctgccacgctcaccgtggcaggccagg atggtaccgcgg-3′ |
| | ODNs for a long stem-loop DNA used in electron microscopy |
| Stem-1 | 5′-$^{P}$ ggcagg ccagcgaggcaggtgcccaggggcaggccaggggcagg atggtaccgcgg-3′ |
| Stem-2 | 5′-gacgccttaggta cctgcccctggcctgcccctgggcacctgcctcgctgg-3′ |
| Loop | 5′-$^{P}$ cctgcc cctgcccctggcctgcct **GGACTTGCCT** ggcctgcc tggggcagg-3′ |

[a] ds, double-stranded. Single-stranded ODNs PRE-p21(lin/s) and PRE-p21(lin/as) represent sense and antisense strands of the PRE-p21 in linear conformation.

acid at 200 V for 2.5 h at room temperature. After electrophoresis gels were dried and subjected to autoradiography.

*DNase I Protection Assay*—DNA binding was performed in the same binding buffer as in EMSA. After the binding step, the samples were supplemented with equal volumes of 10 mM $CaCl_2$ and 20 mM $MgCl_2$ solution and treated with 10 units of DNase I (Promega) for 10 min at 37 °C. Digestion was stopped by adding an inactivating buffer containing 0.15 M NaOAc, 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 0.5% SDS, 5 mM EDTA, 0.1 mg/ml proteinase K, and 0.05 mg/ml yeast RNA. After treatment with phenol-chloroform, labeled DNA was recovered by ethanol precipitation and dried. After resuspension, samples were analyzed by electrophoresis in 20% denaturing gels containing 8 M urea. After electrophoresis, the gels were dried and subjected to autoradiography.

*Cross-linking and Immunoblotting of p53 Oligomers*—The oligomeric status of p53 bound to DNA was analyzed by glutaraldehyde cross-linking as described by Wang *et al*. (17). Briefly, p53 was incubated with radioactively labeled DNA to allow complex formation. The p53-DNA complex was resolved by electrophoresis in a 4% native polyacrylamide gel (TBE buffer, 2 h at room temperature) along with the mixture of p53 oligomers used as a reference for the mobility of different oligomeric forms of p53. Various oligomers of p53 were obtained by incubating purified wt p53 protein with different concentrations of freshly diluted glutaraldehyde for 60 min on ice. Before electrophoresis the cross-linked protein samples were boiled in 2% SDS to reverse cross-linking. After electrophoresis, semidry double-membrane transfer was performed as described by Demczuk *et al*. (18) using two membranes, nitrocellulose and Hybond $N^+$, which selectively bind proteins or DNA, respectively. After blotting, p53 protein bound to nitrocellulose was immunodetected with a mixture of p53-specific antibodies PAb421, PAb240, DO-1, and PAb1801. Radioactively labeled DNA bound to the Hybond $N^+$ membrane was detected by autoradiography.

*Electron Microscopy*—Stem-loop DNA with a 64-bp stem was prepared from four unlabeled oligonucleotides as depicted in Fig. 6*A*. Phosphorylated Stem-1 DNA was annealed with unphosphorylated Stem-2 DNA at a 1:1 molar ratio. 12 nucleotides at the 3′-end of the Stem-1 DNA (56 bp) and 13 nucleotides at the 5′-end of the Stem-2 DNA (51 bp) were complementary to the T3-1 DNA (25 bp) but not to each other. The resulting DNA I contains 12- and 13-nucleotide single-stranded ends and six protruding bases at the 5′-phosphorylated end formed by the Stem-1 DNA. DNA I was ligated with a third, self-complementary oligonucleotide loop, which was self-annealed prior to ligation. Loop DNA was phosphorylated at the 5′-end and contained six protruding nucleotides that were complementary to six nucleotides protruding at the 5′-end of Stem-1 DNA. The presence of phosphorylated 5′-ends in Stem-1 and Loop DNAs permitted unidirectional ligation of compatible ends in DNA I and self-annealed Loop DNA (step 2). After ligation, samples were loaded onto a 1.6% agarose gel, and the slowest migrating DNA band was isolated from the gel (step 3). DNA II was recovered by ethanol precipitation, dissolved in annealing buffer, and quantified by spectroscopy at 260 nm. To confirm that ligation had occurred between the 3′-end of the Stem-2 and the 5′-end of the Loop oligonucleotides, 0.1 μg of isolated DNA II was end labeled with T4 polynucleotide DNA kinase and [γ-$^{32}$P]ATP and sequenced by the Maxam-Gilbert chemical degradation method. DNA II was annealed with T3-1 oligonucleotide at a 1:1 molar ratio (step 4), and the resulting structure (DNA III) was isolated from a 1.6% agarose gel (step 5). After ethanol precipitation DNA III was resuspended in 10 mM Na-Hepes (pH 7.5) buffer and used for electron microscopy experiments. As a linear DNA binding substrate we used a 474 bp fragment obtained from a pPGM1 plasmid containing a specific p53 cognate motive (5′-AGACAT-GCCTAGACATGCCT-3′) located at ~40% from one end of the fragment (19). For electron microscopy, DNA-p53 complexes were prepared by incubating the DNA and p53 in 10 μl of a buffer containing 10 mM Na-Hepes (pH 7.5), 50 mM KCl, and 0.01% Triton X-100 at room temperature for 30–40 min. The molar protein:DNA ratio was as indicated in the legend of Fig. 6*B*. After incubation, an aliquot (1–2 μl) was taken from the mixture, diluted 20-fold in a 10 mM Na-Hepes (pH 7.5), 10 mM KCl-containing buffer, and applied to the surface of carbon film mounted on an EM grid. 3–4-nm carbon films were activated by glow discharge in the presence of pentylamine vapor (residual pressure ~150 millitorr, discharge current 2–3 mA, duration of discharge 30 s as described in Ref. 20). The adsorption continued for 2–4 min, then the grids were rinsed with 2% aqueous uranyl acetate, blotted with filter paper, and air-dried. The samples were analyzed with a Philips CM12 electron microscope in a tilted dark field mode at × 60,000 magnification. The negatives were scanned with an Agfa Studio Scan II*si* scanner at 1,200 dpi. For printing, images were processed using a high pass filter with a radius of 250 pixels.

## RESULTS

### *Wild-type p53 Binds Strongly and Specifically to Target Sites in Nonlinear DNA Conformation*

We selected PREs from the p53-regulated *p21*, *egf-r*, and *tgf-α* promoters to analyze the binding of recombinant human wt p53 protein. The PREs share little homology in sequence and in the number of decamers composing the p53 binding site (Table I). However, they all exhibit a 2-fold symmetry that allows intrastrand base pairing of self-complementary regions either between different decamers (structures (*b*) in Fig. 1) or within the same decamer (structures (*c*)). For example, PRE-p21 has the potential to form two different stem-loop structures. Structure (*b*) would be formed by both decamers aligned symmetrically against each other; in structure (*c*) only one decamer would be fully located within stem-loop, and the other

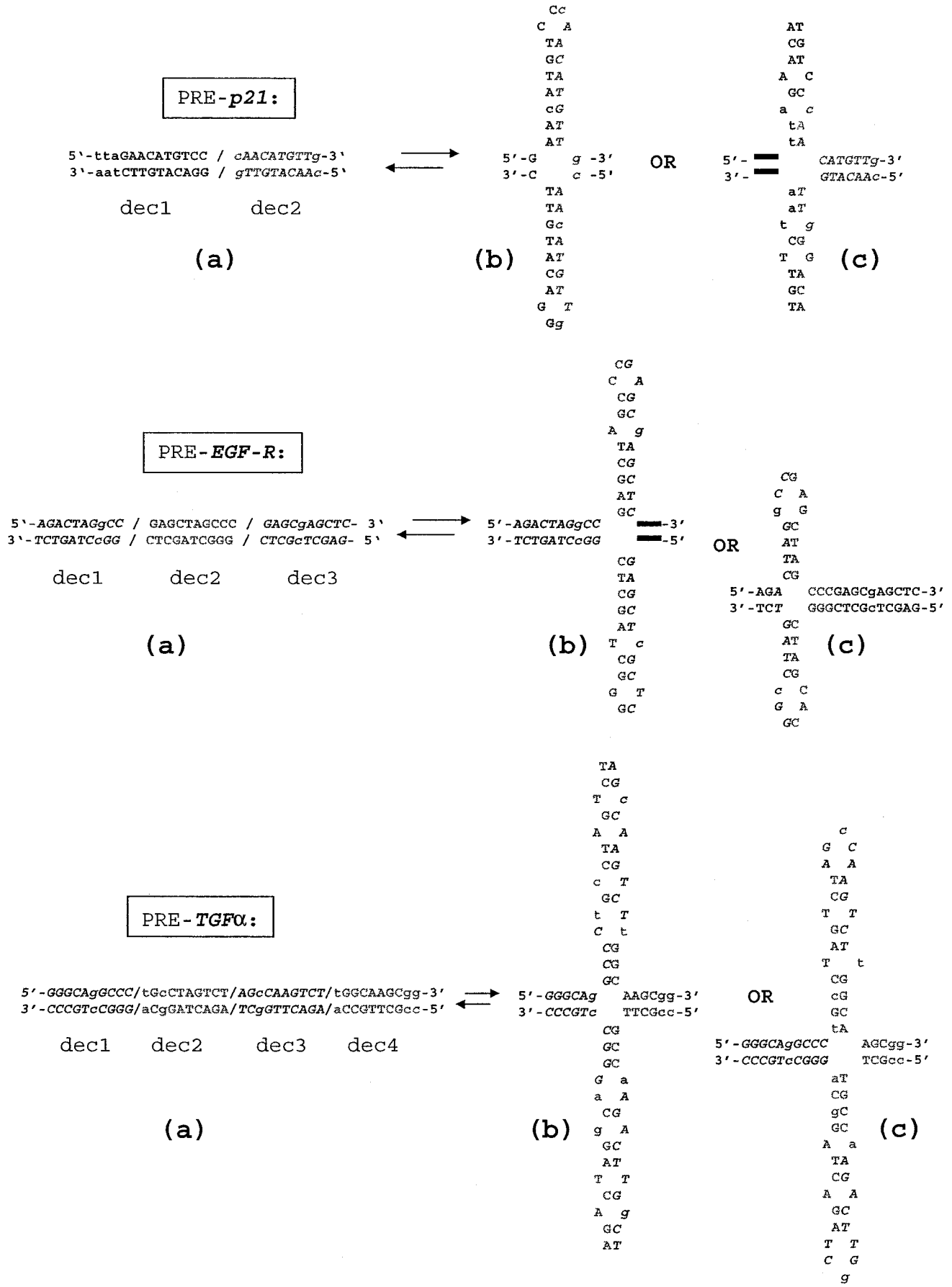


FIG. 1. **Putative stem-loop structure formation by symmetric PREs of the p53-regulated p21, TGF-α, and EGF-R genes.** Individual decamers (designated as *dec*) composing the full PRE in linear form of DNA (*a*) are separated by a *slash*. Bases that violate the consensus rule are shown in *lowercase letters*. Symmetric PREs have the potential to form various secondary structures (DNA isoforms (*b*) and (*c*)).

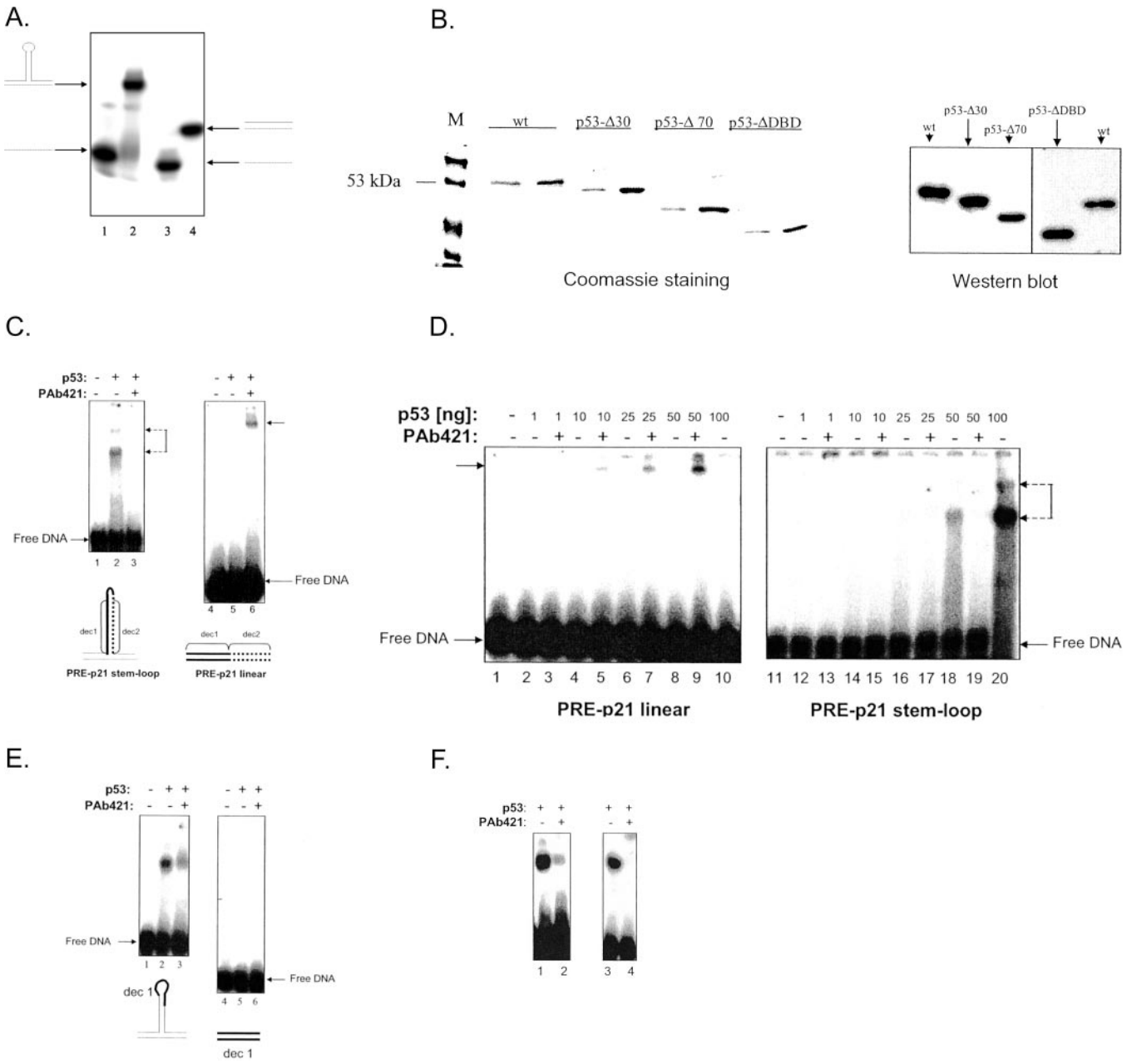


FIG. 2. **Binding of p53 to PRE-p21 present in structurally distinct DNA conformations.** *A*, typical 8% polyacrylamide gel showing the preparation of PRE-p21 in stem-loop (*lane 2*) and linear (*lane 4*) DNA conformation. Radioactively labeled T3·1 lock DNA before (*lane 1*) and after annealing with unlabeled p21-dec1 + 2 DNA (*lane 2*). Annealing of fully complementary ODNs PRE-p21(lin/s) and PRE-p21(lin/as) yields linear duplex DNA (*lane 4*). The mobility of single-stranded ODN PRE-p21(lin/s) is shown in *lane 3*. *B*, purified p53 proteins used in DNA binding experiments. The purity of the proteins was examined by Coomassie staining (shown are 0.5 and 1 $\mu$g of each protein). The integrity of proteins was assessed by Western blot with DO-1 (for human wt p53 and p53-ΔDBD proteins) or with antibody PAb242 (for mouse wt p53, p53-Δ30, and p53-Δ70 proteins). *C*, stem-loop containing a full PRE-p21 composed from two decamers (dec1 and dec2) was prepared by annealing of T3-1 and p21-dec1 + 2 oligonucleotides as described under "Experimental Procedures" and shown in *A*, *lane 2*. PRE-p21 in stem-loop conformation (*lanes 1–3*) or in linear form (*lanes 4–6*) was incubated with wt p53 protein in the absence (*lanes 2* and *5*) or presence of PAb421 (*lanes 3* and *6*). The shifted bands (*broken arrows*) represent specific p53 complexes formed with the DNA in the absence of PAb421. The slower migrating complex is often observed under EMSA conditions and probably represents the complex formed by p53 octamer. The addition of PAb421 results in a supershifted band (*solid arrow*). Binding of p53 to stem-loop and linear DNA was always analyzed in the same experiment as shown in this and in other figures. *D*, analysis of p53 binding to PRE-p21 in linear or in stem-loop conformation using different amounts of the p53 protein with or without PAb421. *Broken arrows* indicate the p53-specific complex formed with stem-loop DNA. *E*, single decamer (dec1) derived from PRE-p21 was prepared in stem-loop (*lanes 1–3*) or in linear DNA conformation (*lanes 4–6*). The binding reactions contained wt p53 protein in the absence (*lanes 2* and *5*) or in the presence of PAb421 (*lanes 3* and *6*). *F*, binding of p53 to different target binding sites present in nonlinear DNA conformation. Stem-loops containing a full PRE-EGF-R (*lanes 1* and *2*) or a single decamer derived from PRE-TGF-α (*lanes 3* and *4*) were prepared as described under "Experimental Procedures" by annealing labeled T3-1 and unlabeled oligonucleotides EGF-R or TGF-α-dec3, correspondingly.

decamer would mostly be located at the junction of the structure. We reconstituted both types of stem-loops that may be formed by either two or by one decamer from PRE-p21 by using an approach commonly used for the preparation of cruciform DNA and stem-loop structures (21, 22). Briefly, two ODNs of different length are annealed, with only the longer ODN containing the PRE-specific sequence. A shorter oligonucleotide is complementary only to the PRE-flanking sequences in the longer oligonucleotide but is lacking the PRE-complementary sequence. Annealing of the longer and the shorter oligonucleo-

tides yields an asymmetric structure in which the upper, PRE-specific sequence is folded back on itself and forms a stem-loop, locked by the shorter, PRE-lacking oligonucleotide (Fig. 2*A*).

We analyzed binding of p53 to linear or to stem-loop DNA by EMSA using recombinant human wt p53 protein purified from insect cells (Fig. 2*B*). The results showed that p53 did not bind to PRE-p21 when it was present in linear DNA conformation unless antibody PAb421, directed against a C-terminal epitope of p53, was added (Fig. 2*C*, compare *lanes 5* and *6*). In contrast, when two decamers of PRE-p21 formed a stem-loop, p53 bound strongly in the absence of PAb421 (Fig. 2*C*, *lane 2*). Moreover, in contrast to the enhancement of p53 binding to linear PRE-p21, PAb421 inhibited binding to stem-loop DNA (compare *lanes 2* and *3*). Complex formation with PRE-p21 in stem-loop conformation was strictly dependent on the p53 concentration. As shown in Fig. 2*D*, nearly all DNA probe representing stem-loop PRE-p21 was bound in the presence of 100 ng of p53 in the absence of PAb421, whereas with linear PRE-p21, no binding could be detected under the same conditions (compare *lanes 10* and *20*). Surprisingly, stem-loop DNA formed by only one decamer of the PRE-p21 was also bound strongly by p53. The binding did not require PAb421 (Fig. 2*E*, *lane 2*); in contrast, addition of PAb421 led to a significant inhibition of p53 binding (*lane 3*). The same decamer in linear DNA form was not bound by p53 at all, regardless of the presence or absence of PAb421 (Fig. 2*E*, *lanes 4–6*). The results demonstrate that the presence of only one specific decamer may already be sufficient for strong binding of p53 provided that it is presented in a stem-loop and not in a linear conformation of DNA. A similar binding pattern was observed with stem-loop structures containing p53 cognate motifs derived from PRE-TGF-$\alpha$ and PRE-EGF-R (Fig. 2*F*), indicating that the interaction of p53 with target binding sites in non-linear DNA conformation is a general phenomenon, and not a peculiar feature of PRE-p21.

### *p53 Binds Stem-Loop DNA as a Tetramer*

Transcriptionally competent p53 must be present as a tetramer (23, 24). Therefore, the question of whether or not p53 binds to stem-loop DNA in its transcriptionally active (tetrameric) form is of physiological relevance. We analyzed the oligomerization status of p53 bound to stem-loop DNA by using an approach optimized for the analysis of the oligomeric status of p53 in complex with DNA (17). The results show that in complex with stem-loop DNA (Fig. 3, *lanes 3* and *3′*) p53 was present predominantly as a tetramer (*lane 3* in the *protein blot panel*). We conclude that the interaction of tetrameric p53 with linear and stem-loop DNA is determined by different modes of DNA binding: p53 tetramer binds readily to stem-loop DNA, but its binding to linear DNA requires modification at the C terminus such as interactions with other proteins, which mimic binding of PAb421.

### *Sequence-specific and Structure-specific DNA Recognition Determines the Strong Interaction of p53 with Target Sites in Stem-Loop Conformation*

An important question arising from the high affinity interaction of p53 with stem-loop DNA is whether stem-loop binding is sequence-specific. To address this question we prepared a set of stem-loops containing either a perfect consensus decamer derived from the RGC-sequence (CON-loop) or its mutated versions in which either one or both highly conserved bases, $C_4$ or $G_7$, were mutated. Specific decamer in CON-loop DNA is derived from the RGC sequence (25), which is capable of mediating p53-dependent transactivation and often used as a model p53 binding site in DNA binding studies. The results show that mutation of the conserved bases reduced binding of

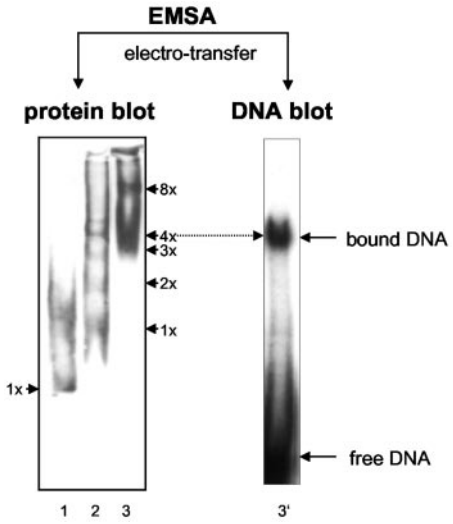


FIG. 3. **Determination of the oligomeric status of p53 protein bound to the PRE-p21 in stem-loop conformation.** Wild-type p53 protein was incubated with labeled PRE-p21 DNA in stem-loop conformation (*lanes 3* and *3′*) and subjected to electrophoresis in native 4% gel along with the mixture of different p53 oligomers obtained by glutaraldehyde cross-linking of a DNA-free wt p53 protein (*lane 2*). To determine the position of a wt monomer a tetramerization-deficient mutant p53-Δ70 was also cross-linked with glutaraldehyde in the absence of DNA and analyzed on the same gel (*lane 1*). p53-Δ70 is mostly monomeric in solution and is not able to form regular oligomers, but it forms irregular unstable oligomers because of stacking interactions of the core domain (17). Such irregular oligomers migrate as a diffuse band above the band representing a monomer of the p53-Δ70 protein (*1x* in *lane 1*). p53-Δ70 is shorter than wt p53, and its monomer migrates faster than a full-length monomer (compare *1x* bands in *lanes 1* and *2*). After electrophoresis, radiolabeled DNA and p53 protein were transferred onto a Hybond $N^+$ or nitrocellulose membrane. p53 was detected by Western blotting (*protein blot panel*), and the labeled DNA was detected by autoradiography of the Hybond $N^+$ membrane (*DNA blot panel*). *Solid arrows* indicate positions of different oligomeric forms, and *numbers* correspond to the number of monomers. The *broken arrow* indicates the p53-DNA complex.

p53 (Fig. 4*A*), indicating that the strong interaction of p53 with stem-loop DNA requires the presence of the p53 cognate motif. Specifically, maintenance of both conserved bases appears crucial to determine strong binding of p53 to stem-loop DNA.

All stem-loop structures tested in our experiments contained single-stranded DNA within the loop. Therefore, the possibility remained that the strong interaction of p53 with stem-loop DNA might have been determined by its ability to bind single-stranded DNA in a sequence-independent fashion (26). Accordingly, enhanced binding of p53 to stem-loop DNA containing the consensus sequence would result from the presence of single-stranded DNA within the loop rather than from specific sequence recognition. If this would be the case, the presence of single-stranded DNA should be the determining parameter for the interaction of p53 with stem-loop DNA, whereas the presence of the specific sequence motif would rather have a modulating effect. We analyzed binding of p53 to various stem-loop structures (depicted in Fig. 4*B*) by DNase I protection assay, which allows a precise mapping of the protein-bound regions in the target DNA. In the absence of p53, DNase I treatment resulted in a characteristic cleavage pattern at hypersensitive bases, which was consistent with the presence of a stem-loop structure (Fig. 4*B*, *lanes 2* and *5*). In the presence of p53, the loop and part of the stem were protected in CON-loop DNA, in which the loop is formed by a perfect consensus decamer (Fig. 4*B*, *lane 3*). Mutation of the conserved bases of the consensus abrogated binding to the loop in MUT-loop DNA (Fig. 4*B*, *lane 6*), indicating that the presence of a specific sequence, but not the presence of single-stranded DNA as such, determined the

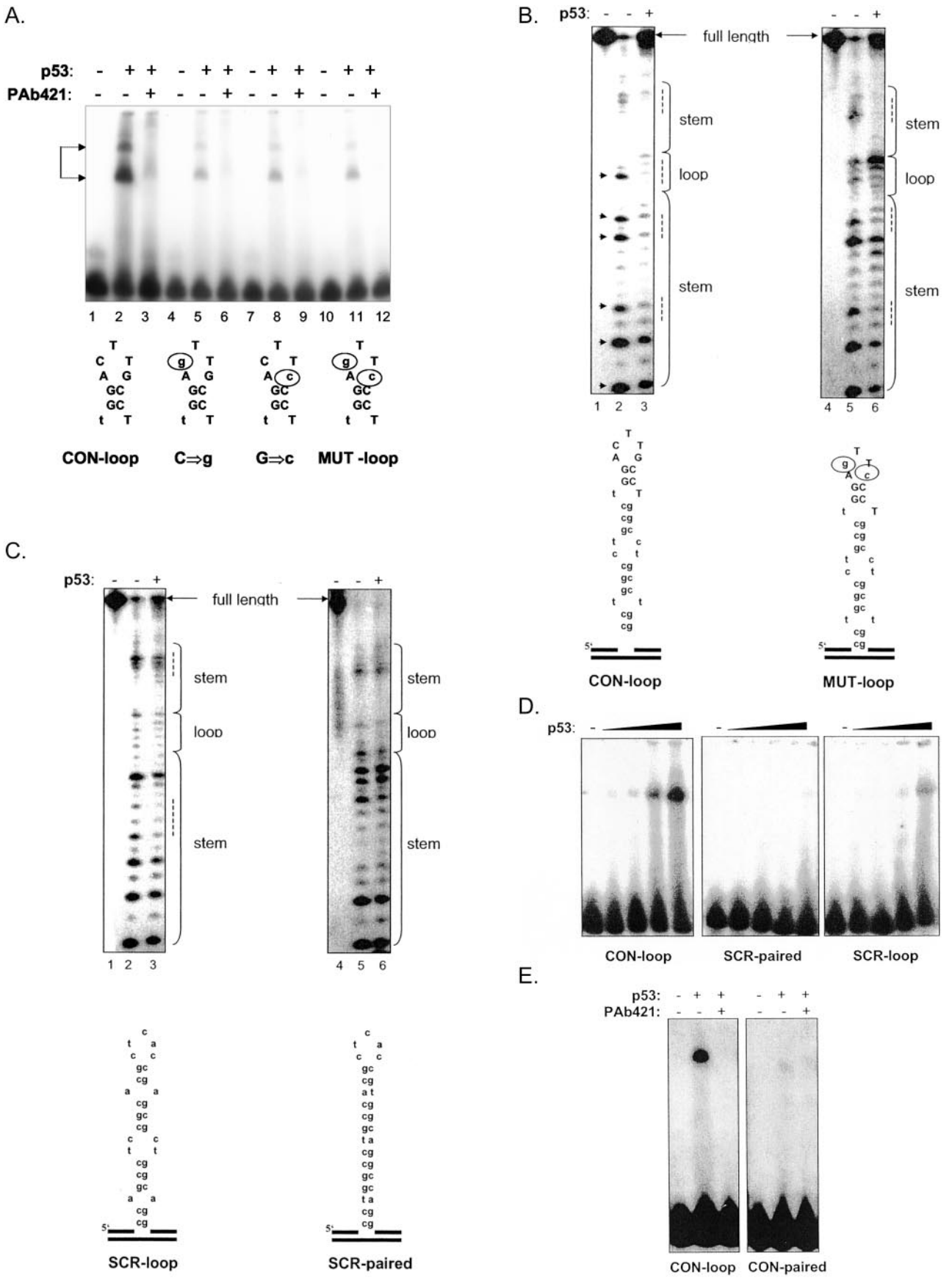


FIG. 4. **Strong interaction of p53 with stem-loop structures is determined by sequence- and structure-specific recognition of DNA.** *A*, binding of p53 to stem-loops containing either a perfect (*lanes 1–3*) or mutated (*lanes 4–6*, *7–9*, and *10–12*) consensus decamer. Loop regions differing in four stem-loops are depicted underneath the gel image. The consensus motif is typed in *capital letters*; mutated conserved bases are *circled*. *Arrows* indicate p53-specific complexes formed with CON-loop DNA. Entire CON-loop and MUT-loop stem-loops are depicted in *B*. *B* and *C*, mapping of p53-bound regions within stem-loop DNAs by DNase I protection. Stem-loop structures are illustrated below the corresponding gel

binding of p53 to the loop region. However, binding to the stem region of stem-loop structures was maintained in CON-loop and MUT-loop DNA (regions designated *stem*), regardless of the presence (CON-loop) or absence (MUT-loop) of the consensus motif. In agreement with our EMSA experiments, the results showed that p53 binds to the stem MUT-loop DNA, albeit much weaker compared with CON-loop DNA (Fig. 4*A*). In addition, the data demonstrate that p53 binds at two independent sites on CON-loop DNA: the consensus motif and at the stem.

We next wanted to identify the parameters that determine stem recognition by p53. A common feature of all stem-loop structures bound by p53 was the presence of unpaired mismatched bases in the stem. The C-terminal and the DNA binding domains of p53 bind mismatched bases in a sequence-independent fashion (27, 28). Therefore, we assumed that mismatched bases might be important structural marks determining the recognition of the stem in stem-loop DNA. To test this possibility, we compared the binding of p53 to stem-loops formed by scrambled sequences and termed SCR-loop and SCR-paired. Both DNAs contain an identical loop that shares no homology to the consensus, but they differ in their stem region: in SCR-paired DNA the stem is fully paired, whereas SCR-loop DNA contains mismatched bases in the stem.

DNase I protection experiments showed that p53 slightly protected the stem in SCR-loop DNA (Fig. 4*C*, *lanes 1–3*), whereas no protection was observed in SCR-paired DNA, in which the stem is fully paired (Fig. 4*C*, *lanes 4–6*). The data suggest that the presence of mismatched bases determines weak and sequence-unspecific binding of p53 at the stem of SCR-loop DNA. EMSA experiments confirmed this conclusion because they also showed weak but reproducible binding of p53 to SCR-loop but not to SCR-paired DNA (Fig. 4*D*). Therefore, we conclude that mismatched bases might be important structural marks determining the recognition of the stem in stem-loop DNA. To test this assumption, we compared the binding of p53 to CON-loop and CON-paired DNA, which both contain a consensus decamer within the loop but differ in the presence of mismatched bases in the stem. EMSA showed that p53 bound strongly to CON-loop but not to CON-paired DNA lacking mismatched bases in the stem (Fig. 4*E*). We conclude that structural recognition of mismatched bases in the stem strongly enhances the overall binding of p53 to stem-loop DNA mediated by sequence-specific recognition of the consensus motif.

### *The C-terminal Domain Positively Regulates Specific Interaction of p53 with Binding Sites in Stem-Loop DNA*

*Analysis of p53 Deletion Mutant Binding by EMSA*—Our EMSA experiments demonstrated that the C terminus plays an important role in the regulation of specific binding to stem-loop DNA. Although strongly enhancing binding of p53 to linear DNA, the C-terminal antibody PAb421 inhibited p53 binding to stem-loops. The inhibition was specific for PAb421 because N-terminal specific antibody DO-1 only retarded the migration of the complex but did not inhibit binding (Fig. 5*A*).

To characterize further the role of the C-terminal domain in stem-loop DNA binding, we analyzed the binding of p53 deletion mutants to stem-loop DNA. Confirming previously reported observations, binding of the C-terminally truncated p53-Δ30 protein to linear PRE-p21 DNA was much stronger compared with full-length p53 (Fig. 5*B*, compare lanes *2′* and *4′* in the *lower panel*). In striking contrast, binding of p53-Δ30 was abolished when PRE-p21 was presented in a stem-loop conformation (compare *lanes 2* and *4* in the *upper panel*). Similar patterns were observed with PRE-TGF-$\alpha$ and PRE-EGF-R stem-loop DNAs (data not shown). Oligomerization-deficient p53-Δ70 did not bind to linear or stem-loop forms of PRE-p21 (*lanes 5* and *5′*). We next analyzed stem-loop DNA binding of the p53-ΔDBD protein, which contains the N- and the C-terminal domains but lacks the DNA binding domain (DBD, amino acid residues 110–280). The results showed that p53-ΔDBD did not bind to PRE-p21 stem-loop DNA at all (*lanes 6*), indicating that stem-loop binding requires the DBD. In addition, the data show that binding of the C terminus to mismatched bases as such is not sufficient to determine binding of p53 to stem-loop DNA in the absence of the DBD.

*Analysis of p53 Deletion Mutant Binding by Electron Microscopy*—Several groups, including ours, reported that the use of different assays might greatly influence the outcome of experiments analyzing the interactions of p53 with DNA. For example, apparently discrepant results were obtained when DNA binding of p53 was analyzed using different methods such as EMSA or DNase protection assay (8, 11, 29). In addition, the DNA size strongly influences the specific interaction with target binding sites because different effects of C-terminal deletion on DNA binding by p53 were observed with short or large DNA templates (8).

We examined binding of p53 to the consensus binding site present in linear and in stem-loop DNA by electron microscopy. The approach allows the analysis of protein-DNA interactions over a wide range of DNA length. A 474-bp fragment, containing a 20-bp consensus binding site located ~40% from one end of the DNA, was used as a linear binding substrate (19). Consensus-containing stem-loop DNA with an elongated 64-bp stem was prepared using the strategy depicted in Fig. 6*A*. Binding of full-length and various deletion mutant p53 proteins was analyzed as described under "Experimental Procedures."

In accordance with previously published results (19), we observed that the isolated DBD (p53-DBD, amino acid residues 94–312) formed a globular complex at the consensus binding site in linear DNA (Fig. 6*B*, *upper panel*, *a–c*). Similarly, the C-terminally truncated p53-Δ30 bound to the consensus binding site in linear DNA (*upper panel*, *d–f*) in agreement with EMSA experiments. With stem-loop DNA, both p53-DBD and full-length wt p53 formed a complex located asymmetrically at one side of the stem-loop (*lower panel*, *b–f* and *g–i*, respectively). The asymmetric location of the p53-DNA complexes most likely reflects binding of p53 at the consensus decamer located within the loop of the stem-loop DNA. The conclusion is supported further by the fact that neither p53-DBD nor full-length wt p53 formed a complex at DNA ends (see *upper panel*). p53-DBD protein that lacks the C and the N terminus formed

images. CON-loop and MUT-loop stem-loops contain either perfect or mutated consensus decamer, respectively. In SCR-loop DNA all bases of the consensus are mutated. SCR-paired DNA is lacking both the consensus motif and mismatched bases in the stem. Control *lanes 1* and *4* show labeled DNA substrates not treated with DNase I. In *lanes 2* and *5* DNA probes were treated with DNase I in the absence of p53. The *arrows* indicate bases hypersensitive to DNase I. The relatively poor detection of bases more distant to the labeled 5′-end reflects the symmetrical nature of stem-loop structures. *Lanes 3* and *6* show DNA samples that were treated with DNase I in the presence of p53. *Dashed lines* indicate regions that become protected in the presence of p53. *Brackets* demarcate bases forming the stem or the loop in stem-loop structures. p53 binding to different target DNAs was analyzed in the same experiment and run on the same gel. *D*, analysis of p53 binding to different stem-loops by EMSA. Increasing amounts of p53 (5–50 ng) were incubated with the indicated stem-loops in the absence of PAb421. *E*, comparison by EMSA of p53 interaction with stem-loop DNA either lacking (CON-paired) or containing (CON-loop) mismatched bases in the stem. Both DNAs contain a perfect consensus motif in the loop.

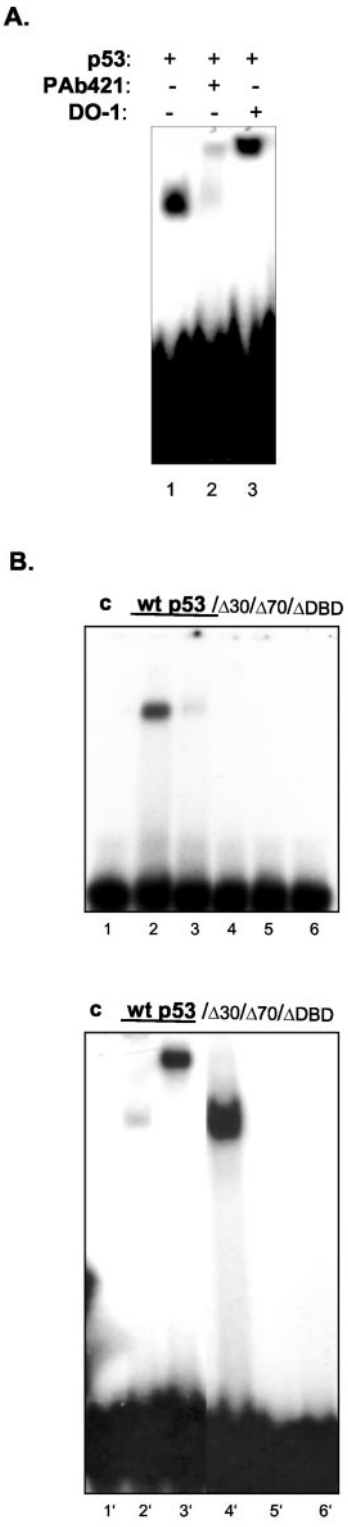


FIG. 5. **Role of the C-terminal domain in regulation of p53 interaction with structurally distinct target binding sites analyzed by EMSA.** *A*, CON-loop DNA was incubated with 50 ng of p53 in the absence (*lane 1*) or in the presence of the C-terminal PAb421 (*lane 2*) or the N-terminal DO-1 (*lane 3*) antibodies. *B*, binding of p53 deletion mutants to PRE-p21 in stem-loop (*upper panel*) or in linear (*lower panel*) conformation. Amino acid residues 360–393 (regulatory C-terminal domain) or 110–280 (DNA binding domain) are deleted in p53-Δ30 and p53-ΔDBD mutants, respectively. The p53-Δ70 mutant is lacking both the C-terminal and the oligomerization domains (amino acid residues 320–393). In all *lanes* except *1* and *1′*, DNA was incubated with 50 ng of the indicated p53 protein. Control *lanes 1* and *1′* show DNA in the absence of p53 or PAb421. In *lanes 3* and *3′*, binding mixtures contained PAb421.

a complex with stem-loop DNA. However, no complex formation with stem-loop DNA was observed with the p53-Δ30 protein lacking only the C terminus. The observation suggests that the C terminus may stabilize the complex formed with the stem-loop DNA by counteracting destabilizing effects of the N terminus (29). In agreement with our EMSA experiments, the results show that the DBD is required for binding to stem-loop DNA, and deletion of the p53 C terminus abrogates binding to target sites in stem-loop conformation but not to linear DNA. Thus, the two domains are equally important for the structure-dependent interaction of p53 with DNA, with the core domain mediating binding to DNA and the C-terminal domain regulating such interaction possibly in cooperation with the N-terminal domain.

## DISCUSSION

The data presented in this study show that the terms *latent* and *activated* p53 with regard to the specific interaction of p53 with its DNA target sites are valid only for certain *in vitro* interactions of p53 with linear target DNA. In fact, very recent evidence obtained by chromatin immunoprecipitation experiments also failed to support the latency model for regulation of p53 activity *in vivo* (30). Previous investigations had already demonstrated that activation of p53 DNA binding activity is not required if other methods of investigation were applied (8, 11, 29). Here we demonstrate that the specific interaction of p53 with target binding sites strongly depends on DNA conformation. Wild-type p53 binds its target sites with a high degree of specificity in linear and non-linear DNA. However, p53 interacts with its target sites very differently when they are presented in different DNA conformations. Interaction with linear binding sites is weak unless the C-terminal regulatory domain is modified or removed and is entirely determined by sequence-specific recognition. Presentation of the same target sites in stem-loop conformation strongly promotes specific DNA binding of p53 and requires the p53 C terminus. The high specificity of the interaction of p53 with stem-loop target DNA results from a dual recognition of the cognate sequence motif and of structural properties of the DNA. Invariability of the conserved bases within PREs is crucial for the specific interaction with target sites in stem-loop conformation, whereas non-conserved bases can be rather variable. This finding could explain how the low conformity of natural PREs to the consensus sequence is compatible with the specificity of p53 interaction with various heterogeneous PREs.

The finding that p53, in the absence of PAb421, binds strongly to PREs in a non-linear conformation of DNA raises the question of whether such binding reflects a physiologically relevant situation. Until recently, the effects of PAb421 on DNA binding of p53 were considered physiologically relevant because binding of PAb421 was thought to mimic a physiological modification required for the p53 DNA binding activity. However, recent *in vitro* studies performed under close to physiological conditions revealed that p53 in the absence of PAb421 was fully competent for DNA binding and transcriptionally active, whereas p53 modified by PAb421 appeared to be much less active (8). Thus, DNA binding of p53 in the absence of PAb421 reflects a physiologically relevant situation that occurs during a p53-mediated transcriptional response.

The binding of p53 to PREs in a non-linear DNA conformation might provide a new explanation for the remarkable sequence versatility of natural PREs as well as the variability in the number of decamers composing the various PREs. Earlier studies performed with linear DNA led to the conclusion that the multidecameric composition of PREs may be important for stable complex formation with p53 (31). It was proposed that the two dimers of the p53 tetramer each bind their own

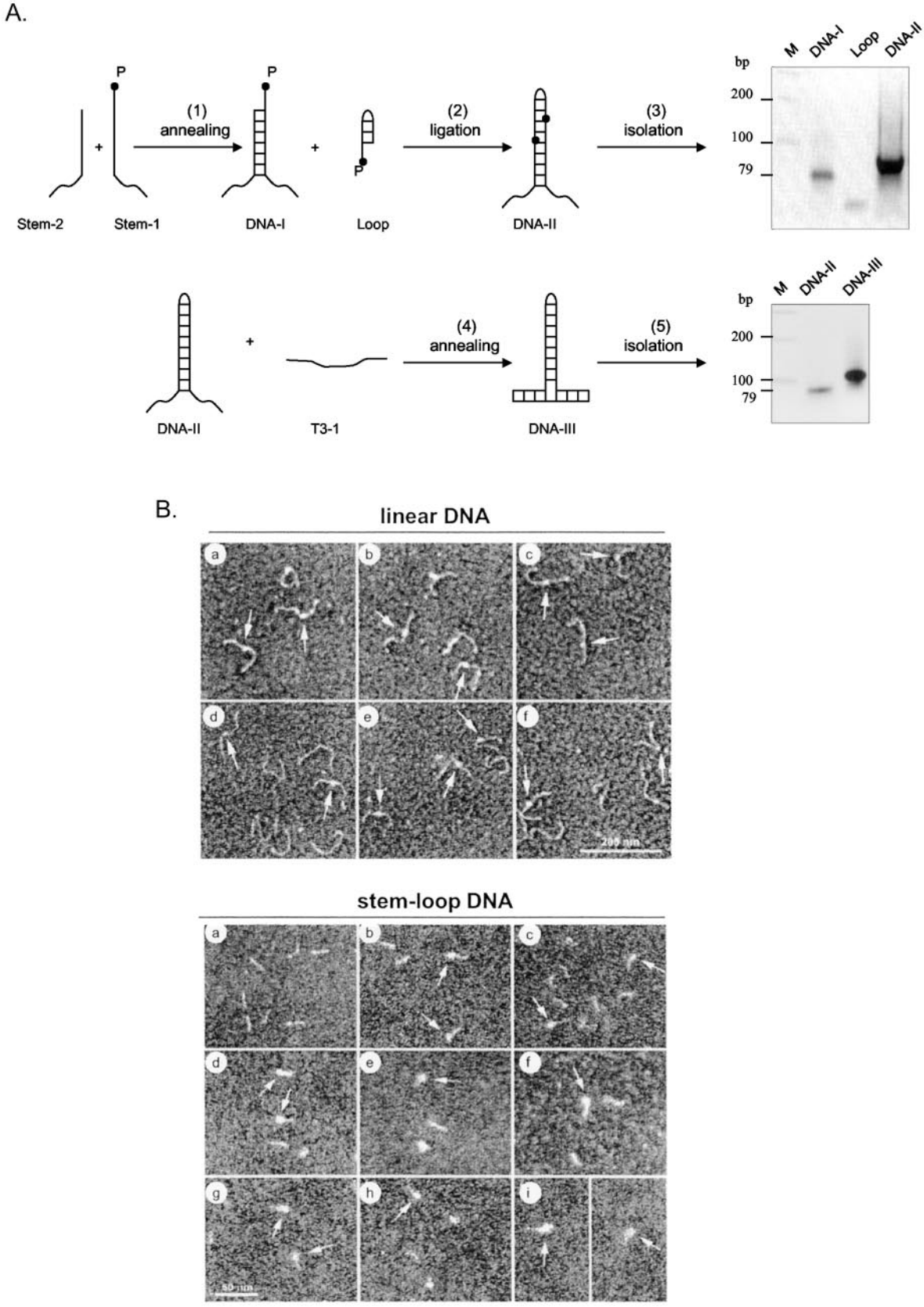


FIG. 6. **Analysis of interactions of p53 with linear and stem-loop DNA by electron microscopy.** *A*, preparation of stem-loop DNA for electron microscopy (for a detailed description, see "Experimental Procedures"). 5′-End phosphorylated oligonucleotides are marked with *P*. DNA III resulting after step 5 was used for electron microscopy. *B*, the *upper panel* represents images of the complexes formed with a linear 474-bp DNA fragment bearing the p53 recognition sequence (19). Complexes were formed by the DNA binding domain p53-DBD (*a–c*) or by p53-Δ30 mutant (*d–f*) at molar protein:DNA ratio of ~4 for p53-DBD and ~3.5 for p53-Δ30 (calculated for p53 tetramer). The *arrows* indicate p53-DBD and p53-Δ30 bound at the specific cognate motif. The *lower panel* represents images of the complexes formed with stem-loop DNA III. Complexes were formed by the DNA binding domain p53-DBD (*b–f*) or by wt p53 (*g–i*) at molar protein:DNA ratio ~0.5–1.0 and ~2.5 (calculated for p53 tetramer), respectively. *a*, DNA III alone.

decamer and that the cooperative interaction between DNA-bound dimers may further stabilize the p53-DNA complex (31). However, the higher stability of the complex was only observed in the presence of PAb421, whereas in the absence of PAb421 p53 did not bind, regardless of the number of decamers. Our finding that a single decamer in stem-loop DNA conformation is sufficient to mediate high affinity binding of p53 in the absence of PAb421 raises the question of why PREs should contain more than one decamer to be recognized by p53. As shown here, p53 bound to a single decamer only when this decamer was present in the loop of a stem-loop structure. Within PREs, the potential to form a secondary structure that is compatible with high affinity binding of p53 depends on the presence of self-complementary regions. The structural plasticity of a single-decameric PRE would be much lower than that of a multidecameric PRE in which the potential to undergo structural changes is provided by complementary regions shared by different decamers (see Fig. 1). Therefore, the multidecameric composition of PREs provides a structurally flexible context that allows PREs to adopt different conformations. Thereby, individual decamers could be converted from poor binding sites into structurally favorable sites strongly bound by p53. Such a mechanism could also explain why in most of PREs only one decamer strictly conforms to the consensus, whereas the others deviate to a variable degree.

We show that the presence of mismatched bases is required for the strong interaction of p53 with target binding sites in stem-loop conformation. Our analysis of DNA binding by various p53 deletion mutants demonstrated that the C-terminally truncated p53 protein bound to linear DNA but was unable to bind to stem-loop DNA. The requirement of the C terminus for stem-loop DNA binding can be explained by the intrinsic affinity of the C terminus to mismatched bases in DNA as demonstrated previously (27, 28). The requirement of the C terminus for p53 to bind to stem-loop DNA was alleviated when the N-terminal domain was also absent, as evidenced by electron microscopy. The observation suggests that the C terminus may only be required for specific interaction of p53 with stem-loop DNA in the presence of the N-terminal domain. It was proposed that the N terminus negatively regulates DNA binding of p53 by increasing the dissociation of the p53-DNA complex (29). Our results are compatible with this hypothesis and suggest that the C- and the N-terminal domains may regulate structure-specific interactions of p53 with target DNA in an interdependent fashion.

An important question relating to the biological relevance of p53 binding to PRE in non-B DNA conformation is whether, and if so, under which physiological conditions, PREs might undergo structural transitions. Thermodynamically, the formation of secondary structures is unfavorable in fully paired double-stranded DNA. However, during actively ongoing transcription or replication, DNA strands must be locally separated. Under such conditions, the energetic constraints that impede secondary structure formation in fully paired DNA will be obviated, thereby promoting the formation of secondary DNA structures by intrastrand pairing. Indeed, active replication and transcription are accompanied by an increased formation of unusual DNA structures that have been detected *in vivo* (32–35). Formation of such structures has been associated with regulation of transcription, replication, and recombination (36, 37; for review, see Refs. 38 and 39).

Several lines of evidence strongly support the idea that the specific interaction of p53 with target binding sites is dependent on DNA structure. First, transcriptionally active p53 binds to target binding sites in highly organized chromatin DNA, whereas it does not bind to linear DNA (8). Second, PREs possess features such as repetitiveness and internal symmetry, which are characteristic for structurally flexible DNA sequences. Third, p53 binds to various target binding sites with different efficiency as observed *in vitro* (40) and *in vivo* (30, 41), but these differences do not correlate with the degree of conformation to the consensus. Fourth, the specific interaction of p53 with target binding sites is enhanced by the high mobility group (HMG) family member, HMG1 protein (42), which influences the helical structure of DNA and binds to unusual structures such as cruciform DNA (21, 43, 44). Regulation of protein-DNA interactions by DNA topology is not unique for p53 and resembles the specific interaction with target sites of other transcription factors, *e.g.* CREB (45, 46). Specific DNA binding of the CREB protein is also enhanced by the formation of stem-loop structures within its binding sites, and the presence of mismatched bases in the stem is required for CREB binding (46).

A plethora of genes with different functions is regulated by p53. Transcriptional regulation of various target genes by p53 must occur in a strictly coordinated manner to avoid the simultaneous triggering of conflicting pathways. The different modes of interaction of p53 with target sites in linear or non-linear DNA conformation represent a plausible mechanism by which p53-regulated promoters could be recognized in a selective fashion. Binding of p53 to various promoters thus would depend on the DNA topology of p53 cognate sites and could be fine tuned by the specific interaction of p53 with DNA topology-modulating proteins that either, like HMG1 (42), stabilize or, like topoisomerases (47, 48) may resolve unusual DNA structures. Structural transitions in DNA mostly occur during the S phase of the cell cycle (32, 49, 50). Our hypothesis that structural transitions in PREs may determine the selective promoter recognition by p53 would predict that p53 binding to some but not all promoters may depend on DNA replication. Indeed, Gottifredi *et al.* (51) recently reported that transcriptional activation by p53 of some, but not all promoters is selectively inhibited when DNA synthesis is blocked. The DNA structure-selective interaction of p53 with target binding sites, as demonstrated here, might provide a new understanding as to how selective promoter recognition by p53 could be achieved.

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