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# Change of Conformation of the DNA-binding Domain of p53 Is the Only Key Element for Binding of and Interference with p73*

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***Xenopus* p53 has biological and biochemical properties similar to those of human p53, except for optimal temperature. The frog protein is fully active at 30 °C and inactive at 37 °C, leading to a temperature-sensitive behavior similar to that of the human mutant p53Ala$^{143}$ and the murine mutant p53Val$^{135}$. Using hybrid proteins between human and *Xenopus* expressed from artificial p53 minigenes, we have been able to demonstrate that change of conformation of the DNA-binding domain is the major determinant of this heat sensitivity. It has been reported that some human tumor-derived p53 mutants can engage in a physical association with p73, thus inhibiting its transactivating properties. The mechanism of this association remains to be elucidated. The nature of the mutant p53 that can engage in this association also remains controversial. Using the unique opportunity of the temperature sensitivity of *Xenopus* p53, we demonstrate that binding of and interference with p73 require a change of conformation in the p53 protein. This interaction occurs through the DNA-binding domain of p53 only when it is in a denatured state. These results reinforce the notion that mutant p53 with a conformational change can act as a down-regulator of the p73 pathway in human cancer and could confer a selective advantage to the tumor.**

The p53 protein plays a crucial role in the cellular response to DNA damage by activating either an apoptotic or a growth-arrest pathway in proliferating cells (1). Among the various biochemical activities linked to the p53 protein, its main function seems to be its ability to activate transcription from genes containing two contiguous monomers of the (Pu)3xC(A/T)(A/T)G(Py)3x sequence (2). DNA-binding sites for p53 are localized either in the promoter region or in introns of many target genes involved in either growth arrest or apoptosis (3, 4). The transcription domain of p53 is localized in the amino-terminal part of the protein (residues 1–42), whereas the DNA-binding domain is localized in the central region of the protein (residues 90–290) (5). The importance of this DNA-binding region is emphasized by the observation that more than 14,000 described point mutations of the p53 gene are clustered in this region (6). Many of these mutations are correlated with loss of wild-type (wt)[1] p53 function.

One important feature of p53 is its flexibility (7). The first line of evidence came from work with various monoclonal antibodies. Historically, it was reported that some p53 epitopes were either revealed or masked, depending on growth culture conditions (8). It has been shown that the central region of the p53 protein contains numerous cryptic epitopes that are revealed either after denaturation of the protein or in specific types of p53 mutants with altered folding such as p53His$^{175}$ (9–11). The second line of evidence comes from the discovery of temperature-sensitive p53 mutants that have a wild-type behavior at 30 °C but are inactive at 37 °C (12). After the initial discovery of the p53Val$^{135}$ mutant in mice, several human p53 mutants were shown to have a similar behavior (13). This temperature sensitivity is often linked with a variation in protein conformation that changes the exposure of several epitopes. The third line of evidence concerns the function of the carboxyl-terminal domain that is involved in negative regulation of specific DNA binding of p53. It is generally admitted that this domain interacts with a specific domain of the central region of the protein and inhibits DNA binding (14). Impairment of this interaction leads to stimulation of specific DNA binding. This flexibility of wild-type and mutant p53 is an important feature, as it has been demonstrated that some small molecules can be used to rescue mutant p53 and restore p53 function (15, 16). The observation that monoclonal antibodies or small peptides directed against the amino or carboxyl terminus of mutant p53 can restore the activity of various p53 mutants suggests that there is important cross-talk between the various domains of the p53 protein. Unfortunately, the crystal structure of the whole p53 has not been elucidated, and there is no evidence concerning the possible function of the amino and carboxyl termini in the architecture of the p53 protein (17).

Two additional p53 family members, p73 and p63, have recently been identified and characterized (18, 19). p73 and p63 both contain regions corresponding to p53 amino-terminal

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[1] The abbreviations used are: wt, wild type; hsp70, generic term for all members of the 70-kDa heat shock protein family; BSA, bovine serum albumin; Xp53, *Xenopus* p53; Hp53, Human p53; mAb, monoclonal antibody; PBS, phosphate buffer saline.

transactivation, central DNA binding, and carboxyl-terminal oligomerization domains (20). Because of their structural similarities, p73 and p63 can bind to p53 consensus sequences, activate transcription of several p53 target genes, and induce apoptosis when overexpressed in cells. However, unlike the p53 gene, which is represented by a single polypeptide, the p73 and p63 genes are more complex and possess at least two major transcriptional promoters, which direct at least 6 products with different activities as transcription factors. All isotypes possess a fully functional DNA-binding domain and the carboxyl terminus oligomerization domain. The two alternate promoters generate isoforms lacking the amino-terminal transactivation domain. These isoforms, known as ΔNp73 and ΔNp63, are likely to act as dominant-negative regulators of their full-length counterparts. Several splicing variants generate different carboxyl termini, some of which contain a sterile alpha motif-like domain at the carboxyl terminus, known to be involved in protein-protein interactions (21, 22).

Although molecular analysis has failed to reveal any mutations in these two genes in human cancer, recent studies have described accumulation of p73 and p63 in various human tumors (21–23). Although wild-type p53 cannot form tetramers with full-length p73 and p63 (24), it has been shown that some p53 mutants can interact with p73 and p63, leading to functional inactivation of their transcriptional activity (25–28). This association is believed to be mediated by a nonspecific interaction via the DNA-binding domain of both proteins. This association interferes with the transcriptional activity of p73 and p63 and their ability to induce apoptosis. Because p73 is phosphorylated in response to the chemotherapeutic agent, cisplatin, binding of mutant p53 to p73 may affect sensitivity to this drug (29–31). These data suggest that a dominant activity of specific mutant p53, associated with a defined genotype, could act via inactivation of the p73 and p63 pathways and could explain a gain in function of some p53 mutants. Although the association between wt p73 and mutant p53 has been repeatedly described, the identity of mutant p53 that could interact with p73 remains controversial. Several studies suggest that only mutant p53 that undergo a conformational change could bind to p73 (25, 28). It has also been suggested that the intragenic polymorphism at position 72 (Arg or Pro) could also modify mutant p53 behavior (27).

We have previously shown that the temperature sensitivity of wild-type *Xenopus laevis* p53 (Xp53) could be associated with a change in protein conformation (32, 33). At 37 °C, wild-type Xp53 is inactive and denatured, as this temperature is well above the permissive temperature for normal frogs (25 °C). This was demonstrated by the binding of hsp70 to the incorrectly folded Xp53 at 37 °C, whereas no binding could be detected at 32 °C (32). Furthermore, the transcriptional activity of Xp53 is normal at 32 °C but is abolished at 37 °C (15, 33). As this behavior is not linked to any mutation, such temperature sensitivity could be theoretically associated with any domain of the protein. The observation that cancer p53 mutations occur in the central domain of the protein suggests that this region could be an important determinant for such temperature sensitivity. Nevertheless, we cannot exclude that other p53 domains could also be involved in this conformational change.

To use this specific property of Xp53 as a tool to analyze the requirement of a change of conformation of human p53 for specific interactions, we first defined the region of Xp53 involved in this temperature sensitivity. We generated a series of hybrids between human and *Xenopus* p53. Our data indicate that the central DNA-binding domain is a key element in the change in p53 conformation in a similar way to that observed in human p53 mutant. By using either Xp53 or hybrid protein, we demonstrate that this conformational change of the DNA-binding domain is the only requirement for binding and interference with the activity of the wild-type form of p73α. These results reinforce the notion that mutant p53 with a conformational change can act as a down-regulator of the p73 pathway.

## EXPERIMENTAL PROCEDURES

*Plasmids*—Artificial p53 genes were constructed using a two-step strategy. In the first step, various virtual p53 genes were designed and analyzed for splicing using several online programs, such as Genscan and Geneview (34). Intronic sequences were derived from the natural human and *Xenopus* p53 genes. Once a gene with correct splicing was obtained, each cassette was synthesized using a combination of PCR and DNA synthesis. Each cassette was subcloned in pT7blue (Novagen) and sequenced on both strands for verification. Each wild-type artificial gene was then assembled in the eukaryotic expression vector pcDNA neo 3.1 (Invitrogen). The two wild-type genes pHC (human p53) and pXC (*Xenopus* p53) were controlled after transfection in Saos-2 cells, as described under "Results." The entire sequence of these artificial genes is available on request. HDM2-luc, Bax-luc, PIG3-luc, and WAF-luc were generously provided by M. Oren, J. Reed, and B. Vogelstein. Recombinant baculovirus expressing human p73α was obtained by using the BaculoGold vector (BD Biosciences). The expression vector for human Ha-Tag p73 alpha was provided by D. Caput.

*Cell Culture and Antibodies*—Saos-2 and H1299 cell lines were obtained from the American Type Culture Collection. Cells were maintained in Dulbecco's modified Eagle's medium with glucose (4.5 g/l), L-glutamine, and sodium pyruvate. The medium was supplemented with 10% heat-inactivated fetal calf serum and antibiotics. Cells were grown at 37 °C in a humidified 5% $CO_2$ atmosphere. Both cell lines are devoid of endogenous p53 and do not express high levels of p73. H1299 cells were used for transient assays, whereas Saos-2 cells were used for long-term experiments. Sf9 insect cells were grown in TC100 medium (Life Science) at 28 °C.

For the transactivation assay, H1299 and Saos-2 cells were plated in 24-well plates (respectively $5 \times 10^4$ and $10^5$ cells per 1.5-cm well). After 24 h, cells were transfected using Superfect Reagent (Qiagen) or LipofectAMINE (Life Science). One μg of reporter gene and 0.05 μg of p53 plasmid were used. The luciferase activity was tested 24 h after transfection. Luciferase activity was measured using the MicroLumat LB96P (Berthold) with automated injection. Each transfection was repeated at least four times.

For labeling and immunoprecipitation, $3 \times 10^5$ cells were plated on 60-mm dishes and transfected the next day by the calcium phosphate procedure with 10 μg of plasmid. The precipitate was left on the cells for 15 h before washing twice with Dulbecco's modified Eagle's medium and feeding with 5 ml of complete medium. Labeling was usually performed 48 h after transfection.

Long-term transfection for cell growth arrest assay was performed as described by Ory *et al.* (10). $3.5 \times 10^5$ Saos-2 cells were plated into 25-$cm^2$ flasks and transfected on the following day by the calcium phosphate procedure using 1.7 μg of plasmid. Two days after transfection, the cells were divided into two flasks in medium containing 0.8 mg/ml geneticin (G418, Life Science). After 3–4 weeks, one flask was stained using Giemsa, whereas the other was assessed for p53 protein expression by immunocytochemistry using X77 or DO7 mAb, as described by Ory *et al.* (10).

Monoclonal antibody (mAb) DO7 recognizes both wild-type and mutant human p53, whereas X77 recognizes both human and *Xenopus* p53. The epitope is localized in the amino terminus of p53 for both antibodies. X77 also reacts strongly with human p73 and could not be used for the study of p53/p73 interaction (35). PAb240 is specific for human p53 with altered conformation. HR231 and X36 recognized both wild-type and mutant human or *Xenopus* p53, respectively. The epitope is localized in the carboxyl terminus of p53 for both antibodies. We have previously demonstrated that these two monoclonal antibodies are able to activate the specific DNA-binding activity of p53 (15). The hsp70 mAb was purchased from Santa Cruz Biotechnology (clone W27: sc-24). The rabbit serum specific for Xp53 has already been described (32). The anti-Ha tag used for the detection of p73 was purchased from Babco.

To establish stable cell lines, Saos-2 cells were transfected with 5 μg of plasmid by the calcium phosphate procedure in 100-mm Petri dishes. After 3 weeks of selection with G418 (0.8 mg/ml) at 37 °C, clones were pooled and used for subsequent studies. Immunocytochemistry analysis indicates that more than 50% of the cells express the protein of interest. For apoptosis analysis by FACS, only p53-expressing cells were tested.

For p73 colony suppression assays, Saos-2 cells were plated and

transiently transfected by the calcium phosphate method in the presence of BES (*N*,*N*-bis(2-hydroxyethyl-2-aminoethanesulfonic acid, sodium salt) (Sigma). The precipitates were left for 12 h, after which the medium was changed. 24 h after the transfection, cells were detached and replated in triplicate at $3 \times 10^4$/60-mm dish. Two days later, puromycin (2 $\mu$g/ml) was added to each plate and maintained for 72 h. Two weeks later, colonies were fixed in methanol and stained with Giemsa solution followed by washing with water.

*Flow Cytometry*—H1299 cells ($4 \times 10^5$ cells per 100-mm Petri dish) were transfected with 10 $\mu$g of plasmid DNA corresponding to wt or chimeric p53s. Seventy-two hours post-transfection, adherent and floating cells were combined and washed in cold PBS. Cells were permeabilized in 1% paraformaldehyde-PBS for 10 min and washed in PBS. Cells were fixed for 7 min at −20 °C in 100% methanol, rehydrated in PBS for 10 min, and then stained for p53 with DO7 monoclonal antibody for p53His$^{273}$, pHC and pHX3, X36 monoclonal antibody for pXC and pXH3, and a mouse purified $IgG_{2b},\kappa$ (BD Biosciences) as isotype control in 0.1% PBS, 0.1% bovine serum albumin, Tween 20 for 1 h at room temperature. Cells were then washed twice in 0.1% PBS, 0.1% bovine serum albumin, Tween 20 and incubated with a goat anti-mouse fluorescein isothiocyanate-conjugated secondary antibody (BD Biosciences) for 30 min at room temperature. After incubation, cells were washed twice in 0.1% PBS, 0.1% bovine serum albumin, Tween 20 and treated with RNase A (500 $\mu$g/ml) and propidium iodide (50 $\mu$g/ml). Samples were analyzed on a standard FACScalibur flow cytometer (BD Biosciences). p53 fluorescein isothiocyanate fluorescence was measured by log amplification into the FL1 channel, and PI DNA fluorescence into the FL3 channel. Two files of 10,000 events were acquired for each sample; the first included all events based on a forward scatter threshold, whereas the second file included p53 fluorescein isothiocyanate-positive cells only, the gate being set on the corresponding isotype control. Apoptosis was calculated as the percentage of sub-$G_1$ events of the total p53-negative or p53-positive population. DNA histograms of all p53-negative and p53-positive cells were analyzed using the MODFIT 2.0 cell cycle analysis program (Verity Software, Topersham, MA).

*Labeling and Immunoprecipitation*—Cells were metabolically labeled for 3 h with 200 $\mu$Ci of [$^{35}$S]methionine and cysteine (Expre$^{35}$S$^{35}$S, PerkinElmer Life Sciences). Proteins were extracted with 0.5 ml of RIPA buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholic acid) and protease inhibitors. After 30 min on ice, extracts were cleared for 30 min at 15,000 revolutions per minute at 4 °C. Cell lysates were precleared twice by adsorption with Protein G-Sepharose (Amersham Biosciences) and non-immune serum. *In vivo*-radiolabeled proteins were immunoprecipitated with specific antibodies for 2 h on ice. Antigen-antibody complexes were collected by using Protein G-Sepharose and washed three times in lysis buffer. Immunoprecipitates were analyzed by SDS-PAGE. For the specific interaction with p73, whole-cell extracts were prepared using the Nonidet P-40 buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 2% Nonidet P-40).

## RESULTS

*Construction and Characterization of p53 Minigenes Expressing Wild-type Human and Xenopus p53*—Human p53 protein (Hp53) can be divided into five domains, each corresponding to specific functions (Fig. 1*A*) (36). 1) The amino-terminal part 1–39 contains the acidic transactivation domain and the murine double minute protein-binding site. 2) Region 40–100 contains series repeated proline residues that are conserved in the majority of p53. 3) The central region (101–306) contains the DNA-binding domain. It is the target of 90% of p53 mutations found in human cancers. 4) The oligomerization domain (307–355) consists of a beta-strand, followed by an alpha-helix necessary for dimerization, as p53 is composed of a dimer of two dimers. A nuclear export signal is localized in this oligomerization domain. 5) The carboxyl terminus of p53 (356–393) contains 3 nuclear localization signals and a nonspecific DNA-binding domain that binds to damaged DNA. This region is also involved in down-regulation of DNA binding of the central domain. We constructed two artificial p53 minigenes to perform swapping experiments between human and *Xenopus* p53 (Fig. 1*B*). Five cassettes, each corresponding to a functional domain described above, were devised. For easy swapping, each cassette was designed as a unique exon flanked by an artificial intron. Unique restriction sites were introduced in each intron so that each p53 cassette could be easily moved from one construct to another. These two constructs allow many swapping experiments to generate various hybrid proteins. We first verified that the p53 expressed by these minigenes did not contain any alteration because of aberrant splicing. The two vectors, pHC and pXC, were transfected in Saos-2 cells. After 48 h, RNA was extracted and PCR amplification was performed using either human or *Xenopus* p53 primers. The cDNA was entirely sequenced on both strands. No alteration was detected, indicating that splicing occurred correctly in the 2 artificial genes (data not shown). During PCR amplification, only one product was observed in each case, indicating that no aberrant splices had occurred. The two proteins, Hp53 and Xp53, were well expressed by these two constructs and behaved as p53 expressed by their corresponding cDNA (data not shown, see also Fig. 2*D*). The constructions used in the present study are summarized in Fig. 1*B*. Western blot and immunoprecipitation with mAb specific for various cassettes confirmed the identity of each protein (data not shown).

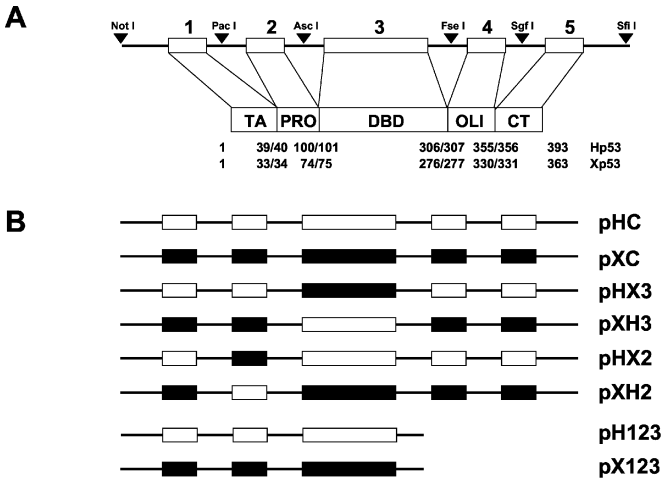


FIG. 1. **Schematic representation of the various p53 constructs.** *A*, structure of the artificial p53 gene divided into 5 exons. The boundary of each domain in amino acid residues is given for human (*Hp53*) and *Xenopus* p53 (*Xp53*); 1 to 5 corresponds to the 5 cassettes: *TA*, transactivation domain; *PRO*, proline-rich domain; *DBD*, DNA-binding domain; *OLI*, oligomerization domain; *CT*, carboxyl-terminal regulation domain. *B*, representation of the various hybrid genes: human exons (*white box*) and *Xenopus* exons (*black box*) are depicted for each hybrid construct used in the present study.

*Hybrid Proteins with the Xenopus p53 DNA-binding Domain Have an Altered Conformation at 37 °C*—The conformational change can be detected either by binding of monoclonal antibody PAb240 or by recognition by cellular chaperone hsp70. To address this point, proteins were labeled and immunoprecipitated after transient transfection in Saos-2 cells. Monoclonal antibody DO7 is specific for Hp53 (wild-type and mutant), PAb240 is specific for an epitope that is released in conformational mutant Hp53, and X77 recognizes both human and *Xenopus* p53. Immunoprecipitation of Xp53 expressed at 37 °C led to the detection of Xp53 (46 kDa) and coprecipitation of a 70-kDa protein (Fig. 2*B*). This coprecipitation was not detected at 32 °C or when wild-type human p53 was expressed at either temperature (Fig. 2, *A* and *B*). Control experiments with the human mutant p53His$^{175}$ indicated that it binds to hsp70 at both temperatures. This observation, previously described in 1989, led us to the hypothesis that the conformation of Xp53 is altered at 37 °C (32). The identity of the 70-kDa protein was confirmed by immunoprecipitation directed against p53 followed by Western blot with hsp70 antibody (Fig. 2*C*). Using similar experimental conditions in H1299 cells, we showed that

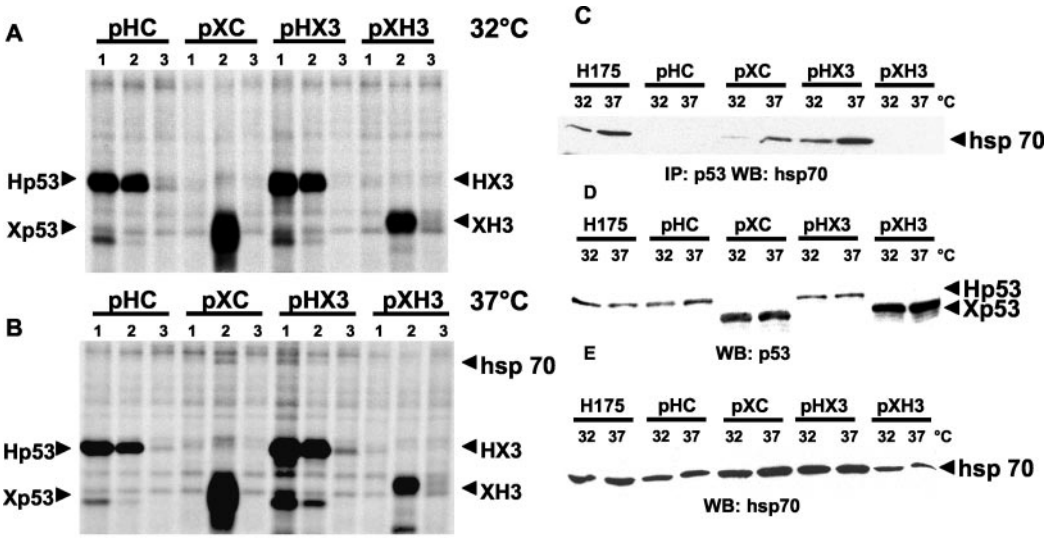


FIG. 2. **Conformational change of p53 construct with the DNA-binding domain of Xp53.** Saos cells ($5 \times 10^5$ in 60-mm Petri dish) were transfected with 10 $\mu$g of the various constructs. After transfection (48 h), cells were metabolically labeled for 3 h as described under "Experimental Procedures." Extracts were prepared and used for immunoprecipitation: *1*, DO7; *2*, X77; *3*, PAb240. *A* and *B*, transfection performed at 32 °C and 37 °C, respectively. *C*, H1299 cells ($4 \times 10^5$ in 60-mm Petri dish) were cotransfected with 5 $\mu$g of the indicated plasmid. Cells were incubated at either 30 or 37 °C and extracted 48 h after transfection. Unlabeled cell extracts were immunoprecipitated with p53 antibody, DO7 or X36 for human and *Xenopus* p53, respectively. After immunoprecipitation and electrophoresis, the proteins were transferred onto a membrane that was probed with a specific hsp70 antibody.

HX3 protein coprecipitates with hsp70 only at 37 °C, whereas XH3 did not show any interaction at either temperature (Fig. 2, *B* and *C*). Control immunoblot shows that a similar amount of each protein was used for each temperature (Fig. 2, *D* and *E*). These observations suggest that the central region of p53 is important for the conformational change of Xp53.

*Hybrid Proteins with the Xenopus p53 DNA-binding Domain Have a Heat-sensitive Transcriptional Activity*—The various constructs were cotransfected in the Saos-2 cell line with various reporter plasmids containing a transcription promoter known to be transactivated by human p53 (Fig. 3*A*). At 32 °C, both wild-type Hp53 and Xp53 were active and transactivated the various promoters. The efficiency of the two proteins was similar, except for PIG3. These results with natural p53 promoters confirm our previous studies using a reporter plasmid with consensus p53 DNA-binding sites (15). At 37 °C, Xp53 was completely inactive with the PIG3 and Bax promoters and was only partially active with murine double minute or the Waf1 promoter. Western blot experiments indicated that these observations are not related to a lack of expression of the various proteins, because expression of each protein was higher at 37 °C, supporting our data concerning the temperature-sensitivity of frog p53 (Fig. 3*B*). XH3, the Hp53 DNA-binding domain inside the Xp53 protein, had a similar behavior to that of Hp53 at both temperatures. Introduction of the human DNA-binding domain into a *Xenopus* framework therefore restores normal p53 behavior, suggesting that temperature sensitivity is linked to this region. This observation is confirmed by the behavior of HX3, a human p53 with the Xp53 DNA-binding domain. This mutant is inactive at 37 °C and partially or fully active at 32 °C. Swapping another region of p53 (cassette 2) had no effect on temperature sensitivity: HX2 was active at both temperatures and XH2 remained temperature-sensitive (Figs. 1 and 3*A*). Similar results are obtained in Saos-2 cells (data not shown and Fig. 6). Taken together, these results indicate that the central DNA-binding domain is an important element for the temperature sensitivity of Xp53.

*Overexpression of Hybrid Proteins with the Xp53 DNA-binding Domain Leads to Growth Arrest and Apoptosis Only at 32 °C*—The ability of the various constructs to cause overall cell growth arrest was assessed in a colony-formation assay at 37 °C. Saos-2 cells were transfected with the vector expressing the indicated p53 proteins. After 3 to 4 weeks of selection with G418, colonies were stained and counted. In some experiments, immunocytochemistry was performed to confirm expression of the corresponding p53. Compared with the control vector (pcDNA 3.1), human wild-type p53 had a strong growth arrest activity (Fig. 4, *A* and *B*). *Xenopus* p53 had no growth-inhibitory activity, as it yielded a similar number of colonies to the empty vector. Most of these clones expressed Xp53 determined by immunocytochemistry analysis (Fig. 4*A*). Because this experiment was performed at 37 °C, the behavior of Xp53 as an inactive p53 was not unexpected. Insertion of the human DNA-binding domain into *Xenopus* p53 (XH3) restored the ability to inhibit colony formation, whereas incorporation of the *Xenopus* DNA-binding domain into human p53 led to a mutant phenotype with no growth inhibition and formation of colonies expressing the hybrid protein (Fig. 4, *A* and *B*). The ability of the various constructs to mediate apoptosis was evaluated in H1299 cells using a transient-transfection assay, as described under "Experimental Procedures." The two proteins with the human p53 DNA-binding domain (Hp53 and XH3) were both able to induce apoptosis at 37 °C, whereas the two proteins with the *Xenopus* p53 DNA-binding domain were unable to induce apoptosis and showed a similar behavior to that of mutant p53His$^{273}$ (Fig. 4*C*). Similar experiments could not be performed at 32 °C because of slow growth of the cells at this temperature. We then established permanent cell lines expressing Xp53 and HX3, the two proteins tolerated at 37 °C. When these cells were incubated at 32 °C, apoptosis could be detected by FACS analysis (Fig. 4*C*). No apoptosis could be detected in cells expressing either the control vector or the mutant p53His$^{273}$. The low yield of apoptosis in these experiments was possibly caused by the slow metabolism of these cells at 32 °C.

We also tested whether this temperature-sensitive behavior could influence the cell cycle. Using FACS analysis of transiently transfected H1299 cells, we observed that, at 32 °C, the four constructs (pHC, pXC, pHX3, and pXH3) induced cell cycle arrest with a decrease of S phase and a significant increase of

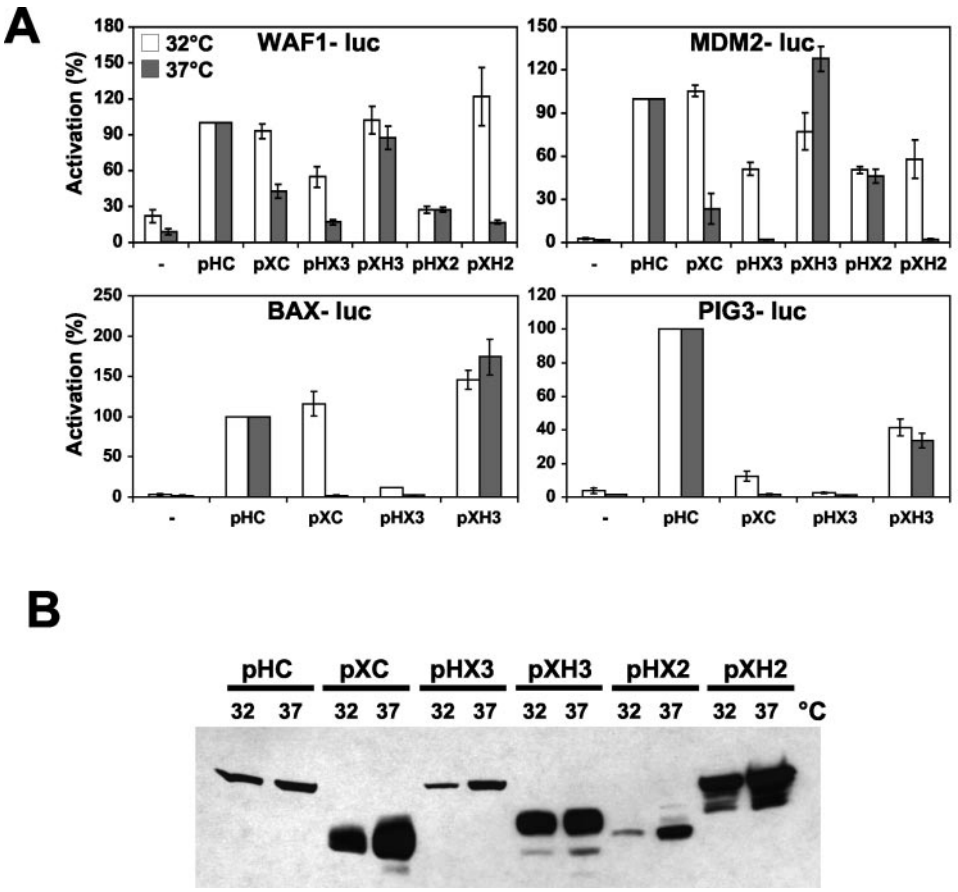


FIG. 3. **The DNA-binding domain of Xp53 determines the temperature sensitivity of the p53 transcriptional activity.** *A*, exponentially growing Saos-2 cells were cotransfected with 50 ng of p53 expression vector and 1 $\mu$g of reporter plasmid. Luciferase assays were performed 24 h after transfection. Results are expressed as percent of activation relative to wt p53 used under the same experimental conditions (32 °C or 37 °C). Expression of wt p53 at 32 °C results in a 20–50% decrease in activity compared with 37 °C, depending on the promoter used (data not shown). *B*, equal amounts of protein extract were analyzed by Western blot with X77 monoclonal antibody to check expression. X77 recognizes both Hp53 and Xp53 with a higher affinity for Xp53. Only the 32 °C/37 °C ratio of each protein can therefore be assessed in this experiment.

phase $G_2$ (Fig. 4*D*). Control experiments with the vector alone (pcDNA 3.1) or a mutant p53 (p53His$^{273}$) indicated that they did not affect cell growth (Fig. 4*D*). At 37 °C, only the two proteins with the human p53 DNA-binding domain (Hp53 and XH3) were able to induce growth arrest, whereas the two constructs containing the *Xenopus* DNA-binding domain were inactive (Fig. 4*D*). All of these experiments indicate that the *Xenopus* p53 DNA-binding domain is temperature-sensitive, both structurally and functionally.

*Hybrid Proteins with Altered Conformation of the DNA-binding Domain Bind to Human p73*—The data presented above indicate that the change of Xp53 conformation after the temperature shift leads to the same properties as those observed in several human p53 mutants, such as p53His$^{175}$. Interactions between mutant p53 and wild-type p73 have been shown to occur via the DNA-binding domain, but this has only been shown by using a mutated core domain (25, 26). To gain greater insight into this interaction, we used Xp53 and the various hybrid proteins as probes for the properties of p53 associated with conformational change without any influence of mutations.

In a first set of experiments, we tested whether the change of conformation of Xp53 could lead to interactions with p73. The four constructs described above were cotransfected into H1299 cells with an expression vector specific for p73. Mutant p53His$^{175}$ was used as a positive control, because it undergoes a drastic conformational change and efficiently binds to p73 (25–27). The transfection experiment was performed at both 30 °C and 37 °C. Preliminary experiments using protein extracts performed in RIPA buffer indicated that all the various p53s were able to interact with p73 (data not shown). This is undoubtedly because of the high detergent content of this buffer, which can alter p53 conformation. It is noteworthy that the PAb240 epitope can also be revealed when an excessively harsh extraction procedure is used (11). Gaiddon *et al.* have already reported that denaturation of wild-type p53 during a purification process can lead to denaturation of a fraction of p53 that reacts with both the monoclonal antibody PAb240 and the p73 protein (25). The use of a milder buffer ensures conditions allowing discrimination of wild-type and mutant p53 behavior. We therefore performed all of our experiments with a milder Nonidet P-40 buffer (see "Experimental Procedures"). Under these conditions, wild-type p53 did not coprecipitate efficiently with p73 (Fig. 5*A*, *pHC*). The human mutant p53His$^{175}$ bound efficiently to p73 at both temperatures (Fig. 5*A*, *H175*). The behavior of Xp53 indicates that it bound more efficiently to p73 at 37 °C than at 30 °C, suggesting a temperature-sensitive behavior for this activity (Fig. 5*A*, *pXC*). Hybrid protein HX3 with the Xp53 DNA-binding site behaved in a similar way, whereas the behavior of the other protein, XH3, was similar to that of Hp53. As indicated by the control Western blot, the same amount of p53 and p73 was expressed and used for all these experiments (Fig. 5, *B* and *C*). This interaction was also analyzed in insect cells. Recombinant baculoviruses were used to express the various recombinant proteins in cells infected at 28 °C. In this system, only mutant p53His$^{175}$ bound efficiently to p73, whereas wt Hp53 and Xp53 did not (Fig. 5*D*, *4 °C*). Hansen *et al.* have shown that incubation of

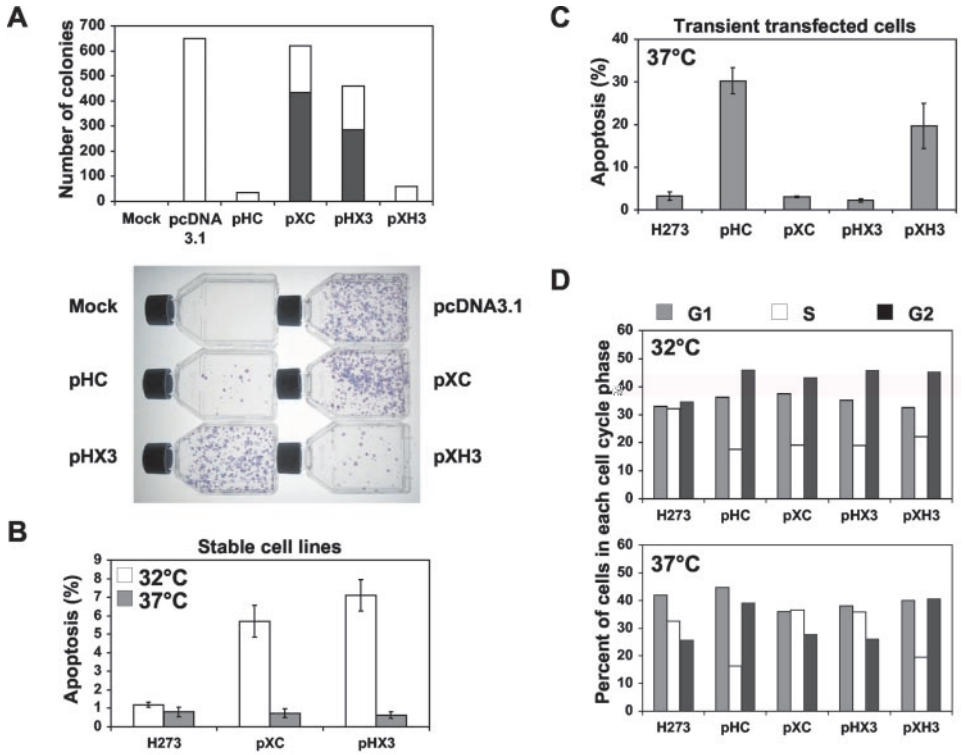


FIG. 4. **Specific inhibition of cellular proliferation by hybrid proteins with the *Xenopus* p53 DNA-binding domain.** *A*, colony-formation assay was performed by transfection of Saos-2 cells with either an empty control vector (pcDNA 3.1) or an expression vector encoding the various hybrid proteins. Stably transfected cells were selected with G418 for 3–4 weeks, and numbers of G418 resistant colonies were counted after staining with Giemsa's solution. In duplicate experiments, colonies were also stained with X77 or DO7 depending on the vector used for transfection. The frequency of clones expressing p53 is indicated in *black* on the figure. Giemsa staining of representative experiments is shown. *B*, stable cell lines expressing mutant p53His$^{273}$, Xp53, HX3, and the empty vector were established. These cells grow well at 37 °C. The 4 cell lines were incubated either at 32 °C or 37 °C for 80 h, and apoptosis was evaluated by FACS analysis as described under "Experimental Procedures." *C*, apoptosis was tested as described under "Experimental Procedures." For transient assay, 100-mm Petri dishes with H1299 cells ($4 \times 10^5$) were transfected with 10 $\mu$g of DNA. Apoptosis was analyzed 72 h after transfection. *D*, H1299 cells ($4 \times 10^5$ cells in 100-mm Petri dishes) were transfected with 10 $\mu$g of various plasmids and were incubated at either 32 °C or 37 °C for 72 h. Cell growth arrest was evaluated by FACS as described under "Experimental Procedures." For *D*, only the results obtained for p53-positive cells are shown. p53His$^{273}$-positive cells showed the same behavior as p53-negative cells (data not shown).

human p53 at 40 °C for 10 min leads to loss of the wild-type conformation (37). In the present study, we show that incubation of the extract at 40 °C for 15 min induced a specific interaction of both human and *Xenopus* p53 to p73 (Fig. 5*D*, *40 °C*).

An important point that must be considered in these experiments is the possibility that binding of Xp53 to p73 could be due to heterooligomerization through the oligomerization domain. Although it has been shown that Hp53 cannot heterooligomerize with p73, this has not been demonstrated for Xp53. The observation that pHX3, which contains the human p53 oligomerization domain, binds to p73 is already a strong indication that heterooligomerization is not involved in this association.

Experiments with glutathione *S*-transferase-p73 and *in vitro*-translated truncated Xp53 confirmed the absence of oligomerization between p73 and Xp53 (data not shown). Using the facility provided by the two artificial genes, we designed two new constructs (Fig. 1*B*). H123 and X123 contain only the amino terminus and the central region of p53 without the oligomerization domain and the carboxyl-terminal region. When cotransfected with p73 in H1299 cells, X123 interacts efficiently with p73 with a higher efficiency at 37 °C compared with 30 °C. For H123, an interaction with p73 can be detected at 37 °C (Fig. 5*E*). In some experiments, this interaction could also be observed at 30 °C (data not shown). The observation that H123 binds to p73 is certainly due to its denaturation status induced by the large deletion.

Control experiments indicate that all these proteins are expressed at similar levels (Fig. 5*F*). Similar findings were observed with other constructs that specifically lack the oligomerization domain (data not shown). These results indicate that the oligomerization domain is not necessary for this interaction and that the conformational change of p53 is a key determinant for binding to p73.

*p53 with a Conformational Change of the DNA-binding Domain Interferes with in Vivo p73 Transcriptional Activity*—Tumor-derived p53 mutants reduce p73 transcriptional activity in mammalian cells, but it is not clear whether only those mutants with a change of conformation can interfere with p73 (25–27). We therefore used a transactivation assay to determine the behavior of the chimeric proteins described above. Experiments were performed in H1299 cells at 3 different temperatures to emphasize the dependence of the p53 change of conformation in this assay. Mutant p53His$^{175}$ was used as a positive control, because its binds efficiently to p73 (25). p53His$^{175}$ interfered with p73 activity and reduced the transcriptional activity of p73 from 5-fold at 32 °C to 10-fold at 37 °C and 39.5 °C (Fig. 6*A*). At 32 °C, the four other proteins had an efficient transcriptional activity (Fig. 6*A*, *left panel*). Cotransfection with p73 led to an increase of transcription compared with p73 alone (Fig. 6*A*, *right panel*). At 37 °C and 39.5 °C, the two constructs with Xp53 DNA-binding domain, XC and HX3, were inactive for transcription (Fig. 6*A*, *left panels*) and when cotransfected with p73 led to a substantial decrease of p73 activity (Fig. 6*A*, *right panels*). Control experiments with Hp53 showed no signs of interference, whereas

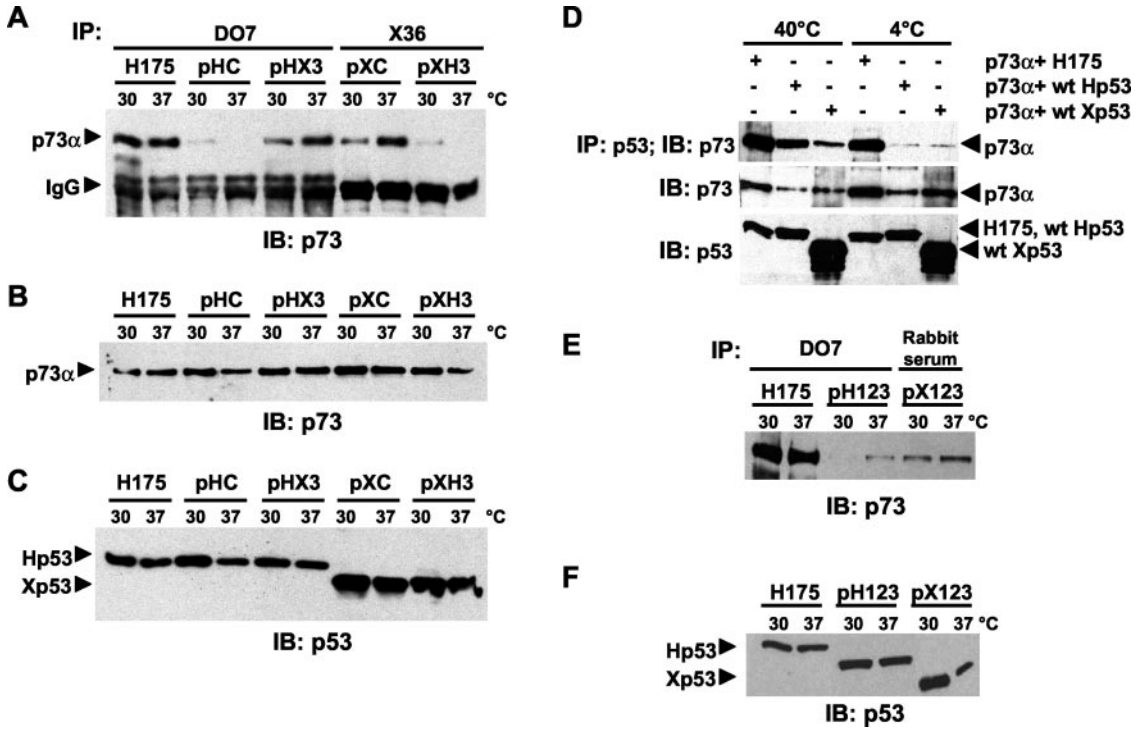


FIG. 5. **Hybrid p53 proteins with change of conformation associate with p73.** H1299 cells were cotransfected with 2.5 $\mu$g of p73-Ha-tagged expression vector and 2.5 $\mu$g of the indicated plasmid. Cells were incubated at either 30 or 37 °C and extracted 48 h after transfection. Before immunoprecipitation, the amount of p53 was normalized by Western blot to ensure immunoprecipitation with an equivalent amount. *A*, cell extracts were immunoprecipitated with a p53 monoclonal antibody (DO7 for p53His$^{175}$, Hp53, and HX3; X36 for Xp53 and XH3). After immunoprecipitation and electrophoresis, the proteins were transferred onto a membrane that was probed with an anti-Ha antibody for p73 detection. *B* and *C*, total extracts were analyzed by Western blot with the Ha-tag (p73) and X77 (Hp53 and Xp53) monoclonal antibodies, respectively. *D*, Sf9 cells were coinfected with various recombinant baculoviruses for 48 h. Cell extracts were incubated at either 4 °C or 40 °C for 15 min before immunoprecipitation with a p53 antibody (DO7 or X36). After immunoprecipitation and electrophoresis, the proteins were transferred onto a membrane that was probed with a specific p73 antibody. Control Western blot with total extract was probed directly with X77 that recognized Hp53, Xp53, and p73. *E*, H1299 cells were cotransfected with 2.5 $\mu$g of p73-Ha tagged expression vector and 2.5 $\mu$g of the indicated plasmid. Cells were incubated at either 32 °C or 37 °C and extracted 48 h after transfection. Cell extracts were immunoprecipitated with a p53 monoclonal antibody or a polyclonal sera that reacts with the amino terminus of Xp53. After immunoprecipitation and electrophoresis, the proteins were transferred onto a membrane that was probed with an anti-Ha antibody for p73 detection and a mix of X77 and DO7 for p53 detection.

XH3 showed a slight inhibition activity at 39.5 °C (Fig. 6*A*). Western blot experiments showed that the various proteins were expressed at similar levels in each experiment (Fig. 6*B*). To exclude the contribution of the oligomerization domain in a possible formation of heterologous tetramers, we also analyzed the behavior of the truncated proteins described above, H123 and X123 (Fig. 7*A*). These 2 proteins are inactive for transcription, because oligomerization is necessary for DNA binding (Fig. 7*A*, *left panel*). When cotransfected with p73, they induced inhibition of p73 transcriptional activity (Fig. 7*A*, *right panel*). Inhibition of p73 activity by H123 was not unexpected, because it also binds to p73 at high temperatures (Fig. 5*E*). Control Western blots indicated that these proteins are expressed at similar levels (Fig. 7*B*). Taken together, these results indicate that the association between proteins with the Xp53 DNA-binding domain and p73 leads to a loss of the transcriptional activity of p73.

*Overexpression of Hybrid Proteins with Xp53 DNA-binding Domain Reduces p73 Growth Suppression*—To further investigate the biological relevance of the association between Xp53 and its derivatives with p73, we investigated whether exogenous expression of these proteins interferes with p73-mediated growth suppression, as demonstrated for human mutant p53. For this purpose, Saos-2 cells were cotransfected with p73 together with a plasmid encoding pHC, pXC, pHX3, pXH3, or p53His$^{175}$. Overexpression of p73 alone suppressed colony formation of Saos-2 cells as compared with that of cells transfected with empty vector (Fig. 8). Conversely, Saos-2 cells regained colony formation when mutant p53 was overexpressed (Fig. 8), confirming the interference behavior of mutant p53. Similarly, pXC and pHX3, which contain the *Xenopus* DNA-binding domain, impaired the growth-suppressive activity of p73. pXH3 and pHC were unable to reactivate this growth suppressive activity (Fig 8). Taken together, these results confirm that proteins expressing the *Xenopus* p53 DNA-binding domain behave like mutant p53.

## DISCUSSION

A better understanding of the heterogeneity of mutant p53 behavior in human cancer is an important challenge to allow more effective tumor evaluation and for the design of specific chemotherapeutic agents. p53 mutations were initially classified according to their potential dominant-negative activity on wild-type p53 (38). Latter, the use of monoclonal antibodies specific for unfolded p53 led to the identification of two classes of mutants. Class I mutants are those with a missense mutation that affects residues of the DNA-binding surface and disrupts protein-DNA contact points with no change in protein conformation. Class II mutants have a disrupted conformation leading to the release of cryptic epitopes. These mutants are also well recognized by chaperone proteins, such as hsp70 (9, 11). More recently, thermodynamic studies have defined at least 5 classes of mutant p53 (39). Extensive studies have tried to link all of these various classes of mutant p53 to different cellular or tumor phenotypes, but with contradictory results and no clear evidence of a gain of function for mutant p53.

The discovery of the various p53 members, such as p73 and p63, has provided new perspectives concerning the potential

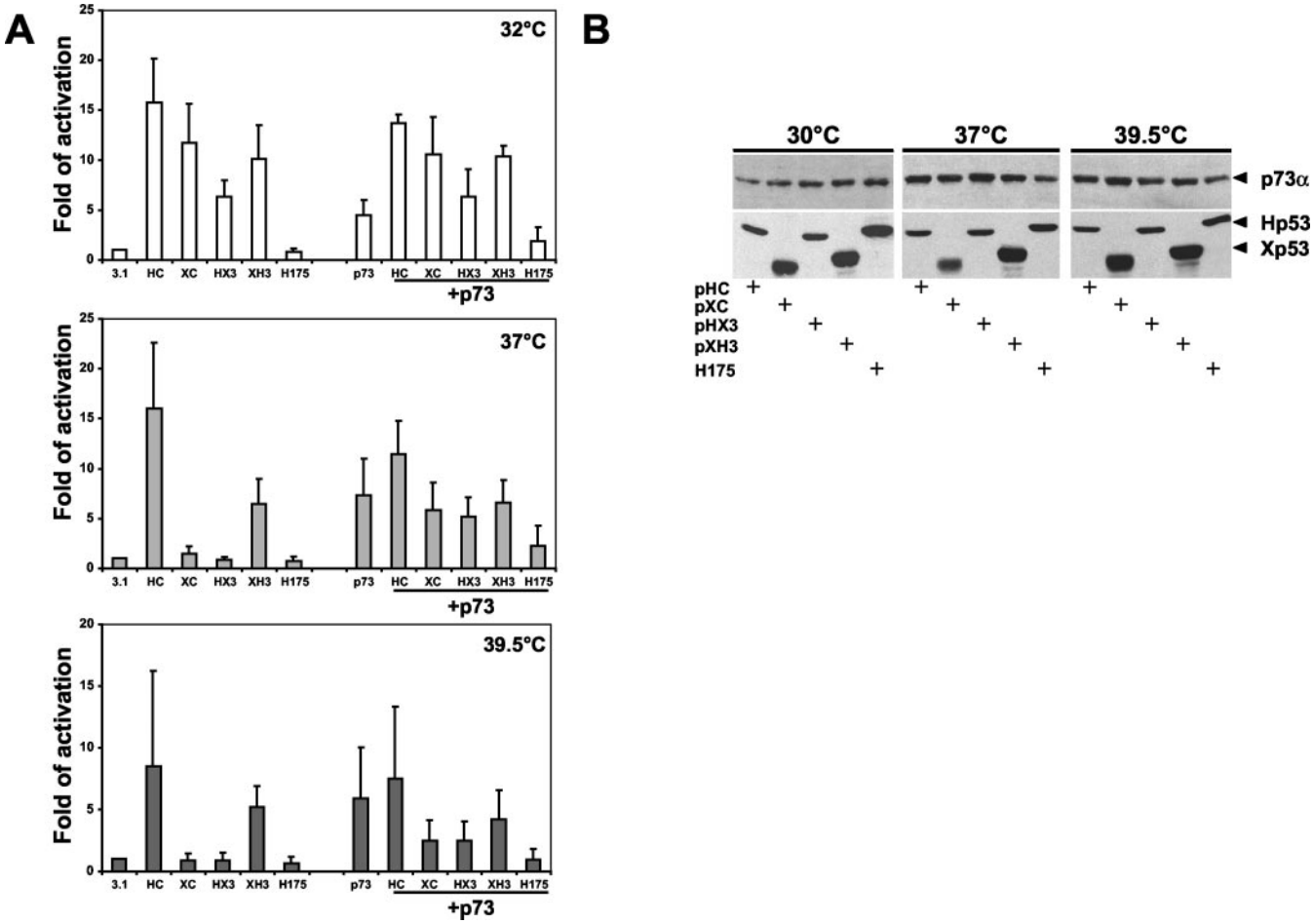


FIG. 6. **Hybrid p53 proteins with change of conformation down-regulate p73 transactivating activity.** H1299 cells were cotransfected with 10 ng of p73 expression vector, 20 ng of the indicated plasmid, and 100 ng of Waf-Luc vector. Luciferase assays were performed 24 h after transfection. *A*, results are presented as percent of activation relative to empty vector used under the same experimental conditions (30 or 37 or 39.5 °C). *B*, control Western blot performed either with anti-Ha antibody for p73 detection and a mix of X77 and DO7 for p53 detection.

gain of function for some mutant p53. Although p63 and p73 are not mutated in human cancer, recent studies indicate that they are important for apoptosis induced by chemotherapeutic agents in combination with wild-type p53 (30, 31, 40).

Functional and structural studies have demonstrated that no heterooligomerization can occur between p53 and p73 or p63 via their oligomerization domain (24). Nevertheless, it has been reported that some mutant p53 could bind to wild-type p73 or p63 and interfere with their activities, such as transcriptional activity or growth suppression (25–28, 41). These observations are important, as they suggest that the gain in function of these p53 mutants could be linked to inactivation of the p73 and/or p63 pathway. This association appears to be restricted to the class of mutant p53 with an altered conformation, but this remains controversial. The observation that this interaction occurs via the DNA-binding domain of p53 is a strong argument in favor of the requirement of a change of conformation associated with specific p53 mutation. However, it is fairly difficult to dissociate the change of conformation from the contribution of the mutation *per se*.

To conduct a structure/function study of p53, we designed two artificial p53 genes allowing easy access to the various functional domains of the protein. These constructs consist of five artificial exons. Rare restriction sites have been added to manipulate each exon individually. To validate this project, we tested this approach by the construction of hybrid proteins between human and *Xenopus* p53 to investigate the temperature sensitivity of the frog protein and to develop specific tools to analyze the contribution of the change of the DNA-binding domain of p53 in certain specific activities. Swapping experiments were performed with the central DNA-binding domain, as it was likely to be involved in this behavior. Control experiments indicated that all these artificial minigenes are fully functional, and no aberrant splicing has been detected.

Transient-transfection assays were used to evaluate the transcriptional activity of the various hybrid proteins. Using the natural p53 response promoter, we confirmed that Xp53 is temperature-sensitive, with an intense activity at 32 °C and no activity at 37 °C. This temperature sensitivity of non-mammalian p53 is not unexpected, as the optimal growth temperature of *X. laevis* cells, like that of insect cells, is 27 °C, and it has been recently shown that the transcriptional activity of Dm p53 is also temperature-sensitive (42). The various hybrid constructs between human and *Xenopus* p53 indicate that temperature sensitivity is only linked to the central DNA-binding domain. When the Xp53 DNA-binding domain is inserted into a human p53 backbone, it induces temperature sensitivity of the hybrid protein. Introduction of the human DNA-binding domain into the Xp53 backbone totally restores normal activity at 37 °C. This temperature sensitivity of the transcriptional activity of the various proteins is totally linked to their functional activity tested for cell cycle arrest or apoptosis. Each protein with the human DNA-binding domain is able to induce apoptosis and inhibit cell growth at 37 °C. The two proteins with the *Xenopus* DNA-binding domain have no such activity at 37 °C, but this activity could be restored at 32 °C. These obser-

vations are also in accordance with our results concerning variations in protein conformation. When expressed at 37 °C, the DNA-binding domain of Xp53 induces binding of hsp70, whether it is expressed in human or *Xenopus* backbone. No such binding is detected at 32 °C. This indicates that inactivation of Xp53 at 37 °C mimics the behavior of various mammalian mutant p53s with a change in folding of the DNA-binding domain. In human p53, this denaturation is always linked to the appearance of specific epitopes that were previously buried in this region (10). There is therefore a good correlation between structural data and biological activity of the various proteins. Our data emphasize the extreme fragility of the DNA-binding domain of the p53 protein. Previous studies have described mouse and human p53 mutants that also have temperature-sensitive behavior (12, 43). All of these mutations are localized in the central DNA-binding domain.

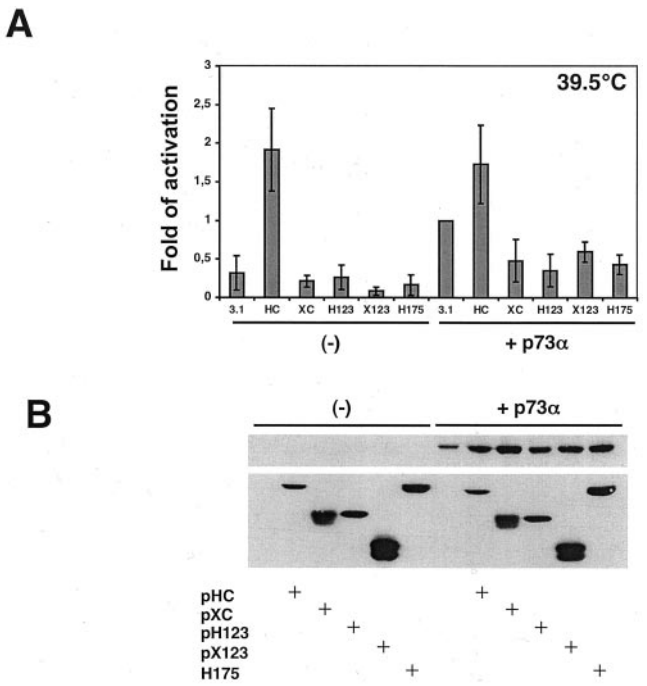


FIG. 7. **The oligomerization domain is not necessary for p53 and p73 interference.** H1299 cells were cotransfected with 10 ng of p73 expression vector, 20 ng of the indicated plasmid, and 100 ng of WAF-Luc vector. Luciferase assays were performed 24 h after transfection. *A*, results are presented as percent of activation relative to p73α used under the same experimental conditions (39.5 °C). *B*, control Western blot performed either with anti-Ha antibody for p73 detection and a mix of X77 and DO7 for p53 detection.

The use of Xp53 and the various hybrid derivatives offers a unique opportunity to verify whether p53 conformational changes are important for p73 binding. Our data show that Xp53 binds to human p73 in a temperature-dependent manner. Hybrid proteins with the *Xenopus* DNA-binding domain behave in a similar way. This interaction does not require the oligomerization domain, and our data suggest that it occurs via the DNA-binding domain only when it is denatured. We would like to emphasize the extreme fragility of the DNA-binding domain of p53, regardless of its origin. During the course of this work, we observed that wild-type Hp53 could interact with p73 under several experimental conditions, such as harsh extraction procedure or repeated freezing/thawing (data not shown). A similar observation has been reported by Gaiddon *et al.* using highly purified wild-type p53 that became slightly denatured during the purification procedure (25). This behavior is also very similar to that already described for Hp53 and its recognition by specific monoclonal antibodies such as pAb240, HO15.4, or HP64 that only react with the denatured protein (11). Although this conformational change of p53 has only been detected in a subclass of mutant p53, we cannot exclude the possibility that wild-type p53 may undergo such a conformational change *in vivo* in response to normal or abnormal specific stimuli. Truncated wt p53 lacking the oligomerization domain also binds weakly to p73. This can be due either to a change of conformation induced by the deletion or exposure of the hydrophobic DNA-binding domain by monomeric p53.

When expressed at 37 °C or 39 °C, XC and HX3 are transcriptionally inactive and are able to inhibit p73 transcriptional and growth-suppressive activity, mimicking the behavior of human mutant p53. The mechanism leading to such inactivation is not known. It has been suggested that the interaction of mutant p53 with p73 could prevent DNA binding to target genes, but this has been difficult to prove, as both p73 and p53 recognize the same response element. Although our studies do not formally demonstrate that this interaction occurs via the DNA-binding domain of p53, there are strong arguments to suggest that this region is essential for this activity: 1) the temperature-sensitivity of the conformation of the DNA binding domain of Xp53; 2) the specific binding of Xp53 to p73 only at high temperatures; 3) the specific inhibition of p73 activity by Xp53 at high temperatures; and 4) the body of literature indicating that the p53 DNA-binding domain is necessary for this interaction (25, 26).

Overall, our data indicate that the swapping experiments described here may be a useful approach to dissecting the various functions of the p53 protein and to provide a better understanding of the diverse properties of these proteins. This

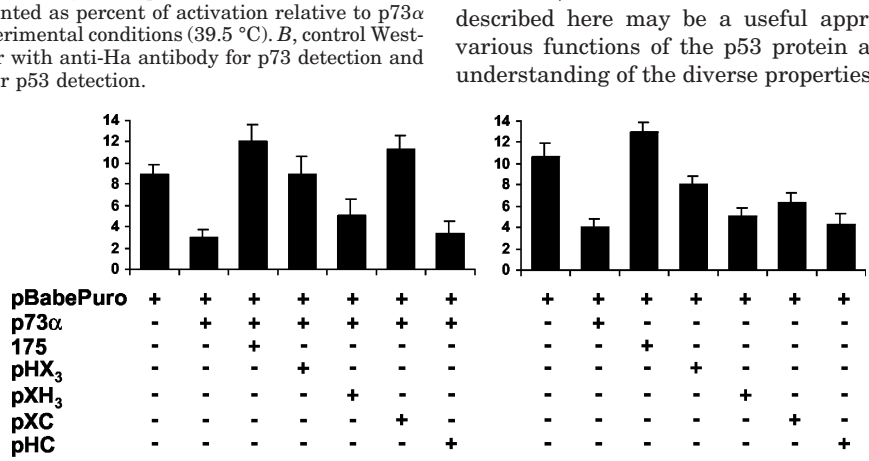


FIG. 8. **Hybrid p53 proteins with change of conformation restore p73-induced growth suppression.** Saos-2 cells were grown in 60-mm dishes and transfected with the indicated plasmids together with a selectable marker plasmid. The ratio between p73 expression plasmid (2 μg/transfection) and plasmids encoding for p53 proteins (6 μg/transfection) and its core domain was 1:3. An equal amount of pBabe-puro (0.5 μg/transfection) was added to each transfection. Cells were replated and selected with puromycin as described under "Experimental Procedures." The data shown represent the average number of colonies formed relative to the cells transfected with the marker alone. *Error bars* indicate S.D. of a representative experiment of three experiments performed in triplicate.

approach could be applied to fly p53, whose normal temperature is similar to *Xenopus*, and to the newly discovered p53 homologues such as p73 and p63. In recent studies, for example, Chen *et al.* produced and studied p53-p73 hybrids for identification of the p53 domain involved in murine double minute-dependent degradation (44).

Our data clearly demonstrate, for the first time, that only the conformational change in p53 is required for p73 binding and that the mutation *per se* is not the major determinant. This is an important issue when considering the heterogeneous behavior of mutant p53 in human tumors. If indirect inactivation of the p73 pathway by conformational mutant p53 is confirmed, this will have important clinical implications.

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REFERENCES

1. Levine, A. J. (1997) *Cell* **88,** 323–331
2. El-Deiry, W. S., Kern, S. E., Pientenpol, J. A., Kinzler, K. W., and Vogelstein, B. (1992) *Nat. Genet.* **1,** 45–49
3. Zhao, R., Gish, K., Murphy, M., Yin, Y., Notterman, D., Hoffman, W. H., Tom, E., Mack, D. H., and Levine, A. J. (2000) *Genes Dev.* **14,** 981–993
4. Yu, J., Zhang, L., Hwang, P. M., Rago, C., Kinzler, K. W., and Vogelstein, B. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 14517–14522
5. Soussi, T., and May, P. (1996) *J. Mol. Biol.* **260,** 623–637
6. Soussi, T., and Béroud, C. (2001) *Nat. Rev. Cancer* **1,** 233–240
7. Milner, J. (1995) *Trends Biochem. Sci.* **20,** 49–51
8. Milner, J. (1984) *Nature* **310,** 143–145
9. Gannon, J. V., Greaves, R., Iggo, R., and Lane, D. P. (1990) *EMBO J.* **9,** 1595–1602
10. Ory, K., Legros, Y., Auguin, C., and Soussi, T. (1994) *EMBO J.* **13,** 3496–3504
11. Legros, Y., Meyer, A., Ory, K., and Soussi, T. (1994) *Oncogene* **9,** 3689–3694
12. Michalovitz, D., Halevy, O., and Oren, M. (1990) *Cell* **62,** 671–680
13. Hainaut, P., Butcher, S., and Milner, J. (1995) *Br. J. Cancer* **71,** 227–231
14. Hupp, T. R., Meek, D. W., Midgley, C. A., and Lane, D. P. (1992) *Cell* **71,** 875–886
15. Hardy-Bessard, A. C., Garay, E., Lacronique, V., Legros, Y., Demarquay, C., Houque, A., Portefaix, J. M., Granier, C., and Soussi, T. (1998) *Oncogene* **16,** 883–890
16. Hupp, T. R., Sparks, A., and Lane, D. P. (1995) *Cell* **83,** 237–245
17. Cho, Y. J., Gorina, S., Jeffrey, P. D., and Pavletich, N. P. (1994) *Science* **265,** 346–355
18. Yang, A. N., Kaghad, M., Wang, Y. M., Gillett, E., Fleming, M. D., Dotsch, V., Andrews, N. C., Caput, D., and McKeon, F. (1998) *Mol. Cell* **2,** 305–316
19. Kaghad, M., Bonnet, H., Yang, A., Creancier, L., Biscan, J. C., Valent, A., Minty, A., Chalon, P., Lelias, J. M., Dumont, X., Ferrara, P., McKeon, F., and Caput, D. (1997) *Cell* **90,** 809–819
20. Yang, A., and McKeon, F. (2000) *Nat. Rev. Mol. Cell. Biol.* **1,** 199–207
21. Ikawa, S., Nakagawara, A., and Ikawa, Y. (1999) *Cell Death Differ.* **6,** 1154–1161
22. Levrero, M., De Laurenzi, V., Costanzo, A., Gong, J., Melino, G., and Wang, J. Y. (1999) *Cell Death Differ.* **6,** 1146–1153
23. Hibi, K., Trink, B., Patturajan, M., Westra, W. H., Caballero, O. L., Hill, D. E., Ratovitski, E. A., Jen, J., and Sidransky, D. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 5462–5467
24. Davison, T. S., Vagner, C., Kaghad, M., Ayed, A., Caput, D., and Arrowsmith, C. H. (1999) *J. Biol. Chem.* **274,** 18709–18714
25. Gaiddon, C., Lokshin, M., Ahn, J., Zhang, T., and Prives, C. (2001) *Mol. Cell. Biol.* **21,** 1874–1887
26. Strano, S., Munarriz, E., Rossi, M., Cristofanelli, B., Shaul, Y., Castagnoli, L., Levine, A. J., Sacchi, A., Cesareni, G., Oren, M., and Blandino, G. (2000) *J. Biol. Chem.* **275,** 29503–29512
27. Marin, M. C., Jost, C. A., Brooks, L. A., Irwin, M. S., O'Nions, J., Tidy, J. A., James, N., McGregor, J. M., Harwood, C. A., Yulug, I. G., Vousden, K. H., Allday, M. J., Gusterson, B., Ikawa, S., Hinds, P. W., Crook, T., and Kaelin, W. G., Jr. (2000) *Nat. Genet.* **25,** 47–54
28. Strano, S., Fontemaggi, G., Costanzo, A., Rizzo, M. G., Monti, O., Baccarini, A., Del Sal, G., Levrero, M., Sacchi, A., Oren, M., and Blandino, G. (2002) *J. Biol. Chem.* **272,** 18817–18826
29. Yuan, Z. M., Shioya, H., Ishiko, T., Sun, X. G., Gu, J. J., Huang, Y. Y., Lu, H., Kharbanda, S., Weichselbaum, R., and Kufe, D. (1999) *Nature* **399,** 809–813
30. Agami, R., Blandino, G., Oren, M., and Shaul, Y. (1999) *Nature* **399,** 806–809
31. Jost, C. A., Marin, M. C., and Kaelin, W. G. (1999) *Nature* **399,** 814–817
32. Soussi, T., Caron de Fromentel, C., Stürzbecher, H. W., Ullrich, S., Jenkins, J., and May, P. (1989) *J. Virol.* **63,** 3894–3901
33. Wang, Y., Farmer, G., Soussi, T., and Prives, C. (1995) *Oncogene* **10,** 779–784
34. Burge, C., and Karlin, S. (1997) *J. Mol. Biol.* **268,** 78–94
35. Le Bras, M., Delattre, V., Bensaad, K., Blandino, G., and Soussi, T. (2002) *Oncogene* **21,** 1304–1308
36. Ko, L. J., and Prives, C. (1996) *Genes Dev.* **10,** 1054–1072
37. Hansen, S., Hupp, T. R., and Lane, D. P. (1996) *J. Biol. Chem.* **271,** 3917–3924
38. Milner, J., and Medcalf, E. A. (1991) *Cell* **65,** 765–774
39. Bullock, A. N., Henckel, J., and Fersht, A. R. (2000) *Oncogene* **19,** 1245–1256
40. Flores, E. R., Tsai, K. Y., Crowley, D., Sengupta, S., Yang, A., McKeon, F., and Jacks, T. (2002) *Nature* **416,** 560–564
41. DiComo, C. J., Gaiddon, C., and Prives, C. (1999) *Mol. Cell. Biol.* **19,** 1438–1449
42. Waddell, S., Jenkins, J. R., and Proikas-Cezanne, T. (2001) *Oncogene* **20,** 6001–6008
43. Zhang, W., Guo, X. Y., Hu, G. Y., Liu, W. B., Shay, J. W., and Deisseroth, A. B. (1994) *EMBO J.* **13,** 2535–2544
44. Gu, J., Chen, D., Rosenblum, J., Rubin, R. M., and Yuan, Z. M. (2000) *Mol. Cell. Biol.* **20,** 1243–1253