Physical interaction of tumour suppressor p53/p73 with CCAAT-binding transcription factor 2 (CTF2) and differential regulation of human high-mobility group 1 (HMG1) gene expression

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The CCAAT-binding transcription factor (CTF)/nuclear factor I (NF-I) group of cellular DNA-binding proteins recognizes the sequence GCCAAT and is implicated in eukaryotic transcription, as well as DNA replication. Molecular analysis of human CTF/NF-I cDNA clones revealed multiple mRNA species that contain alternative coding regions, apparently as a result of differential splicing. Expression and functional analysis established that individual gene products can bind to GCCAAT recognition sites and serve as both promoter-selective transcriptional activators and initiation factors for DNA replication. The interaction between CTF2 and p53/p73 was shown to modulate their ability to regulate transcription of their respective target genes. In the present paper, we report that p53 down-regulates the activity of the high mobility group 1 (HMG1) gene promoter, whereas p73α up-regulates the activity of this promoter. Furthermore, CTF2 transactivates p53-induced p21 promoter activity, but inhibits p73α-induced p21 promoter activity. Using deletion mutants, we found that the DNA-binding domains of both p53 and p73α are required for physical interaction with CTF2 via the regions between amino acid residues 161 and 223, and 228 and 312 respectively. CTF2 enhances the DNA-binding activity of p53 and inhibits the DNA-binding activity of p73α. These results provide novel information on the functional interplay between CTF2 and p53/p73 as important determinants of their function in cell proliferation, apoptosis, DNA repair and cisplatin resistance.

Key words: apoptosis, cell-cycle arrest, DNA damage, tumour suppressor gene.

INTRODUCTION

The expression of genes transcribed by RNA polymerase II (class II genes) is mediated by a complex set of DNA–protein and protein–protein interactions [1]. The CCAAT-binding transcription factor (CTF)/nuclear factor I (NF-I) group of cellular DNA-binding proteins recognizes the sequence TTGGC(N_5)-GCCAA and is required for the efficient transcription and initiation of adenovirus replication [2–7]. The CTF/NF-I binding sites are present within the promoter of viral [8–10] and cellular [11–15] genes. The diversity of the CTF/NF-I protein family is generated by four distinct, but closely related, genes by differential splicing and post-translational modification by glycosylation [16,17]. CTF/NF-I forms stable homo- and heterodimers in the absence of DNA [18]. The growth conditions of cells affect the form of CTF/NF-I, suggesting that regulation of CTF/NF-I in cells is sensitive to changes in the cellular environment [19].

High-mobility group 1 (HMG1) is ubiquitously expressed in higher eukaryotes and preferentially binds to cisplatin-modified DNA [20–22]. We have previously shown that CTF2, one of the splice variants of CTF/NF-I, is overexpressed in cisplatin-resistant cells, and overexpression of this transcription factor might be responsible for the transactivation of the HMG1 genes [23]. Cellular levels of HMG1 may have an important role in directing the response of tumour cells to cisplatin.

Cellular response to DNA damage involves the tumour suppressor gene products p53/p73 [24–30]. These proteins accumulate in nuclei after DNA damage and control cell proliferation through their activity as transcription factors [29,31]. Although p53/p73-dependent apoptosis suggests how cisplatin kills tumour cells, the relationship between cisplatin sensitivity and cellular expression of p53/p73 remains unclear. The success of cisplatin-based chemotherapy may be due in part to apoptosis controlled by cellular levels of both DNA damage recognition proteins and tumour suppressors. In the present study, we report that HMG1 gene expression is differentially regulated by tumour suppressors via their physical interaction with CTF/NF-I.

EXPERIMENTAL

Cell lines

The Saos-2 human osteosarcoma cell line and the COS-1 monkey kidney cell line were grown in Dulbecco's modified Eagle's medium (Nissui, Tokyo, Japan), the A2780 human ovarian cancer cell line was grown in RPMI 1640 (Nissui), and the PC3 human prostate cancer cell line was grown in Eagle's minimal essential medium, supplemented with 10% foetal bovine serum. These cell lines were maintained in a 5% CO_2 atmosphere at 37 °C.

Abbreviations used: CTF, CCAAT-binding transcription factor; DTT, dithiothreitol; ECL®, enhanced chemiluminescence; EMSA, electrophoretic mobility shift assay; GST, glutathione S-transferase; HA, haemagglutinin; HMG, high-mobility group; NF-I, nuclear factor I; NP40, Nonidet P40; TBE, Tris/borate/EDTA; TBP, TATA-binding protein; TNT, transcription and translation; YB-1, Y-box-binding protein 1.

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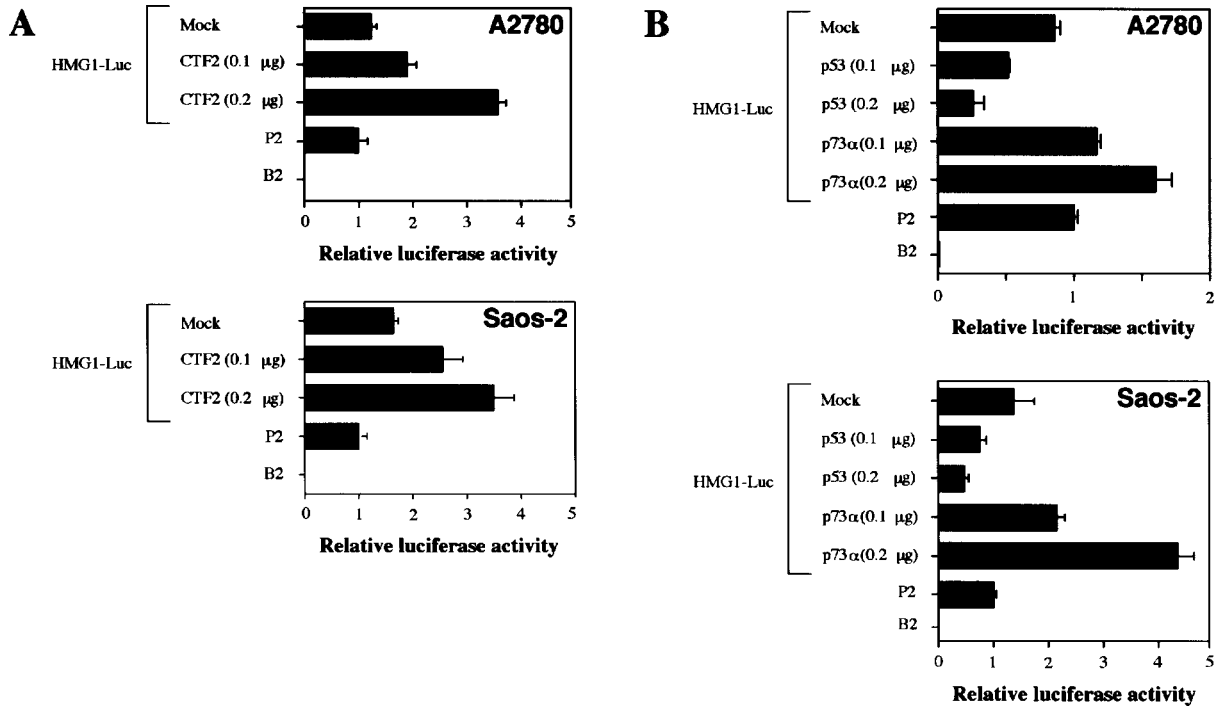


Figure 1 p53 and p73α differentially regulate the CTF2-dependent HMG1 promoter activity

(**A**) HMG1-Luc was transiently transfected into A2780 or Saos-2 cells with increasing amounts of a CTF2-expression plasmid, along with a β-galactosidase reporter gene (pCH110) as an internal control. A pGL3 promoter vector (P2) containing a simian virus 40 (SV40) promoter and a pGL3 basic vector (B2) lacking promoter were also transfected to normalize luciferase activity. Results are means ± S.D. of promoter activity for triplicate samples, relative to the luciferase activity of the cell lysates transfected with P2, which was set at 1. (**B**) HMG1-Luc was transiently transfected into A2780 or Saos-2 cells with increasing amounts of a p53/p73α-expression plasmid, along with a β-galactosidase reporter gene (pCH110) as an internal control. The luciferase activity and their values are as for (**A**).

Antibodies

Anti-haemagglutinin (anti-HA; clone F-7) monoclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Anti-HA–peroxidase (clone 3F10) and anti-FLAG (clone M2) monoclonal antibodies were purchased from Roche Molecular Biochemicals (Mannheim, Germany) and Sigma Chemical Co. (St. Louis, MO, U.S.A.) respectively. Antibodies against p53 and PAb421 were purchased from Calbiochem (San Diego, CA, U.S.A.). Anti-Thio monoclonal antibody was purchased from Invitrogen (Groningen, The Netherlands).

Plasmid constructs

HMG1-Luc plasmid, an *Xho*I/*Eco*NI fragment including nt −506 to +147 of the HMG1 promoter utilized for the luciferase assay has been described previously in [23]. p21-Luc plasmid, utilized for the luciferase assay, has been described previously in [32]. Following the synthesis of cDNA, full-length human p53 cDNA was amplified using the primer pair, 5′-CCATGGAGGAGCCGCAGTCAGATCC-3′ and 5′-GAAGTGGAGAATGTCAGTCTGAGTCAGGCCC-3′. The PCR product was cloned into pGEM-T Easy (Promega, Madison, WI, U.S.A.). For construction of HA–p53, plasmid DNA was purified and digested with *Not*I, and full-length p53 cDNA was cloned into N-terminal HA-tagged pcDNA3 (Invitrogen) for expression in mammalian cells. Full-length ThioHis–p53, ThioHis–p53 deletion mutants N376, N362, N322, C224 and 161/223, and glutathione S-transferase (GST)–p53, expressed in *Escherichia coli*, were described in [32]. ThioHis–p53 deletion mutants were constructed from the ThioHis–p53 full-length plasmid by digestion with *Eco*81I for N223, *Nco*I for N160 and *Ava*II for C60. The pertinent restriction endonuclease sites for all deletion mutants are shown in Figure 3(A). ThioHis–p53 161/223 was cloned by PCR using the primer pair, 5′-GGTACCGGCCATCTACAAGCAGTCACAGC-3′ and 5′-TGATCAAGGCGGCTCATAGGGCACCACC-3′. To prepare the FLAG- or HA-tagged fusion protein expressed in *E. coli*, ThioHis–FLAG vector and ThioHis–HA vector were prepared by digesting the pThioHis vector (Invitrogen) with *Nde*I and *Acc*65I, self-ligating after T4 DNA polymerase treatment to delete the thioredoxin, and inserting the following double-stranded oligonucleotides: for FLAG, 5′-ATGGACTACAAGGACGATGATGACAAGGGC-3′, and for HA, 5′-ATGGGTTATCCGTATGATGTTCCTGATTATGCTAGCCTCGGT-3′. N-terminal HA-tagged pcDNA3-HA–p73α expressed in mammalian cells was kindly provided by Dr G. Melino, University of Rome, Italy [33]. HA–p73α expressed in *E. coli* was obtained to ligate the HA–p73α fragment to the ThioHis vector in which thioredoxin was deleted previously. HA–p73α deletion mutants Δ545–636, Δ396–636, Δ313–636, and Δ228–636, expressed in *E. coli*, were obtained to delete the fragment of ThioHis–HA–p73α from the *Sda*I, *Bfm*I, *Eco*O109I and *Psy*I sites to the C-terminus respectively, with complete or partial digestion. The pertinent restriction endonuclease sites are shown in Figure 4(A). ThioHis–p73 228/312 was cloned by PCR using the primer pair, 5′-GGATCCTG-

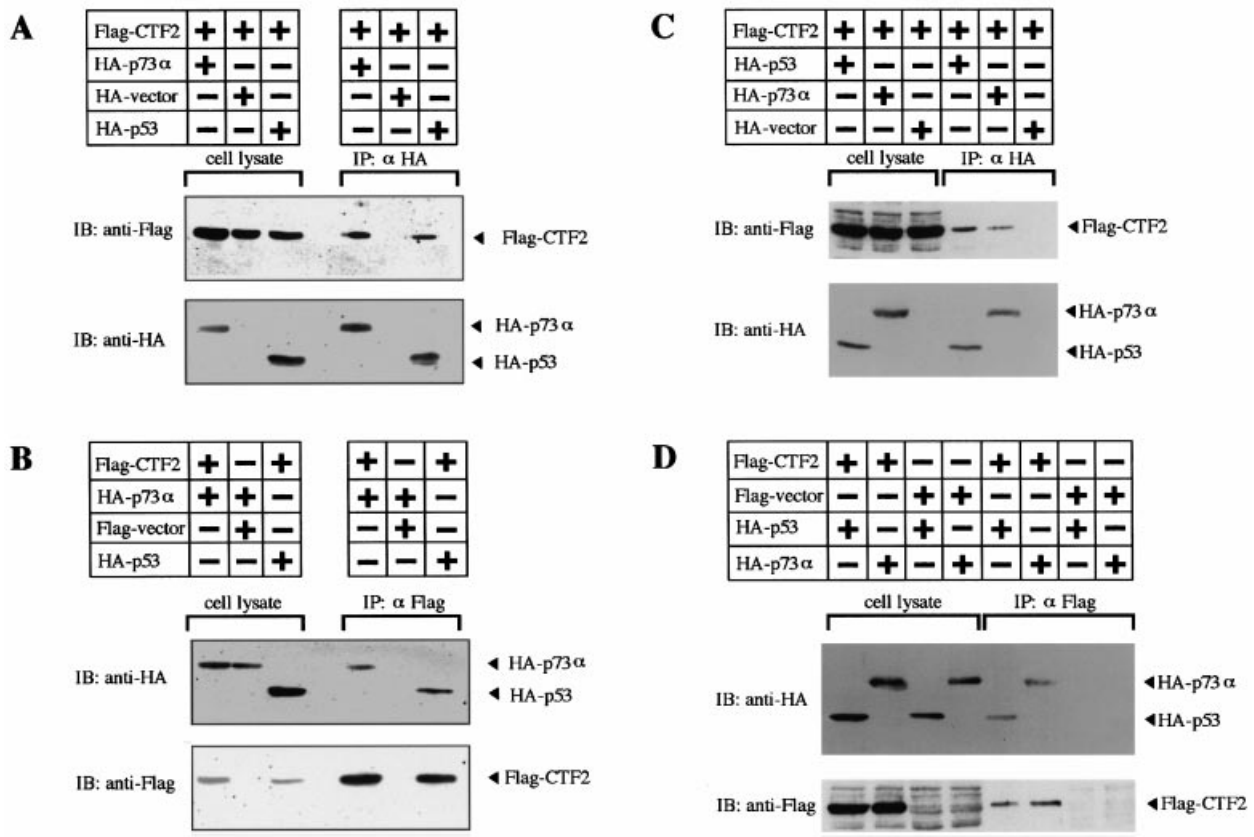


Figure 2 CTF2 interacts with p53/p73α

Whole-cell extracts were prepared from COS-1 (**A** and **B**) or PC3 (**C** and **D**) cells transfected with FLAG–CTF2, HA–p53, HA–p73α or empty vector and immunoprecipitated (IP) with 2 μg of anti-HA or anti-FLAG antibody, and the resulting immunocomplex and 10% of the input were subjected to SDS/PAGE and analysed by Western blotting (IB) with anti-HA and anti-FLAG (M2) antibodies.

ACCCTGTCACCGGCAGGCAG-3′ and 5′-TGATCACTGC-TCCCGGTAGTGGTCCTCATC-3′. Following synthesis of cDNA, full-length human CTF2 cDNA was amplified using the primer pair, 5′-GCAGCGCCATGGATGAGTTCCACCC-3′ and 5′-GGCGAGGGAAGAAGACCTTTGCTATCCC-3′. The PCR product was cloned into pGEM-T Easy. For construction of FLAG–CTF2, plasmid DNA was purified and digested with *Eco*RI, and full-length CTF2 cDNA was cloned into N-terminally FLAG-tagged pcDNA3 (Invitrogen) for expression in mammalian cells. For construction of GST–CTF2, plasmid DNA was purified and digested with *Eco*RI, and full-length CTF2 cDNA was cloned into pGEX-4T (Pharmacia Biotech Inc., Tokyo, Japan). GST–CTF2 deletion mutants were constructed from the GST–CTF2 full-length plasmid by digestion with *Bgl*II for N407, *Bgl*I for N285, *Xcm*I for N245, *Eco*NI for N150, and *Bpu*1102I for N84 and C84, as shown in Figure 5(A).

Promoter reporter assay (DNA transient transfection and luciferase assay)

For transient transfections, Saos-2 cells were plated at a density of 5×10^4 cells/well on the day before transfection. At approx. 50% confluence, the cells were co-transfected with 0.2 μg of reporter plasmid, 0.1–0.4 μg of expression plasmid and 0.3 μg of pCH110 (a β-galactosidase expression plasmid; Promega) using 2 μl of SuperFect® (Qiagen, Hilden, Germany) according to the manufacturer's protocol. After 3 h, the cells were washed twice with PBS and incubated in fresh medium. Each expression plasmid and reporter plasmid were standardized individually to a molar ratio of 1:1, and the total amount of DNA per well was adjusted to 1.0 μg by the addition of a mock DNA plasmid. After 48 h, the cells were lysed with 100 μl/well of reporter lysis buffer (Promega). After centrifugation at 10000 ***g*** for 2 min, the luciferase activity in the resulting supernatants was assayed using a Picagene kit (Toyoinki, Tokyo, Japan), as described in [34]. The light intensity was measured for 15 s with a luminometer (Dynatech ML 1500, JEOL, Tokyo, Japan). All cells were co-transfected with pCH110 as a control for transfection efficiency, and the β-galactosidase activity was measured using an Aurora GAL-XE kit (Costa Mesa, CA, U.S.A.) and was expressed as a ratio of the corrected luciferase activity of cells co-transfected with vector. Results were normalized to β-galactosidase activity and were representative of at least three independent experiments.

In vivo binding assay (transient transfection and co-immunoprecipitation assay)

COS-1 cells were seeded in six-well plates at a density of 1×10^5 cells/well. On the following day, the cells were co-transfected with 1.5 μg of FLAG–CTF2 and HA–p53 or HA–p73α expression plasmid with 6 μl of SuperFect® according to the manufacturer's protocol. At 3 h after transfection, the cells

were washed with PBS and the medium was replaced with fresh medium. After 48 h, cells were washed twice with PBS and lysed in Binding Buffer X containing 50 mM Tris/HCl, pH 8.0, 1 mM EDTA, 120 mM NaCl, 0.5 % (v/v) Nonidet P40 (NP40), 10 % (v/v) glycerol and 1 mM PMSF. After incubating for 30 min on ice, the lysates were centrifuged at 21 000 ***g*** for 10 min at 4 °C. The supernatants (1 mg) were incubated with 2 μg of anti-HA (F-7) antibody or anti-FLAG (M2) antibody for 60 min at 4 °C and the beads were washed three times with Binding Buffer X. The immunoprecipitated samples and starting material were separated by SDS/PAGE (10 % gels), and blotted on to a PVDF membrane. The membranes were immunoblotted with anti-FLAG (M2) antibody or anti-HA–peroxidase and developed using an enhanced chemiluminescence (ECL®) kit (Amersham Biosciences, Uppsala, Sweden).

Expression of GST, HA, and FLAG fusion proteins in *E. coli*

Expression of recombinant GST, HA, and FLAG fusion proteins was induced by 1 mM isopropyl 1-thio-β-D-galactopyranoside (Boehringer Mannheim, Mannheim, Germany) for 1 h at 25 °C as described in [34]. The bacteria were collected by centrifugation at 3000 ***g*** for 10 min at 4 °C, before lysis in Binding Buffer X and subsequently sonication (TAITEC sonicator, Tokyo, Japan) for 10 s at 4 °C. After incubation on ice for 30 min, the lysates were centrifuged at 21 000 ***g*** for 15 min at 4 °C and the supernatants were stored at −80 °C until use.

In vitro binding assay (pull-down assay)

GST and full-length GST–CTF2 or its deletion mutants were immobilized on glutathione–Sepharose beads for 1 h at 4 °C. After the immobilized GST fusion proteins were washed three times with Binding Buffer X, soluble ThioHis–p53 or its deletion mutants, HA–p73α or its deletion mutant fusion proteins, were added with 1 mM dithiothreitol (DTT), and were incubated further for 2 h at 4 °C. The binding samples were washed three times with Binding Buffer X and separated by SDS/PAGE (10 % gel), transferred on to a PVDF membrane, immunoblotted with anti-HA–peroxidase or anti-ThioHis antibody, and developed by ECL® as described above.

Purification of GST fusion proteins and *in vitro* translation reactions

To prepare the purified GST fusion proteins of GST–CTF2 or its deletion mutants, GST fusion proteins immobilized on glutathione–Sepharose beads were eluted with elution buffer containing 50 mM Tris/HCl at pH 8.0 and 20 mM GSH, according to the manufacturer's protocol (Amersham Biosciences). The identity of each GST fusion protein was verified by Coomassie Blue (R-250) staining of a gel following SDS/PAGE, and the concentration of each protein was equalized with GST elution buffer. FLAG–CTF2, HA–p53 and HA–p73α proteins were made by using a coupled transcription and translation (TNT) system (Promega). Briefly, 0.5 μg of DNA was added directly to 20 μl of TNT rabbit reticulocyte lysate with 0.5 μl of methionine, and reactions were carried out at 30 °C for 90 min. The translated products were stored at −80 °C until use.

Preparation of nuclear extracts

Nuclear extracts were prepared as described in [35]. Briefly, 2×10^7 cells were collected with PBS, resuspended in 2 ml of ice-cold 10 mM Hepes/KOH (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT and 0.5 mM PMSF, and incubated on ice for 15 min. The cells were lysed by adding NP40 to a final concentration of 0.5 % (v/v), and the lysate was centrifuged at 500 ***g*** for 10 min. The resulting nuclear pellet was resuspended in 300 μl of ice-cold 20 mM Hepes/KOH (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT and 1 mM PMSF, and incubated for 15 min on ice with frequent gentle mixing. Following centrifugation at 21 000 ***g*** for 5 min at 4 °C in a microcentrifuge to remove insoluble material, the supernatant (nuclear fraction) was stored at −80 °C until use. Protein concentrations were determined by the method of Bradford [36].

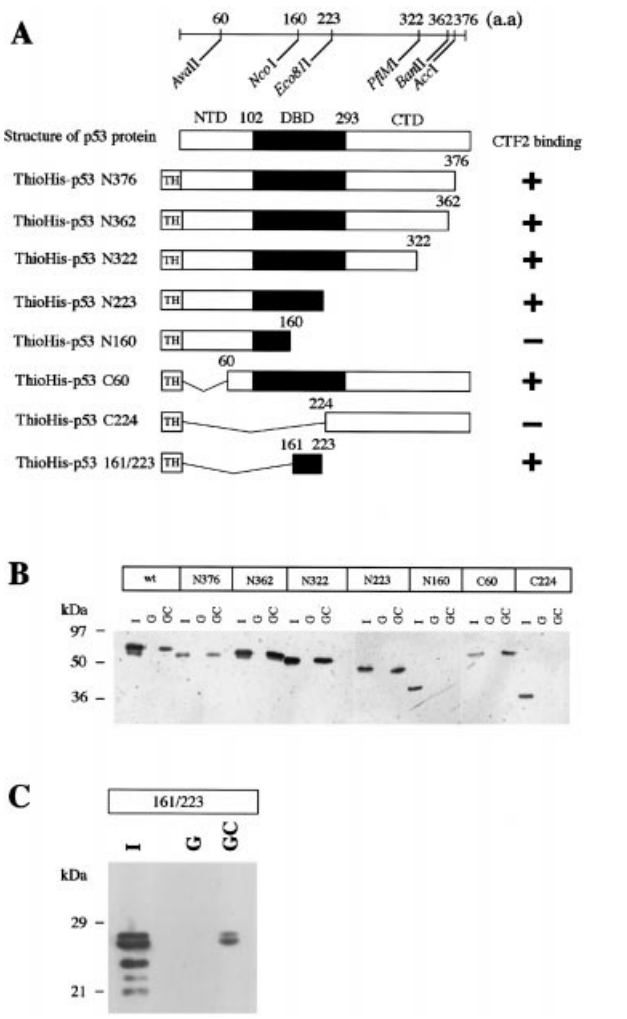


Figure 3 Identification of the CTF2-binding domain in p53

(**A**) Schematic illustration of the ThioHis–p53 deletion mutants. Human p53 protein contains: NTD, N-terminal domain (amino acids 1–101); DBD, DNA-binding domain (amino acids 102–293); CTD, C-terminal domain (amino acids 294–393). (**B**) Interaction of the p53 deletion mutants with GST–CTF2. GST alone (G) and GST–CTF2 (GC) fusion proteins were immobilized on 15 μl of glutathione–Sepharose 4B, and the resin was incubated with a 20-fold excess of ThioHis–p53 (wt) or its deletion mutants. The bound protein samples and 5% of the input (I) were analysed by Western blotting using an anti-Thio antibody. (**C**) Interaction of amino acid residues 161–223 of p53 with GST–CTF2. GST alone (G) and GST–CTF2 (GC) fusion proteins were incubated with a 20-fold excess of the ThioHis–p53 161/223, and analysed by Western blotting using an anti-Thio antibody as described above.

Electrophoretic mobility shift assay (EMSA)

Double-stranded oligonucleotides for the CTF/NF-I consensus binding site and p53 consensus binding site were used as probes.

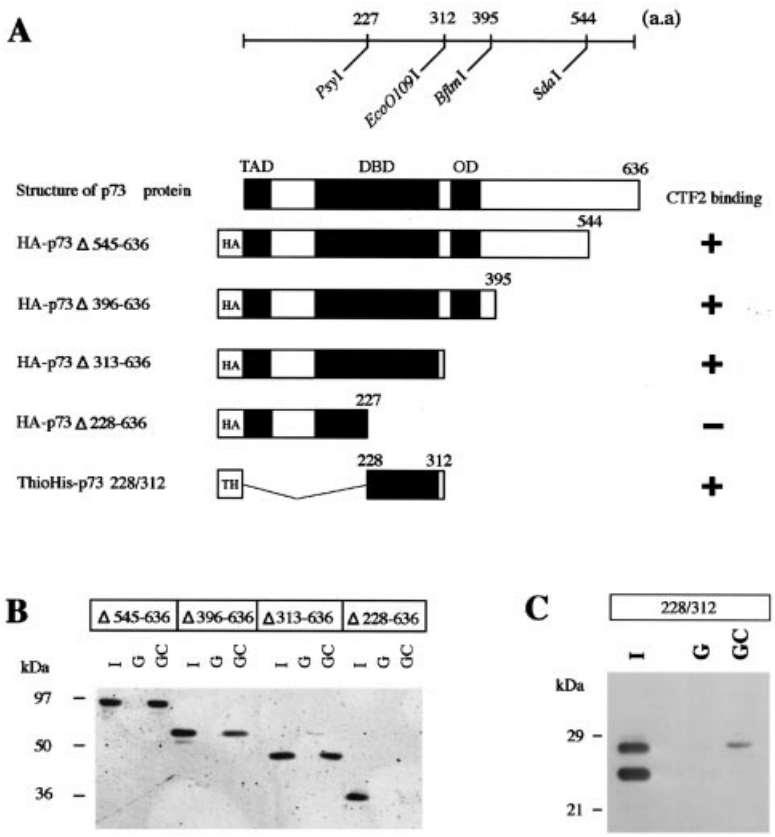


Figure 4 Identification of the CTF2-binding domain in p73α

(**A**) Schematic illustration of the p73α deletion mutants. Human p73α protein contains: DBD, a DNA-binding domain (amino acids 131–310); and OD, an oligomerization domain (amino acids 345–380). The amino acids 1–54 correspond to the transactivation domain (TAD). (**B**) Interaction of the p73α deletion mutants with GST–CTF2. GST alone (G) and GST–CTF2 (GC) fusion proteins were immobilized on 15 μl of glutathione–Sepharose 4B and the resin was incubated with a 20-fold excess of the HA–p73α deletion mutants. The bound protein samples and 5% of the input (I) were analysed by Western blotting using an anti-HA antibody. (**C**) Interaction of amino acid residues 228–312 of p73 with GST–CTF2. GST alone (G) and GST–CTF2 (GC) fusion proteins were incubated with a 20-fold excess of the ThioHis–p73 228/312 and analysed by Western blotting using an anti-Thio antibody as described for Figure 3(C).

The sequences were CTF/NF-I consensus, 5′-GGCTTTGGATTGAAGCCAATATGAGG-3′ [23] and p53 consensus, 5′-TCAGGAACATGTCCCAACATGTTGAGCT-3′ [32]. The probes were labelled with [γ-^{32}P]ATP using T4 polynucleotide kinase and purified on 15% polyacrylamide gels in 1 × Tris/borate/EDTA (TBE) buffer. EMSA was performed as described in [34]. Briefly, eluted GST fusion proteins and 4 ng of radiolabelled oligonucleotides were mixed in reaction buffer containing 25 mM Hepes at pH 7.5, 50 mM KCl, 0.5 mM EDTA, 5% (v/v) glycerol, 10 mM DTT, 0.1% (v/v) NP40, and 0.5 mg/ml BSA, and incubated for 20 min at room temperature (25 °C). Binding reactions were analysed on a 4% polyacrylamide gel in 0.5 × TBE buffer, followed by autoradiography as described previously in [34]. For competition experiments or supershift assay, preincubation was performed in the presence of 10–30 × unlabelled competitor DNA or 0.5 μg of anti-HA antibody for 15 min at 20°C, before the addition of radiolabelled oligonucleotides.

RESULTS

p53 and p73α differentially regulate the HMG1 promoter

The HMG1 gene is often up-regulated in cisplatin-resistant cell lines [23]. The cellular expression of p73 and mutants of p53 correlates with cisplatin sensitivity [37–42]. Since both p53 and p73 function as transcription factors, we tested the effect of p53/p73α on HMG1 promoter activity. To show that CTF2 is responsible for HMG1 promoter activity, we utilized A2780 and Saos-2 cells, which express low levels of CTF2 [23]. We co-transfected the HMG1 promoter–luciferase reporter gene along with increasing amounts of CTF2-expression plasmids. CTF2 was able to activate HMG1-promoter activity in a dose-dependent manner in both A2780 and Saos-2 cells (Figure 1A). To show that p53 or p73α is responsible for HMG1 promoter activity, we utilized Saos-2/p53-null cells and A2780/p53 wild-type cells, which also express low levels of p73α (results not shown). We co-transfected the HMG1 promoter–luciferase reporter gene along with increasing amounts of p53 or p73α expression plasmids. p73α was able to activate HMG1-promoter activity in a dose-dependent manner, but p53 down-regulated promoter activity in a dose-dependent manner in both Saos-2 and A2780 cells (Figure 1B).

CTF2 interacts with p53/p73α

To confirm that p53/p73α interacts with CTF2 to affect its function *in vivo*, we performed co-immunoprecipitation assays using COS-1 and PC3 cells. Because p73α is expressed at low levels in cancer cell lines [43], we transiently transfected an

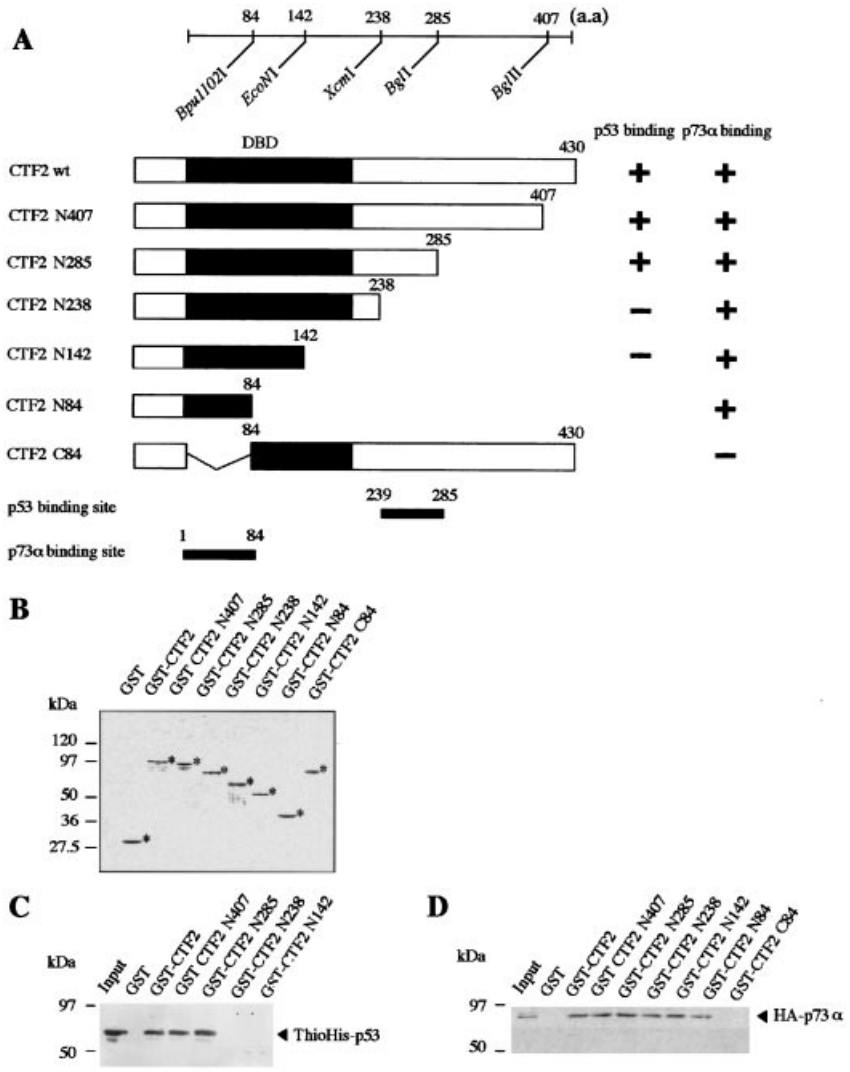


Figure 5 Identification of the p53/p73α binding domain in CTF2

(**A**) Schematic illustration of GST–CTF2 (wt) and its deletion mutants. Human CTF2 protein contains DBD, a DNA binding domain (amino acids 1–220). (**B**) Purification of the recombinant proteins. Recombinant proteins were expressed in bacteria, purified on 15 μl of glutathione–Sepharose 4B and analysed by Coomassie Blue staining after SDS/PAGE. Asterisks indicate the full-length GST or GST–CTF2 protein or its deletion mutant proteins. (**C**) Interaction of GST–CTF2 and its deletion mutants with ThioHis–p53. GST alone and GST–CTF2 or its deletion mutant fusion proteins were immobilized on 15 μl of glutathione–Sepharose 4B, and the resin was incubated with a 20-fold excess of the ThioHis–p53 protein. The bound protein samples and 5% of the input were analysed by Western blotting using an anti-Thio antibody. (**D**) Interaction of CTF2 and its deletion mutants with HA–p73α. GST alone and GST–CTF2 or its deletion mutant fusion proteins were immobilized on 15 μl of glutathione–Sepharose 4B, and the resin was incubated with a 20-fold excess of the HA–p73α protein. The bound protein samples and 5% of the input were analysed by Western blotting using an anti-HA antibody.

expression plasmid of p73α fused to an HA-tag. COS-1 cells were transfected with expression constructs for both HA–p53 or HA–p73α and CTF2 fused to a FLAG-tag because an effective anti-CTF2 antibody for immunoprecipitation is not available at the present time. Whole-cell extracts were immunoprecipitated with antibody against either HA or FLAG. Complexes that immunoprecipitated with anti-HA antibody included FLAG–CTF2 (Figure 2A). Reciprocally, the complexes that immunoprecipitated with anti-FLAG antibody also contained HA–p53/p73α (Figure 2B). Furthermore, to confirm *in vivo* interaction, we employed another cancer cell line, prostate PC3 cells. The results again demonstrate the ability of CTF2 and p53/p73α proteins to form complexes *in vivo* (Figures 2C and 2D).

To identify the CTF2-binding sites of p53, we used an *in vitro* GST pull-down assay. Bacterially expressed ThioHis–p53 with various constructs (Figure 3A) were incubated with GST–CTF2. Pull-down assays demonstrated that p53 interacts with GST–CTF2 through the region between amino acid residues 161 and 223 (Figure 3B). The region between amino acid residues 161 and 223 of p53 alone can bind to CTF2 (Figure 3C).

To identify the CTF2-binding sites of p73α, bacterially expressed HA–p73α with various constructs (Figure 4A) were incubated with GST–CTF2. Pull-down assays demonstrated that p73α interacts with GST–CTF2 through the region between amino acid residues 228 and 312 (Figure 4B). The region between amino acid residues 228 and 312 of p73 alone can bind to CTF2 (Figure 4C).

Using various deletion constructs for CTF2 (Figures 5A and 5B), the interaction domain of CTF2 with p53/p73α was identified. As shown in Figure 5(C), p53 interacted with CTF2

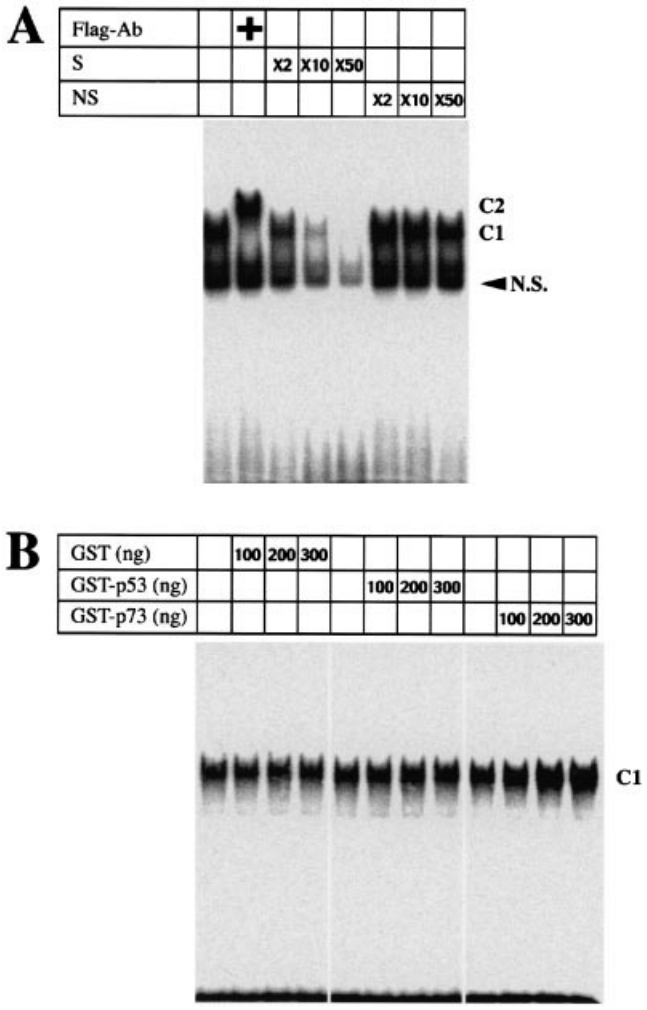


Figure 6 p73α stimulates CTF2 binding to DNA

FLAG–CTF2 translated using the *in vitro* TNT system (**A**) and FLAG–CTF2 translated *in vitro* with GST fusion proteins (**B**) were incubated with double-stranded oligonucleotides containing CTF/NF-I consensus elements, and EMSA was performed. C1, CTF2–DNA complex formed; C2, anti-HA antibody–CTF2–DNA complex formed, S, unlabelled CTF/NF-I consensus element oligonucleotide used as a specific competitor; NS, unlabelled E-box on the YB-1 promoter used as a non-specific competitor. A2-, 10- and 50-fold molar excess of the unlabelled oligonucleotides was added in the competition assay.

between amino acid residues 239 and 285. As shown in Figure 5(D), p73α interacted with CTF2 between amino acids residues 1 and 84.

p73α, but not p53, enhances DNA binding of CTF2

Our binding assays support a model in which p53/p73α may stimulate or stabilize CTF2 functions (such as its DNA-binding activity) by directly interacting with p53/p73α. In order to examine the ability of p53/p73α to enhance CTF2 DNA-binding activity, we used FLAG–CTF2 translated *in vitro* for EMSA using a CTF2 consensus oligonucleotide because GST–CTF2 does not bind to DNA. To evaluate the influence of p53/p73α on the DNA binding of CTF2, increasing amounts of p53/p73α were added to the DNA-binding reaction. p73α enhanced the DNA binding of CTF2, but p53 did not (Figure 6). Addition of p73 to the CTF2 DNA-binding reaction did not alter the electrophoretic mobility of the complex, indicating that interaction is unstable and that p73 may dissociate from the CTF2–DNA complex during electrophoresis.

Effect of CTF2 on both p53 and p73α DNA binding

Since the CTF2-binding site in p53/p73α is located in the DNA-binding domain (Figures 3B and 4B), we next examined the effect of CTF2 on the sequence-specific binding activity of p53/p73α. Although GST–p53/p73α did not bind to DNA, *in vitro* translated p73α did bind to DNA very well. Sequence-specific DNA-binding activity of the p53 was stimulated in the presence of PAb421, an anti-p53 monoclonal antibody. Thus we used *in vitro* translated p53 with PAb421 and p73α for EMSA. The DNA-binding activity of p53 was markedly increased by the addition of CTF2 in the reaction mixture (Figure 7A). CTF2 did not alter the electrophoretic mobility of the p53–DNA complex again. In contrast, the DNA-binding activity of p73α was markedly decreased by the addition of CTF2 in the reaction mixture (Figure 7B).

CTF2 increases the p21 promoter activity activated by p53, but decreases promoter activity activated by p73α, in Saos-2 cells

We co-transfected the p21 promoter–luciferase reporter gene along with increasing amounts of p53 or p73α expression plasmids into cell lines. p53 and p73α were able to activate p21 promoter activity in a dose-dependent manner in Saos-2 cells, but CTF2 was not. Interestingly, CTF2 was able to increase the p21-promoter activity activated by p53, but decreased its promoter activity when activated by p73α in Saos-2 cells (Figure 7C). Thus there is a functional interplay between CTF2 and p53/p73.

DISCUSSION

Although many cellular proteins bind to cisplatin-modified DNA, the first such protein to be identified belongs to the HMG family of non-histone chromosomal proteins [34,44–47]. HMG1 is often overexpressed in cisplatin-resistant cell lines [23]. One report demonstrated that the HMG1 gene was up-regulated by oestrogen treatment of MCF-7 cells, and treatment of MCF-7 cells with oestrogen followed by exposure to cisplatin caused a 2-fold increase in sensitivity to the drug [48]. The relationship at the cellular level of HMG1 and cisplatin sensitivity remains to be solved. We have previously shown that the HMG1 gene is up-regulated at the transcriptional level and that this probably occurs through the enhanced expression of CTF/NF-I [23].

The CTF/NF-I family of ubiquitous transcription factors was initially discovered as part of an adenovirus DNA replication complex [2–7], but was later found to be involved in the transcriptional regulation of various cellular genes [11–15]. There are four different CTF/NF-I genes [16] and various isoforms of each gene, generated by alternative splicing, have been identified [7]. We have previously found that CTF2 is up-regulated in cisplatin-resistant cells [23].

On the other hand, the tumour suppressors p53 and p73 have been shown to modulate cellular sensitivity against anti-cancer agents. Cancer cells carrying mutant p53 [37–41] or those overexpressing p73 [42] are less sensitive to anti-cancer agents. Thus p53 or p73 might modulate the expression of drug-resistance-related genes [31,49]. We have now demonstrated that a physical interaction between CTF2 and tumour suppressor gene products is essential for the reciprocal regulation of their target genes in transient transfection systems (Scheme 1). How-

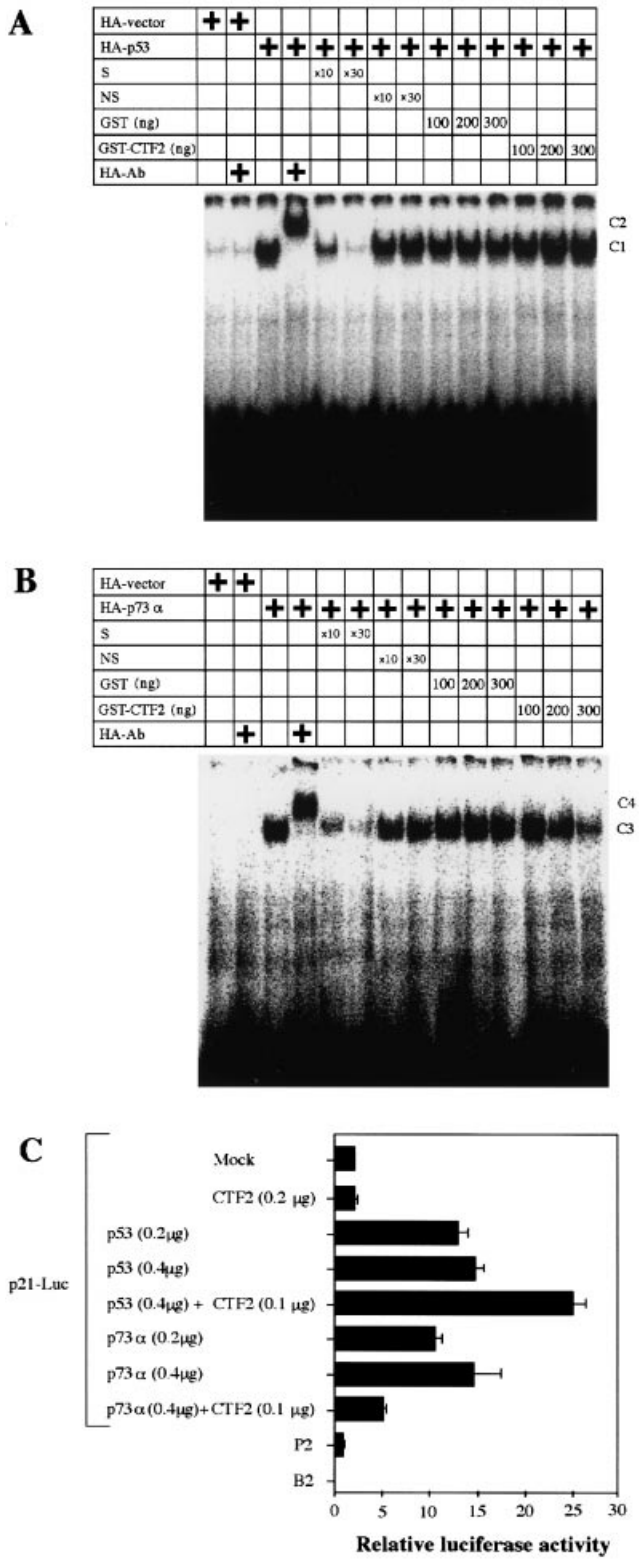
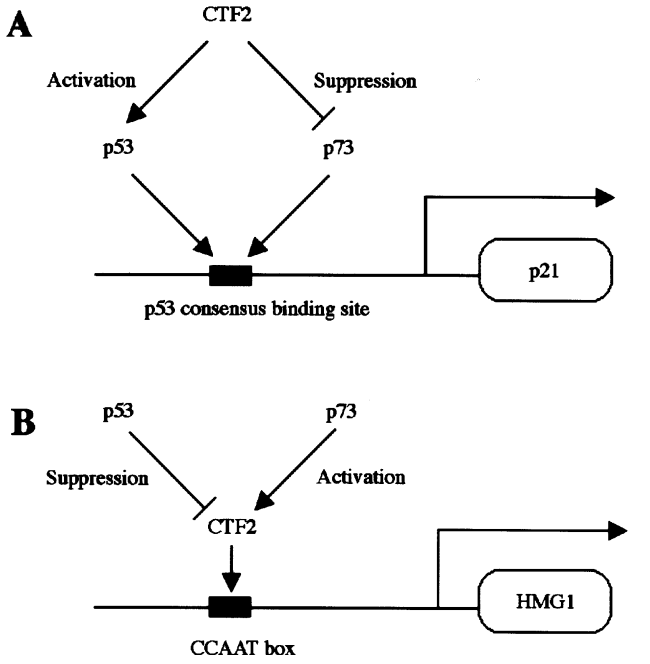


Figure 7 CTF2 stimulates p53 binding to DNA and inhibits p73α binding to DNA, and CTF2 increases the p21 promoter activity activated by p53, but decreases its promoter activity activated by p73α in Saos-2 cells

(**A**) HA or HA–p53 translated using the Promega *in vitro* TNT system with Pab421 were incubated with double-stranded oligonucleotides containing p53 consensus elements on the p21 promoter with GST alone and GST fusion proteins, and EMSA was performed. C1, p53–Pab421–DNA complex formed; C2, anti-HA antibody–p53–Pab421–DNA complex formed; S, unlabelled p53 consensus element on the p21 promoter oligonucleotide used as a specific competitor; NS, unlabelled E-box element on the YB-1 promoter oligonucleotide used as a non-specific competitor. The competition assay was performed as described in the legend to Figure 6. (**B**) HA or HA–p73α translated using the Promega *in vitro* TNT system were incubated with double-stranded oligonucleotides containing p53 consensus elements on the p21 promoter with GST alone and GST fusion proteins, and EMSA was performed. C3, p73–DNA complex formed; C4, anti-HA antibody–p73–DNA complex formed. The competition assay was performed as described above. (**C**) p21-Luc was transiently transfected into Saos-2 cells with increasing amounts of a p53/p73α-expression plasmid and a CTF2-expression plasmid, along with a β-galactosidase reporter gene (pCH110) as an internal control. The luciferase activity and their values are as described in the legend to Figure 1. A pGL3 promoter vector (P2) containing a simian virus 40 (SV40) promoter and a pGL3 basic vector (B2) lacking promoter were also transfected to normalize luciferase activity.



Scheme 1 Model of reciprocal regulation of target gene expression through the interaction of p53/p73 with CTF2

Reciprocal regulation of p21 (**A**) and HMG1 (**B**) gene expression through the interaction of p53/p73 with CTF2.

ever, it is difficult to show whether interactions of CTF2 with p53/p73 can modulate endogenous gene expression, because these transcription factors may interact with other molecules in nuclei. The results of our transfection experiments in Saos-2 cells are in agreement with those of EMSA. Although p53 down-regulates the HMG1 promoter, p53 has no inhibitory activity for CTF2 DNA binding. p53 has been shown to interact with basal transcriptional factors, such as TATA-binding protein (TBP) [50]. p53 might inhibit the interaction of TBP or a cofactor with CTF2.

The DNA-binding domain of both p53 and p73α is involved in CTF2 binding; this region is highly conserved. There is significant similarity of a nine-amino-acid stretch in the CTF2-binding region between p53 and p73α. However, CTF2 inhibits DNA binding of p53 and enhances the DNA binding of p73α, suggesting that p53 and p73 interact with CTF2 through a different binding region. As expected, we confirmed that the binding region of p53 is completely different from that of p73. We have previously shown that Y-box-binding protein 1 (YB-1) interacts with the

C-terminus of p53 and that this interaction inhibits the DNA binding of p53 [32]; however, it is hard to explain this intriguing observation.

The CTF2-binding region of p53 is identical with the 'hot spot' of the mutation in cancer cells. It would be of interest to examine whether CTF2 interacts with mutant p53. The functional interaction between p53/p73 and CTF/NF-I has not been described previously. Both p53/p73 and CTF/NF-I have been implicated in cell-specific expression [19]. Furthermore, there are four different CTF/NF-I genes with several splice variants of each gene [16]. The 220-amino-acid and N-terminal regions of the CTF/NF-I proteins are highly conserved and are responsible for DNA binding [51], indicating that p73 can interact with all of the CTF/NF-I proteins, but that p53 cannot.

DNA-damage-recognition proteins, such as HMG1 [52] and YB-1 [34], interact with p53. However, CTF2 does not bind to cisplatin-modified DNA (results not shown). CTF2 can serve as both a transcription factor and an initiator of DNA replication and is overexpressed in cisplatin-resistant cells. These data may provide a simple explanation for the co-ordinate involvement of CTF2 in both transcription and DNA repair. Another possibility is that overexpression of CTF2 may be required to stimulate DNA replication in cisplatin-resistant cells in which the growth rate is significantly reduced.

It is interesting that CTF2 differentially regulates the p53/p73-dependent activation of the p21 promoter (Figure 7C). Multiple mRNA species encoding NF-I family proteins have been detected from various tissues [11–15]. Cell-specific differences in both the form and the amount of NF-I family proteins might be responsible for the cellular differences in responses to DNA damage. p73 was initially cloned from neuroblastoma cell lines [49] and is involved in the development of the central nervous system, indicating that major roles of p73 might be different from those of p53 in tumours.

Failure to regulate the cell cycle upon DNA damage, thus allowing damaged DNA to be replicated, could be a putative factor responsible for cancer incidence [24–26]. Tumour suppressor gene products accumulate after DNA damage and control cellular proliferation through their activity as transcription factors [31]. The expression of the downstream gene p21 induces cell-cycle arrest and allows the cell time to repair damage [26]. One might predict from the dual function of CTF2 that CTF2 is a component of the p53/p73-containing complex in linking DNA-repair events and transcriptional regulation to deal with damaged lesions.

Our findings suggest that CTF2 functions in concert with tumour suppressor gene products to co-ordinate critical DNA-damage-signalling processes, including cell-cycle arrest, DNA repair and drug resistance. Further studies may reveal that CTF/NF-I is one of the fundamental components for DNA repair.

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