High-Affinity Binding of Tumor-Suppressor Protein p53 and HMGB1 to Hemicatenated DNA Loops†

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ABSTRACT: We have recently observed that chromatin architectural protein HMGB1 (previously reported to be involved in numerous biological processes such as DNA replication, recombination, repair, tumor growth, and metastasis) could bind with extremely high affinity (K_d < 1 pM) to a novel DNA structure that forms a DNA loop maintained at its base by a hemicatenane (hcDNA). The loop of hcDNA contains a track of repetitive sequences derived from CA-microsatellites. Here, we report using a gel-retardation assay that tumor-suppressor protein p53 can also bind to hcDNA. p53 is a crucial molecule protecting cells from malignant transformation by regulating cell-cycle progression, apoptosis, and DNA repair by activation or repression of transcription of its target genes by binding to specific p53 DNA-binding sites and/or certain types of DNA lesions or alternative DNA structures. The affinity of p53 for hcDNA (containing sequences with no resemblance to the p53 DNA consensus sequence) is >40-fold higher (K_d ∼ 0.5 nM) than that for its natural specific binding sites within its target genes (*Mdm2* promoter). Binding of p53 to hcDNA remains detectable in the presence of up to ∼4 orders of magnitude of mass excess of competitor linear DNA, suggesting a high specificity of the interaction. p53 displays a higher affinity for hcDNA than for DNA minicircles (lacking functional p53-specific binding sequence) with a size similar to that of the loop within the hcDNA, indicating that the extreme affinity of p53 for hcDNA is likely due to the binding of the protein to the hemicatenane. Although binding of p53 to hcDNA occurs in the absence of the nonspecific DNA-binding extreme carboxy-terminal regulatory domain (30-C, residues 363−393), the isolated 30-C domain (but *not* the sequence-specific p53 "core domain", residues 94−312) can also bind hcDNA. Only the full-length p53 can form stable ternary complexes with hcDNA and HMGB1. The possible biological relevance of p53 and HMGB1 binding to hemicatenanes is discussed.

The p53 protein is a sequence-specific transcription factor that protects cells from malignant transformation by regulating cell-cycle progression, apoptosis, and DNA repair following genotoxic stress and oncogene activation (reviewed in refs *1* and *2*). p53 exerts its function by activation or repression of transcription of its target genes (such as *p21*, *Mdm2*, *Bax*, and *GADD45*) by binding to specific DNA binding sites consisting of two-half-site decamers 5′-PuPuPuC(A/T)(T/A)GPyPyPy-3′ (where Pu represents purine and Py represents pyrimidine) linked by a 0−13 nucleotide spacer.

p53 protein contains 393 amino acids and consists of three main domains (Figure 1B): the N-terminal transactivation domain (amino acids 1−99); the central ("core") domain or DBD[1] (DNA-binding domain; amino acids 98−312), which binds to the DNA sequence both specifically and nonspecifically (reviewed in ref *2*); and the C-terminal domain (CTD; amino acids 323−393), which includes the tetramerization domain (amino acids 325−356) and the extreme C-terminal regulatory region (30-C; amino acids 363−393). p53 binds to DNA as a tetrameric complex, and the protein contains two DNA-binding sites. The p53 core domain binds to specific DNA sequences and is mutated in most human cancers. The 30-C region of p53 binds with a high affinity to ssDNA or distorted DNA structures (see below).

In "normal" unstressed cells, p53 is present at low levels or in a latent form, but various types of stress (such as oncogene activation, DNA damage, and hypoxia) can lead to the rapid induction of p53 activity. Modulation of p53 activity is in most cases associated with changes in the sequence-specific DNA binding of the p53 core domain that

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[1] Abbreviations: HMG, high-mobility group; dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; cccDNA, supercoiled DNA; hcDNA, hemicatenated DNA loops; WT, wild type; TAD, transactivation domain of p53; DBD, DNA-binding domain of p53; CTD, C-terminal domain of p53; 30-C, amino acid residues of the extreme C terminus of p53; GST, glutathione-*S*-transferase; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay.

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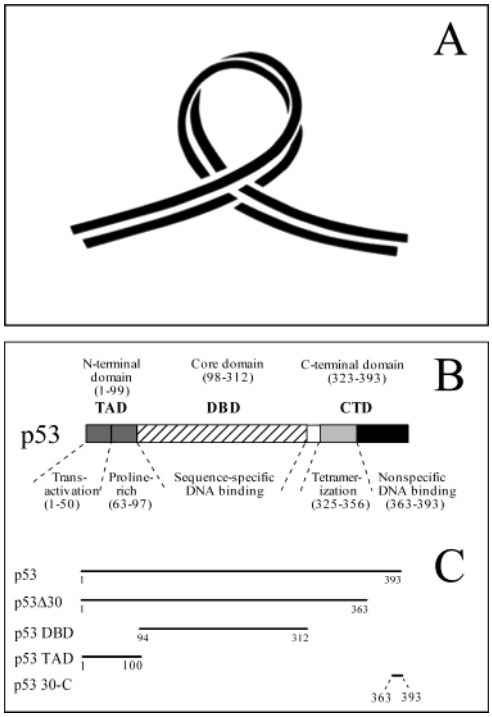

FIGURE 1: (A) Schematic drawing of hcDNA: a DNA loop (containing repetitive sequences from CA-microsatellites) and a hemicatenated DNA junction. (B) Domain structure of p53. TAD, transactivation domain; DBD, DNA binding domain; and CTD, C-terminal domain. (C) p53 and peptides used in EMSA experiments (numbers indicate positions of the amino acids in context with the full-length p53 protein). From top to bottom: p53, full length; p53Δ30, p53 peptide lacking 30 amino acids at the extreme terminus (30-C); p53 DBD, DNA binding or "core" domain; p53 TAD, transactivation domain; and p53 30-C, 30 amino acid residues of the extreme CTD.

include alterations in the regulation by the extreme CTD (reviewed in ref *2*). Functioning of p53 in DNA repair, recombination, and replication includes interactions of p53 with a host of proteins such as XPD, XPB, WRN, Rad51, RecA, RPA, and DNA polymerase α (*3*−*7*), as well as direct nonsequence-specific binding of the p53 CTD to intermediates of the above pathways such as ssDNA ends of dsDNA (*8*), Holliday junctions (cruciform structures), and DNA containing mismatched and bulged bases (*9*−*11*). The nonspecific DNA binding of p53 also occurs via the p53 core domain as demonstrated for binding to ssDNA or to linear dsDNA or cccDNA lacking the p53 DNA consensus sequence (*12*, *13*). All of these DNA lesions or alternative DNA structures occur as a result of DNA recombination, replication, and damage/repair. The DNA lesions need to be repaired before the cell-cycle progression to occur. It was also reported that binding of p53 to damaged DNA (such as single-stranded or cisplatin-modified DNA) results in the cleavage of the protein by formation of p53 fragments competent for sequence-specific DNA binding (*14*, *15*). It is possible that recognition and binding of p53 to these lesions serves to transactivate downstream genes involved in cell-cycle arrest/apoptosis or signal-repair pathways (*1*, *2*).

We have recently observed that chromatin architectural protein HMGB1 (previously reported to be involved in numerous biological processes such as DNA replication, recombination, repair, tumor growth, and metastasis; reviewed in refs *16* and *17*) could bind with extremely high affinity (K_d < 1 pM) to a novel DNA structure (hcDNA) that forms a DNA loop maintained at its base by a hemicatenane (*18*−*20*). In this paper, we demonstrate that recombinant human p53 can also bind to hcDNA and that the affinity of p53 for hcDNA (lacking sequences with any similarity to the p53 consensus sequence) is >40-fold higher than that for its natural specific DNA-binding sites within one of its target genes (*Mdm2* promoter). Although binding of p53 to hcDNA occurs in the absence of the nonspecific DNA-binding extreme carboxy-terminal regulatory domain (30-C), the isolated 30-C domain can also bind hcDNA, and the presence of the domain within p53 is required for the formation of stable ternary complexes of p53-hcDNA with HMGB1. Our results demonstrate that hcDNA (in addition to previously shown DNA minicircles containing the p53-specific binding sequences, ref *21*) constitute the highest affinity substrates known to date for p53 and HMGB1.

MATERIALS AND METHODS

Antibodies. Monoclonal antibodies to human p53 (DO-7, epitope 21−25 amino acid residues within the TAD of p53) were prepared as previously reported (*22*). Affinity-purified polyclonal anti-HMGB1 antibodies were generated by immunization of rabbits with synthetic HMG peptide corresponding to amino acids 166−181 of human HMGB1 (BD Biosciences Pharmingen). The concentration of both p53 and HMGB1 antibodies was 1 μg/μL.

Purification of HMGB1, p53, and Truncated Forms. HMGB1 protein (untagged) was isolated either from calf thymus or expressed in *Escherichia coli* using rat HMGB1 cDNA and extensively purified to near homogeneity by FPLC chromatography as previously described (*23*, *24*). GST-fused recombinant rat HMGB1 and its domains were expressed in *E. coli* and purified essentially as described in ref *24* but without removal of the GST moiety by thrombin digestion. Recombinant human WT p53 (full length) was expressed in baculovirus-infected Sf9 insect cells or in *E. coli* DH5α cells carrying pT7-7 (p53) plasmid as detailed in ref *25*. Cell lysates (from bacteria or insect cells) were centrifuged at 14 000 rpm for 30 min, and the supernatant was diluted 5-fold in a low-salt purification buffer (15% glycerol, 15 mM Hepes-KOH at pH 8.0, 0.04% Triton X-100, 5 mM DTT, 2 mM benzamidine, and 1 mM β-glycerolphosphate), filtered, and loaded onto a 5-mL Heparin-Sepharose column (Amersham Biosciences). The p53 protein was eluted by a KCl gradient (0−1 M), and the peak fractions that eluted between 0.5 and 0.6 M KCl were pooled together, dialyzed against a low-salt purification buffer for 12 h at 4 °C, and loaded onto an anion-exchange HQ column of the BioCad Sprint perfusion chromatography system (PerSeptive Biosystems, Inc.) as described in ref *25*. Segments of human p53 were expressed in *E. coli* either with the GST moiety (TAD, residues 1−112; 30-C, residues 362−393) or without any tag (full-length p53, residues 1−393; p53Δ30, residues 1−362; DBD, residues 96−312) and extensively purified by FPLC chromatography.

hcDNA. hcDNA was prepared and purified as previously described (*18*), with modifications indicated below. An 120-bp *Cla*I − *Eco*RI restriction fragment containing a 60-bp tract of poly(CA)·poly(TG) was labeled at their 5′ termini

by [γ-^{32}P]ATP. Approximately 1 μg of the DNA fragment was dissolved in 50 μL of 10 mM Tris-HCl at pH 7.5 and 1 mM EDTA and heat-denaturated at 100 °C for 2 min. The denaturated DNA fragment was quickly added to 190 μL of a reassociation solution (50 mM NaCl, 25 mM Tris-HCl at pH 7.5, 1 mM EDTA, and 1 mM DTT) containing ~7 μg of calf thymus HMGB1 protein. The renaturation proceeded at 37 °C for 30 min. The DNA−HMGB1 complexes were purified on nondenaturing 4% polyacrylamide gels in 0.5× TBE at 4 °C, followed by electroelution into 1 M NaCl, 10 mM Tris-HCl at pH 7.5, and 1 mM EDTA (Undirectional Electroeluter, IBI). The hcDNA−HMGB1 complexes were then deproteinized by chloroform extraction in 1% SDS, and the hcDNA was precipitated by ethanol in the presence of 0.045% linear polyacrylamide (*19*, *26*). Precipitated hcDNA was redissolved in 140 mM NaCl, 10 mM Tris-HCl at pH 7.5, and 1 mM EDTA and stored in small aliquots at −70 °C.

Preparation of DNA Minicircles. The DNA duplex of 66 bp containing an active p53-binding site (GADD45) was prepared by the annealing of oligonucleotides 1 (5′-CTAGCTGATATCGAATTCTCGAGCAGAACATGTCAAGCATGCTGGGCTCGAGAATTCCTGCAGCG-3′) and 2 (5′-CTAGCGCTGCAGGAATTCTCGAGCCCAGCATGCTTAGACATGTTCTGCTCGAGAATTCGATATCAG-3′). The DNA duplex of 66 bp containing a mutated p53-binding site (mutGADD45) was prepared by the annealing of oligonucleotides 3 (5′-CTAGCTGATATCGAATTCTCGAGCAGAAAATTTCTAAGAATTCTGGGCTCGAGAATTCCTGCAGCG-3′) and 4 (5′-CTAGCGCTGCAGGAATTCTCGAGCCCAGAATTCTTAGAAATTTTCTGCTCGAGAATTCGATATCAG-3′). Sequences 1−4 were derived from ref *21* with some modifications as shown above. All oligonucleotides were highly purified by denaturing polyacrylamide gel electrophoresis and subsequently labeled at their 5′ termini by [γ-^{32}P]ATP. T4 DNA ligase-mediated circularization was carried out as described earlier (*24*) with the following modifications. DNA ligations were carried out at a DNA concentration of ~10 nM (66-bp DNA duplexes) and recombinant rat HMGB1 protein, at 0.5 μM. Ligations were allowed to proceed for 30 min at 30 °C using 0.1 unit/μL of T4 DNA ligase (Promega). The ligation products were deproteinized, followed by their resolution by electrophoresis on a 5% polyacrylamide gel in 0.5× TBE as detailed for electrophoretic mobility shift assay (EMSA) experiments. DNA minicircles were detected by autoradiography, followed by electroelution from the polyacrylamide gel, and ethanol precipitation as indicated for the preparation of hcDNA. Purified DNA minicircles were finally dissolved in a 1× EMSA buffer and stored at −70 °C.

EMSA. EMSA was carried out in a total volume of 25 L in a 1× EMSA buffer containing 50 mM NaCl, 25 mM Tris-HCl at pH 7.5, 1 mM EDTA, 1 mM DTT, 100 μg/mL acetylated BSA, and 3% glycerol. Reaction mixtures containing ^{32}P-labeled hcDNA and added proteins were typically preincubated on ice (unless otherwise indicated) for 30 min. Some EMSA experiments were carried out in the presence of competitor DNAs, typically by the addition of the competitor DNA to the preincubated protein−hcDNA mixtures, followed by incubation for 20 min on ice (inclusion of competitor DNA directly with labeled hcDNA prior to the addition of proteins had no significant impact on the EMSA results). Supershift experiments were carried out by the addition of 1 μL each of the indicated antibodies to the preincubated protein−hcDNA complexes (in the presence of a 100-fold mass excess of linearized pBluescript DNA (Stratagene) over labeled hcDNA to avoid nonspecific binding of the antibodies to hcDNA), followed by incubation for 20 min on ice. Reaction mixtures were finally loaded (without the addition of dye) on prerun and precooled 5% polyacrylamide gels in 0.5× TBE containing 0.05% Nonidet NP-40 and 1 mM EDTA at 250 V (4 °C) for 2−4 h. After electrophoresis, the gels were dried and the DNA was visualized and quantified on PhosphorImager Storm (Molecular Dynamics) using ImageQuant 4.1 for data processing.

Dissociation Constants. The K_d was estimated from the gel-mobility shift assays (using a fixed concentration of the labeled hcDNA and varying amounts of the proteins) as the protein concentration at the point in the titration where half of the input DNA had been complexed with protein (i.e., protein concentration at which 50% of the DNA was shifted; refs *24* and *27*).

GST Pull-Down Assay. The full-length p53 was synthesized in vitro from the corresponding cDNA (cloned into the mammalian expression vector pcDNA3; Invitrogen) in the presence of L-[^{35}S]methionine (Amersham Cat AG1094; >37 TBq/mmol) using the TNT T7 Polymerase Quick Coupled Transcription/Translation Reticulocyte Lysate System (Promega). The lysate with labeled proteins was precleared with glutathionine-Sepharose beads. The precleared lysate was mixed with GST−HMGB1 or truncated forms of HMGB1 fused with GST, followed by rotation for at least 2 h at 4 °C in a PD buffer [50 mM Tris-HCl at pH 8.0, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM DTT, 1 mM PMSF, and protease inhibitors cocktail (Sigma)], as previously reported (*28*). The glutathionine-Sepharose glutathione beads were then added, and the samples were further rotated for at least 1 h at 4 °C. The beads were washed 5 times with the PD buffer, mixed with 40 μL of 4× concentrated Laemmli buffer, and subsequently boiled for 5 min. The proteins bound to glutathionine-Sepharose were then resolved by electrophoresis on a SDS/10% polyacrylamide gel. After electrophoresis, the gel was stained in Coomassie blue R-250, destained, and soaked in an Amplify solution (Amersham Pharmacia Biotech) for 30 min. The dried gel was finally exposed to X-ray films at −70 °C.

RESULTS AND DISCUSSION

High-Affinity Binding of Tumor-Suppressor Protein p53 to hcDNA. Recently, a novel DNA structure was reported, hemicatenated DNA loops (hcDNA). hcDNA is formed by reassociation of the strands of a DNA fragment containing a track of repetitive poly(CA)·poly(TG) sequences from CA-microsatellites (*18*). The repetitive sequence is arranged in hcDNA in a DNA loop at the base of which the two DNA duplexes cross, with one of the strands of one duplex passing between the strands of the other duplex (refs *18* and *29* and also Figure 1A). hcDNA is specifically recognized by chromatin architectural protein HMGB1 (refs *19* and *20* and also Figure 2A), with the affinity 3−4 orders of magnitude higher (K_d < 1 pM) than that for the highest affinity DNA structures for HMGB1 so far reported, the synthetic four-way (Holliday) junctions and DNA minicircles (*30*, *31*).

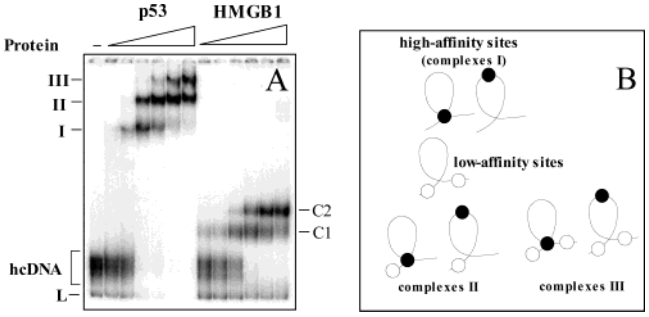

FIGURE 2: (A) Binding of p53 and HMGB1 to hcDNA. Increasing amounts of the p53 protein were added to ^{32}P-labeled hcDNA (37 pM, ~70 pg). The molar ratios of p53 tetramer/hcDNA were (left to right) 1:1 (p53 at 0.14 nM), 2:1 (p53 at 0.28 nM), 4:1 (p53 at 0.56 nM), 6:1 (p53 at 0.84 nM), 8:1 (p53 at 1.1 nM), and 10:1 (p53 at 1.4 nM). HMGB1 was at 16, 32, 80, 160, 320, and 800 pM. p53−hcDNA complexes are marked I−III, whereas HMGB1−hcDNA complexes are denoted as C1 and C2. Binding experiments were carried out in the absence of competitor DNA. Protein−DNA complexes were resolved by electrophoresis on 5% polyacrylamide gels (EMSA) and visualized by autoradiography as detailed in the Materials and Methods. (B) Putative binding sites of p53 within hcDNA. Black and white circles indicate high- and low-affinity sites on hcDNA for p53, respectively.

To identify hcDNA binding protein(s) other than HMGB1, we have searched for proteins exhibiting similarities with HMGB1 in binding to DNA. There is growing evidence that tumor-suppressor protein p53 shares with HMGB1 a similar preference for binding to alternative DNA structures such as extra-base bulges, UV-irradiated DNA, DNA modified with anticancer drug cisplatin, three-stranded DNA structures, Holliday junctions, and DNA minicircles (*9*, *11*, *21*, *31*−*34*; reviewed in refs *2* and *16*). EMSA was therefore employed to study whether p53 could interact with hcDNA. As shown in Figure 2, recombinant human p53 (isolated from baculovirus-infected insect cells, denoted as $p53_i$) could bind ^{32}P-labeled hcDNA by forming up to 3 bands of lower mobility (complexes I−III in Figure 2A), likely reflecting an existence of different p53-binding sites on hcDNA (Figure 2B; see also below).

Specificity of p53 binding to hcDNA was challenged in EMSA experiments with varying amounts of different types of unlabeled competitor DNAs. As shown in Figure 3A, addition of increasing amounts of competitor DNA to preformed complexes of p53 with ^{32}P-labeled hcDNA (complexes I−III) resulted in a preferential disappearance of complexes II and III. However, complex I was detectable up to 4 orders of magnitude mass excess of unlabeled nonspecific dsDNA (salmon sperm dsDNA was a slightly more efficient competitor than linearized plasmid, possibly because of the presence of DNA sequences for which p53 could bind with increased affinity. As expected, linearized plasmids containing sequences derived from *Bax* or *Mdm2* gene promoters were better competitors than the corresponding empty linearized vector). From these experiments, we propose the existence of high-affinity (DNA loop and hemicatenane; complex I) and low-affinity (linear segments outside the DNA loop) sites of hcDNA for p53 (see Figure 2B). Thus, complexes II and III most likely originate from a simultaneous binding of p53 to high- and low-affinity sites within the same hcDNA molecule (see below).

We have noticed that nonspecific ssDNA or cccDNA was a ~3−5-fold more efficient competitor of p53 binding to hcDNA than the corresponding linear DNA (Figure 3A). This finding was in agreement with previous reports indicating that p53 exhibited a higher affinity to ssDNA or cccDNA than to the corresponding linear dsDNA lacking the p53 consensus binding sequence (*2*, *35*, *36*). Collectively, the above competition experiments indicated that binding of p53 to hcDNA (formation of complex I) was highly specific.

We have estimated the K_d for $p53_i$ binding to hcDNA from EMSA by using two fixed concentrations of the labeled hcDNA and varying amounts of the proteins as the protein concentration at the point in the titration where half of the input DNA had been complexed with the protein (i.e., protein concentration at which 50% of the DNA was shifted) and calculated using the formula $[P] = K_d + [D]/2$, where [P] and [D] are the total protein and DNA concentrations, respectively. The K_d for specific p53 binding to hcDNA (formation of complex I in Figure 2B) was ~0.5 nM (approximately four tetramers of p53 per one molecule of hcDNA), which was a >40-fold higher affinity than that for

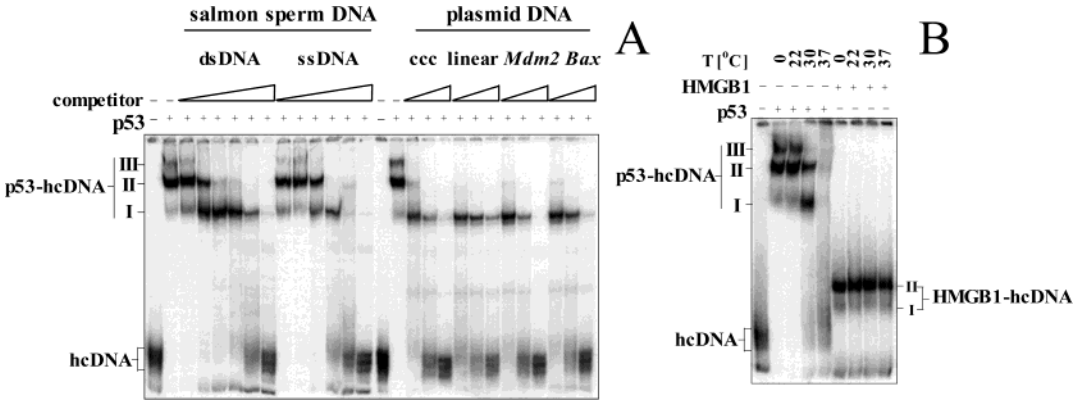

FIGURE 3: (A) Binding of p53 to hcDNA is highly specific as revealed by competition experiments. A fixed amount of p53 (3.7 nM) was incubated with ^{32}P-labeled hcDNA (37 pM), and the resulting complexes were challenged by increasing amounts of various competitor DNAs [dsDNA, double-stranded salmon sperm DNA; ssDNA, single-stranded salmon sperm DNA (prepared by heat-denaturation of dsDNA, followed by rapid cooling); cccDNA, supercoiled pBluescript plasmid DNA; linear DNA, linearized pBluescript plasmid DNA; *Mdm2*, linearized pBluescript plasmid DNA containing p53-binding site from the human *Mdm2* promoter; and *Bax*, linearized pBluescript plasmid DNA containing p53-binding site from the *Bax* promoter]. Mass-fold excess of salmon sperm competitor DNA over hcDNA was 2, 10, 50, 100, 1000, and 10 000 (left to right). Mass-fold excess of plasmid competitor DNA over hcDNA was 100, 1000, and 10 000 (left to right). (B) Effect of temperature on p53 and HMGB1 binding to hcDNA. p53 (3.7 nM) and HMGB1 (0.87 nM) were incubated with labeled hcDNA (37 pM) at indicated temperatures, and the protein−DNA complexes were then analyzed by EMSA as in Figure 2.

the binding of p53 to its natural specific binding sites within the *Mdm2* promoter (*37*).

We have found that binding of p53 to hcDNA was temperature-sensitive. Whereas p53 binding to hcDNA was not affected between 0 and 22 °C, incubation of p53 with hcDNA at 30 °C resulted in the reduced formation of low-affinity binding complexes II and III, with no binding observed at 37 °C (Figure 3B). Impaired binding of p53 to hcDNA at 37 °C is reminiscent of the effect of the mutation of p53 on binding of the protein to specific DNA-binding sites at this temperature (*38*). On the other hand, binding of HMGB1 to hcDNA was not affected within the above temperature range studied (Figure 3B).

Main Determinant of High-Affinity Binding of p53 to hcDNA Is the hcDNA. p53, like HMGB1 (*31*, *39*), has previously been reported to bind with high affinity to synthetic four-way (Holliday) junctions (*9*) and DNA minicircles lacking the p53-specific binding sequences (*21*). Formation of the high-affinity complex I in Figure 2A may therefore correspond to the binding of p53 either to the DNA loop and/or the base of the DNA loop, where the two DNA duplexes cross in hcDNA (hemicatenane, Figure 2B), similarly to the formation of *structure*-specific complex I upon binding of p53 or HMGB1 to the *DNA crossover* of the four-way junctions (*9*, *39*, *40*). On the other hand, complexes II and III in Figure 2A may arise from the binding of additional one or two molecules, respectively, to the linear arms of hcDNA having p53 already bound at high-affinity sites (Figure 2B), similarly to the formation of complex II or higher by binding of additional p53 or HMGB1 molecules to four-way junction arms within the *structure*-specific complex I (*9*, *39*, *40*). A possibility of a simultaneous binding of two or more p53 molecules to high-affinity sites within hcDNA (both to the hemicatenane and the loop) is not supported from results of our competition experiments (Figure 3A) and is also unlikely due to sterical restrains.

Our next experiments were aimed at finding out whether the main determinant of the high preference of p53 for hcDNA (formation of complex I) is the hemicatenane or the loop. One possible approach to tackle this problem was to introduce a restriction site in the center of the DNA loop of hcDNA and to compare the binding of p53 to cut hcDNA (only hemicatenane) with that of uncut hcDNA (hemicatenane plus the DNA loop). Because we have not succeeded in cleaving the introduced restriction sites within the loop by corresponding restriction enzymes (possibly because of impaired accessibility of the restriction nuclease recognition sequences within the DNA loop), we have compared the affinity of p53 for hcDNA with that for DNA minicircles (lacking functional p53-specific binding sequence), a size similar to that of the DNA loop within the hcDNA. DNA minicircles were prepared by ligase-mediated cyclization of 66-bp DNA duplex in the presence of HMGB1 (*21*, *24*). Purified DNA minicircles were then mixed with hcDNA and titrated with p53. As seen in Figure 4A, p53 could bind to hcDNA with a clear preference over the minicircles, suggesting that the extreme affinity of p53 for hcDNA is likely due to the binding of the protein to the hemicatenane rather than to the loop of the hcDNA. Interestingly, the affinity of p53 for hcDNA was very similar to that of p53 for linear DNA or DNA minicircles containing the WT p53-specific binding sequence (Figure 4B). However, we have noticed

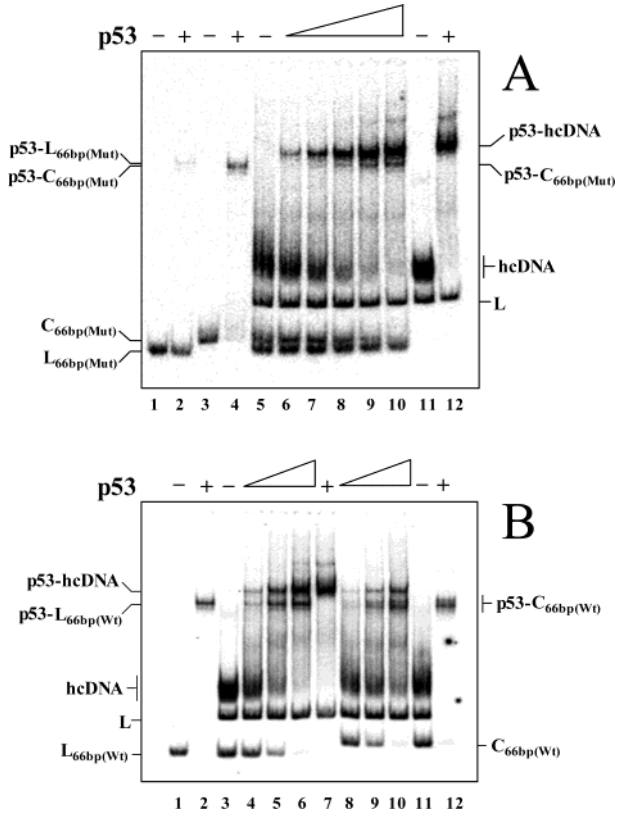

FIGURE 4: (A) DNA hemicatenane is the main determinant of high-affinity binding of p53 to hcDNA. Radioactively labeled linear and circular DNAs (66 bp) lacking functional p53-binding sites were mixed with ^{32}P-labeled hcDNA (37 pM) and titrated with increasing amounts of p53 (2.5, 5, 10, 20, and 30 nM, lanes 6−10, respectively). Individual forms of different DNA were mixed with p53 at 30 nM (lanes 2, 4, and 12). L_{66bp}(Mut), 66-bp linear DNA duplex containing the mutated GADD45 p53-binding site; C_{66bp}(Mut), 66-bp circular DNA duplex containing the mutated GADD45 p53-binding site; and L, 120-bp linear DNA used to prepare hcDNA. (B) p53 binds with similar affinity to DNA hemicatenane and DNA minicircles containg p53-binding sites. Linear [L_{66bp}(WT)] or circular [C_{66bp}(WT)] DNA of 66 bp containing the functional p53-binding GADD45 sites were mixed with hcDNA (37 pM) and titrated with increasing amounts of p53 (2.5, 10, and 20 nM), lanes 4−6 or 8−10, respectively). Individual forms of different DNA were mixed with p53 at 30 nM (A, lanes 2, 4, and 12 or B, lanes 2, 7, and 12). All binding reactions in A and B contained nonspecific unlabeled competitor (linearized plasmid pBluescript) DNA at 100-fold mass excess (~7 ng) over the hcDNA. The protein−DNA complexes were analyzed by EMSA as indicated in Figure 2.

that addition of p53 to hcDNA in the presence of linear DNA with p53-binding sequences resulted in a visibly higher formation of p53−hcDNA complexes as compared to EMSA experiments, where p53 was added to hcDNA and DNA minicircles with p53-specific binding sequences (Figure 4B). This finding is in agreement with previous reports indicating that p53 has much higher affinity to DNA minicircles containing the p53-specific binding sequence than to the corresponding specific linear DNA sequences (*21*). Thus, hcDNA (this paper) and DNA minicircles containing the p53-specific binding sequence (*21*) constitute the highest affinity substrates known to date for p53.

Extreme 30 Amino Acids of the C Terminus of p53 Bind with High Affinity to hcDNA. We have compared p53 expressed in insect cells (using the baculovirus expression system, which supports the majority of posttranslational modifications required for proper functioning of the protein,

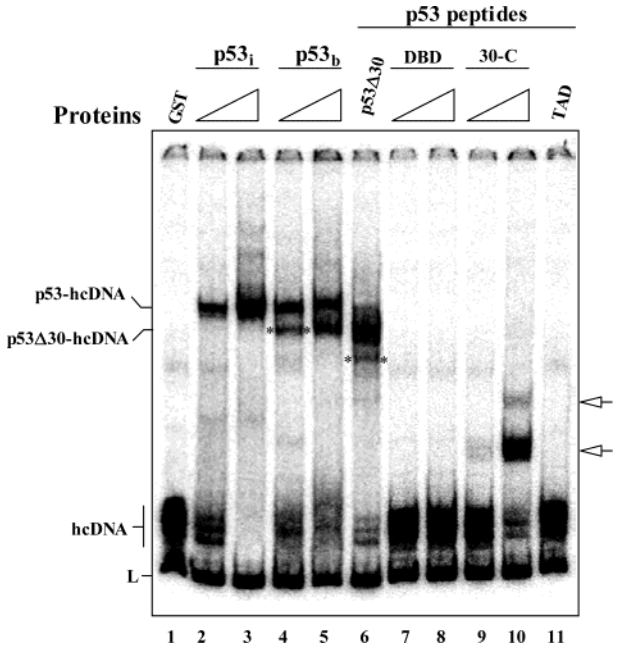

FIGURE 5: CTD is involved in the binding to hcDNA. Radioactively labeled hcDNA (37 pM) was mixed with full-length p53 expressed either in baculovirus-infected insects cells ($p53_i$) or in bacterial cells ($p53_b$), (5 nM, lanes 2 and 4 or 30 nM, lanes 3 and 5). All truncated forms of p53 were expressed in bacterial cells and the purified p53 peptides were mixed with ^{32}P-labeled hcDNA and analyzed by EMSA. p53Δ30 (30 nM, lane 6); DBD (5 and 30 nM, lanes 7 and 8, respectively); GST−30-C (3 and 30 nM, lanes 9 and 10, respectively); GST−TAD (30 nM, lane 11); GST (30 nM, lane 1). Asterisks mark position of complexes of hcDNA with degradation products of p53. Void arrows indicate position of complexes of hcDNA with p53 30-C. All binding reactions contained nonspecific unlabeled competitor (linearized plasmid) DNA at 100-fold mass excess (~7 ng) over the hcDNA. The protein−DNA complexes were analyzed by EMSA as indicated in Figure 2.

referred as to "active" p53 or $p53_i$) with p53 expressed in *E. coli* (lacking posttranslational modifications, referred as to "latent" p53 or $p53_b$) for their abilities to bind hcDNA. First, we verified using EMSA that both $p53_i$ and $p53_b$, as well as truncated p53 proteins, expressed in *E. coli* (p53Δ30 and p53 DBD) could bind a 40-bp DNA duplex containing the specific p53-binding sequence from the *Mdm2* gene promoter (results not shown). In agreement with the expected latency of $p53_b$, the specific binding of $p53_b$ was much weaker than that of $p53_i$.

As shown in Figure 5 (lanes 2−5), both $p53_b$ and $p53_i$ could bind hcDNA in the presence of 100-fold mass excess of linear competitor DNA, suggesting that postsynthetic modifications of p53 may not be critical for specific binding of the protein to hcDNA. We have noticed that the addition of purified recombinant $p53_b$ (and also p53Δ30) to hcDNA resulted in the appearance of additional (faster) migration bands (marked by asterisks, lanes 4−6 in Figure 5). This is likely due to the presence of small amounts of degradation products in $p53_b$ and p53Δ30 preparations before addition to hcDNA because no apparent degradation of $p53_b$ was visible by Coomassie blue staining of polyacrylamide gels upon binding of repurified $p53_b$ to hcDNA. We have also ruled out a possibility that binding of $p53_b$ to hcDNA had triggered autoproteolysis of the protein (data not shown). Thus, unlike previously reported N- or C-terminal cleavage of p53 upon binding to single-stranded or cisplatin-damaged DNA (15), binding of p53 to hcDNA does not promote degradation of the protein.

p53 contains two regions involved in DNA binding (Figure 1B): (i) the "core" domain or DBD, responsible for both sequence-specific binding as well as nonsequence-specific binding to internal parts of dsDNA and ssDNA as well as to alternative (non-B-type) DNA structures, and (ii) 30-C (30 amino acids of the extreme C terminus of p53), involved in nonsequence-specific binding to DNA lesions (such as irradiated DNA, cisplatin-modified DNA, and the single-strand ends of DNA), cccDNA, and four-way (Holliday) DNA junctions (*8*, *12*, *13*, *35*, *41*−*44*). To delineate the region of p53 responsible for binding to hcDNA, EMSA experiments were carried out with $p53_b$ and its truncated forms: p53Δ30 (p53 lacking the 30-C domain), p53 DBD, GST-p53 30-C (the isolated 30-C domain of p53 fused with GST), and GST-p53 TAD (the isolated transactivation domain fused with GST).

As shown in Figure 5, both the full-length p53 and p53Δ30 could bind hcDNA, suggesting that the 30-C domain may not be involved in hcDNA binding. However, the isolated extreme 30 amino acids carboxy-terminal regulatory region (fused with GST) did bind hcDNA even in the presence of 100-fold mass excess of the linear (unlabeled) competitor. The importance of the 30-C domain for p53 binding to hcDNA became further evident from its requirement for the formation of ternary complexes of p53−hcDNA with the HMGB1 protein (see below, Figure 6). Interestingly, no binding of the isolated core domain of p53 to hcDNA was detected within the protein/DNA molar range studied (up to >50-fold molar protein excess relative to hcDNA; lanes 7 and 8 of Figure 5), and as expected, no binding to hcDNA was detected with the p53 TAD peptide (lane 11 of Figure 5). All of these results strongly indicated that the 30-C domain as well as a part of CTD region between the core domain and the beginning of the 30-C domain are likely engaged in high-affinity binding of p53 to hcDNA.

p53 and HMGB1 Can Simultaneously Bind hcDNA. Although both p53 and HMGB1 proteins could bind separately to hcDNA (Figure 2), the affinity of HMGB1 for hcDNA was ~3-orders of magnitude higher than that of p53 (K_d for the specific binding of HMGB1 or p53 to hcDNA was <1 pM or ~0.5 nM, respectively). We have therefore asked whether HMGB1 could displace p53 from binding to hcDNA. In the absence of any competitor DNA, a gradual retardation of complexes I and II was observed upon addition of increasing amounts of HMGB1 to p53−hcDNA complexes (lanes 2−5 of Figure 6A). Similarly, addition of p53 to HMGB1−hcDNA complexes brought about a complete disappearance of the HMGB1−hcDNA complex leading to formation of complexes I and II (lanes 7−9 of Figure 6A; the apparent lack of retardation of complex I in lanes 8−9 is due to the faster transition of complex I into complexes II−III relative to lanes 3−5). These results were likely indicative of the formation of ternary p53−HMGB1−hcDNA complexes. To conclusively verify the existence of ternary HMGB1−p53−hcDNA complexes, similar experiments were carried out in the presence of 100-fold mass excess of competitor (unlabeled) linear DNA. Addition of HMGB1 (HMGB1 expressed in *E. coli* or native HMGB1, isolated from calf thymus, were equally efficient) to preformed p53−hcDNA complexes resulted in the formation of HMGB1−

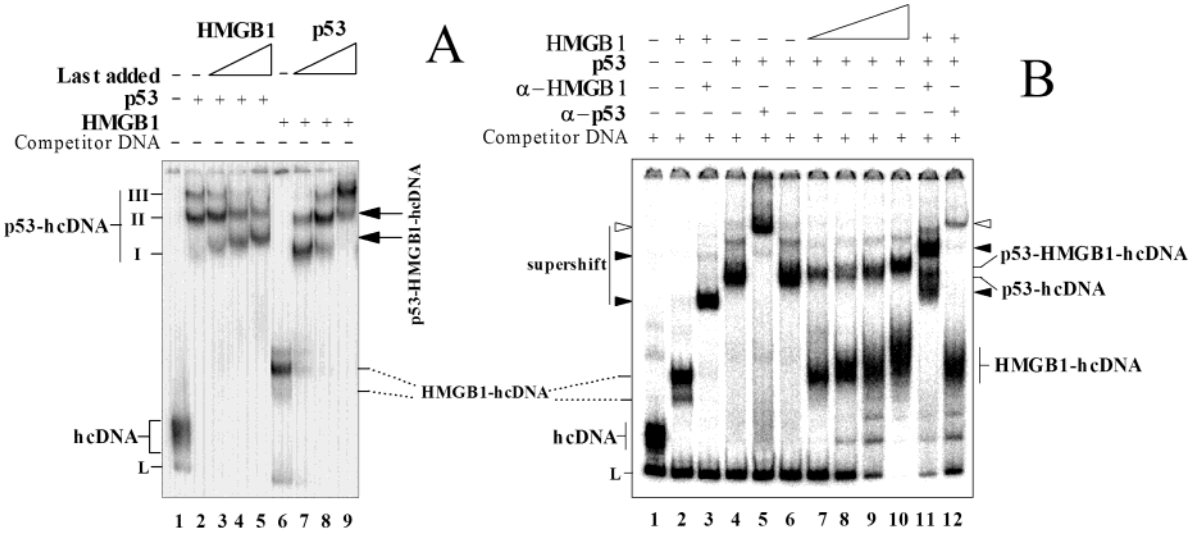

FIGURE 6: Formation of ternary p53−HMGB1−hcDNA complexes. (A) Binding experiments in the absence of competitor DNA. Either a fixed amount of p53 (7 nM) was preincubated with hcDNA (37 pM, lane 2) and the resulting complexes were titrated with increasing amounts of HMGB1 (7, 70, and 700 nM, lanes 3−5), or a fixed amount of HMGB1 (0.87 nM) was preincubated with hcDNA (37 pM, lane 6) and then the resulting complexes were titrated with increasing amounts of p53 (7, 17, and 70 nM, lanes 7−9). (B) Binding experiments in the presence of nonspecific competitor DNA (unlabeled linearized plasmid) at 100-fold mass excess (∼7 ng) over the hcDNA. A fixed amount of p53 (30 nM) was preincubated with hcDNA (37 pM, lanes 4 and 6), and the resulting complexes were titrated with increasing amounts of HMGB1 (3, 30, 150, and 450 nM, lanes 7−10). Black arrowheads indicate the positions of the complexes containing HMGB1 (HMGB1−hcDNA, lane 2 or HMGB1−p53−hcDNA, lanes 7−10) that were supershifted by polyclonal antibodies (1 μL) against HMGB1 (lanes 3 and 11, respectively). Void arrowheads indicate the positions of the complexes containing p53 (p53−hcDNA, lane 4 or p53−HMGB1−hcDNA, lanes 7−10) that were supershifted by monoclonal antibodies against p53 (D0−7), lanes 5 and 12, respectively. The protein−DNA complexes were analyzed by EMSA as indicated in Figure 2.

hcDNA complexes (lanes 7−10 of Figure 6B), presumably because of the partial displacement of p53 from binding to hcDNA because no free hcDNA had been present in the initial p53−hcDNA complexes (lanes 4 and 6 of Figure 6B). However, the intensity and mobility of the complex I (p53−hcDNA) was visibly reduced upon addition of increasing amounts of HMGB1 (lanes 7−10 of Figure 6B). The presence of both p53 and HMGB1 within the retarded complex I was verified by the supershift of the latter complex with specific antibodies against HMGB1 or p53 (lanes 11 and 12 of Figure 6B, respectively; a partial supershift of the retarded complex I in lane 11, the ternary complex, is likely due to the presence of high amounts of HMGB1 relative to the amount of the added HMGB1 antibody. A small magnitude of antibody shift of the HMGB1−p53−hcDNA complex relative to the complex p53−hcDNA may be a reflection of different molecular masses of both proteins and/or changes in the conformation of the supershifted complexes affecting their electrophoretic mobilities). Although both p53Δ30 and isolated p53 30-C peptides could interact with hcDNA (Figure 5), only traces of ternary p53Δ30−HMGB1−hcDNA complexes and no ternary p53 30C−HMGB1−hcDNA complex could be detected upon addition of HMGB1 to the complexes containing hcDNA and truncated p53 (Figure 7). The latter results gave evidence that the formation of stable ternary p53−HMGB1−hcDNA complexes requires the intact (full-length) p53 protein.

Formation of ternary HMGB1−p53−hcDNA complex I may be in principle a consequence of direct protein−protein interactions of free p53 (displaced from the complex by HMGB1) with DNA-bound HMGB1 and/or DNA binding of HMGB1 to the p53−hcDNA complex (e.g., via other binding sites on hcDNA). We favor the hypothesis that the formation of stable p53−HMGB1−hcDNA complexes occurs via protein−protein interactions, most likely via the 30-C domain, and also via other regions of the CTD. The existence of physical interactions of HMGB1 with p53 in free solution (unaffected by the presence of DNA) have previously been reported by several groups (*28*, *45*, *46*), and the binding region of p53 with HMGB1 was mapped within the 30-C domain (amino acid residues 363−376; ref *46*). Direct cross-linking experiments are needed to conclusively decipher the nature of interactions within the ternary p53−HMGB1−hcDNA complex.

To understand whether both HMGB1 domains, A and B, could interact with p53 (which was the case of another member of the p53 family, p73; ref *28*), we have carried out a pull-down assay with lysates containing in vitro synthesized ^{35}S-labeled p53. As shown in Figure 8, the full-length HMGB1 as well as both individual HMGB1 domains, A (residues 1−84) and B (residues 85−180, designated in Figure 8A as B7), could bind p53. We believe that the inability of previous authors to detect any binding of the HMGB1 domain B to p53 (*46*, *47*) is most likely to be explained by the absence of the extended N terminus of the domain B (residues ^{85}TKKKFKD^{91}), as well as because of the attachment of the acidic C terminus to the domain B peptides (residues 99−215) used by the authors cited above. The extended N terminus of the domain B has previously been shown to be essential both for DNA binding (*24*, *48*) and for the interaction of the HMGB1 domain B with p73 (*28*). In accordance with this suggestion, we were not able to detect any binding of the short HMGB1 domain B (residues 92−180, designated as B in Figure 8A) to p53 using the pull-down assay. In addition, the presence of the acidic C terminus within the HMGB1 domain B peptides used in refs *42* and *44* most likely resulted in the loss of secondary and tertiary structures and alternation of the

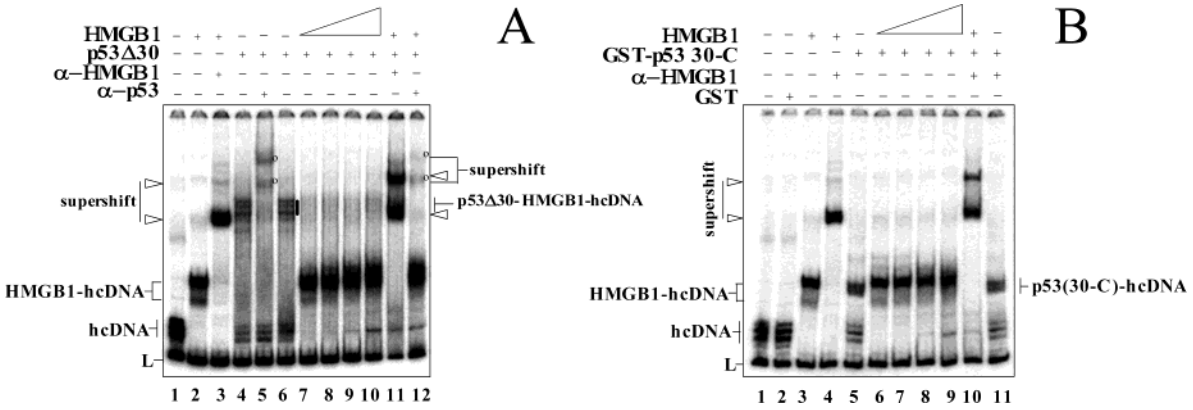

FIGURE 7: Full-length p53 is required for stable ternary p53−HMGB1−hcDNA complexes. (A) Binding experiments with p53Δ30. A fixed amount of p53Δ30 (6 nM) was preincubated with hcDNA (37 pM, lanes 4 and 6) and the resulting complexes were titrated with increasing amounts of HMGB1 (3, 10, 30, and 150 nM, lanes 7−10 or 150 nM, lanes 11−12). (B) Binding experiments with p53 30-C. A fixed amount of GST−p53 30-C (30 nM) was preincubated with hcDNA (37 pM, lane 5), and the resulting complexes were titrated with increasing amounts of HMGB1 (3, 10, 30, and 150 nM, lanes 6−9 or 30 nM, lanes 10−11). Void arrowheads indicate the positions of HMGB1−hcDNA complexes supershifted by polyclonal antibodies against HMGB1 (lanes 3 and 11 in A and lane 4 in B). The vertical line in lane 6 (A) indicates the position of the p53Δ30−hcDNA complex. Open circles indicate the positions of supershifted p53Δ30−hcDNA (lane 5, A) or p53Δ30−HMGB1−hcDNA (lanes 12, A) complexes by monoclonal antibodies against p53 (D0−7). Complexes of p53Δ30−hcDNA or p53−30C−hcDNA containing HMGB1 were probed with polyclonal antibodies against HMGB1 as indicated in lane 11 (A) and lane 10 (B). All experiments in A and B were carried out in the presence of nonspecific competitor DNA (unlabeled linearized plasmid pBluescript) at 100-fold mass excess (∼7 ng) over the hcDNA. The protein−DNA complexes were analyzed by EMSA as indicated in Figure 2.

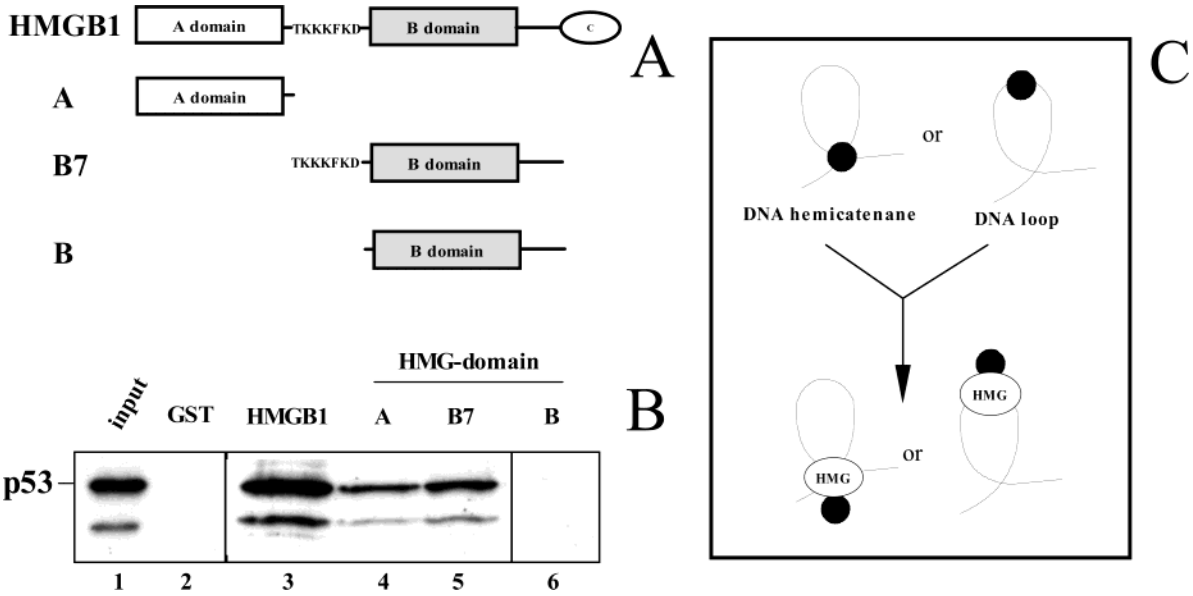

FIGURE 8: (A) Schematic structure of HMGB1 protein and design of HMGB1 domains used for the pull-down assay. (B) Identification of HMGB1 domains involved in binding to p53 ("pull-down" assay). The reticulocyte lysate containing ^{35}S-labeled p53 was mixed with GST (negative control; 1 μg), GST−HMGB1 (6 μg), or truncated forms of HMGB1 fused with GST (A, B, or B7; ∼2 g each), rotated with glutathionine-Sepharose beads, followed by extensive washing of the beads. The beads were then boiled in a SDS-containing electrophoretic sample buffer, and the released proteins were resolved on a SDS/10% polyacrylamide gel as detailed in the Materials and Methods. Input, 1/100 of the total amount of labeled p53 used for the "pull-down" assay. (C) Putative high-affinity binding sites of p53 (circles) to hcDNA and the proposed formation of ternary complexes of hcDNA and p53 via protein−protein interactions of p53 (circles) with DNA-bound HMGB1 (ellipse).

binding properties of the HMG box as it had been reported previously (*49*).

Biological Relevance of p53 and HMGB1 Binding to the hcDNA Loop. p53 is a key molecule in response to various cellular stress such as DNA damage, subsequently inducing cell-growth arrest, or apoptosis by transcriptional regulation of a number of downstream genes by sequence-specific DNA binding and thereby preventing tumorogenesis (*42*, *50*).

p53 can bind via its CTD to the ssDNA ends of dsDNA (*8*), Holliday junctions (cruciform structures), and DNA containing mismatched and bulged bases (*9*−*11*), as well as nonspecifically via the core domain to ssDNA or internal linear dsDNA or cccDNA lacking the p53 DNA consensus sequence (*8*, *12*, *13*). DNA lesions and/or non-B-type DNA structures may occur in vivo not only as a consequence of DNA damage, but also they may originate in the course of recombination, replication, and DNA repair. Binding of p53 to damaged (single-stranded or cisplatin-modified) DNA results in the cleavage of the protein by formation of p53 fragments competent for sequence-specific DNA binding (*14*, *15*). The fact that no autoproteolysis of p53 was observed upon binding to hcDNA (this paper) may suggest that

hemicatenanes are recognized by p53 as natural sites on DNA, rather than damaged DNA. The observed high affinity of p53 for hcDNA raises immediately the question of the existence and of the possible role of DNA hemicatenanes in the cell. Little is known about hemicatenanes, because they have been much less-studied than the Holliday junction, the other kind of DNA junction between dsDNA molecules that are specifically recognized and bound by p53 (*9*, *11*, *32*). The lack of knowledge of DNA hemicatenanes does not reflect a lack of interest but is merely due to technical reasons, because four-way junctions can be easily prepared in vitro from synthetic oligonucleotides, whereas hemicatenanes are much more difficult to obtain. Despite that difficulty, the possibility for dsDNA molecules to be associated in hemicatenanes has been mentioned by many authors and hemicatenanes have been studied both in vitro [as alternative DNA conformations (*18*) or as substrates for recombination enzymes (*51*−*54*)] and in vivo [mainly in studies dealing with recombination (*55*−*59*) or replication (*60*−*64*) in living cells or in *Xenopus* egg extracts]. In particular, many of these studies done by bidimensional electrophoresis in neutral/neutral agarose gels showed clearly the existence of junctions between DNA molecules. It has often been difficult to determine unambiguously whether these junctions were Holliday junctions, double Holliday junctions, or hemicatenanes. However, some articles suggest extremely strongly that hemicatenanes can be found under physiological conditions (*60*−*62*, *64*). In vitro techniques used presently for hemicatenane preparation require repetitive sequences, and only hemicatenanes containing the CA/TG sequence have been studied so far. However, in theory, it should be possible to have stable hemicatenanes between any two DNA fragments, and we are currently trying to develop techniques for the preparation of hemicatenanes with any DNA sequences, repetitive or nonrepetitive, which should allow us to study the interactions of p53 and HMGB1 with hemicatenanes containing different DNA sequences.

CONCLUDING REMARKS

The most obvious possibility deduced from refs *2*−*15* is that hcDNA represents DNA intermediates occurring during recombination or replication. However, we can view DNA hemicatenanes having other functions. For example, on the basis of the fact that hemicatenanes possess the unique property of stably associating two DNA molecules, we can speculate that they could play a role in the architectural organization of the genome in large domains and in chromosomal loops, in association with specific proteins in charge of controlling, regulating, and maintaining this architecture. In this respect, p53 might have a specific role in the control of the integrity of this architecture. This idea is not incompatible with recently published data on the functioning of p53 as a chromatin accessibility factor mediating (UV-induced) global chromatin relaxation (*65*). In accordance with the latter finding, a large number of p53-binding sites present in the human genome (*66*, *67*), including some of the alternative DNA structures bearing no resemblance to the p53-consensus sequences, may in fact represent sites of chromatin decondensation, independent of transcriptional activation (*65*, *68*). In a different way of functioning, binding of p53 CTD to DNA lesions or alternative DNA structures including DNA hemicatenanes may result in either (i) releasing of the core domain for sequence-specific binding of p53 to enable (transcription-dependent) cell-cycle arrest or apoptosis or (ii) signaling repair pathways. Whatever the consequence of p53 binding to non-B-type DNA structures may be, it is obvious that to secure further functioning of p53, the protein should be released from its (nonsequence-specific) binding sites in chromatin, possibly via direct interactions of p53 CTD with another protein(s). An ideal molecule for this purpose might be a highly abundant general chaperone like HMGB1. HMGB1 is the most mobile nuclear protein (<1.5 s are sufficient for one molecule to traverse the entire nuclear compartment; ref *69*), scanning the nucleus for appropriate binding sites and subsequently facilitating the (transient) formation of a number of multiprotein/DNA complexes (enhanceosomes), involving specific DNA sequences and dozens of different proteins (reviewed in ref *17*), including p53 (*28*, *45*). In accordance with this idea, HMGB1 can easily displace p53 from binding to DNA hemicatenanes in vitro by formation of ternary complexes (Figures 6 and 8). Thus, binding of HMGB1 to p53 may have different outcomes depending on the nature of DNA: (i) promotion of *sequence*-specific DNA binding of p53 (and consequently transcription-dependent p53 functions) by HMGB1-mediated prebending of p53-consensus sites without formation of stable ternary complexes (possibly because of the transient nature of these complexes; refs *17*, *21*, *28*, *45*) or (ii) displacement of p53 from *non*sequence-specific DNA binding sites (including alternative DNA structures such as hemicatenanes) and/or formation of ternary complexes (this paper). Additional work is required to assess the above outlined hypotheses taking advantage of recently developed techniques (ref *18* and unpublished data) to purify significant amounts of DNA hemicatenanes.

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