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Tumor Suppressor p53 Binds with High Affinity to CTG·CAG Trinucleotide Repeats and Induces Topological Alterations in Mismatched Duplexes*[S]

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DNA binding is central to the ability of p53 to function as a tumor suppressor. In line with the remarkable functional versatility of p53, which can act on DNA as a transcription, repair, recombination, replication, and chromatin accessibility factor, the modes of p53 interaction with DNA are also versatile. One feature common to all modes of p53-DNA interaction is the extraordinary sensitivity of p53 to the topology of its target DNA. Whereas the strong impact of DNA topology has been demonstrated for p53 binding to sequence-specific sites or to DNA lesions, the possibility that DNA structure-dependent recognition may underlie p53 interaction with other types of DNA has not been addressed until now. We demonstrate for the first time that conformationally flexible CTG·CAG trinucleotide repeats comprise a novel class of p53-binding sites targeted by p53 in a DNA structure-dependent mode *in vitro* and *in vivo*. Our major finding is that p53 binds to CTG·CAG tracts by different modes depending on the conformation of DNA. Although p53 binds preferentially to hairpins formed by either CTG or CAG strands, it can also bind to linear forms of CTG·CAG tracts such as canonic B DNA or mismatched duplex. Intriguingly, by binding to a mismatched duplex p53 can induce further topological alterations in DNA, indicating that p53 may act as a DNA topology-modulating factor.

Cells are equipped with extraordinarily sensitive surveillance systems, which are able to monitor genomic DNA for the appearance of any unprogrammed alterations that may pose a danger to the structural integrity of the genome (1–4). The appearance of just one or two double strand breaks in a cell is sufficient for rapidly putting the system into a "high alert" mode, marked by the activation of signaling pathways that are responsive to DNA damage (5–7). The ability to function either in a "stand by" or in an activated high alert mode is also a hallmark of the tumor suppressor p53, one of the key factors involved in the maintenance of genomic integrity (8–11). Activation of p53 is an essential component of the global response of the cell to acute genotoxic insults that leads to rapid stabilization of the p53 protein and to p53 functioning in a high alert mode (12, 13). As a DNA damage-inducible factor, p53 can be potently activated by various types of genotoxic stimuli (9, 14, 15). A causative relationship between the occurrence of damaged DNA and the activation of the p53 response has been established by studies in a cell-free system demonstrating that sequence-specific DNA binding of p53 (p53-SSDB) can be potently stimulated by free DNA ends (16). *In vitro* DNA binding analyses further revealed that the C-terminal DNA-binding domain of p53 can bind selectively to some types of aberrant DNA structures in a sequence-independent manner (17–19). These findings led to the proposal that p53 may recruit cellular repair factors to the sites of damage by directly binding to DNA lesions in a DNA structure-dependent fashion (20, 21). Notably, it appears that such sequence-independent and DNA structure-selective DNA binding of p53 (p53-DSSB) may not necessarily be exclusively associated with damaged DNA, because some types of DNA structures to which p53 binds with high affinity *in vitro* can form in cells under physiological conditions. Indeed, non-canonic DNA structures such as cruciforms, hemicatenated DNA, DNA bulges, three- and four-way junctions, or telomeric t-loops can all be bound by p53 (18, 19, 22–25).

Previous studies provided valuable mechanistic insights into the principal features that appear to be characteristic of p53-DSSB. p53-DSSB is mediated by the p53 core DNA-binding domain in cooperation with the p53 C terminus, whereas the N-terminal domain may have some modulating effect (26–28). Although apparently unaffected by mutations in the p53 core domain, p53-DSSB requires an intact tetramerization domain indicating that the tetramer is the major active form not only in p53-SSDB (29–31) but also in p53-DSSB (32, 33). In contrast to p53-SSDB, which is determined by specificity to both sequence and DNA structure (32, 34), the specific architecture of DNA is the major common determinant underlying p53 interaction with DNAs that share no apparent sequence homology. DNA junctions such as those formed in hairpins, cruciforms, Holliday structures, or recombination intermediates are important structural elements determining p53 binding (20, 22, 23, 32, 33). Mismatched bases comprise another type of structural element to which p53 can bind with varying affinity depending on the type of mismatch (21). The findings indicate that the specific three-dimensional structure of DNA determines substrate specificity of p53-DSSB. However, neither the physiological relevance of p53-DSSB nor its significance to known p53 activities is fully understood. This is largely due to the fact that, with the exception of telomeric t-loops (18, 35), no other naturally occurring genomic sequences are known so far whose recognition by p53 would be determined exclusively by the structure of DNA. The identification of naturally existing genomic sequences with known functions that can be targeted by p53 via DSSB is of paramount importance for elucidating whether there is any physiological significance of p53-DSSB and its relation to p53 functions.

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Considering that p53 binds to unusual DNA structures, we wondered whether there might be an interaction between p53 and structures formed by CTG·CAG tracts, which represent conformationally flexible genomic DNA sequences with the propensity to form unusual structures (36–38). Like many types of repeat sequences, CTG·CAG tracts have a high propensity to be genetically unstable. There is much interest in the mechanisms leading to genetic instability of CTG·CAG tracts, because expansions within them cause a number of different hereditary neurological diseases, including myotonic dystrophy, Huntington disease, and several spinocerebellar ataxias (38, 39). Although the molecular details leading to expansion are not completely understood, it has become clear that the genetic stability of repeat tracts can be influenced by most aspects of DNA metabolism, including various DNA repair pathways (38, 39). It is thought that formation of non-linear DNA structures formed by self-folding of the CTG or the CAG strand is an important causative factor of instability associated with CTG·CAG repeats (40–43). Indeed, there is a strong causative relationship between the formation of hairpin structures by CTG·CAG tracts and the occurrence of DNA breakpoints (reviewed in Ref. 38). Furthermore, expansion of CAG repeats can activate the DNA damage checkpoint pathway (44), indicating that cellular factors responsive to DNA damage may be involved in the control of the CTG·CAG DNA stability.

We report in this study that trinucleotide CTG·CAG repeats comprise a novel physiological p53 target binding site, to which p53 binds via p53-DSSB. Our data show for the first time that p53 interacts with CTG·CAG tracts in naked DNA as well as in the context of chromatin and that p53 binds to alternative conformations adopted by CTG·CAG tracts via different binding modes. Whereas the physiological relevance of the interaction of p53 with CTG·CAG tracts remains to be elucidated, our finding that CTG·CAG DNA can be targeted by p53 under physiological conditions is intriguing, because it points to the possibility that replication-dependent instability of genomic DNA containing CTG·CAG tracts might be influenced by p53.

EXPERIMENTAL PROCEDURES

Preparation of DNA Substrates—DNA-substrates were prepared from synthetic oligonucleotides shown in TABLE ONE. 100 pmol of CTG_{11}, CAG_{11}, p53BS-2, or the appropriate sense oligonucleotide were 5′-labeled by T4-DNA polynucleotide kinase and [γ-^{32}P]ATP. To obtain double-stranded DNA substrates, the 5′-labeled sense DNA strand was annealed with the corresponding unlabeled antisense DNA strand in 100 μl of annealing buffer (10 mM Tris·HCl, pH 7.8, 50 mM KCl, 0.1 mM EDTA) at a molar ratio of 1:2. Structured DNA substrates were obtained by annealing the 5′-labeled CTG_{11}, CAG_{11}, or p53BS-2 DNA with the unlabeled Lock oligonucleotide in 100 μl of annealing buffer at a molar ratio of 1:4. The annealed DNA was purified by electrophoresis in a 8% polyacrylamide gel. Single-stranded labeled oligonucleotides were loaded alongside DNA hybrids as a reference for electrophoretic mobility. The band corresponding to the hybrid DNA resulting from annealing was excised, submerged into the annealing buffer, and incubated at 4 °C overnight. Eluted DNA was concentrated by acetone precipitation and resuspended in the annealing buffer.

Electrophoretic Mobility Shift Assay—DNA binding experiments were performed using 50 ng of recombinant human p53 proteins expressed in insect cells and purified as described (32) with or without 1 μg of monoclonal antibody in 15 μl of DNA binding buffer (10 mM Tris·HCl, pH 7.8, 50 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 20% (v/v) glycerol) containing 5 ng poly(dI-dC) × poly(dI-dC) and 2 μg of bovine serum albumin. After 20 min of preincubation at room temperature, 20 kcpm of the labeled DNA probe (1–5 ng) in 5 μl of DNA binding buffer were added, and the incubation was continued for another 20 min at room temperature. DNA binding of SSB was analyzed under essentially the same conditions except that the probe was incubated with 25 ng of the SSB protein (Promega, Mannheim, Germany) either alone or in the presence of p53. The samples were analyzed on a 4% native polyacrylamide gel (10 mM Tris·CH_3COOH, pH 7.8, 0.1 mM EDTA, 1.25 mM NaOAc, and 10% (v/v) glycerol) and separated at 200 V for 2 h at room temperature. Gels was dried and subjected to autoradiography.

DNase I Footprinting—DNA binding by p53 was performed under the same conditions as described in the previous section. After the binding step, 30 μl of DNase I solution (10 mM Tris·HCl, pH 7.8, 50 mM KCl, 8.3 mM $CaCl_2$, 8.3 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, and 20% (v/v) glycerol) and 0.1 unit of DNase I (Promega) were added, and the samples were incubated for 10 min at room temperature. The reaction was stopped by adding 50 μl of stop solution (100 mM Tris·HCl, pH 8.0, 300 mM NaOAc, 100 mM NaCl, 1% (w/v) SDS, 10 mM EDTA, 200 μg/ml proteinase K, 100 μg/ml yeast t-RNA). After 15 min of incubation at 37 °C, the DNA was extracted by phenol-chloroform and recovered by ethanol precipitation. DNA pellets were resuspended in formamide loading buffer and analyzed by electrophoresis on a 20% denaturing polyacrylamide gel.

Cell Culture—LNZ308/2024 clone (kindly provided by Dr. E. G. Van Meir) is derived from the human glioma cell line LNZ308 and expresses wild type p53 in the presence of doxycycline (45). The human osteosarcoma cell line SaOs-2 is null for p53 (ATCC). The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum at 37 °C in a humidified incubator (5% CO_2).

Transient Transfection and Luciferase Reporter Gene Assay—Transient transfection was performed essentially as described previously (46). Briefly, LNZ308/2024 cells were plated into a six-well tissue culture plate (Nunc, Roskilde, Denmark) at a density 0.2×10^6/well and transfected by nucleoporation (Amaxa, Köln, Germany). The transfections were done in triplicates using 0.5 μg of reporter DNA/well. Transfected cells were incubated either in the absence or the presence of doxycycline (1 μg/ml) and harvested 24 h after transfection. Luciferase activity was measured in lysates prepared from the transfected cells by using the luciferase assay system from Promega. Luciferase activity was normalized against total protein amounts determined by using the Bio-Rad protein assay.

Cloning and Stable Transfections—Nineteen CTG·CAG_{19} repeats were cloned into the AflII site of the pEYFP-C1 vector (Clontech, Heidelberg, Germany), which is suitable for transfection of eukaryotic cells and enables selection of transfected cells by resistance to neomycine. p53-null SaOs-2 cells were stably transfected with 2.5 μg of either pEYFP-C1 or pEYFP-CTG·CAG_{19} plasmids using Effectene™ transfection reagent (Qiagen). Transfected cells were maintained for 3 weeks in the selection medium containing 0.5 mg/ml G418. Genomic DNA was isolated from the selected clones resistant to G418 and analyzed for the presence of pEYFP-C1 or EYFP-CTG·CAG_{19} sequences by PCR using EYFP-for (5′-CATGGTCCTGCTGGAGTTCGTG-3′) and EYFP-rev (5′-GGAACAACACTCAACCCTATCTCG-3′) primers. The identities of the resulting PCR products were confirmed by sequencing. Two of the recombinant clones termed SaOs-2/pEYFP and SaOs-2/pEYFP-CTG·CAG_{19} were selected for ChIP[2] experiments.

ChIP—Wtp53 or mutant R273H proteins were expressed in SaOs-2/pEYFP or in SaOs-2/pEYFP-CTG·CAG_{19} cells by transient transfection

[2] The abbreviations used are: ChIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility shift assay; 3WJ, three-way junctions; SSB, single-stranded DNA-binding protein; ssDNA, single-stranded DNA.

TABLE ONE

Oligonucleotides used in the study

Sequences flanking CTG or CAG tracts are shown in bold type. The underlined sequences correspond to the regions that are complementary to the Lock sequence. The sequences shown in italics correspond to p53-binding sites from the *mdm2*(P2) promoter.

Oligonucleotide	Sequence	DNA substrates
Lock	5′-**CCGCGGTACCATTACCTAAGGCCCC**-3′	$CTG_{hairpin}$ and $CAG_{hairpin}$ and p53BS-2
CTG_{11}	CTGCTGCTGCTGCTGCTGCTGCTGCTGCTG 5′-**GGGGCCTTAGGTA** **ATGGTACCGCGG**-3′	$CTG_{hairpin}$
CAG_{11}	CAGCAGCAGCAGCAGCAGCAGCAGCAGCAG 5′-**GGGGCCTTAGGTA** **ATGGTACCGCGG**-3′	$CAG_{hairpin}$
$CTG_{13\text{-sense}}$	CTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG 5′-CGGAATTC CTTAAGGC-3′	CTG·CAG and $CTG{\cdot}CTG_{T\cdot T}$
$CTG_{13\text{-antisense}}$	CTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG 5′-GCCTTAAG GAATTCCG-3′	$CTG{\cdot}CTG_{T\cdot T}$
$CAG_{13\text{-sense}}$	CAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAG 5′-CGGAATTC CTTAAGGC-3′	$CAG{\cdot}CAG_{A\cdot A}$
$CAG_{13\text{-antisense}}$	CAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAG 5′-GCCTTAAG GAATTCCG-3′	CTG·CAG and $CAG{\cdot}CAG_{A\cdot A}$
p53BS-2	*AGTTAAGTCCTGACTTGTCT* 5′-**GGGGCCTTAGGTA** **ATGGTACCGCGG**-3′	$p53BS\text{-}2_{hairpin}$
$p53BS\text{-}2_{sense}$	5′-CGGAATTC *AGTTAAGTCCTGACTTGTCT* CTTAAGGC-3′	$p53BS\text{-}2_{lin}$
$p53BS\text{-}2_{antisense}$	5′-GCCTTAAG *AGACAAGTCAGGACTTAACT* GAATTCCG-3′	$p53BS\text{-}2_{lin}$
$DNA_{SPEC\text{-}sense}$	5′-GAGCT*GGTCAAGTTCAGACACGTTC*CGAAACTGCAGTAAAAGG*AGTTAAGTCCTGACTTGTCTC*CAGC-3′	DNA_{SPEC}
$DNA_{SPEC\text{-}antisense}$	5′-GCTGG*AGACAAGTCAGGACTTAACTC*CTTTTACTGCAGTTTCG*GAACGTGTCTGAACTTGACC*AGCTC-3′	DNA_{SPEC}

using p53 expression vectors pCMV-wtp53 (47) or pCMV-Tag-R273H (kindly provided by S. Dehde), respectively. 2×10^6 cells were plated in 10-cm dishes and transfected the next day with 2 μg of pCMV-wtp53 or pCMV-Tag-R273H using Effectene^{TM} transfection reagent (Qiagen). 36 h after transfection, ChIP was performed with the polyclonal antibody p53(FL-393) (Santa Cruz, Heidelberg, Germany) as described previously (46). Semi-quantitative PCR was performed in 100 μl of PCR buffer (Eppendorf, Hamburg, Germany) using 100 nM primers, 200 μM dNTP mix, 2 units of *Taq* DNA polymerase and 10% (v/v) of the immunoprecipitated DNA or 1% (v/v) of input DNA. PCR protocol included an initial denaturation step (2 min at 95 °C) followed by 35 cycles of 50 s at 95 °C, 50 s at 55 °C, and 1 min at 72 °C. Primers for *GAPDH* and *mdm2*(P2) were those described in Ref. 48. EYFP-for and EYFP-rev primers were used to amplify stably integrated pEYFP-C1 or pEYFP-CTG·CAG$_{19}$ (specific PCR products of 520 or 628 bp, respectively).

RESULTS

In Vitro Preparation and Analyses of DNA Structures Formed by CTG·CAG Triplets—Formation of non-canonical DNA structures such as slipped strand DNA or slipped intermediate DNA are thought to be the cause of the CTG·CAG DNA expansion associated with some neurodegenerative diseases (41, 42, 49). Slipped strand or slipped intermediate DNA can result from a misaligned annealing of CTG and CAG DNA strands that can fold back to form asymmetric hairpin structures branching out from three-way junctions (3WJ) (36). Therefore, 3WJ structures that contain hairpin and mismatched duplex formed by self-annealed CTG or CAG strands correspond to the basic structural elements of the biologically relevant DNA structures such as slipped strand or slipped intermediate DNA. We prepared DNA templates that recapitulate CTG·CAG tracts either in the canonical B-form (CTG·CAG$_{\text{B-duplex}}$), or in non-canonical DNA conformations (3WJ structures CTG$_{\text{hairpin}}$ and CAG$_{\text{hairpin}}$ contain hairpins, whereas CAG·CAG$_{\text{A·A}}$ or CTG·CTG$_{\text{T·T}}$ DNAs correspond to a mismatched homoduplexes formed by CTG or CAG repeats, respectively). 3WJ structures were designed as depicted in supplemental Fig. S1 (shown for CTG$_{\text{hairpin}}$). CTG·CTG$_{\text{T·T}}$ and CAG·CAG$_{\text{A·A}}$ structures contain multiple pairs of T·T or A·A mismatches, respectively. To ensure that the expected structures were formed, we assessed the DNA structure either enzymatically, using endonucleases (restriction enzymes or T7-EndoI) or chemically, with OsO_4 and diethyl pyrocarbonate (supplemental Fig. S1). The patterns of the T7-EndoI cleavage or reactivity with OsO_4 and diethyl pyrocarbonate were fully concordant with the formation of the expected structures and the stereochemical features established in structural analyses of hairpins formed by CTG or CAG triplets (50–53).

Interaction of Wild Type p53 with CTG·CAG Tracts in Alternative Conformations of DNA—Having confirmed the structure of our DNA substrates, we examined p53 binding to different conformations of CTG·CAG tracts by EMSA. Note that 3WJ structures that contain hairpins formed by CTG or CAG triplets appear in 4% native polyacrylamide gels as two bands migrating with differing mobility (Fig. 1, *A*, *lanes 9* and *17*, and *B*, *lane 17*). Whereas the faster migrating band comprises the major population of 3WJ structures containing a hairpin, the form of the DNA contained within the slower migrating band is unknown. We infer that the slower migrating band corresponds to a conformational isomer formed by CTG or CAG repeats. Such an interpretation would be concordant with the fact that the slower migrating band appeared in the preparations of 3WJ structures *after* they have been initially isolated as a single band from the preparative 8% gel. EMSA experiments showed that all of the DNA structures were bound by wtp53, which formed a single major complex (Fig. 1*A*, p53·DNA complex in *lanes 2*, *6*, *10*, *14*, and *18*). The complex was specific for p53, as is evident from the retardation of its mobility in the presence of the p53-specific antibody DO-1 (*lanes 4*, *8*, *12*, *16*, and *20*). The specificity of p53 binding was also evident from the characteristic inhibitory effects of the p53-specific antibody PAb421 (*lanes 3*, *7*, *11*, *15*, and *19*), which binds at the C-terminal domain of p53 and is a known *in vitro* modulator of both p53-SSDB (54) and p53-DSSB (32, 55). The inhibitory effect of PAb421 on p53 binding has been noted previously with different types of DNA templates and is indicative of the involvement of the C-terminal DNA-binding domain (32–34, 55, 56). The reduced binding in the presence of PAb421 thus suggests that the C-terminal DNA-binding domain of p53 is also essential for p53 interaction with the CTG·CAG DNA. Although the pattern of p53 binding to all three DNAs structures appeared similar, there was a marked quantitative difference in that the p53 complex formed with 3WJ structures containing a hairpin (*lanes 10–12* and *18–20*) was significantly more abundant compared with the complex formed with linear forms of CTG·CAG DNA, such as canonical B-duplex (*lanes 2–4*), or irregular homoduplexes formed by CTG or CAG strands (*lanes 6–8* and *14–16*, respectively). Importantly, p53 binding to different isoforms of CTG·CAG DNA is specific because linear p53BS-2$_{\text{lin}}$ DNA, which contains p53-binding site from the *mdm2*(P2) promoter (57) but lacks CTG·CAG repeats, did not bind p53 under the same experimental conditions (Fig. 1*A*, *right panel*, compare *lane 8* with *lanes 2* and *5*). Again, PAb421 strongly inhibited p53 binding to CTG·CAG DNA (*lanes 3* and *6*), whereas p53 binding to p53BS-2$_{\text{lin}}$ was activated (*lane 9*) as expected (54).

To further corroborate the conclusion that p53 binds preferentially to CTG·CAG DNA in hairpin conformation, we compared the potential of CTG$_{\text{hairpin}}$ or CTG·CAG$_{\text{B-duplex}}$ DNAs to displace specific DNA from p53 complexes formed with the canonic (sequence-specific) p53-binding sites. DNA$_{\text{SPEC}}$ contains two p53-binding sites, p53BS-1 and p53BS-2 (TABLE ONE), that comprise full p53 response element from the *mdm2*(P2) promoter (57). Whereas p53BS-2 alone does not bind p53 in the absence of PAb421 (Fig. 1*A*, substrate p53BS-2$_{\text{lin}}$ in the *right panel*), DNA$_{\text{SPEC}}$ binds p53 strongly even in the absence of PAb421 because of the presence of two p53-binding sites, p53BS-1 and p53BS-2 (Fig. 1*B*, *lane 2*). The p53 complex with DNA$_{\text{SPEC}}$ was challenged by increasing amounts of unlabeled CTG$_{\text{hairpin}}$ or CTG·CAG$_{\text{B-duplex}}$ DNA (Fig. 1*B*, *lanes 1–10*). Whereas DNA$_{\text{SPEC}}$ was efficiently displaced from the p53-SSDB complex by CTG$_{\text{hairpin}}$ DNA at a molar competitor:probe ratio of 50 (compare *lanes 2* and *8*), an at least 4-fold higher ratio was required to achieve a comparable effect with CTG·CAG$_{\text{B-duplex}}$ DNA as competitor (*lane 6*). Coherent results were obtained in the reciprocal experiments, in which unlabeled DNA$_{\text{SPEC}}$ was used as a competitor to challenge p53 complexes formed with labeled CTG·CAG$_{\text{B-duplex}}$ (*lanes 11–16*) or CTG$_{\text{hairpin}}$ (*lanes 18–22*) DNAs. As seen in *lane 13*, CTG·CAG$_{\text{B-duplex}}$ DNA was efficiently displaced by DNA$_{\text{SPEC}}$ already at a molar ratio of 10 (compare with *lane 12*), whereas an at least 10-fold higher amount of DNA$_{\text{SPEC}}$ was required to achieve a similar displacement of CTG$_{\text{hairpin}}$ DNA from its complex with p53 (*lane 21*). Altogether, the results demonstrate that a hairpin represents the preferred conformation to which p53 binds more efficiently than to a linear form of CTG·CAG DNA.

Wtp53 Binds to Alternative Conformations of CTG·CAG Tracts by Different Binding Modes—To obtain more detailed insight into the interaction of p53 with CTG tracts, we employed DNase I protection assay, which allows the identification of protein-binding sites at a single base resolution. p53 complexes formed with conformationally different CTG·CAG tracts were subjected to limited hydrolysis by DNase I, and the resulting patterns of DNA protection were compared.

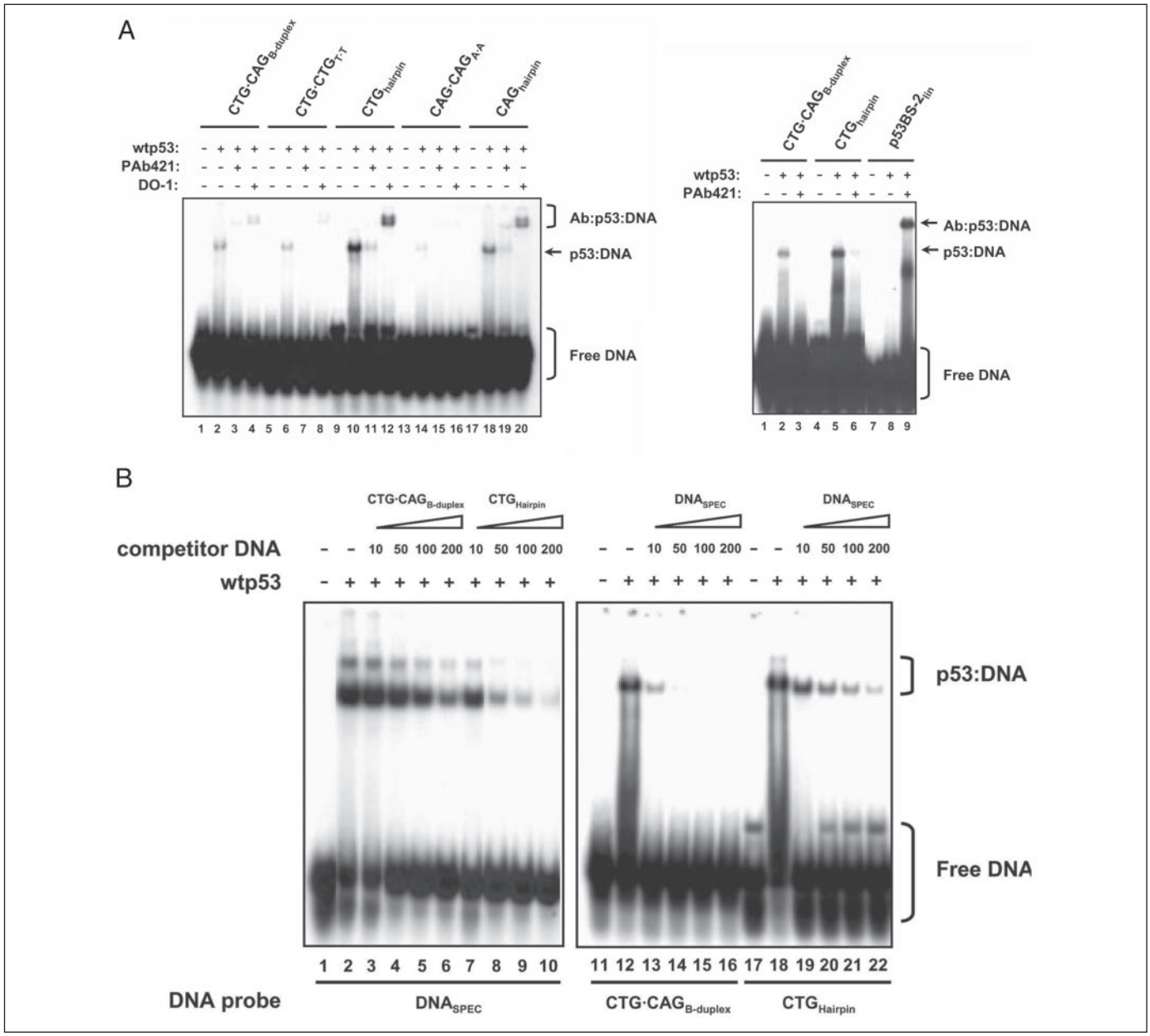

FIGURE 1. **Analyses of wtp53 binding to CTG·CAG tracts in different conformations by EMSA.** *A*, *left panel*, DNA structures formed by CTG·CAG tracts were incubated with 50 ng of recombinant human wtp53 protein with or without 1 μg of purified p53 specific antibodies DO-1 or PAb421. Note that the intensity of bands corresponding to the trimeric DNA·p53·DO-1 complexes appears decreased in *lanes 4*, *8*, *12*, *16*, and *20* compared with *lanes 2*, *6*, *10*, *14*, and *18*, respectively. This is due to the partition of intensity between two complexes formed in the presence of DO-1 compared with a single complex formed by p53 in the absence of antibody. *Right panel*, comparison of p53 binding to CTG·CAG tracts (*lanes 1–6*) and to linear p53BS-2_{lin} DNA, which lacks CTG·CAG tracts (*lanes 7–9*). *B*, wtp53 binds with different affinities to linear and non-linear conformations of CTG·CAG DNA. Relative affinities of p53 binding were analyzed by reciprocal competition assays. wtp53 (50 ng) was incubated with radioactively labeled DNA probes (indicated below the corresponding images) in the presence or absence of increasing amounts of unlabeled competitor DNA. The *numbers* indicate the molar excess of competitor DNA relative to the amounts of DNA probe (1 ng).

We first analyzed the interaction of p53 with CTG·$CAG_{B\text{-}duplex}$, which represents a canonical B DNA (Fig. 2*A*). p53 binding resulted in the protection of a large area that covered almost the entire length of the CTG tract in CTG·$CAG_{B\text{-}duplex}$ DNA (Fig. 2*A*, *lanes 4–8*). At the highest p53 concentration, a large region comprising 10 CTG triplets (bases enclosed within the *boxed area*) became completely protected (*lane 8*). Such a pattern is characteristic for non-sequence-specific DNA binding and indicates that p53 interaction with the CTG·$CAG_{B\text{-}duplex}$ is unlikely to be mediated by sequence-specific recognition of individual CTG·CAG triplets.

A different pattern of DNA protection was observed in the $CTG_{hairpin}$ DNA formed by 11 CTG triplets. Bases that became protected by the bound p53 protein were located within two clearly defined regions, each covering about three CTG triplets. One of the protected regions spanned three triplets proximal to the 5′-end, (CTG_1-CTG_3, numbered from the 5′-end onwards), whereas the other region included triplets CTG_9–CTG_{11} proximal to the 3′-end (Fig. 2*B*, *lanes 4–7*, triplets enclosed within the *boxed area*). Superimposition of the protected bases with the presumed structure of $CTG_{hairpin}$ showed that the protected CTG triplets were the ones engaged in the formation of the stem of the hairpin structure (depicted next to the gel image in Fig. 2*B*). The two protected regions were separated by an intervening region that remained unprotected (*lanes* 4–7). Although a slight protection within the intervening region emerged at the highest amount of p53 (*lane 8*), the data clearly demonstrate that the primary p53-

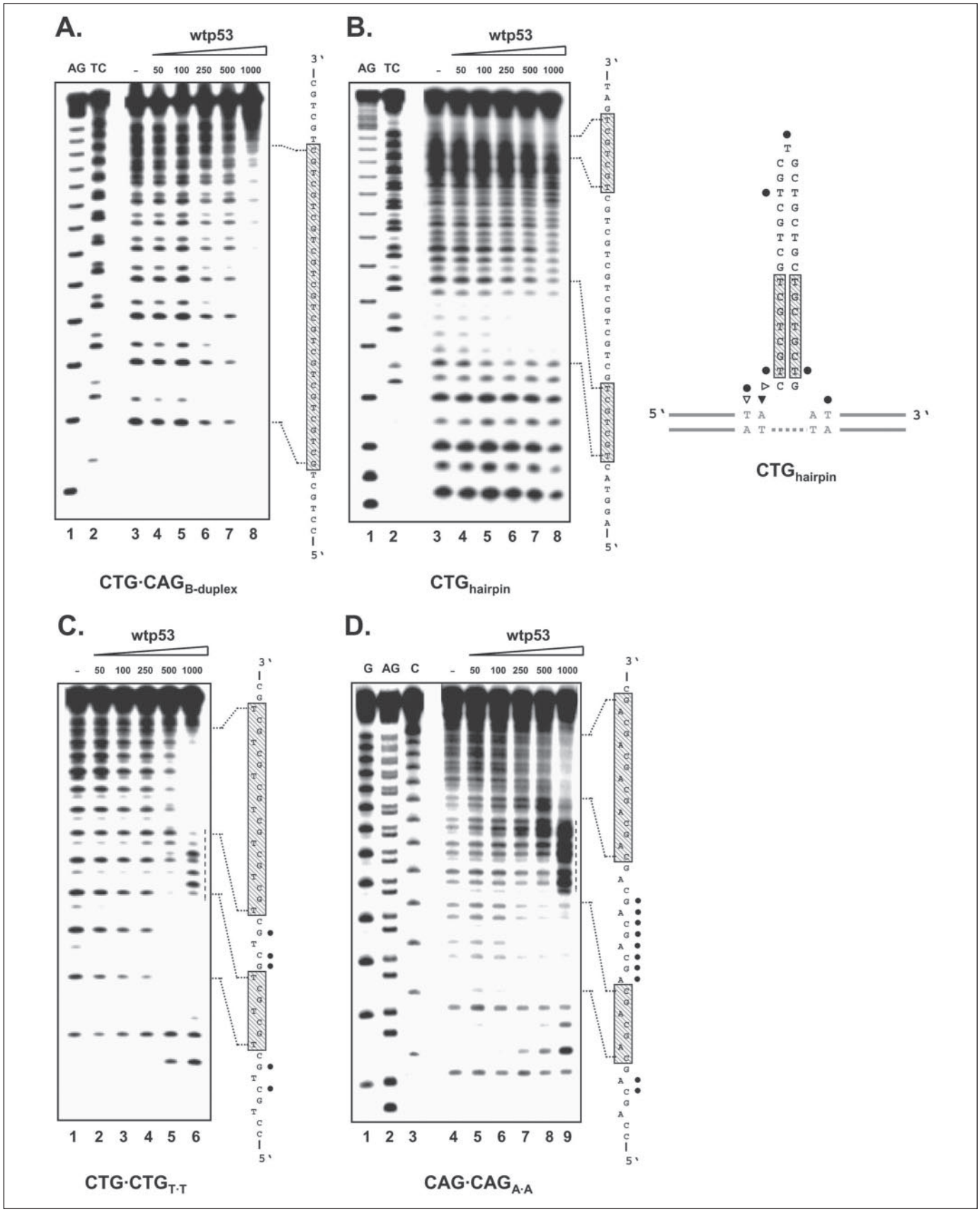

FIGURE 2. **Analyses of wtp53 interaction with CTG·CAG DNA by DNase I protection.** Images of 20% denaturing polyacrylamide gels showing patterns of wtp53 binding to different structures formed by CTG·CAG tracts (as indicated below gel images). Maxam-Gilbert sequencing ladders corresponding to the labeled strands from each DNA structure were run alongside to allow the precise identification of individual bases cleaved by DNase I within CTG or CAG tracts. *A*, p53 "footprints" on the CTG·CAG$_{B\text{-}duplex}$ DNA. Control sample was treated by DNase I in the absence of p53 (*lane* 3). Sequence of the labeled strand is shown on the *right side* of the gel image. Bases enclosed within the *boxed area* are those that

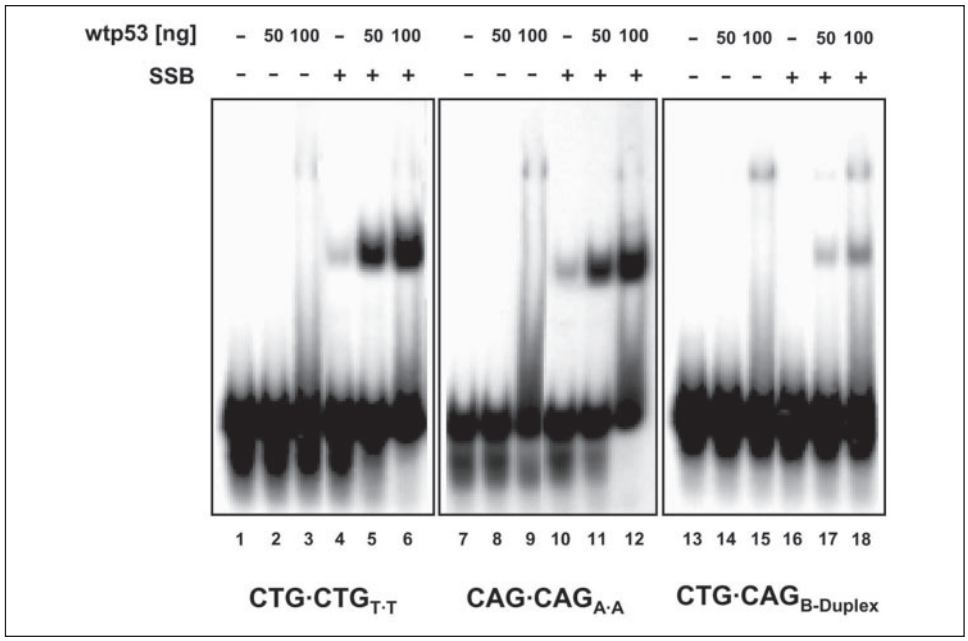

FIGURE 3. **Wtp53 stimulates DNA binding of SSB.** Effects of p53 on DNA binding of SSB analyzed by EMSA. DNA was incubated in the presence of p53, SSB, or both proteins. The amount of SSB was kept constant (25 ng), whereas wtp53 amounts varied (as indicated).

binding site is located within the stem of the hairpin adjacent to a three-way junction. Such a pattern is characteristic for site-specific binding and is reminiscent of the pattern resulting from p53-SSDB, in which a specific sequence determines the site of p53 binding (supplemental Fig. S2). Because the region protected by p53 in CTG$_{hairpin}$ DNA lacks any resemblance to the p53 consensus (58) and in its base composition is identical to sequences that remain unprotected, sequence-specific recognition cannot explain why p53 binds selectively to some but not all CTG triplets within the CTG$_{hairpin}$ DNA. However, such binding pattern is compatible with a stereo-specific DNA recognition whereby the structure of DNA determines the binding site for p53. Thus we conclude that p53 binds to CTG$_{hairpin}$ in a site-specific binding mode, in which the specificity of the interaction is determined not by a specific sequence but by the three-dimensional configuration of DNA.

We next analyzed the pattern of p53 binding to the CTG·CTG$_{T·T}$ DNA, which contains multiple pairs of regularly spaced mismatching T·T bases (Fig. 2*C*). Surprisingly, the "signature" of p53 bound to CTG·CTG$_{T·T}$ was very different from those observed with either CTG·CAG$_{B\text{-}duplex}$ or CTG$_{hairpin}$ DNAs. At lower concentrations of p53, two regions weakly protected by p53 emerged that did not span throughout the entire CTG tract but localized within CTG triplets located closer to DNA ends (shown inside the *boxed area* in Fig. 2*C*). Interestingly, the protected regions were separated by two triplets that initially remained unprotected (indicated by a *broken line* in Fig. 2*C*) but became hypersensitive to DNase I as the concentration of p53 increased (Fig. 2*C*, bases indicated by *dots*). Because the susceptibility to DNase I strongly depends on structural variations along DNA duplex (59), the result suggests that binding of p53 might have induced a change in the structure of CTG·CTG$_{T·T}$ DNA. To test whether this type of mismatch matters, we also analyzed the patterns of p53 binding to CAG·CAG$_{A·A}$ DNA that contained multiple pairs of A·A mismatches. The results show that, similar to the pattern seen in CTG·CTG$_{T·T}$ DNA, p53 binding rendered CAG·CAG$_{A·A}$ hypersensitive to DNase I within a region located in the center of the molecule, between the two areas protected by p53 (Fig. 2*D*). Importantly, the structural change induced by p53 was specific to CTG·CTG$_{T·T}$ and CAG·CAG$_{A·A}$ DNA, because it did not occur in CTG·CAG$_{B\text{-}duplex}$ nor in CTG$_{hairpin}$ DNA (Fig. 2, *A* and *B*).

Altogether, the DNase I protection experiments revealed that p53 interacts with CTG·CAG tracts by different modes; it binds in a site-specific mode to the hairpin structure formed by CTG repeats (CTG$_{hairpin}$) but not to the canonical B-form of CTG·CAG DNA (CTG·CAG$_{B\text{-}duplex}$). In mismatched duplexes formed by CTG or by CAG strands, p53 binding induces a topological change in DNA exhibited by an increased sensitivity to DNase I.

p53 Promotes Binding of Single-stranded Binding Protein to Irregular Homoduplexes Containing T·T or A·A Mismatches—Distortions of the DNA duplex are often accompanied by local separation of DNA strands. We considered the possibility that local disturbances of the DNA duplex induced by p53 in CTG·CTG$_{T·T}$ or CAG·CAG$_{A·A}$ DNAs might lead to the appearance of regions of single-stranded DNA (ssDNA). To test the hypothesis, we assessed the ability of single-stranded DNA-binding protein (SSB) to bind DNA in the absence or the presence of p53. The rationale behind this experiment was that SSB, which binds preferentially and with high affinity to ssDNA, should be able to "trap" distorted regions in trinucleotide repeat DNA. In our experiments using limiting amounts of SSB, fully paired CTG·CAG$_{B\text{-}duplex}$ DNA did not bind considerably (Fig. 3, *lane 16*), whereas CTG·CTG$_{T·T}$ and CAG·CAG$_{A·A}$ DNAs bound only weakly to SSB (SSB·DNA complex in *lanes 4* and *10*). However, upon the addition of p53, SSB binding to CTG·CTG$_{T·T}$ and CAG·CAG$_{A·A}$ DNAs increased dramatically in a p53 dose-dependent manner (*lanes 5*, *6*, *11*, and *12*, respectively). In contrast, SSB binding to CTG·CAG$_{B\text{-}duplex}$ was influenced only modestly by p53 (*lanes 17* and *18*). The results support the idea that the enhanced formation of SSB·DNA complexes is due to a change in DNA structure induced by p53. In accordance with such an explanation, the effects of p53 were considerably more pronounced with CTG·CTG$_{T·T}$ and CAG·CAG$_{A·A}$

became protected at the highest amounts of p53 (*lane* 8). *B*, p53 binds to a well defined site in the CTG$_{hairpin}$ DNA. Sequence of the labeled strand is shown as plain (along the gel image) or in hairpin conformation corresponding to its actual arrangement in the CTG$_{hairpin}$ DNA (most right image). *C*, and *D*, patterns of p53 binding to irregular duplexes CTG·CTG$_{T·T}$ and CAG·CAG$_{A·A}$ homoduplexes containing multiple T·T or A·A mismatches, respectively. Ts or As engaged in the formation of mismatches are shown as protruding in the corresponding sequences next to the gel images. *Broken lines* mark the areas that became hypersensitive to DNase I in the presence of p53. Individual bases that became hypersensitive are marked by *dots*.

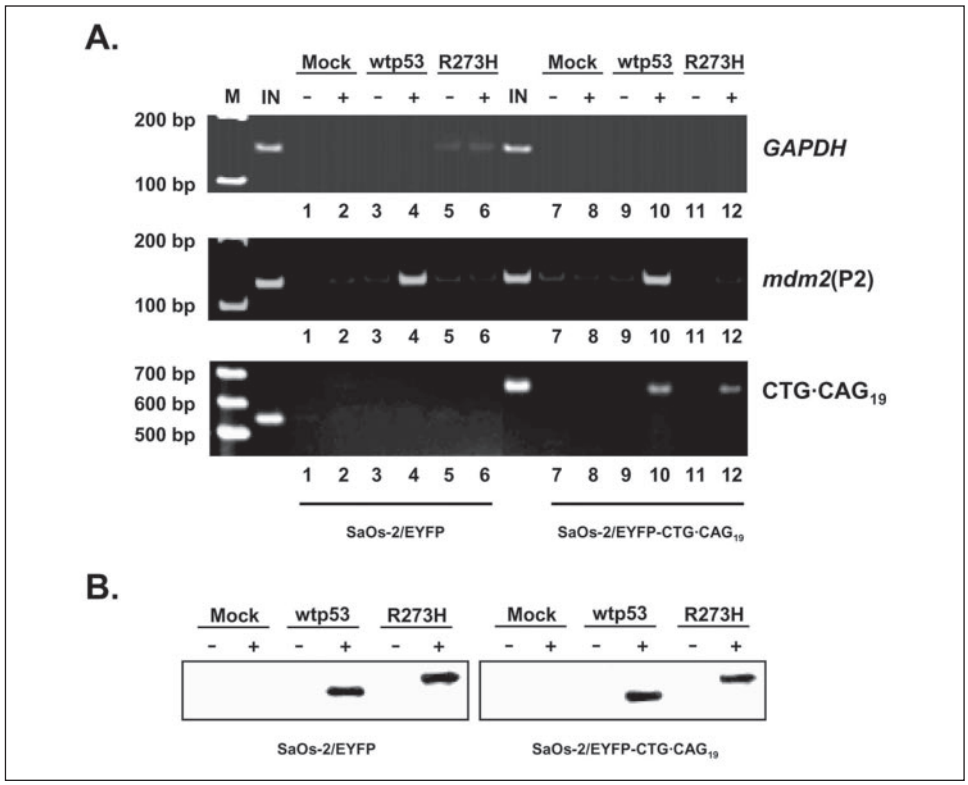

FIGURE 4. **Wtp53 and R273H mutant bind to CTG·CAG tracts in the chromatin context.** *A*, ChIP analyses of genomic DNA bound by p53 in chromatin. ChIP experiments were performed with SaOs-2 cells that contained stably integrated a model CTG·CAG tract (clone SaOs-2/EYFP-CTG·CAG_{19}) or an "empty" pEYFP-C1 vector (clone SaOs-2/EYFP). The cells were transiently transfected with Mock DNA (pUC18) or with expression plasmids that encode wtp53 or R273H. Input DNA was subjected to PCR with each primer used (samples in *lanes IN*). Genomic DNA was recovered from immunoprecipitates obtained either with anti-p53 antibody (+) or without antibody (−). Control PCRs were set with PCR primers specific for *GAPDH*-DNA (negative control) or *mdm2*(P2) promoter DNA (positive control) (shown in the *upper* and *middle panels*, respectively). *B*, comparable levels of wtp53 and of R273H expression in SaOs-2/EYFP-CTG·CAG_{19} or SaOs-2/EYFP cells was confirmed by Western blot analysis using anti-p53 antibody DO1.

homoduplexes (*lanes 1–12*), which are highly prone to topological distortions, than with the more stable CTG·$CAG_{B\text{-}duplex}$ DNA (*lanes 13–18*). That p53 did not induce larger SSB·DNA complexes as would be expected if more SSB homotetramers would bind cooperatively to the same DNA molecule is also concordant with a scenario in which p53 facilitates SSB binding by increasing the population of DNA molecules containing ssDNA regions. Notably, SSB-specific complexes, which can be easily distinguished from the slower migrating p53 complex, migrated with the same mobility either in the absence or presence of p53 (compare *lanes 4* and *10* with *lanes 5* and *6* and *lanes 11* and *12*, respectively). The finding that either SSB·DNA complexes or p53·DNA complexes are seen in EMSA but no ternary complexes (containing DNA, p53, and SSB) indicates that p53, while promoting SSB binding to ssDNA, can be displaced by SSB from DNA. Such a mechanism has been described for the dynamic interaction between p53 and other DNA structure-dependent proteins such as HMGI(Y), which displaces p53 from Holliday junctions (24). Stimulation of SSB binding to CTG·$CTG_{T\cdot T}$ or CAG·$CAG_{A\cdot A}$ DNAs by p53 without formation of a ternary complex is reminiscent of the interaction between p53 and the mismatch repair protein complex hMSH2/6, which enhances p53 binding to Holliday junctions or to bulged DNA without direct participation in the p53·DNA complex (24).

p53 Proteins Bind Specifically to CTG·CAG Tracts in the Context of Chromatin—To further test the idea that CTG·CAG tracts represent novel type of p53-binding sites, we next analyzed p53 interaction with CTG·CAG DNA *in vivo*. To address the question we established stable clones carrying stretches of 19 uninterrupted CTG·CAG repeats integrated into the chromatin of SaOs-2 cells (clone SaOs-2/EYFP-CTG·CAG_{19}). Clones carrying EYFP vector DNA lacking transgenic CTG·CAG repeats (clone SaOs-2/EYFP) were analyzed in parallel as a negative control. p53 binding to genomic DNA was assessed by ChIP in cells that were transiently transfected with an expression vector encoding wtp53 (Fig. 4). Expression of p53 was ascertained by Western blot analysis of lysates prepared from the transfected cells (Fig. 4*B*). *GAPDH*-DNA, to which p53 does not bind was included as a negative control in our PCR analyses of the recovered genomic DNA (*upper panel* in Fig. 4*A*). As expected, wtp53 bound specifically to the *mdm2*(P2) promoter in cells containing both SaOs-2/EYFP and in SaOs-2/EYFP-CTG·CAG_{19} (Fig. 4*A*, *middle panel*, *lanes 4* and *10*, respectively). Having ascertained that our experimental conditions allow detection of specific p53-DNA interactions, we tested whether p53 binds to CTG·CAG DNA in the context of chromatin. The results show that wtp53 bound to the CTG·CAG_{19} tract in SaOs-2/EYFP-CTG·CAG_{19} cells, as evidenced by PCR using the EYFP-for and EYFP-rev primers that flank the CTG·CAG_{19} insert (*lower panel*, *lane 10*). Confirming the specificity of binding, no PCR product was obtained with the same primers in the control reactions with genomic DNA from SaOs-2/EYFP cells, which lack the transgenic CTG·CAG_{19} sequence (Fig. 4*A*, *lower panel*, *lane 4*). The results of ChIP analyses thus demonstrate that wtp53 binds specifically to CTG·CAG tracts *in vivo*.

An important question was whether upon integration into the genome of SaOs-2 cells the model CTG·CAG_{19} repeats had retained a linear or assumed a non-linear conformation. Because this question is difficult to analyze by direct means, we took advantage of our previous finding that binding to linear DNA is completely lost by mutant p53 proteins (33, 60). Supplemental Fig. S3*A* shows that this also true in the case of linear CTG·CAG DNA, which does not bind the R273H p53 mutant, as evidenced by DNase I protection experiments. In contrast, R273H mutant did bind to $CTG_{hairpin}$ and CTG·$CTG_{T\cdot T}$ DNAs, and the resulting patterns of DNA protection were similar to those seen with wtp53 (supplemental Fig. S3, *B* and *C*). Analyses of DNA binding by ChIP revealed that R273H bound to the transgenic CTG·CAG_{19} repeats in the context of chromatin and with the efficacy comparable with that of wtp53 (Fig. 4*A*, *lower panel*, *lane 12*). Considering that mutant p53 proteins are deficient for binding to linear DNA, these results indicate that transgenic CTG·CAG_{19} repeats most likely adopt a non-B DNA

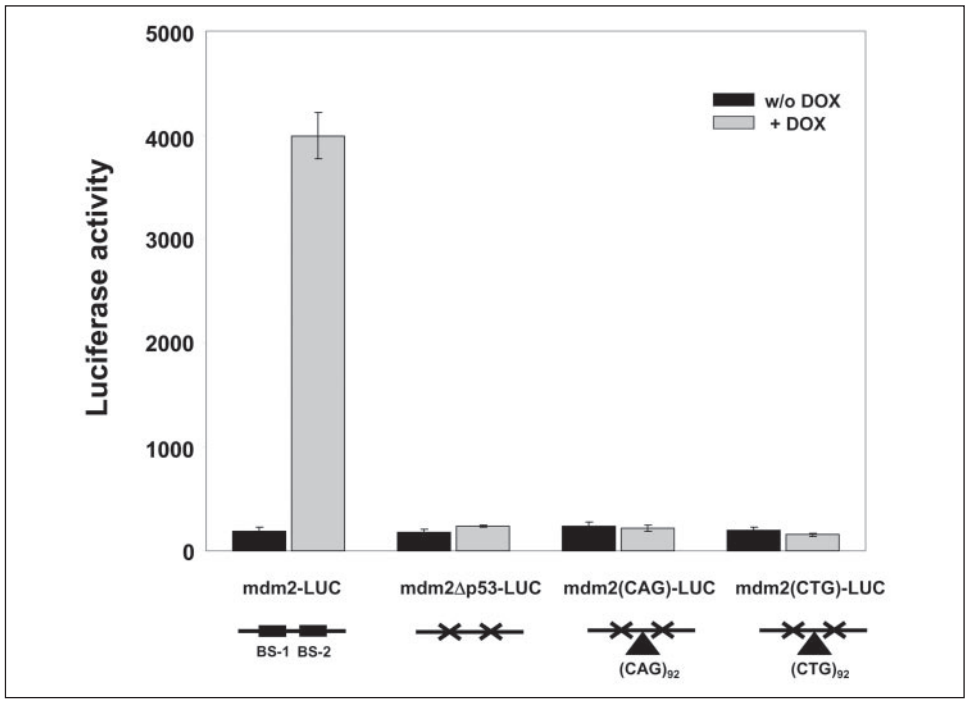

FIGURE 5. **Assessment of the potential of CTG·CAG tracts to function as p53 response elements by the reporter assay.** Mdm2-LUC plasmid (74) contains a luciferase gene under the control of the *mdm2*(P2) promoter (57), which contains two p53-binding sites (p53BS-1 and p53BS-2). Mdm2Δp53BS-LUC contains a derivative of the *mdm2*(P2) promoter, from which both p53BS-1 and p53BS-2 sites have been deleted. The derivatives of mdm2Δp53BS-LUC, mdm2(CTG)-LUC, and mdm2(CAG)-LUC contain 92 (CTG·CAG_{92}) repeats in sense or antisense orientation, respectively, cloned instead of BS-1 and BS-2. Luciferase activity (counts normalized to the total protein amounts) reflects activities of the *mdm2*(P2) promoter and its derivatives in the absence (*black bars*) or the presence (*gray bars*) of p53.*DOX*, doxycycline.

conformation in the context of chromatin. As expected, R273H mutant did neither bind to the endogenous *mdm2*(P2) promoter (*middle panel*, *lanes 6* and *12*) nor to the *GAPDH*-DNA (*upper panel*, *lanes 6* and *12*), indicating that R273H binds to the integrated CTG·CAG_{19} repeat DNA specifically. Notably, DNase I protection experiments showed that not only was R273H potent for binding to mismatched homoduplexes *in vitro*, it also, similarly to wtp53, induced local hypersensitivity to DNase I in the CTG·$CTG_{T\cdot T}$ DNA (supplemental Fig. S3*C*, *lane 12*). However, the potential of R273H to bind to CTG·$CTG_{T\cdot T}$ DNA and to induce structural changes in DNA duplex was considerably weaker than that of wtp53 (compare *lanes 7–12* with *lanes 1–6*, respectively). Thus p53 mutant R273H, which is impaired for binding to the canonic B-form of DNA, retains the potential to bind non-canonic structures formed by CTG repeats such as hairpin or mismatched duplex.

DISCUSSION

In this study, we demonstrate that CTG·CAG repetitive sequences comprise a novel type of specific p53-binding site, to which p53 binds *in vitro* and *in vivo*. Interaction between p53 and CTG·CAG tracts is determined not by a specific sequence but by the structure of DNA, thereby identifying CTG·CAG tracts as a binding target in p53-DSSB. Our *in vitro* analyses reveal that p53 can interact with different conformations of CTG·CAG tracts by distinct modes. In the canonical B-form, CTG·CAG repeat DNA is bound by p53 in a non-sequence-specific manner. However, upon formation of a hairpin conformation within the CTG·CAG tracts, structural features are created in the DNA that promote p53 binding in a site-specific manner. The identification of CTG·CAG tracts as a novel type of p53-specific target site provides the first evidence that the specific interaction of p53 with DNA is not restricted to p53-SSDB but may be mediated also by p53-DSSB under physiological conditions. An important implication from these findings is that the pool of p53-specific binding sites might be much larger than estimated from searches that were based exclusively on the analysis of p53 consensus sequences (61–63).

Identification of CTG·CAG tracts as a novel p53 target DNA raises the question of a physiological significance of such interaction. Considering that specific DNA binding is a prerequisite for transcriptional activation mediated by p53, one obvious possibility to consider would be that CTG·CAG tracts may act as non-canonical p53 response elements. We have examined such a possibility by the reporter assay using a chimeric promoter derived from the *mdm2*(P2) promoter, from which the intrinsic (sequence-specific) p53 response element was deleted and replaced by 92 CTG·CAG repeats in either orientation (Fig. 5, mdm2(CAG)-LUC and mdm2(CTG)-LUC). The results showed that the CTG·CAG_{92} tract did not render either of the chimeric promoters responsive to wtp53 (Fig. 5), indicating that p53 interaction with CTG·CAG tracts may be not relevant for regulation of transcription. However, more studies are required to ascertain the potential of CTG·CAG tracts to act as p53 response elements unambiguously.

Another possibility is that p53 interaction with CTG·CAG tracts (or possibly more general, p53-DSSB) may be important in processes other than regulation of transcription. In this regard, p53 activities associated with DNA repair should be considered. Our finding that p53 interacts with different conformational isoforms of CTG·CAG tracts in various modes reveals striking parallels with findings made for the mismatch repair protein hMSH2, which also binds to CTG·CAG tracts in a DNA structure-dependent mode (64). Similarly to our findings with p53, hMSH2 binds to CTG·CAG tracts specifically and with wide ranging affinity, depending on the type of structure, with slipped strand DNA and linear DNA being the most and the least preferred conformations, respectively (64). Furthermore, there is a direct mechanistic link between p53-DSSB and mismatch repair processes as revealed by earlier studies demonstrating that hMSH2 stimulates p53 binding to Holliday junctions *in vitro* (24) and co-localizes with p53 at discrete chromatin loci in living cells (65). In conjunction with the fact that CTG·CAG tracts represent a common binding target for both hMSH2 (64) and for p53 (this study) and that DNA is thought to be the connecting component in the complex and interdependent interplay between p53 and hMSH2 activities (24), it is tempting to speculate that the activities of p53 in DNA repair may be relevant for its interaction with CTG·CAG tracts. In this regard, it is important that instability associated with CTG·CAG tracts arises during normal DNA metabolic processes, such as replication and/or transcription (38). Considering that p53 binds to

CTG·CAG tracts in cells in the absence of DNA damage (this study), the possibility that maintenance of CTG·CAG tract stability may be one of the-stand by-functions of non-activated p53 must be considered (see the Introduction). Such an interpretation would be in accordance with the general idea that maintenance of genomic integrity may be one of the functions of "latent" p53 (8, 66). Considering that there is a causative relationship between human diseases and different types of DNA triplet repeats including CTG·CAG triplets (41), an obvious direction for future investigations would be to test whether p53 may interact with different types of DNA triplet repeats that are relevant for human pathologies known as triplet repeat diseases. A connection between p53 activity and triplet repeat diseases has been revealed in a recent study demonstrating that p53 single nucleotide polymorphism at codon 72 may be involved in the modulation of the age of onset of Huntington disease patients (67). Furthermore, there is also evidence for a direct mechanistic link between p53 and Huntington disease at the level of protein interaction; p53 and Huntington disease proteins interact with each other, and intriguingly, expansion of CAG triplets from the *huntingtin* gene impairs binding of mutant htt protein to p53 (68).

Our finding that p53 induces local distortions in mismatched CTG·$CTG_{T\cdot T}$ and CAG·$CAG_{A\cdot A}$ duplexes suggests that p53 may be involved in the maintenance of the CTG·CAG tracts stability. One possible scenario is that non-canonical DNA structures that contain T·T or A·A mismatches may be targeted by p53, which then binds to and destabilizes such DNA structures. Such scenario would be concordant with the notion that p53, by binding to DNA, can affect DNA topology as demonstrated previously. p53 can influence DNA topology by different means, of which bending, twisting, or denaturation are some that occur on double-stranded DNA (69), whereas the re-annealing activity of p53 prevails with ssDNA (70, 71). Our observation that p53 stimulates ssDNA binding of SSB suggests that destabilization of unusual and potentially recombinogenic structures formed by CTG·CAG tracts may involve the concerted action of p53 and single-stranded DNA-binding proteins, which can resolve secondary structures from ssDNA (72, 73). In such a scenario, the co-operative action of p53 and ssDNA-binding proteins may provide an efficient mechanism to prevent formation of potentially "dangerous" DNA structures in unstressed cells, even when the amounts of non-activated p53 are limited.

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