p73 Interacts with c-Myc to Regulate Y-box-binding Protein-1 Expression*

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YB-1 is a member of the cold shock domain family of proteins that is important for signaling DNA damage and cell proliferation. YB-1 is induced by DNA damage and can also recognize cisplatin-modified DNA. In this study we observed a 6-fold increase in the steady-state level of YB-1 mRNA in response to cisplatin exposure in cells of the human cancer cell line KB. We present evidence from cotransfection experiments for a critical role of c-Myc and p73 in the transactivation of the YB-1 promoter. p73 transactivated the YB-1 promoter in experiments with Saos-2 cells, which express c-Myc, but not with HO15.19 cells, which lack c-Myc. In turn, c-Myc transactivated an intact YB-1 promoter but not a YB-1 promoter with a mutant E-box, indicating that the E-box is necessary for the response of the promoter to cisplatin. We also found that p73 interacts with c-Myc in vitro and in vivo. Using deletion mutants we showed that the DNA-binding domain of p73 and the C-terminal region of c-Myc are required for the interaction. Furthermore, p73 stimulated the interaction of Max with c-Myc and promoted binding of the c-Myc-Max complex to its target DNA. Our data suggest that p73 stimulates the transcription of the YB-1 promoter by enhancing recruitment of the c-Myc-Max complex to the E-box.

YB-1,1 a transcription factor first identified by its ability to bind to the inverted CCAAT box (Y-box), has been implicated in gene transcription, cell proliferation (1), and cisplatin resistance (2). YB-1 contains a unique DNA-binding domain, the cold shock domain, that is highly conserved in prokaryotes and eukaryotes (1, 3, 4). YB-1 is mainly cytoplasmic, but it is translocated to the nucleus when cells are treated with anticancer agents (5). Furthermore, YB-1 mRNA accumulates when cells are treated with UV irradiation or anticancer agents (6). YB-1 can bind to cisplatin-modified DNA and interacts with PCNA (7). Cancer chemotherapeutic agents such as cisplatin exert their cytotoxic effect by inducing DNA damage and activating apoptosis (11). The p53 tumor suppressor gene family is central to the pathway that inhibits cell cycle progression following damage (8–10). It consists of transcription factors that bind to DNA in a sequence-specific manner (12), and their activation in response to DNA damage elicits apoptosis and cell cycle arrest. We have recently found that YB-1 interacts with p53 and can regulate gene expression (13). We have also identified multiple consensus 5’-CACGTT-3’ sequences, the so-called E-boxes (15) in the YB1 promoter (14).

c-Myc is a transcription factor that has also been found to induce apoptotic cell death under certain conditions and to modulate cellular susceptibility to anticancer agents such as cisplatin (16, 17). Thus, both YB-1 and c-Myc may play key roles in cell proliferation as well as in the DNA damage signaling pathway (18, 19). Interestingly, c-Myc is known to bind to and transactivate via E-boxes (15). The transcriptional apparatus responsible for signaling DNA damage is poorly understood, and YB-1 may provide useful insight into this process. We show that p73, a close relative of p53, interacts with c-Myc and stimulates the E-box binding activity of the c-Myc-Max complex. Both c-Myc and p73 activate YB-1 transcription and may regulate important biological processes via their effect on YB-1 gene expression. Our data may help to account for the dual function of c-Myc in cell proliferation and apoptosis.

EXPERIMENTAL PROCEDURES

Cells Lines and Drugs—The KB human epidermoid cancer cell line was grown in modified Eagle’s medium (Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum. The Saos-2 human osteosarcoma cell line, the HO15.19 rat fibroblast cell line, and the COS-1 monkey kidney cell line were grown in Dulbecco’s modified Eagle’s medium (Nissui) supplemented with 10% fetal bovine serum. HO15.19 cells were kindly provided by Dr. J. M. Sedivy (Brown University, Boston, MA) (20, 21). The cell lines were maintained in a 5% CO2 atmosphere at 37 °C. Cisplatin was from Sigma. Drugs were added directly to the culture medium at the indicated times.

Antibodies—An anti-YB-C antibody to the C-tail domain of YB-1 was generated as described previously (5). Anti-c-Myc (N262) polyclonal antibody, anti-c-Myc (9E10) monoclonal antibody, and anti-HA (F7) monoclonal antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-p73a antibody, anti-HA (3F10) (hemagglutinin)-peroxidase, and anti-FLAG (M2) monoclonal antibody were purchased from Wako (Osaka, Japan), Roche Molecular Biochemicals, and Sigma, respectively.

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§ The abbreviations used are: YB-1, Y-box-binding protein-1; GST, glutathione S-transferase; HA, hemagglutinin; PBS, phosphate-buffered saline; EMSA, electrophoretic mobility shift assay.
Interaction of c-Myc with p73 and YB-1

Plasmid Constructs—The YB-1-Luc and YB-1-m-Luc utilized in the luciferase assays were prepared by digesting pYB-1-CAT (23). To obtain pYB-1-Luc, the pYB-1-CAT plasmid was amplified by PCR using the following primer pairs: for YB-1-Luc, 5′-TCAGTGGATCT-3′ and 5′-TTACGTCGTCTTCCTGAATCC-3′, and for YB-1-m-Luc, 5′-TCAGTGGATCT-3′ and 5′-TTACGTCGTCTTCCTGAATCC-3′. The PCR products were cloned into pGEM-T Easy (Promega). For construction of GST-Max, TH-FLAG-Max, and TH-FLAG-TBP, the cDNA plasmid, a NheI fragment including nucleotides 661 to 1324 of the YB-1 promoter was ligated into the NheI site of vector pGL3-basic (Promega, Madison, WI). To generate the YB-1-Luc plasmid, a Bgl II-Bgl IIIII fragment including nucleotides 661 to +295 of the YB-1 promoter was ligated into the Bgl II-Bgl IIIII site of vector pGEM-T Easy (Promega, Madison, WI). To generate the YB-1-m-Luc plasmid, which contains a mutation in the E-box, a Bgl II-Bgl IIIII fragment including nucleotides 661 to +295 of the YB-1 promoter was ligated into the Bgl II-Bgl IIIII sites of vector pGEM-T Easy (Promega). To obtain the YB-1-m-Luc plasmid, the polylinker of the pBluescript vector was replaced by a NheI fragment amplified by PCR from pGEM-T Easy (Promega). The PCR products were cloned into pGEM-T Easy (Promega). The YB-1-Luc and YB-1-m-Luc utilized in the luciferase assays were prepared by digesting pYB-1-CAT (23). To obtain pYB-1-Luc, the pYB-1-CAT plasmid was amplified by PCR using the following primer pairs: for YB-1-Luc, 5′-TCAGTGGATCT-3′ and 5′-TTACGTCGTCTTCCTGAATCC-3′, and for YB-1-m-Luc, 5′-TCAGTGGATCT-3′ and 5′-TTACGTCGTCTTCCTGAATCC-3′. The PCR products were cloned into pGEM-T Easy (Promega). 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dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride and incubated on ice for 15 min. The cells were lysed with Nonidet P-40 to a final concentration of 0.5%, and the lysate was centrifuged at 50,000 × g for 10 min. The resulting nuclear pellet was resuspended in 300 μl HEPES-KOH, pH 7.9, 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride 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. The protein concentrations were determined by the method of Bradford (25).

Western Analysis—Either nuclear extracts (see Fig. 3A) or whole cell extracts (see Fig. 3C) were separated on a 10% SDS-PAGE gel. The proteins were transferred to a polyvinylidene difluoride membrane (Millipore) using a semi-dry blotter. A prestained protein marker was used as a molecular weight standard. The membrane was immunoblotted with anti-c-Myc (N262), anti-Max, anti-p73α, anti-YB-1, and anti-HA antibodies and then developed by chemiluminescence using an ECL kit according to the manufacturer (Amersham Biosciences).

Promoter Reporter Assay (DNA Transient Transfection and Luciferase Assay)—For transient transfections, KB, HO15.19, and Saos-2 cells were plated at a density of 5 × 10^4 cells/well 1 day before transfection. The cells were cotransfected at ∼50% confluence with 0.2 μg of reporter plasmid, 0.2–0.4 μg of expression plasmid, and 0.3 μg of pCH110 (a β-galactosidase expression plasmid; Promega) using 2 μl of SuperFect according to the manufacturer’s protocol (Qiagen). Three hours later, the cells were washed twice with PBS and incubated in fresh medium. Each expression plasmid and reporter plasmid was standardized individually to a molar ratio of 1:1, and the total amount of DNA/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 a brief centrifugation, the luciferase activity in the resulting supernatants was assayed using a Picagene kit (Toyoink, Tokyo, Japan) as described previously (7). Light intensity was measured for 15 s with a luminometer (DynaTech ML 1500, JEOL, Tokyo, Japan). All of the cells were cotransfected with pCH110 as a control for transfection efficiency; β-galactosidase activity was measured using a β-galactosidase enzyme assay system (Promega) and expressed as a ratio of the corrected luciferase activity of cells cotransfected with vector. The results shown are normalized to β-galactosidase activity and are representative of at least three independent experiments.

Chromatin Immunoprecipitation Assay—Protein-DNA cross-linking was performed by incubating KB cells with formaldehyde at a final concentration of 1% for 10 min at room temperature. The cells were washed with PBS and collected by centrifugation at 500 × g for 5 min. They were then lysed in Buffer X (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 120 mM NaCl, 0.5% Nonidet P-40, 10% glycerol, and 1 mM phenylmethylsulfonyl fluoride) for 15 min on ice. The lysate was sonicated with 10 pulses of 10 s each at 50–60% of maximum power with a sonicator (TAITEC, Tokyo, Japan) equipped with a micro tip to reduce the chromatin fragments to an average size of less than 500 bp. Soluble chromatin was precleared by addition of 10 μg of protein A-Sepharose. An aliquot of precleared chromatin containing 1 × 10^7 cells was removed and used in the subsequent PCR analysis. The remainder of the chromatin was divided into lots each corresponding to 1 × 10^7 cells and diluted with Buffer X. The protein-DNA was incubated overnight at 4 °C with 2 μg of antibody, mouse IgG, or rabbit preimmune serum in a final volume of 800 μl. Immune complexes were collected by incuba-
tion with 15 μl of protein A/G-agarose for 1 h at 4 °C. Protein A/G-agarose pellets were washed once with 1 ml of Buffer X, once with high salt Buffer X (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 500 mM NaCl, 0.5% Nonidet P-40, 10% glycerol, and 1 mM phenylmethylsulfonyl fluoride), once with LiCl buffer (10 mM Tris, 1 mM EDTA, 0.25 M LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, pH 8.1), and twice with 10 mM Tris, 1 mM EDTA, pH 8.0. The immune complexes were then eluted twice with 250 μl of elution buffer (0.1 M NaHCO₃, 1% SDS). To reverse protein-DNA cross-linking, the eluted samples were incubated with 0.2 M NaCl for 4–5 h at 65 °C. The samples were digested with proteinase K (0.04 mg/ml) for 2 h at 45 °C and then with RNase A (0.02 mg/ml) for 30 min at 37 °C. DNA was purified with phenol-chloroform followed by ethanol precipitation. The purified DNA was resuspended in 10 μl of H₂O. Aliquots of 2 μl of serial dilutions were analyzed by PCR with the appropriate primer pairs. The YB-1 promoter primers were 5'-AGAT-CCTATACACGTTGCGTCCG-3' and 5'-AAGCTTTATGTCCTCCAT-TCTCATGTT-3'. The HMG1 promoter primers were 5'-GCTGTTGCC-3' and 5'-GCTGTTGGAAACAATCCC-3'. Amplification was performed for a predetermined optimal number of cycles. PCR products were separated by electrophoresis on 2% agarose gels, which were stained with ethidium bromide.

In Vitro Binding Assay (Transient Transfection and Coimmunoprecipitation Assay)—COS-1 cells were seeded in 6-well plates at a density of 1 × 10⁶ cells/well. The following day the cells were cotransfected with 1.5 μg of c-Myc and HA-p73α expression plasmids along with 6 μl of SuperFect according to the manufacturer's protocol (Qiagen). 3 h following transfection, the cells were washed with PBS, and the medium was replaced with fresh medium. After 48 h the cells were washed twice with PBS and lysed in Buffer X. 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 for 60 min at 4 °C with 2 μg of mouse IgG, preimmune rabbit IgG, anti-HA (F-7) antibody, anti-c-Myc (N262) antibody, or anti-p73α antibody, in each case together with 1 μl of GST fusion proteins and 4 ng of radiolabeled oligonucleotides were added with 1 mM dithiothreitol and further incubated for 3.5 h at 4 °C. The binding samples were washed three times with Buffer X and separated on a 10% gel by SDS-PAGE, transferred to a polyvinylidene difluoride membrane, immunoblotted with anti-HA-peroxidase, and developed by chemiluminescence as described above.

Purification of GST Fusion Proteins and in Vitro Translation—To prepare purified GST fusion proteins, GST-Myc and GST-Max immobilized on glutathione-Sepharose beads were eluted with elution buffer containing 50 mM Tris-HCl, pH 8.0, and 20 mM reduced glutathione according to the manufacturer's protocol (Amersham Biosciences). The concentrations of the GST fusion proteins were equalized with GST elution buffer. HA-p73α protein was made in a coupled transcription and translation system (Promega). Briefly, 0.5 μg of DNA was added directly to 20 μl of transcription and translation rabbit reticulocyte lysate with 0.5 μl of methionine, and the reactions were carried out at 30 °C for 90 min. The translated products were stored at −80 °C until use.

Electrophoretic Mobility Shift Assay (EMSA)—Oligonucleotides from the E-box and p53 consensus binding sites were used as probes. The sequences were: YB-1 E-box (E-box Y), 5'-GGCCCTCTCTATACAGTGGCTGGTG-3'; E-box consensus (E-box C), 5'-GGTCGACCGACGTTGGCAGTGGC-3'; and Y-box, 5'-GGTGAGGCTGCTGGCGAGAG-3'. The E-box probe was labeled with [γ-32P]ATP using T4 polynucleotide kinase and purified on a 15% polyacrylamide gel in 1× TBE buffer. EMSA was performed as described previously (7). Briefly, eluted GST fusion proteins and 4 ng of radiolabeled oligonucleotides were mixed in reaction buffer containing 25 mM HEPES, pH 7.5, 50 mM KCl, 0.5 mM EDTA, 5% glycerol, 10 mM dithiothreitol, 0.1% Nonidet P-40, and 0.5 mg/ml bovine serum albumin and incubated for 20 min at 20 °C (26, 27). The binding reactions were analyzed on a 4% polyacrylamide gel in 0.5× TBE buffer, followed by autoradiography as described previously (7). For the competition experiments, preincubation was performed in the presence of 10× or 40× unlabeled competitor DNA for 15 min at 20 °C before the addition of radiolabeled oligonucleotides.

RESULTS

DNA Damage Induces Expression of YB-1 in KB Cells—We first examined YB-1 gene expression in the in the human cancer cell line KB and found that YB-1 mRNA increased in

Interaction of c-Myc with p73 and YB-1

FIG. 5. Interaction of c-Myc with p73. A, whole cell extracts prepared from COS-1 cells transfected with or without c-Myc, HA-p73α, or empty vector were immunoprecipitated (IP) with 2 μg of mouse IgG, preimmune rabbit IgG, or anti-HA. The resulting immunocomplexes and 10% of the input were subjected to SDS-PAGE and analyzed by Western blotting with an anti-c-Myc (N262) antibody. B, a reciprocal immunoprecipitation assay was performed with an anti-c-Myc (N262) antibody and analyzed by Western blotting with an anti-HA antibody. C and D, 100 μg of nuclear extracts from KB cells were immunoprecipitated with 2 μg of preimmune rabbit IgG, an anti-p73α antibody, or an anti-c-Myc (N262) antibody, and the resulting immunocomplexes and 10% of the input were subjected to SDS-PAGE and analyzed by Western blotting with anti-p73 antibody.

Determination of the Optimal Number of Amplification cycles. PCR products were separated by electrophoresis on 2% agarose gels.
response to cisplatin treatment (Fig. 1A). We tested whether cisplatin could induce luciferase expression from reporter constructs in cells that express high levels of c-Myc and p73. Significant induction of luciferase activity was observed when intact YB-1 Luc was transfected into KB cells but not when YB-1-m-Luc, whose promoter contains a mutant E-box, was introduced (Fig. 1, B and C). The E-box that is proximal to the transcriptional start site of the YB-1 promoter has the nucleotide sequence 5'-CACGTG-3' that is bound with high affinity by c-Myc (28–31). It therefore seemed possible that c-Myc expression might affect the outcome of such reporter assays. To show that c-Myc binds specifically to the YB-1 promoter in vivo, we utilized a chromatin immunoprecipitation assay. PCR amplification of the YB-1 promoter was carried out with DNA extracted from the immunocomplex obtained with anti-c-Myc antibody. Fig. 2 shows that the YB-1 promoter sequence was significantly enriched in this complex. In controls, no HMG1 promoter sequence was detected in the immunocomplex (Fig. 2B), and enrichment of the YB-1 promoter sequence was not observed with preimmune serum or mouse IgG.

Western blots with c-Myc antiserum indicated that the level of c-Myc protein increased dramatically after cisplatin treatment (Fig. 3A). Cisplatin treatment has been shown to stabilize p73 protein (32), and as expected, treatment of KB cells with cisplatin increased p73 protein levels (Fig. 3A). We also examined whether overexpression of p73 can induce the expression of endogenous YB-1 in a c-Myc-dependent manner. As expected, a significant increase of endogenous YB-1 was observed when p73 was overexpressed in Saos-2 cells (Fig. 3C) but not in rat HO15.19/c-Myc null cells (19–21) (data not shown).

c-Myc and p73 Positively Regulate YB-1 Expression via the E-box—Because the induction of p73 after treatment with cisplatin correlates with the induction of c-Myc, we tested the effect of p73 and c-Myc on YB-1 promoter activity. To determine whether p73 or c-Myc is responsible for the YB-1 promoter activity, we utilized two cell lines: Saos-2/p53 null cells, which express low levels of p73 (data not shown), and HO15.19/c-Myc null cells (19–21). The SV40 promoter-Luc plasmid (P2) served as a control, and we transected the YB-1 promoter-luciferase reporter, together with increasing amounts of p73 or c-Myc expression vectors, into these cell lines. c-Myc activated the intact YB-1 promoter but not the reporter with an E-box mutation in HO15.19 cells (Fig. 4A). Interestingly, p73 was able to increase YB-1 promoter activity in Saos-2 cells (Fig. 3B) but not in HO15.19 cells (Fig. 4B). Because there is no p73-binding site in the YB-1 promoter, this suggests that p73 regulates expression indirectly by promoting binding of c-Myc to the E-box.
p73 Interacts with c-Myc—To examine whether p73 interacts with c-Myc, we performed coimmunoprecipitation assays. Because p73α is expressed at low levels in tumor cell lines (33), we transiently transected COS-1 cells with expression constructs for both HA-p73α and c-Myc, and extracts were immunoprecipitated with antibody against either HA or c-Myc. The complexes immunoprecipitated with HA-antibody contained c-Myc (Fig. 5A), and conversely, the anti-c-Myc complexes contained HA-p73α (Fig. 5B). To demonstrate that endogenous p73 interacts with c-Myc, we performed coimmunoprecipitation assays. The nuclear extracts of KB cells were immunoprecipitated with anti-p73α or an anti-c-Myc; as before, complexes immunoprecipitated with anti-p73α contained c-Myc (Fig. 5C), and vice versa (Fig. 5D). To determine whether the interaction between p73 and c-Myc is enhanced by cisplatin treatment, similar amounts of nuclear extracts from KB cells with or without cisplatin treatment were immunoprecipitated with anti-c-Myc. The interaction between p73 and c-Myc was clearly stimulated by cisplatin treatment (Fig. 5E). We identified the domain of c-Myc that interacts with p73 using c-Myc deletion constructs (Fig. 6A). As shown in Fig. 6B, the C-terminal of c-Myc, between amino acid residues 347 and 382, was required for interaction with p73α. There exist seven additional p73 isoforms (α, β, δ, γ, ε, ζ, and ΔNα) (34, 35), and using pull-down experiments, we have found that the p73α, β, δ, and γ isoforms also interact with c-Myc (data not shown).

To identify the c-Myc binding sites in p73, we used an in vitro GST pull-down assay. Intact HA-p73 and various deletion derivatives were expressed in bacteria and incubated with GST-c-Myc fusion protein (Fig. 6C). Pull-down assays demonstrated that p73 interacts with GST-c-Myc via the region between amino acid residues 228 and 312 (Fig. 6D), a region also present in p73α, β, δ, γ, ε, ζ, and ΔNα. Because the DNA-binding domain of p73 is required for c-Myc binding, it seemed possible that p73 might stimulate the c-Myc-dependent transcription of the YB-1 promoter, even if the transactivating domain of p73 was deleted. To test this, we constructed p73ΔN, which lacks residues 1–56, and found that this construct was capable of transactivating the YB-1 promoter (but not the p21 promoter) (Fig. 7A). Moreover transactivation was clearly E-box-dependent (Fig. 7B), suggesting that the transactivation domain of p73 is not involved in the transactivation. Taken together, the results of our binding assays suggest that p73 may enhance the DNA binding activity of c-Myc by binding to it. To evaluate this possibility we purified GST-Max and performed an EMSA using a YB-1 E-box oligonucleotide as a binding target. Binding was not detected with GST-Myc or GST-Max alone (data not shown) but did occur when both were present (Fig. 8A). Increasing amounts of p73α (but not p53) enhanced this binding (Fig. 8B). Similar stimulation was observed with GST-p73αΔ313–636, but not with GST-p73αΔ228–636 (Fig. 8B).

Finally, to investigate the effect of p73 on c-Myc-Max complex formation, we performed double pull-down assays. Recombinant TBP was used as a control because it interacts with c-Myc via a region different from p73. Increasing amounts of p73α promoted the association of Max with c-Myc (Fig. 9A) but did not affect the association of TBP with c-Myc (Fig. 9B). To determine whether p73 stimulates Myc-Max complex formation or instead stabilizes complexes once they are formed, we preincubated c-Myc with p73 and found that this increased subsequent Myc-Max complex formation. On the other hand, no such enhancement was observed when c-Myc was preincubated with Max (Fig. 9, C and D). It appears therefore that p73 must first bind to c-Myc to stimulate subsequent Myc-Max complex formation.

DISCUSSION

We have shown previously that human cancer cell lines that are resistant to cisplatin overexpress YB-1 (5). YB-1 binds to cisplatin-modified DNA, interacts with PCNA (7), and has 3′-5′ DNA exonuclease activity, suggesting that it may be involved in DNA repair (36). We have also found that decreasing YB-1 levels in cells reduces cellular growth both in vitro and in vivo (data not shown), pointing to a role for YB-1 in cell proliferation.

Cells overexpressing the p53-related protein p73 show increased expression of many genes involved in the nucleotide excision repair and mismatch repair pathways. Like p53 itself (37, 38), p73 induces apoptosis (35, 40–42). Cellular levels of p73 protein increase in response to cisplatin treatment (32), and p73-overexpressing clones have increased resistance to cisplatin (39). We have reported above that p73 overexpression increases cellular levels of YB-1 (Fig. 3), in agreement with the observation that the cellular level of YB-1 affects the cellular response to DNA-damaging agents (6). However, the results of our p73 transfection experiments showed that the endogenous YB-1 protein was increased, but rather weakly in Saos-2 cells. This may be due to the following: 1) Transfection efficiency of p73 expression plasmid is critical for the induction of endogenous YB-1 protein. 2) The expression of other transcription factors, such as c-Myc, is responsible for the induction of YB-1 protein. The expression of c-Myc is relatively low in Saos-2 cells (data not shown). 3) p73 may be activated through protein modification in response to the cisplatin treatment. 4) The steady-state level of endogenous YB-1 protein is relatively high in Saos-2 cells leading to difficulties in detecting only a small fraction of YB-1 induced by p73. The results presented here indicate that YB-1 gene expression is up-regulated after cispla-
tin treatment and that this occurs through an interaction of p73 with c-Myc. We further show that p73 promotes the formation of c-Myc-Max complexes involved in transactivation of c-Myc target genes, including YB-1.

Because c-Myc and p73 seem to be involved in the transcriptional control of many cellular functions (43), it is important to identify their various binding partners. Transcriptional activation by p73 is reduced by interaction with certain mutants of p53 (44). Furthermore, in experiments with p53-deficient Saos-2 cells, p73/H9251(1–427) stimulated transcription of luciferase reporters harboring three p53-responsive promoters, but p73/H9251(1–247) did not (45). In addition, p73/H9251(1–247) did not suppress colony formation of Saos-2 cells. These results indicate that the region of p73 between amino acids 248 and 427 is critical for transactivation and the induction of apoptosis. The pull-down assays reported above showed that c-Myc interacts with the DNA-binding domain of p73/H9251, between amino acids 228 and 312. GST and the GST fusion proteins were incubated with double-stranded oligonucleotides containing E-box elements of the YB-1 promoter, and EMSA was performed. A plus sign indicates ~ 100 ng of GST fusion protein. The arrowhead indicates the c-Myc-Max-DNA complex formed. An unlabeled E-box element on the YB-1 promoter oligonucleotide and a c-Myc consensus oligonucleotide were utilized as specific competitors: E-box Y and E-box C, respectively. MDR1 Y-box was used as a nonspecific competitor (7). 10- and 40-fold molar excess of the unlabeled oligonucleotides were added in the competition assays. B, stimulation of Myc-Max complex binding to the E-box requires the region of p73 between amino acid residues 228 and 312. GST and the GST fusion proteins were incubated with double-stranded oligonucleotides containing E-box elements of the YB-1 promoter, and EMSA was performed. A plus sign indicates ~ 25 ng of GST fusion protein.

We have also identified the domain of c-Myc that interacts with p73. The C-terminal region of c-Myc is known to associate with various cellular proteins such as Max (46), Miz (47), AP-2 (48), and YY-1 (49). Most of those proteins have a helix-loop-helix-leucine zipper structure, which is needed for dimerization and exposure of the DNA-binding domain. Binding of p73 to c-Myc did not increase the size of the Myc-Max complex that bound to DNA as seen in the EMSA (Fig. 8); moreover, the Myc-Max complex with DNA could not be supershifted by anti-p73 antibody (data not shown). p73 does not have a helix-loop-helix structure and does not compete with Max for binding to c-Myc, suggesting that its Myc interaction domain may be specific to p73. Additionally we found that p73 enhanced the DNA binding of the c-Myc-Max complex. In view of this it seems possible that p73 changes the conformation of c-Myc in such a way as to increase the affinity of the Myc-Max complex for DNA. As shown in Fig. 9 (C and D), p73促进s the interaction of c-Myc with Max rather than stabilizing the Myc-Max complex once it is formed. Interestingly, c-Myc has been shown to induce expression of p73 (50). Therefore, it is likely that these two molecules are coregulated in such a way as to control the expression of a number of genes important for cell growth and cell death.

Both YB-1 and c-Myc can affect the rate of cell proliferation and resistance to cisplatin, suggesting that c-Myc-dependent growth and apoptosis are separately regulated. Genetic suppressor elements driven by putative transcription factors have
been shown to confer resistance to anticancer agents, and transcription profiling of genetic suppressor element-induced drug-resistant cells using cDNA arrays showed that both c-Myc and YB-1 are up-regulated (51). These data also suggest that stimulation of YB-1 by c-Myc is involved in drug resistance.

In conclusion, our results indicate that signaling of DNA damage by a process involving p73 is responsible for c-Myc-dependent activation of YB-1 in response to cisplatin treatment.

The requirement for c-Myc and its association with p73 suggest that these molecules may form an important complex necessary for conferring transient resistance to anticancer agents.

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FIG. 9. Increased amounts of p73α stimulate association of Max with c-Myc. A, the effect of p73α on the interaction of GST-c-Myc with FLAG-Max. GST proteins immobilized on Sepharose beads were incubated with increasing amounts of bacterial extract containing HA-p73α and equal amounts of FLAG-Max for the double pull-down assay. Proteins bound to the resin were analyzed by Western blotting using an anti-HA antibody and an anti-FLAG antibody. B, the effect of p73α on the interaction of GST-c-Myc with FLAG-TBP. Purified GST proteins were incubated with increasing amounts of bacterial extract containing HA-p73α and an equal amount of FLAG-TBP for the double pull-down assay. Proteins bound to the resin were analyzed by Western blotting using an anti-HA antibody and an anti-FLAG antibody. C, schematic illustration of the delayed pull-down assay. D, stimulation of Myc-Complex formation requires prior incubation of p73 with c-Myc. Purified GST-c-Myc was incubated with HA-p73α and an equal amount of FLAG-Max for the delayed pull-down assays described in the legend of Fig. 9C. Proteins bound to the resin were analyzed by Western blotting using anti-FLAG antibody. IB, immunoblot.
