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E1A Activates Transcription of p73 and Noxa to Induce Apoptosis*

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p73, a member of the p53 family of proteins, transcriptionally activates a number of genes involved in the control of cell cycle and apoptosis. Overexpression of p73 was detected in a large number of primary head and neck cancers, and in the established cell lines examined, these all contained inactivating p53 mutations. The significance of p73 overexpression in the pathogenesis of head and neck cancer is currently unclear. We have shown that the expression of adenovirus 5 E1A in a panel of head and neck cancer cell lines induces apoptosis independently of their p53 status. In this study we examined the role of p73 and its transcriptional targets in E1A-mediated induction of apoptosis. E1A expression resulted in significant activation of the TAp73 promoter but had no effect on the alternative, ΔNp73 promoter. E1A also increased expression of endogenous TAp73 mRNA and protein. E1A mutants lacking the p300- and/or pRB-binding sites showed reduced ability to activate the TAp73 promoter. Additionally, mutations in the E2F1-binding sites in the TAp73 promoter impaired activation by E1A. Importantly, expression of the 13S isoform of E1A substantially induced the p53 apoptotic target Noxa in several p53-deficient cancer cell lines. Our results indicate that E1A activation of p73 and the p53 apoptotic target Noxa can occur in the absence of a functional p53. This activation is likely to play a key role in the mechanism of p53-independent apoptosis induced by E1A in some cancers and may provide an avenue for future cancer therapies.

The *p73* gene, a member of the *p53* family, is overexpressed in many types of cancer (for a recent review see Ref. 1). The p73 protein shares 63% homology with the DNA-binding region of p53 with full conservation of all DNA contact residues. It also has 38% identity with the tetramerization domain and 29% identity with the transactivation domains of p53. However, in contrast to p53, human p73 transcripts result in multiple C-terminal splice variants, p73α to p73Φ. The *p73* gene also has an alternative promoter within intron 3, from which a truncated p73 mRNA, known as ΔNp73, is transcribed. This form lacks the transactivation domain and acts as a dominant negative suppressor of p73 (2, 3).

p73 function has been shown to be required for p53-dependent apoptosis in response to DNA damage (4). p73 cooperates with p53 to induce specific targets such as PERP, Bax, and Noxa, which are involved in the p53-mediated induction of apoptosis. p73 has also been shown to be involved in p53-independent apoptosis induced by E2F1, because disruption of p73 function inhibits E2F1-induced apoptosis in p53-defective tumor cells and in $p53^{-/-}$ mouse embryo fibroblasts (5, 6). The p73 promoter is strongly activated in cells expressing exogenous E2F1 and suppressed by exogenous pRB (7). Several functional E2F1-binding sites are located upstream of p73 exon 1; two of these sites, located at positions −155 and −132, have been shown to be important in the E2F1-mediated transactivation of p73 (7). The ability of E1A to direct apoptosis is thought to be related to its ability to cause the release of transcription factor E2 from pRB (8).

As discussed above, p73 transcriptionally activates target genes, some of which are also p53 targets, leading to apoptosis and growth arrest (1). In addition, heterozygous deletion of p73 has been found in neuroblastoma tumors, suggesting a role for p73 as a tumor suppressor gene (9). However, data from a wide range of human tumors and p73-deficient mice argue against a classical Knudson-type tumor suppressor role for the p73 gene (reviewed in Ref. 10). Only the wild type form of p73 has been identified in all tumors and tumor cell lines tested, and inactivating mutations in human tumors are extremely rare (10). In addition, p73-deficient mice lack a spontaneous tumor phenotype (11). Furthermore, overexpression of p73 has been shown with high frequency in human cancers (reviewed in Ref. 12). Recently the oncogenic ability of p73 has been associated with the expression of ΔNp73, and this isoform has been shown to be up-regulated in a variety of primary tumors including cancers of the breast and genital tract (13).

The E1A (early region 1A) gene of human adenovirus type 5 is essential for adenoviral replication. The primary E1A transcript is processed by differential splicing to yield five distinct isoforms; 13S, 12S, 11S, 10S and 9S (14). The E1A proteins, particularly the major ones of 289R and 243R, regulate transcription of both viral and cellular genes in adenovirus-infected cells. E1A activates the transcription of a number of genes including c-Fos, c-Jun, JunB, and c-Myc. E1A also represses

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the transcription of several cellular genes including, *c-erbB2/neu* (15) and epidermal growth factor receptor (16). E1A proteins can interact and alter the function of several proteins including pRB, KIP1/p27, cyclin A-CDK2, cyclin E-CDK2, CtBP, and p300/CBP (17–21). Expression of E1A was shown to immortalize primary rodent cells and to transform these cells in cooperation with specific oncogenes (17). However, there is no evidence for E1A involvement in human cancers because epidemiological studies have found no linkage between adenovirus infection and occurrence of human malignancies (for review see Ref. 14). A number of studies have demonstrated the ability of E1A to induce cell death in a range of human cancer cell lines (15, 16). Expression of the 12S E1A is associated with the stabilization of p53, which was thought to be the cause of E1A-dependent apoptosis (18). We have shown E1A induction of apoptosis to be independent of the p53 status (16).

Overexpression of E2F1, c-Myc, and E1A has been shown to result in the up-regulation of endogenous p73 proteins and activation of p73 transcription targets in $p53^{-/-}$ tumor cells (6). These data show that in the absence of p53, oncogenes can signal to p73 to induce apoptosis in tumor cells. In contrast, two recent reports have shown E1A to inhibit p73-activated transcription (19, 20). An N-terminal deletion mutant of E1A, which does not bind to the p300/CBP family of co-activators, failed to inhibit p73-mediated transcription, suggesting that the transcriptional activation function of p73 is specifically targeted by E1A through a mechanism involving p300/CBP proteins (20).

We have recently shown efficient induction of cell death by E1A of adenovirus 5 in a panel of head and neck cancer cell lines with mutated or deleted p53 (16). In this study we have investigated whether p73 plays a role in the E1A-mediated induction of apoptosis. Our results demonstrate that E1A strongly activates expression of the transcriptional activating form of p73 (TAp73) but not the dominant negative suppressive form (ΔNp73). Both the p300- and pRB-binding sites of the E1A proteins seem to contribute to p73 activation, because mutations in these regions significantly reduced E1A-mediated transactivation of p73. In addition, the presence of both E2F1-binding sites in the p73 promoter seems to be required for efficient activation of the p73 promoter by E1A. This study also shows that the expression of E1A 13S strongly activates expression of the p53 apoptotic target gene Noxa in p53-deficient cell lines. In summary, our results demonstrate that in the absence of functional p53, E1A activates apoptotic pathways involving p73 and p53 targets such as Noxa.

MATERIALS AND METHODS

Cell Lines and Plasmids—Human HNSCC cell lines HN5, SCC25, HSC-3 and its metastatic match HSC-3 M3, HN30 and its metastatic match HN31 as well as non-small lung cancer cell line H1299, osteosarcoma Saos-2, neuroblastoma SH-SY5Y, cervical carcinoma cell line HeLa, normal human fibroblasts 1BR3, and human embryonal kidney 293A adenoviral packaging cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2.5 μg/ml streptomycin, 5 μg/ml penicillin, and 1 mM sodium pyruvate, all purchased from Sigma. HNSCC cell lines H103, H357, and H376 were cultured in Nut mix (Invitrogen) supplemented with 5% fetal calf serum, 4 mM L-glutamine, and 25 μg/liter hydrocortisone. Normal human keratinocytes Kf4 were cultured in collagen-treated wells in FAD medium (3 parts Dulbecco's modified Eagle's medium and 1 part Ham's F-12 medium) containing 10% fetal calf serum, supplemented with 2.5 μg/ml streptomycin, 5 μg/ml penicillin, 0.5 μg/ml hydrocortisone, 5 μg/ml insulin, 10^{-10} M cholera enterotoxin, and 10 ng/ml epidermal growth factor. Cultures were grown in a 95% air, 5% CO_2 atmosphere in a humidified incubator at 37 °C.

PCR products encoding the various human adenovirus type 5 E1A isoforms were cloned into the pcDNA4-HA expression vector. E1A13S mutants with specific small in-frame deletions were similarly expressed from pcDNA4-HA (21). Three E1A conserved regions, CR1 (residues 1–82), CR2 (residues 93–139), and CR3 (residues 139–204) were expressed from pcDNA4-HA. The TAp73 and ΔNp73 luciferase reporter plasmids, pcDNA3.1-TAp73, and luciferase reporter constructs of p53/p73 target genes were provided by Prof. Gerry Melino. The TAp73PvuII minimal reporter luciferase construct and the E2F1 mutant constructs derived from the TAp73PvuII minimal promoter were provided by Dr. Wanguo Liu (Department of Laboratory Medicine and Pathology, Mayo Clinic and Mayo Medical School, Rochester, NY). The pcDNA3.1-p53 expression construct was obtained from Prof. Bert Vogelstein (Howard Hughes Medical Institute and Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University, Baltimore, MD). pcDNA3.1 was purchased from Invitrogen. pTAT-GFP[1] and pTAT-E1A (13S) were obtained from Dr. Steven Dowdy (Howard Hughes Medical Institute and Department of Cellular and Molecular Medicine, University of California San Diego School of Medicine, La Jolla, CA). The following replication-incompetent adenoviruses were used: Ad-E1A (dl324) containing functional E1A (both E1A12S and E1A13S) but with complete deletion of E1B and E3 (22), Ad-Del (dl312) as a control containing a complete deletion of E1A and E3 (22), Ad-p73 expressing TAp73 fused to GFP (obtained from Prof. Karen Vousden, Beatson Institute for Cancer Research, Glasgow, UK), and Ad-p53 expressing p53 fused to GFP (obtained from Prof. Bert Vogelstein).

Adenovirus Amplification and Purification—Approximately 1×10^9 293A cells were infected with different adenovirus constructs. The cells were harvested when a complete cytopathic effect was observed, *i.e.* 95–100% of the cells were rounded and 5–10% were floating. The cells were lifted from the flask by pipetting gently and collected by centrifuging for 10 min at $200 \times g$. Purification and titration of the adenovirus was essentially done as described by Graham and Prevec (23).

MTT Cell Proliferation Assay—The cells were cultured in 96-well plates at a density of 1.8×10^4 cells/well. The following day cells were infected with adenovirus at an MOI of 10 or treated with 1 μM TAT fusion proteins. Cell survival was measured by MTT assay as described previously (16). Briefly, 20 μl of 5 mg/ml MTT in PBS was added to each well. Cells were incubated with the MTT solution for 2 to 4 h at 37 °C and 5% CO_2; subsequently 150 μl of solubilization solution (50% dimethylformamide, 0.2% glacial acetic acid, 20 mM HCl, 20% SDS) was added to each well. The plates were incubated for a further 16–24 h at 37 °C, and the A_{595} was measured. Statistical analysis was performed by Student's *t* test.

Protein Isolation and Transduction—TAT-E1A and TAT-GFP fusion proteins were isolated and purified as described previously (24). Briefly, fusion protein encoding plasmids were transformed into chemically competent *Escherichia coli* BL21(DE3)pLysS cells (Novagen). A single colony was expanded, and bacterial pellets were resuspended in 10 ml of Buffer Z (8 M urea, 100 mM NaCl, 20 mM HEPES, pH 7.0). The bacterial cell walls were disrupted by sonication on ice, and cell debris was removed by centrifugation. TAT fusion proteins were isolated in Buffer Z supplemented with 10 mM imidazole by nickel ion affinity chromatography (Amersham Biosciences) and eluted in Buffer Z with increasing imidazole concentrations. Urea was eluted through PD-10 desalting columns (Amersham Biosciences) using PBS with 10% glycerol in the presence of protease inhibitors (Complete Mini Roche). The cells were seeded in Falcon 8-well culture slides and transduced at 50–80% confluence with TAT fusion proteins diluted in the culture medium.

Transient Transfection and Fluorescence Microscopy—The cells were seeded in Falcon 8-well culture slides (Becton Dickinson) and transfected at 50–80% confluence with 400 ng of plasmid DNA preincubated with 1.4 μl of Lipofectamine 2000™ reagent (Invitrogen), according to the manufacturer's protocol. Alternatively, they were transduced with TAT fusion proteins. The cells were washed in PBS and directly fixed in acetone for 20 min at −20 °C. TAp73 expression was detected with anti-p73 monoclonal mouse antibody clone GC15 (Oncogene), followed by horse anti-mouse Texas Red (Vector Laboratories). TAT-E1A was detected using anti-adenovirus type 5 E1A monoclonal mouse antibody clone M58 (BD Biosciences Pharmingen) and anti-mouse IgG (whole

[1] The abbreviations used are: GFP, green fluorescent protein; CR, conserved region; DAPI, 4′,6-diamino-2-phenylindole; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HIV, human immunodeficiency virus; HNSCC, human head and neck squamous cell carcinoma; MOI, multiplicity of infection; MTT, 3-(4,5-dimethylthiazolyl-2)-2.5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; RT, reverse transcription; CBP, cAMP-responsive element-binding protein-binding protein; PML, promyelocytic leukemia protein; TAT, transacting activator of transcription; PARP, poly(ADP-ribose) polymerase.

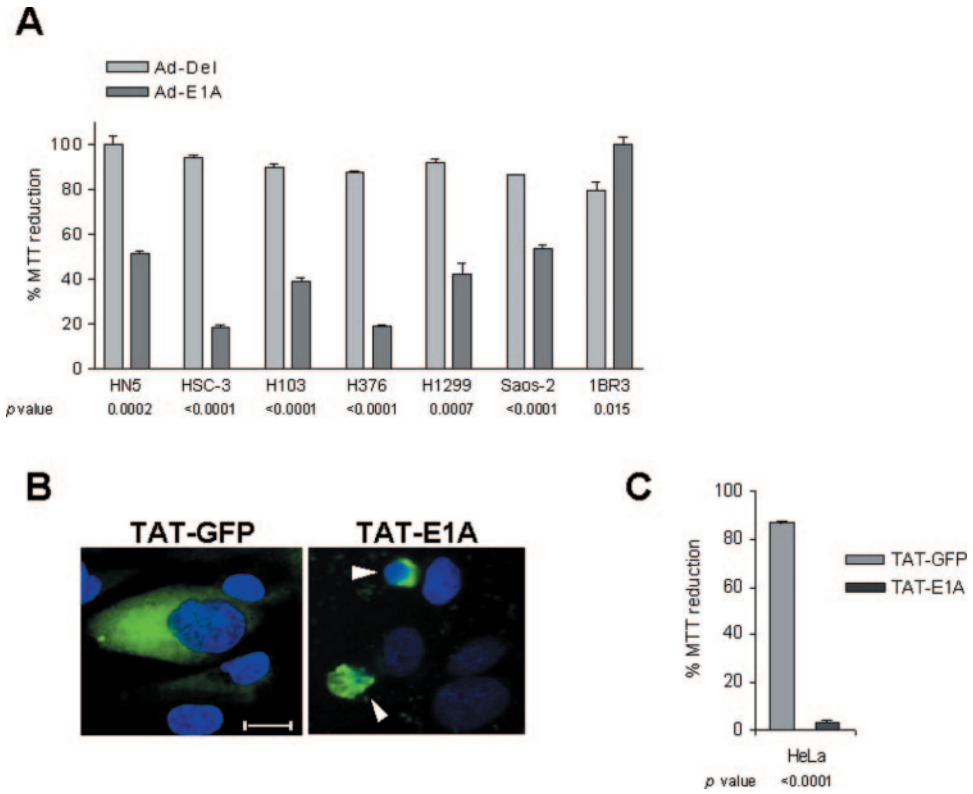


FIG. 1. **Ad-E1A induces cell death in a range of tumor cell lines.** *A*, cell survival was measured by MTT assay 48 h after infection with Ad-E1A expressing functional E1A (E1A12S/E1A13S) or control Ad-Del with a deletion of E1A at an MOI of 10. The results are shown as percentages of viable cells with respect to noninfected cells. Statistical analysis was performed by Student's *t* test; *p* values are shown for each cell line. *B*, induction of cell death in cells exposed to TAT-E1A. Saos-2 cells were treated with 1 μM of either TAT-GFP or TAT-E1A; after 24 h cells were fixed and stained with E1A antibody (clone M58, BD Biosciences Pharmingen). *Arrowheads* indicate apoptotic nuclei. The *scale bar* represents 10 μm. *C*, decreased cell survival in cells treated with TAT-E1A. HeLa cells were treated with 1 μM TAT-E1A or TAT-GFP. Cell survival was measured by MTT assay after 1 day. The results are shown as percentages of viable cells with respect to nontreated cells. Statistical analysis was performed by Student's *t* test, and the *p* value is shown. All of the experiments were performed in triplicate, and the *error bars* indicate the standard deviations.

antibody) fluorescein isothiocyanate conjugate (Sigma). For detection of TAT-GFP and TAT-E1A the cells were fixed in 2% paraformaldehyde for 20 min at room temperature and permeabilized 15 min in 0.2% Triton X-100. Subsequently, the cells were mounted in Vectashield mounting medium (Vector Laboratories) containing 2,4-diamidino-2-phenylindole (DAPI), for the detection of cell death. The cells were visualized using an Olympus AX70 fluorescence microscope at high power (×1000).

Analysis of Promoter Activation by Luciferase Assays—The cells were seeded at 1×10^5 per well in 48-well tissue culture dishes. At 80–90% confluence the cells were transfected with 100 ng of luciferase reporter plasmid and 300 ng of inducer expression plasmid, preincubated with 1.5 μl of Lipofectamine 2000 Reagent (Invitrogen). Twenty-four h post transfection the medium was removed, and the cells were lysed for 10 min in 40 μl of 1:1 Bright-Glo lysis buffer:Bright-Glo Luciferase assay reagent (Promega). The lysates were transferred to a 96-well plate, and luciferase activity was measured in a Wallac 1450 Trilux luminometer (PerkinElmer Life Sciences). The experiments were carried out at least in triplicate, and the data shown are representative of at least two independent experiments. Statistical analysis was performed using the GraphPad Prism software.

Expression Analysis by RT-PCR—One day prior to infection with Ad-Del or Ad-E1A at an MOI of 5, the cells were seeded at 5×10^5/well in 6-well tissue culture dishes. At 24 h after infection the cells were harvested by trypsinization. Cell pellets were flash-frozen in liquid N_2. Messenger RNA was isolated using the RNeasy Mini Kit (Qiagen), according to the manufacturer's instructions. First strand cDNA was generated using the Superscript™ First-Strand Synthesis System for RT-PCR and oligo(dT)$_{12-18}$ (Invitrogen). Specific cDNA strands were amplified by PCR using *Taq* DNA polymerase (HT Biotech). For the amplification of TAp73 cDNA, primers p73-F1 (5′-TCT GGA ACC AGA CAG CAC CT-3′) and p73-R1 (5′-GTG CTG GAC TGC TGG AAA GT-3′) were used. ΔNp73 cDNA was amplified using primers ΔNp73-F1 (5′-CGC CTA CCA TGC TGT ACG TC-3′) and p73-R1. GAPDH cDNA was amplified as an internal control for the RT-PCR procedure using primers GAPDH-F1 (5′-ACC TGA CCT GCC GTC TAG AA-3′) and GAPDH-R1 (5′-TCC ACC ACC CTG TTG CTG TA-3′). Thirty cycles were performed consisting of denaturation at 94 °C, annealing at 58 °C, and extension at 72 °C (each step 30 s). For amplification of Noxa cDNA, primers Noxa-F1 (5′-AGG ACT GTT CGT GTT CAG CTC-3′) and Noxa-R1 (5′-GTG CAC CTC CTG AGA AAA CTC-3′) were used, and twenty-four cycles were performed, consisting of denaturation at 94 °C (30 s), annealing at 59 °C (30 s), and extension at 72 °C (1 min).

Western Blot Analysis—The cells and tissues were washed in cold PBS and lysed in Laemmli sample buffer (62.5 mM Tris-Cl, pH 6.7, 100 mM β-mercaptoethanol, 2% SDS, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 100 μg/ml phenylmethylsulfoxide). The lysate was passed through a 25-gauge needle and boiled for 4 min. Lysates were resolved by gel electrophoresis for 1.5–2 h at 100 volts on 6–12% SDS-polyacrylamide gels. Western blotting was performed as described previously (25). The antibodies used for Western blot analysis were: mouse anti-E1A clone M58 (BD Biosciences Pharmingen) in a 1:500 dilution, rabbit anti-PARP p85 fragment clone G734A (Promega) in a 1:2000 dilution, mouse anti-TAp73 clone GC15 (Oncogene) in a 1:100 dilution, mouse anti-TAp73 and anti-p53 clone X77 (gift from Prof. Thierry Soussi, Laboratoire de Génotoxicologie des Tumeurs, Institut Curie, Paris, France) in a 1:1000 dilution, mouse anti-p53 clone DO-7 (Nova Castra Laboratories) in a 1:1000 dilution, mouse anti-β-actin (Sigma) in a 1:500 dilution, rabbit anti-Cbl clone c-15 (Santa Cruz Biotechnology) in a 1:500 dilution, and mouse anti-Noxa clone 114C307 (Oncogene) in a 1:250 dilution. Secondary anti-mouse and anti-rabbit antibodies linked to horseradish peroxidase (Amersham Biosciences) were used diluted 1:1000 and 1:2000, respectively.

Flow Cytometry Analysis—5×10^5 HN5 cells were plated in 35-mm dishes and infected the next day with Ad-GFP, Ad-p73, and Ad-p53 at an MOI of 5. After 24 and 48 h, the cells were trypsinized and centrifuged for 5 min at 200 × *g* together with the medium in which the cells were grown. The cells were washed in 2 ml of PBS and fixed in 2 ml of 70% ethanol. The cells were stored at −20 °C for a minimum of 1 h. Before analysis the cells were centrifuged for 10 min at 200 ×

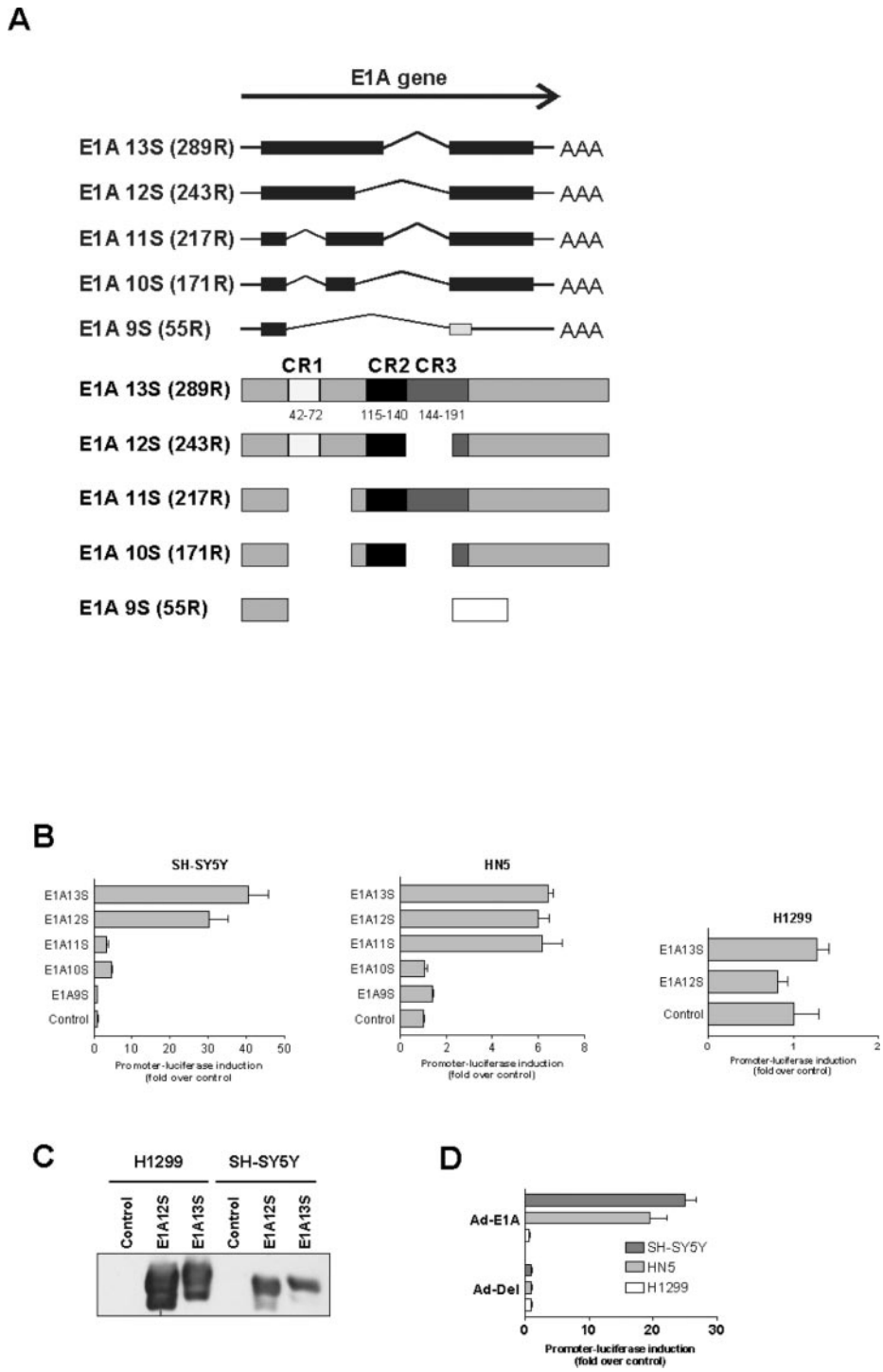


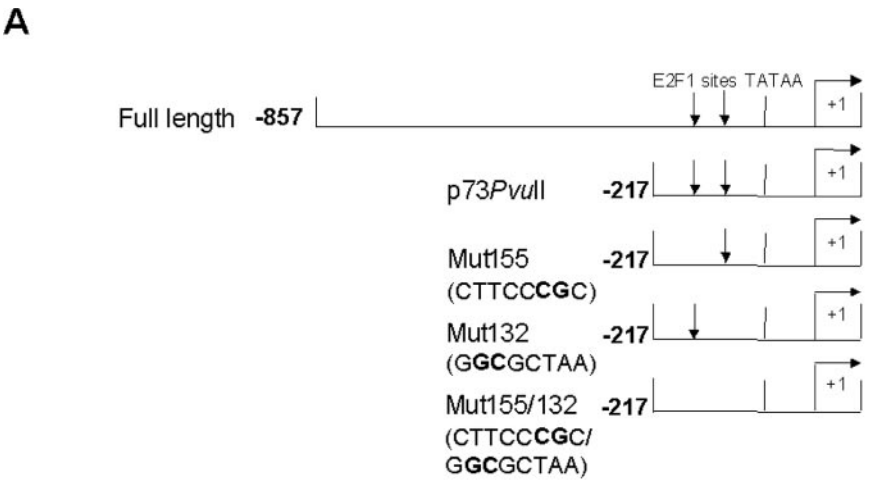
FIG. 2. **E1A transactivates TAp73 promoter.** *A*, different splice variants of the E1A gene are schematically depicted. The *boxes* represent coding sequences. The coding sequence represented by the *light gray box* in E1A9S is in a different frame from the other coding sequences. The number of amino acids of the encoded E1A isoforms and the positions of the conserved regions CR1, CR2, and CR3 in the different E1A splice forms are indicated (CR1, CR2, and CR3 positions have previously been described in Ref. 45). *B*, transactivation of the TAp73 promoter by E1A isoforms in various tumor cell lines. The cells were co-transfected with the TAp73 luciferase reporter plasmid and equal molar amounts of either empty vector DNA or with the E1A isoform expression plasmids. Luciferase activity was measured at 24 h post-transfection, and the result is shown as fold induction compared with basal luciferase level after transfection with empty vector DNA. The *error bars* indicate the standard deviations. *C*, Western blot showing expression of E1A12S and E1A13S in SH-SH5Y and H1299 cells 24 h post-transfection. E1A isoforms were detected using mouse anti-adenovirus 2 E1A (Ab-1, clone M73; Oncogene). *D*, induction of the TAp73 promoter by E1A expressed using adenovirus infection. The cells were transfected with the TAp73 luciferase construct and subsequently infected with Ad-Del or Ad-E1A. Luciferase activity was measured at 24 h post-transfection, and the result is shown as fold induction compared with basal luciferase activity level after infection with Ad-Del. The *error bars* indicate the standard deviations.

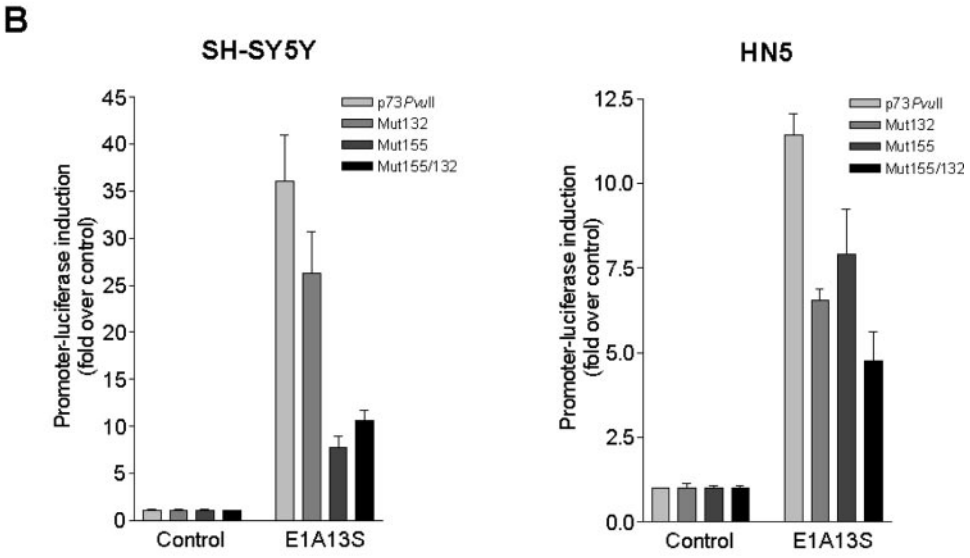
g, and the pellet was resuspended in 1 ml of staining solution (40 μg/ml propidium iodide and 500 μg/ml RNase A in PBS). The cells were incubated 30 min at 37 °C and analyzed by flow cytometry using a Becton Dickinson FACSCalibur.

RESULTS

E1A Induces Cell Death in p53-deficient Tumor Cells—The effect of E1A on cell survival of a panel of head and neck cancer cell lines including HN5, HSC-3, H103, H376, as well as non-small lung cancer H1299 cells, osteosarcoma Saos-2 cells, and 1BR3 normal fibroblasts was investigated. Triplicate cultures of cells were infected with replication-incompetent adenovirus that expresses both E1A12S and E1A13S isoforms (Ad-E1A) or an E1A deleted adenovirus as a control (Ad-Del). The cells were infected at an MOI of 10, and survival was measured by MTT assay after 48 h. As shown in Fig. 1*A*, in all of the cell lines a significant reduction in cell survival was observed in cells infected with Ad-E1A compared with cells infected with the control Ad-Del. Overall cell survival was reduced on average by more than 40% after Ad-E1A infection. More than 80% of HSC-3 and H376 cells were killed 48 h after Ad-E1A infection, whereas virtually all of the cells infected with Ad-Del were alive. On the other hand normal 1BR3 fibroblasts were not sensitive to E1A-induced cell death. In fact, these cells showed a significant increase ($p = 0.0105$) in cell survival after 48 h of infection with Ad-E1A compared with Ad-Del-infected cells.

To verify that the cytotoxic effect of Ad-E1A was specific to the E1A protein and not due to other viral elements, we used a TAT-E1A fusion protein. This fusion protein contains an 11-



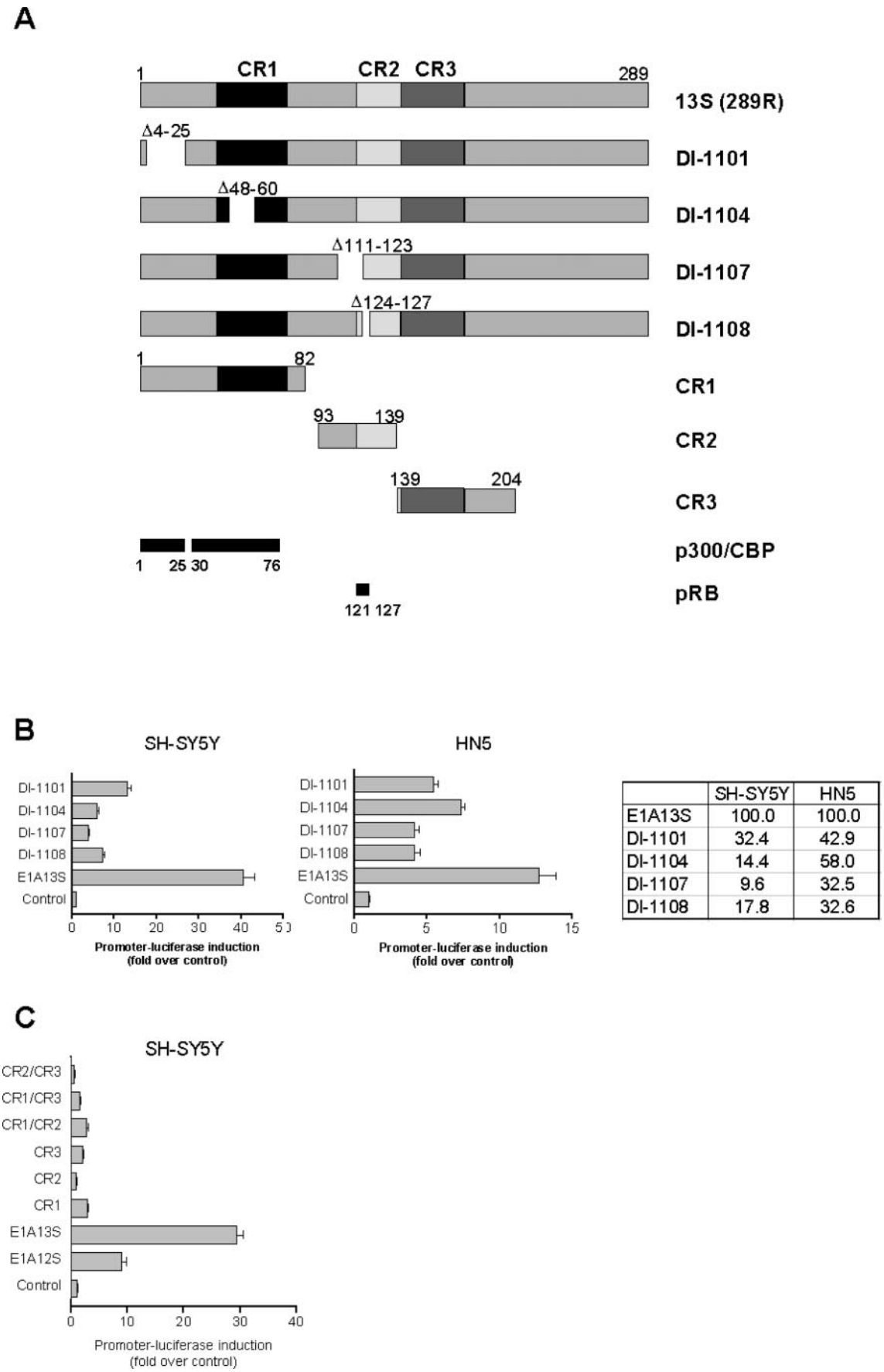


	SH-SY5Y	HN5
TAp73*Pvu*II	100.0	100.0
Mut132	72.7	57.3
Mut155	21.4	69.0
Mut132/155	29.3	41.4

FIG. 3. **Efficiency of E1A-induced activation of the TAp73 promoter is dependent on functional E2F1 binding sites.** The cells were co-transfected with TAp73 luciferase constructs or E2F1 mutant constructs in combination with empty vector DNA or E1A13S. *A*, map showing p73 full-length promoter, TAp73PvuII minimal promoter, and TAp73PvuII-derived mutations, Mut155 and Mut132, which are inactive mutants of the two important E2F-binding sites inside the p73 promoter at positions −155 and −132, respectively, as well as a double mutant Mut155/132 of the p73 promoter was used. The position of substitutions by AT dinucleotides is indicated in *bold type* adapted from Seelan *et al.* (7). *B*, luciferase activity was measured at 24 h post-transfection, and the result is shown as fold induction compared with basal luciferase activity level after transfection with empty vector DNA. The *error bars* indicate the standard deviations. The percentages indicated are deduced from the level of activation using promoters harboring mutated E2F1-binding sites relative to activation of the TAp73PvuII promoter, which was arbitrarily set at 100%.

amino acid positively charged peptide (YGRKKRRQRRR) that has been named a protein transduction domain and that is derived from the HIV-TAT protein. The TAT-protein transduction domain allows efficient and rapid uptake of the chimeric protein by cells both *in vitro* and *in vivo* (26).

Saos-2 cells were treated with 1 μM TAT-E1A or TAT-GFP



	SH-SY5Y	HN5
E1A13S	100.0	100.0
DI-1101	32.4	42.9
DI-1104	14.4	58.0
DI-1107	9.6	32.5
DI-1108	17.8	32.6

FIG. 4. **Deletions in the pRB- and p300-binding sites reduces the ability of E1A to activate TAp73 promoter.** *A*, map showing E1A13S deletion mutants, CR1, CR2, CR3, and the regions required for binding of E1A to p300 and pRB (14, 46). *B*, SH-SH5Y and HN5 cells were co-transfected with the TAp73 luciferase reporter plasmid and equal molar amounts of either empty vector DNA or plasmids expressing E1A mutants. Luciferase activity was measured at 24 h post-transfection, and the result is shown as fold induction compared with basal luciferase

(Fig. 1*B*). After 30 min of exposure TAT-E1A was detected in the cytoplasm of most cells. At 6 h after exposure TAT-E1A was translocated into the nucleus of some transduced cancer cells. DAPI staining of the DNA showed the appearance of apoptotic nuclei in cells that contained TAT-E1A but not TAT-GFP (Fig. 1*B*). Induction of cell death induced by TAT-E1A was further confirmed by MTT assay. Transduction of HeLa cells with TAT-E1A resulted in a significant decrease of cell survival ($p < 0.0001$), compared with HeLa cells exposed to TAT-GFP (Fig. 1*C*).

We have previously reported that E1A expressed from an adenoviral vector is localized to the cytoplasm of normal fibroblast cells, whereas in tumor cells, which are sensitive to E1A-induced apoptosis, E1A expression is nuclear (16). Interestingly, similar differences in the pattern of E1A localization was observed using the TAT-E1A fusion protein (data not shown).

E1A Transactives TAp73 Promoter—Cell death induced by E1A was independent of p53 (Fig. 1), because all the cancer cell lines tested are known to contain either mutations or deletions in the p53 gene. We therefore examined the role of p53 family member p73 in E1A-induced cell death and analyzed the ability of E1A to transactivate the TAp73 promoter. As a result of alternative splicing, E1A is expressed as five isoforms shown in Fig. 2*A*. SH-SH5Y neuroblastoma cells with wild type but nonfunctional p53 (27) and HN5 cells with mutant stabilized p53 were co-transfected with plasmids encoding various E1A isoforms together with a luciferase reporter construct under the control of the TAp73 promoter. In SH-SY5Y cells the TAp73 promoter was activated 30- and 40-fold by E1A12S and E1A13S, respectively. Expression of E1A11S and E1A10S induced the TAp73 promoter 3–4-fold, whereas no transactivation was detected with expression of E1A9S in this cell line (Fig. 2*B*). In HN5 cells the TAp73 promoter was substantially activated by E1A11S, E1A12S, and E1A13S. The other E1A isoforms did not activate the TAp73 promoter (Fig. 2*B*). These data suggest that depending on the cellular background type, CR2 may co-operate either with CR1 or CR3 to induce E1A-induced activation of the p73 promoter. Activation of TAp73 promoter by E1A12S or E1A13S was shown also in several other HNSCC cell lines and the osteosarcoma cell line Saos-2 (data not shown). In the lung carcinoma cell line H1299, no induction of the TAp73 promoter by E1A12S or E1A13S was observed (Fig. 2*B*). Western blot analysis showed that both these E1A isoforms were expressed at a higher level in H1299 cells compared with SH-SH5Y cells that showed a substantial 30–40-fold activation of the TAp73 promoter by E1A12S and E1A13S (Fig. 2*C*).

Infection with Ad-E1A virus resulted in a substantial induction of TAp73 promoter in SH-SY5Y and HN5 cells compared with infection with E1A-deleted adenovirus Ad-Del. Similar to the results obtained using plasmids encoding the different E1A isoforms, E1A expression after infection with adenovirus did not result in stimulation of the TAp73 promoter in H1299 cells (Fig. 2*D*).

Efficient Activation by E1A Is Dependent on the Functional E2F1-binding Sites in the p73 Promoter—E2F1 has been shown to stimulate TAp73 promoter, and the region between bases −113 and −217 of TAp73 promoter harbors the highest promoter activity (7). This region also contains several E2F1-binding sites and two of these at positions −155 and −132 have been shown to be important for E2F1-induced activity of the *p73* gene because mutation of either of these sites results in a dramatic drop in the level of the p73 promoter activation by E2F1 (7). Binding of E1A to pRB results in the release of E2F1, which may lead to transactivation of the TAp73 promoter. The importance of E2F1-binding sites of TAp73 promoter for its activation by E1A was studied using promoter constructs with inactivating mutations in either individual or combined E2F1-binding sites, Mut132, Mut155, and Mut155/132 (Fig. 3*A*). These mutations were introduced in the minimal TAp73 promoter (TAp73PvuII), which has been described to have promoter activity comparable with that of the full-length TAp73 promoter (7). SH-SY5Y and HN5 cells were co-transfected with E1A13S together with luciferase reporter plasmids. E1A13S efficiently induced the TAp73PvuII promoter in all of the cell lines tested. Activation of Mut132 promoter was reduced by ~30% in SH-SY5Y cells, whereas transactivation of Mut155 promoter was significantly reduced by 80% (Fig. 3*B*). Induction of the TAp73 promoter with combined mutations, Mut155/132, was reduced by ~70% in SH-SY5Y cells. In HN5 cells, E1A transactivation of the Mut132 and Mut155 promoters was reduced by 45 and 30%, respectively. Induction of the TAp73 promoter with combined mutations, Mut155/132, was ~60% lower in HN5 cells (Fig. 3, *B* and *bottom panel*). These results suggest that both E2F1-binding sites are involved in transactivation of the TAp73 promoter by E1A and mutation of the E2F1-binding site at position 155 had a greater effect on transactivation by E1A in SH-SY5Y cells, whereas in HN5 cells an active E2F1-binding site at position 132 is more important for efficient E1A-mediated induction of the TAp73 promoter. The combined loss of E2F1-binding sites from the TAp73 promoter further reduced its transactivation by E1A in HN5 cells compared with the TAp73 promoter containing single E2F1-binding site mutations, whereas in SH-SY5Y cells combined mutations of the E2F1-binding sites had an effect comparable with Mut155, which showed a significantly lower activation by E1A (Fig. 3*B*). These results suggest that the involvement of various E2F1-binding sites may depend on cell type-specific factors.

The Role of pRB and p300 in the E1A-mediated Activation of p73—pRB and p300 have been shown to be involved in regulating p73 transcriptional activation function (28). In fact, we recently showed that p300 in the PML-containing nuclear body is required to acetylate p73 and therefore allows its transcriptional activity (29). The N-terminal region of p73 has been shown to directly interact with the N-terminal CH1 domain of p300/CBP to activate transcription of p73 target genes (28), whereas the p73 promoter was strongly suppressed by exogenous pRB (7). Furthermore, E1A has been shown to both physically and functionally interact with p300 and pRB (30, 31).

To study the role of p300 and pRB in E1A-mediated transactivation of the TAp73 promoter, E1A13S mutant constructs with deletions of specific binding sites for p300 and pRB were used in luciferase assays. The cells were transfected with the TAp73 luciferase reporter plasmid in combination with the E1A13S mutant Dl-1101 (lacking the p300-binding region because of deletion in the N terminus; deletion also includes the p400-binding region), Dl-1104 (deletion in CR1 including part of the p300-binding sequences), and Dl-1107 and Dl-1108 (deletion in the pRB-binding regions) (Fig. 4*A*). As shown in Fig. 4*B*, the E1A mutants had substantially reduced transactiva-

activity level after transfection with empty vector DNA. The *error bars* indicate the standard deviations. The percentages of activation of the TAp73 promoter by the E1A mutants, relative to activation by E1A13S (which was arbitrarily set at 100%) are indicated. *C*, SH-SH5Y cells were co-transfected with the TAp73 luciferase reporter plasmid and equal molar amounts of either empty vector DNA or plasmids expressing E1A12S, E1A13S, CR1, CR2, CR3, and combinations of CR1/CR2, CR1/CR3, and CR2/CR3. Empty vector DNA was added when necessary to equalize the final DNA concentration. Luciferase activity was measured at 24 h post-transfection, and the result is shown as fold induction compared with basal luciferase activity level after transfection with empty vector DNA. The *error bars* indicate the standard deviations.

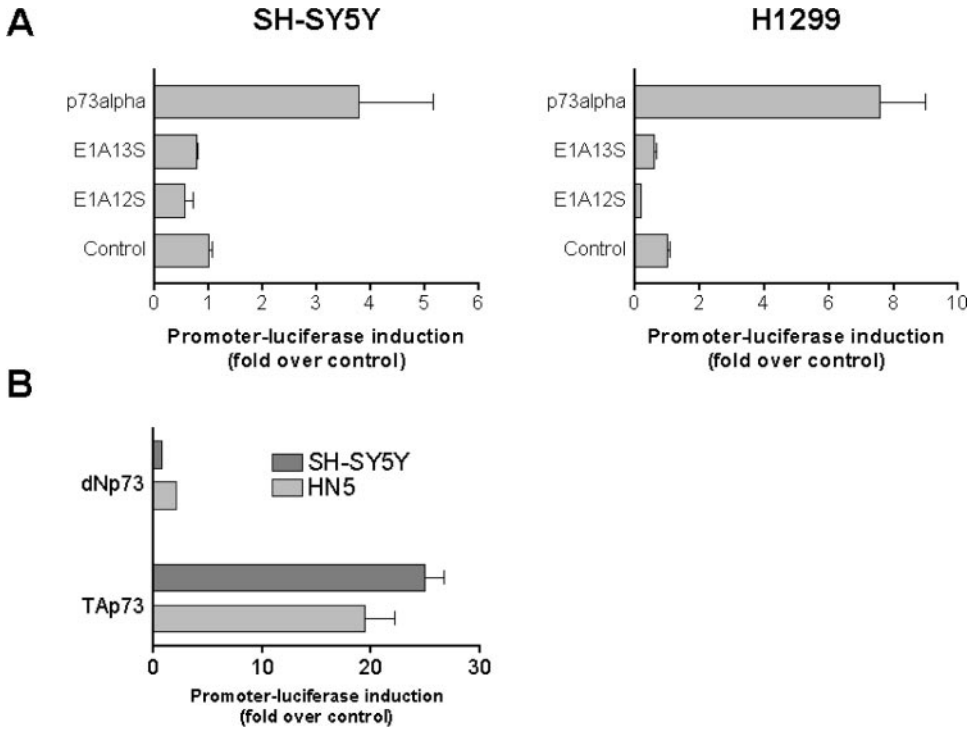


FIG. 5. **E1A has no effect on the ΔNp73 promoter.** *A*, luciferase assay showing the effect of E1A on the induction of ΔNp73 promoter. The cells were co-transfected with the ΔNp73 luciferase reporter plasmid together with equal molar amounts of either empty vector DNA, E1A12S, E1A13S, or pcDNA$_{3.1}$TAp73 plasmids. Luciferase activity was measured at 24 h post-transfection, and the result is shown as fold induction compared with basal luciferase activity level after transfection with empty vector DNA. The *error bars* indicate the standard deviations. *B*, Ad-E1A infection showed no transactivation of the ΔNp73 promoter. The cells were transfected with the ΔNp73 luciferase reporter plasmid and subsequently infected with Ad-Del or Ad-E1A. Luciferase activity was measured at 24 h post-transfection, and the result is shown as fold induction compared with basal luciferase activity level after infection with Ad-Del. The *error bars* indicate standard deviations.

tion ability compared with full-length E1A13S. In SH-SY5Y cells induction of the TAp73 promoter by Dl-1101 was over 3-fold lower compared with E1A13S, whereas transactivation by the other E1A mutants was further reduced. In HN5 cells the level of luciferase induction by Dl-1101 and Dl-1104 was almost 50% less than that obtained by wild type E1A13S, whereas Dl-1107 and Dl-1108 showed about 70% less induction (Fig. 4, *B* and *right panel*). These results suggest that both p300 and pRB are involved in the E1A-mediated activation of the TAp73 promoter. We further investigated the ability of the individual E1A conserved domains to activate the TAp73 promoter. SH-SY5Y cells were transfected with the TAp73 luciferase reporter plasmid together with constructs expressing E1A12S, E1A13S, CR1, CR2, and CR3 as well as combinations of CR1/CR2, CR1/CR3, and CR2/CR3 (Fig. 4*A*). As shown in Fig. 4*C*, the TAp73 promoter was activated by E1A12S and highly induced by E1A13S, and a 3-fold induction was observed with the expression of CR1 but not by CR2 or CR3. In addition, combined expression of CRs did not have any extra effect on TAp73 promoter (Fig. 4*C*).

E1A Has No Effect on the ΔNp73 Promoter—Transcription from a second promoter, located in intron 3 of the p73 gene, results in the expression of the dominant negative ΔNp73 isoform. The ΔNp73 promoter has been shown to be stimulated by TAp73, resulting in a negative feedback loop for regulation of the intracellular level of TAp73 protein (2). To investigate whether expression of E1A isoforms resulted in the activation of the ΔNp73 promoter, several cell lines were co-transfected with a plasmid encoding the luciferase gene under the control of ΔNp73 promoter, together with the plasmids expressing either E1A12S, E1A13S, or TAp73. In SH-SH5Y and H1299 cells expression of TAp73 resulted in 3–8-fold induction of the ΔNp73 promoter. In contrast, in all of the cells tested the expression of E1A12S or E1A13S had no effect on the activity of the ΔNp73 promoter (Fig. 5*A*). Furthermore, infection of SH-SY5Y and HN5 cells with Ad-E1A did not result in the induction of luciferase activity driven by ΔNp73 promoter (Fig. 5*B*).

E1A Expression Results in Induction of TAp73 Expression and Induction of Apoptosis—Activation of the TAp73 promoter by E1A resulted in an increased level of TAp73 mRNA in both SH-SY5Y and HN5 cells. Using RT-PCR, basal levels of endogenous TAp73 mRNA were detected in untreated SH-SY5Y cells and cells infected with Ad-Del. This is not surprising because TAp73 has been shown to be expressed at high levels in various neuroblastoma cell lines and tumors (32). Infection with Ad-E1A clearly resulted in an increase in the level of endogenous TAp73 mRNA in both HN5 and SH-SY5Y cells. In HN5 cells endogenous TAp73 mRNA was not detected in control or Ad-Del infected cultures, whereas infection with Ad-E1A resulted in a clear induction of TAp73 mRNA. In both cell lines a slight increase in the level of ΔNp73 mRNA level was detected, this is possibly due to the increased level of TAp73 protein by E1A (Fig. 6*A*).

The effect of E1A expression on endogenous TAp73 protein level was further investigated in HN5 and SH-SY5Y cells infected with Ad-E1A and control Ad-Del virus. Cells infected with an MOI of 5 were lysed at 24 and 48 h post-infection, and protein expression was examined by Western blot analysis using mouse GC-15 antibody specific to TAp73 (Fig. 6*B*). As positive control human embryonal kidney 293A cells were used because it has previously been reported that 293 cells, which are transformed with adenovirus type 5 E1A and E1B, express high levels of p73 (33). At 24 and 48 h after infection up-regulation in TAp73, the protein level was observed in Ad-E1A-infected HN5 and SH-SY5Y cells that compared with noninfected or Ad-Del-infected HN5 cells. In agreement with our reporter gene assay, E1A expression was unable to induce endogenous p73 protein levels in H1299 cells (data not shown).

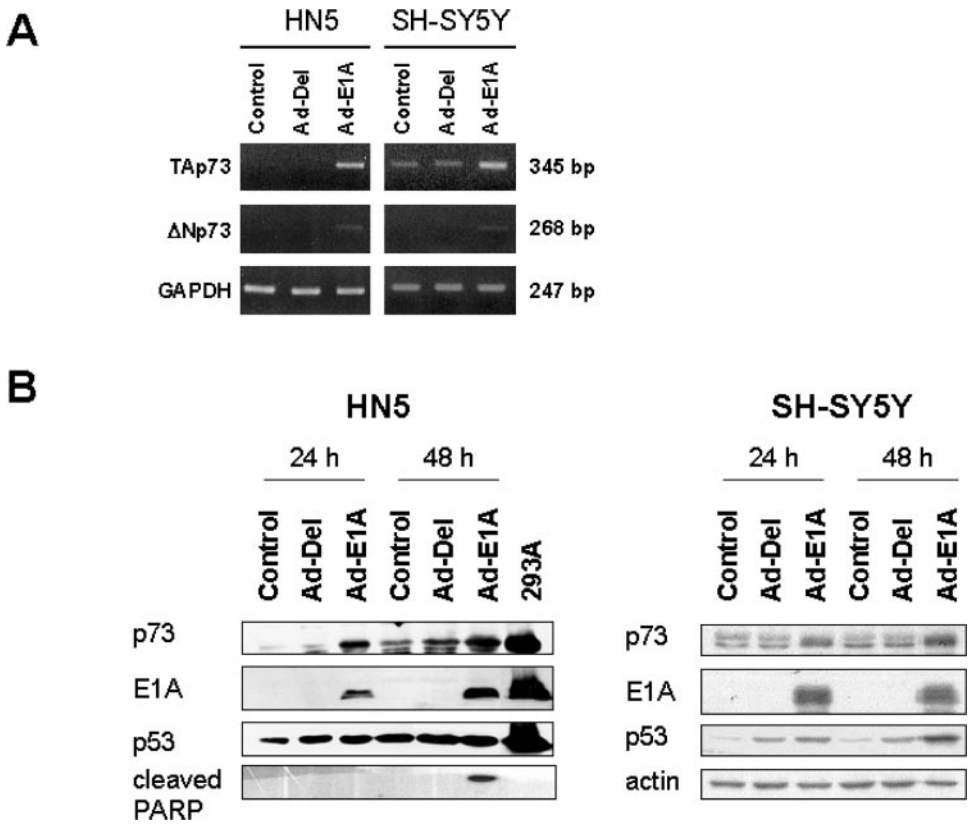


FIG. 6. **Expression of E1A results in up-regulation of TAp73 in HN5 and SH-SY5Y.** *A*, E1A-mediated induction of p73 mRNA. The cells were either untreated or infected with either Ad-Del or Ad-E1A at an MOI of 5 and harvested 24 h after infection. PCR was performed on cDNA generated from whole mRNA. Specific TAp73 and ΔNp73 sequences were amplified using GAPDH as an internal control. The sizes of amplified fragments are indicated. *B*, Western blot analysis showing expression of cleaved PARP, TAp73, E1A, p53, and actin proteins using G734A, GC-15, M58, DO-7, and anti-β-actin (Sigma) primary antibodies, respectively. HN5 and SH-SY5Y cells were infected with Ad-E1A and as a control with Ad-Del adenoviral vectors at an MOI of 5. As positive control for TAp73 expression, 293A cells that express high levels of TAp73 were used. The cells were lysed 24 and 48 h after infection; equal amounts of total protein from each sample was separated by SDS-PAGE, transferred to nitrocellulose, and hybridized to different antibodies as described under "Materials and Methods."

The presence of an 85-kDa, apoptosis-specific, caspase-cleaved PARP fragment only in HN5 cells infected with Ad-E1A confirmed the E1A-mediated induction of apoptosis (Fig. 6*B*).

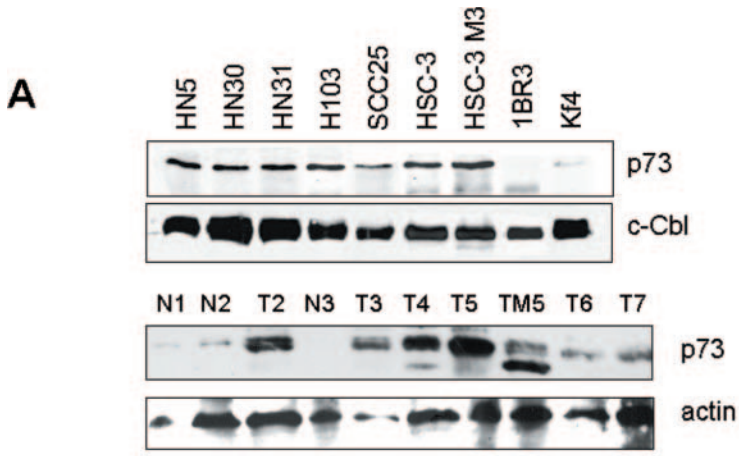
HNSCCs Overexpress p73 and Are Killed by Exogenous p73 Expression—Over 30% of head and neck (9 of 29) tumors and all seven HNSCC cell lines were found to express high levels of endogenous p73 protein compared with normal fibroblasts 1BR3 and keratinocytes Kf4. Fig. 7*A* shows representative tumor and normal samples. Expression of c-Cbl and β-actin protein was used as loading controls.

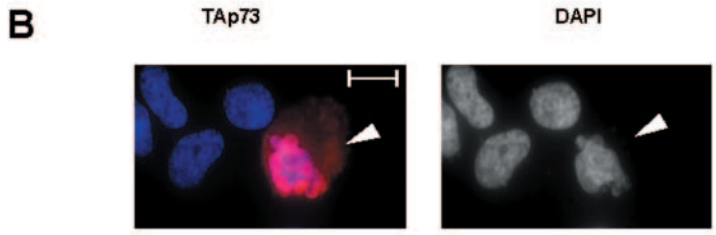
The ability of TAp73 to induce cell death was studied using a p73 expression plasmid. Expression of TAp73 in HN5 cells 48 h after transfection was confirmed by indirect immunofluorescence. At this time point DAPI staining showed that cells that expressed exogenous p73 had an irregular nucleus with condensed chromatin indicative of apoptotic cells (Fig. 7*B*).

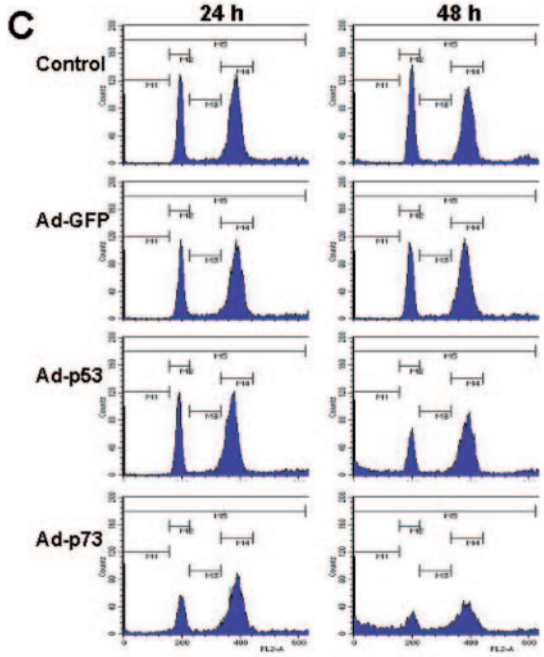
TAp73-mediated induction of apoptosis was also studied by FACSCAN. HN5 cells were infected with recombinant adenovirus expressing p53 fused to GFP (Ad-p53) or p73 fused to GFP (Ad-p73) using an MOI of 5. As a control, recombinant adenovirus expressing GFP (Ad-GFP) was used. At 24 h HN5 cells infected with Ad-p73 showed a significant increase in the sub-G_1 DNA content compared with controls and cells infected with the same MOI with Ad-p53 (Fig. 7*C*). At 48 h both Ad-p73- and Ad-p53-infected cells showed a significant increase of the number of cells with a sub-G_1 DNA content compared with noninfected or control-infected cells. Apoptosis was induced earlier and more efficiently in HN5 infected with p73 compared with p53, suggesting that this p53 mutant cell line is highly sensitive to p73-induced apoptosis. Expression of exogenous p53 and p73 in Ad-p53- and Ad-p73-infected HN5 cells was confirmed by immunofluorescence microscopy. A high proportion of HN5 cells infected with Ad-p73 were observed to contain double nuclei, suggesting that p73 may induce an arrest in the M phase of the cell cycle, whereas cells infected with Ad-p53 did not show such phenotype (data not shown). The G_1:G_2/M ratio calculated by FACSCAN was considerably decreased in HN5 cells infected with Ad-p73 compared with noninfected HN5 cells or HN5 cells infected with Ad-GFP or Ad-p53 (Fig. 7, *C* and *right panel*). These results suggest that HN5 cells infected with Ad-p73 accumulate in the G_2/M phase.

Western blot analysis using the X77 primary antibody, which detects both p53 and p73, showed expression of p53 in Ad-p53-infected cells and expression of p73 in Ad-p73-infected cells after 24 h (Fig. 7*D*). Although the same MOI was used, exogenous p73 expression seemed higher than exogenous p53 expression. This is probably due to induction of Mdm2 by wild type p53, resulting in the degradation of p53. Interestingly, in Ad-p73-infected HN5 cells endogenous mutant p53 expression was undetectable compared with noninfected control or Ad-GFP- or Ad-p53-infected cells (Fig. 7*D*). Down-regulation of p53 in this sample could be due to suppression of p53 transcription or activation of p53 degradation pathways induced by exogenous p73 expression.

The effects of p53 or p73 expression on cell survival varied in different cell types (Fig. 7*E*). Cancer cell lines H357, SH-SY5Y, HN5, and Saos-2 with different p53 status were infected with either Ad-p53 or Ad-p73 at an MOI of 10, and cell survival was measured after 24 h by MTT assay. Significant cell death was observed in most cancer cell lines infected with Ad-p53 and

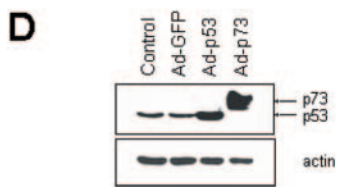






24 h	control	Ad-GFP	Ad-p53	Ad-p73
All	100	100	100	100
Sub-G_1	4.1	6.6	3.7	20.4
G_1	21.4	18.8	21.5	14.4
S-phase	3.2	3.8	4.5	3.8
G_2/M	47.3	44.6	46.1	37.7
G_1: G_2/M	0.45	0.42	0.47	0.38

48 h	control	Ad-GFP	Ad-p53	Ad-p73
All	100	100	100	100
Sub-G_1	7.9	5.4	22.3	34.7
G_1	27.7	22.6	14.3	9.7
S-phase	1.9	2.5	3	5.8
G_2/M	42.8	45.3	37.4	24.7
G_1: G_2/M	0.65	0.5	0.38	0.39



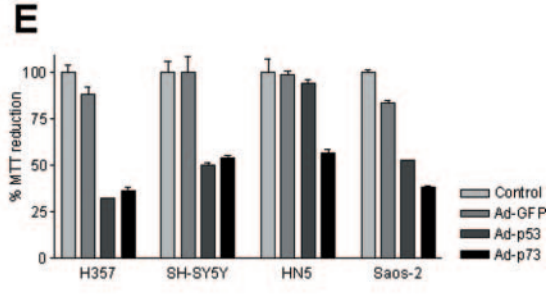


FIG. 7. **p73 expression induces apoptosis in tumor cell lines.** *A*, all HNSCC cell lines and a high percentage of HNSCC tumors expressed high levels of p73. The cells or tissue sections were lysed, and equal amounts of protein were separated by SDS-PAGE, transferred to nitrocellulose, and hybridized to TAp73 using TAp73-specific primary monoclonal antibody clone GC-15- and c-Cbl-specific primary polyclonal antibody clone c-15 (Santa Cruz Biotechnology). *B*, overexpression of TAp73 results in induction of apoptosis. HN5 cells were transfected with pcDNA$_{3.1}$TAp73 and stained after 48 h using monoclonal mouse anti-p73 antibody clone GC-15 and DAPI. The *white arrowheads* indicate apoptotic cells expressing exogenous TAp73. The *scale bar* represents 10 μm. *C*, cell cycle analysis of HN5 by FACSCAN measured at 24 and 48 h after infection with Ad-GFP, Ad-p53, and Ad-p73 at an MOI of 5. The cell cycle distribution represented as percentages of the total cell population (indicated by the marker *M5*)

Ad-p73. H357 cells, which have a p53 mutation in codon 110 in exon 4, were found to be extremely sensitive to both Ad-p53 and Ad-p73 infection because there were decreases in cell survival of almost 70 and 65%, respectively, compared with controls (Fig. 7*E*). HN5 cells, which express a highly stabilized p53 with a mutation in codon 238 in exon 7, did not show significant apoptosis with Ad-p53, and cell survival remained similar to HN5 cells infected with Ad-GFP. However, these cells were efficiently killed by p73. In Saos-2 cells with a deleted p53, expression of p73 resulted in a more effective induction of apoptosis than p53, and in SH-SY5Y cells both Ad-p53 and Ad-p73 resulted in almost 50% reduction in cell survival. The efficiency of infection with both Ad-p53 and Ad-p73 was very high and similar among the different cell lines used (data not shown).

E1A-mediated Induction of p73 Target Genes—Similar to p53, its family member TAp73 is a transcriptional activator of genes involved in apoptosis and cell cycle regulation. It is therefore important to determine whether E1A-induced activation of TAp73 results in the activation of the promoters of such target genes. SH-SY5Y cells were co-transfected with a number of luciferase reporter constructs in combination with pcDNA$_{3.1}$p53, pcDNA$_{3.1}$p73, E1A12S, or E1A13S. As shown in Fig. 8*A*, p53 expression resulted in the efficient induction of Bax and Pig-3 and an almost 3-fold induction of the MDM2 promoter. In contrast, the Noxa and Waf-1 promoters were not activated in these cells. Expression of TAp73 resulted in the induction of the Bax and Pig-3 promoters, whereas the MDM2, Noxa, and Waf-1 promoters were not activated (Fig. 8*B*).

Expression of E1A13S resulted in a slight increase in the activation of Bax and MDM2 promoters, whereas expression of E1A12S reduced the activity of some promoters, in particular Waf-1 (Fig. 8*C*). Remarkably, the promoter of Noxa was highly activated by E1A13S expression (Fig. 8*C*). Induction of Noxa by E1A12S was over four times lower compared with its activation by E1A13S (Fig. 8, *C* and *F*). Analysis of Noxa mRNA by RT-PCR demonstrated a significant increase in Noxa mRNA levels in Ad-E1A-infected SH-SY5Y cells after 48 h compared with untreated or Ad-Del-infected SH-SY5Y cells (Fig. 8*D*). Furthermore, Western blot analysis showed increased levels of Noxa protein in Ad-E1A infected SH-SY5Y cells after 48 h compared with untreated or Ad-Del-infected SH-SY5Y cells in which Noxa was undetectable (Fig. 8*E*). Because the induction of Noxa by E1A13S, which contains the complete CR3 region, was much higher than E1A12S, which lacks CR3, we examined whether the CR3 region alone was able to induce Noxa promoter. SH-SY5Y cells were co-transfected with the Noxa luciferase reporter plasmid together with E1A12S, E1A13S, CR1, CR2, or CR3 expression vectors. As shown in Fig. 8*F*, compared with E1A13S, which highly activated Noxa, expression of CR3 alone was unable to induce Noxa. Furthermore, expression of other conserved domains, CR1 and CR2 alone (Fig. 8*F*), or combined expression of CR1/CR2, CR1/CR3, and CR2/CR3 had no effect on Noxa promoter (data not shown). Similar to the results shown in Fig. 8*C*, expression of E1A12S resulted in the suppression of Waf-1 promoter. Also conserved region CR1 seemed to inhibit Waf-1 promoter activation in SH-SY5Y cells, possibly by sequestering p300 (Fig. 8*G*).

The effect of E1A on the activation of Noxa promoter was also investigated in Saos-2 cells, which have a complete deletion of the *p53* gene. Exogenous expression of both p53 and p73 in Saos-2 cells resulted in the activation of p53 apoptotic target Bax (Fig. 9*A*). Substantial induction of Noxa was observed in cells infected with adenovirus vector expressing wild type (Ad-E1A) but not deleted E1A (Ad-Del) at an MOI of 5 (Fig. 9*B*). Wild type E1A expressed by adenovirus resulted in a moderate induction of the Bax, Waf-1, Mdm-2, and Pig-3 promoters but over 12-fold induction of the Noxa promoter (Fig. 9*B*). Similar to SH-SY5Y cells expression of E1A13S but not E1A12S resulted in a significant induction of Noxa promoter (Fig. 9*C*). Expression of conserved regions CR1, CR2, or CR3 alone (Fig. 9*C*) or in combination (data not shown) did not have an effect on Noxa promoter activity.

DISCUSSION

In this study, the expression of adenovirus type 5 E1A was found to induce apoptosis in a range of p53-deficient cancer cells. We demonstrate that E1A elevates intracellular TAp73 protein levels via stimulation of transcription from the TAp73 promoter in several cancer cell lines with a variety of p53 backgrounds. Among the isoforms expressed from the *E1A* gene, the 12S and 13S encoded products were found to be strong stimulators of TAp73 promoter activity in a range of tumor cell types. In general E1A13S was a stronger transactivator of the p73 promoter than E1A12S. The difference between these two isoforms is the absence of most of the CR3 region from the 12S isoform. However, expression of CR3 alone had no significant effect on the induction of the p73 promoter.

Consistent with its ability to induce apoptosis, E1A expression did not enhance transactivation of the ΔNp73 promoter in various tumor cells. The ΔNp73 product acts as a dominant negative inhibitor of apoptosis induced by TAp73 and p53. Activation of TAp73 by E1A suggests a possible role for p73 in E1A-mediated induction of apoptosis in p53-deficient tumor cells. However, H1299 cells in which E1A expression was unable to activate p73 promoter were still killed by E1A expressed from an adenovirus vector. These results suggest that depending on the cellular background E1A may induce alternative apoptotic pathways.

E1A induction of the TAp73 promoter seems to be dependent on interaction with p300 and pRB, because mutations in the p300- and pRB-binding sites of the TAp73 promoter substantially reduced its activation by E1A. Mutations within the E2F1-binding sites inside the TAp73 promoter also decrease its E1A-mediated activation. Furthermore, we demonstrate that E1A13S expression strongly induces the transcription of p53 apoptotic target Noxa in several p53-deficient cell lines. Interestingly, suppression of p53 target Waf-1 was observed by E1A12S isoform; similar results have recently been reported by Najafi *et al.* (34). Our data suggest that E1A12S and E1A13S isoforms of adenovirus type 5 may have different roles in cell cycle regulation. E1A12S function may be to suppress p53/p73 cell cycle arrest targets such as Waf-1, allowing cell cycle progression, which is required for the replication of adenovirus in the infected cell. Whereas E1A13S may function to activate p53/p73 apoptotic targets such as Noxa, resulting in the killing

is shown in the *right panel*, *M1* indicates the proportion of the cell population in the sub-G_1 phase, *M2* indicates the proportion in the G_1 phase, *M3* indicates the proportion in the S phase, and M4 indicates the proportion in the G_2/M phase. *D*, Western blot analysis of p53 and TAp73 expression in HN5 cells 24 and 48 h after infection with Ad-GFP, Ad-p53, and Ad-p73 at an MOI of 5. Equal amounts of total protein from each sample was separated by SDS-PAGE, transferred to nitrocellulose, and hybridized to TAp73 and actin using p53- and TAp73-specific primary monoclonal antibody clone X77 and β-actin specific primary monoclonal antibody (Sigma). *E*, differences in sensitivity to p53 and p73 in a panel of cancer cell lines as shown by MTT assay measured at 24 h after infection with Ad-GFP, Ad-p53, or Ad-p73 at an MOI of 10. The results are shown as percentages of viable cells with respect to noninfected cells. The experiments were performed in triplicate. The *error bars* indicate the standard deviations.

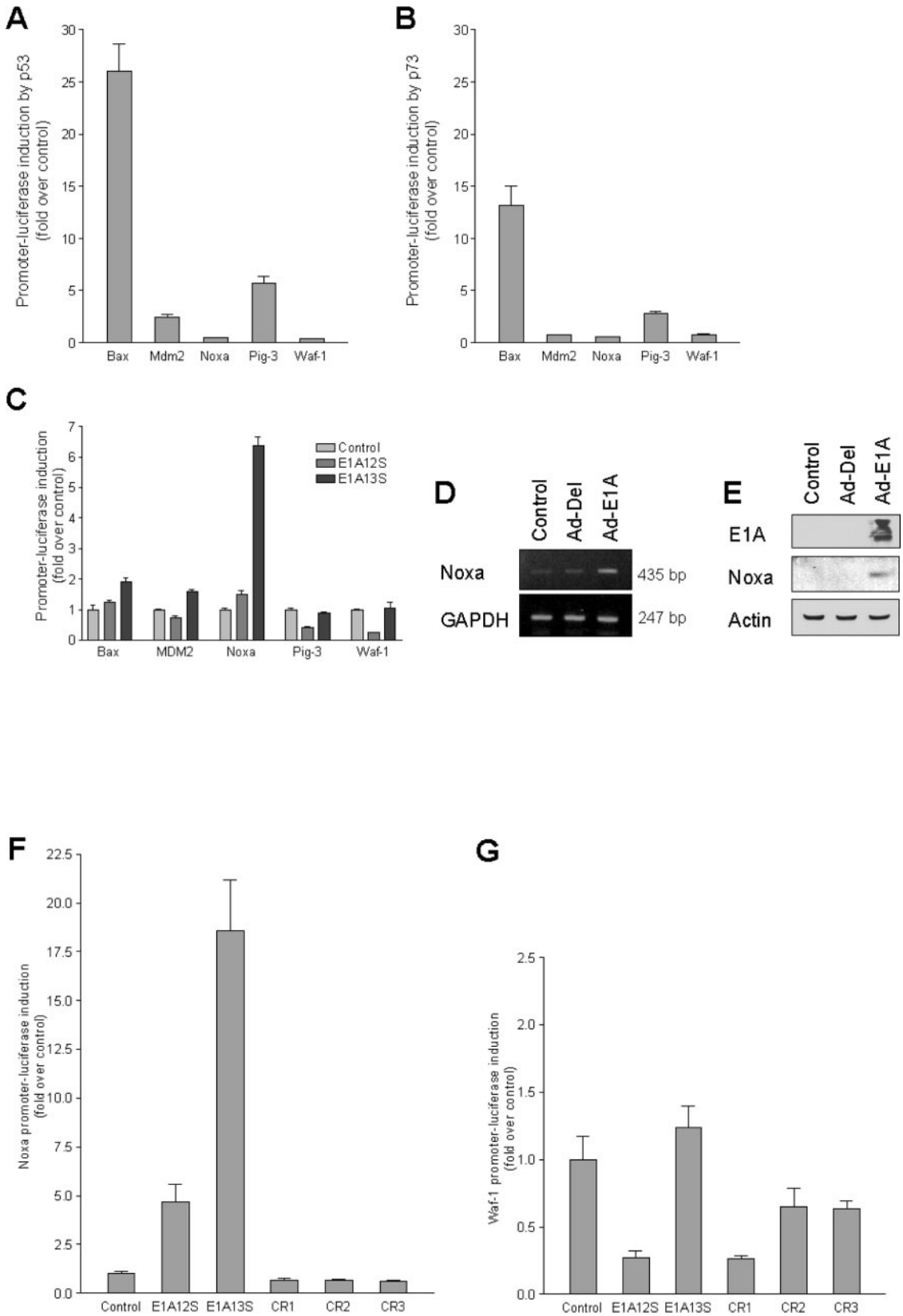


FIG. 8. **E1A-mediated transactivation of p53/p73 target genes.** SH-SY5Y cells were co-transfected with various luciferase reporter plasmids together with equal molar amounts of either empty vector DNA, pcDNA$_{3.1}$p53 (*A*), pcDNA$_{3.1}$TAp73 (*B*), and E1A12S or E1A13S plasmids (*C*). Luciferase activity was measured at 24 h post-transfection, and the result is shown as fold induction compared with basal luciferase activity level after transfection with empty vector DNA. The *error bars* indicate the standard deviations. *D*, E1A-mediated induction of Noxa mRNA. SH-SY5Y cells were left untreated or infected either with Ad-Del or Ad-E1A at an MOI of 5 and harvested 48 h post infection. PCR was carried out on cDNA generated from whole mRNA. Specific Noxa sequences were amplified using GAPDH as an internal control. The sizes of amplified fragments are indicated. *E*, E1A-mediated induction of Noxa protein. SH-SY5Y cells were left untreated or infected either with Ad-Del or Ad-E1A at an MOI of 5 and lysed 48 h post infection. Equal amounts of total protein from each sample were separated by SDS-PAGE, transferred to nitrocellulose, and hybridized to Noxa and actin using Noxa-specific primary monoclonal antibody clone 114C307 (Oncogene) and β-actin-specific primary monoclonal

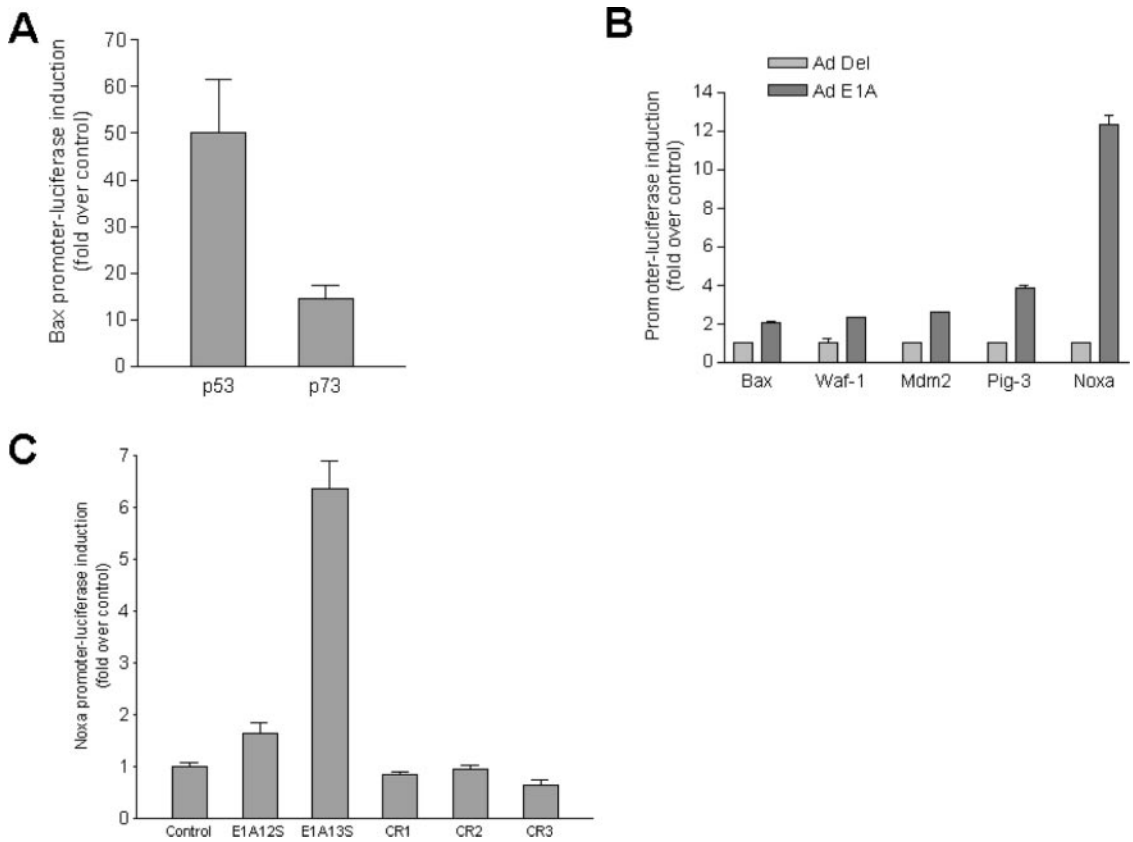


FIG. 9. **E1A-mediated transactivation of p53/p73 target genes in p53-deleted Saos-2 cell line.** Saos-2 cells were co-transfected with various luciferase reporter plasmids and equal molar amounts of either empty vector DNA, $pcDNA_{3.1}p53$, $pcDNA_{3.1}TAp73$, E1A12S, E1A13S, CR1, CR2, or CR3. Luciferase activity was measured at 24 h post-transfection, and the result is shown as fold induction compared with basal luciferase activity level after transfection with empty vector DNA. The *error bars* indicate the standard deviations. *A*, activation of Bax promoter by p53 and TAp73. *B*, the effect of E1A expression on the activation of promoters of p53 target genes. Luciferase activity in cells transfected with reporter plasmids and infected with either Ad-Del or Ad-E1A at an MOI of 5. Luciferase activity was measured at 24 h post-transfection, and the result is shown as fold induction compared with basal luciferase activity level after infection with Ad-Del. The *error bars* indicate the standard deviations. *C*, the effects of E1A12S, E1A13S, CR1, CR2, and CR3 on the activation of Noxa promoter. The cells were co-transfected with Noxa luciferase reporter plasmid together with equal molar amounts of plasmids expressing E1A12S, E1A13S, CR1, CR2, and CR3. Luciferase activity was measured at 24 h post-transfection, and the result is shown as fold induction compared with basal luciferase activity level after transfection with empty vector DNA. The *error bars* indicate the standard deviations.

of adenovirus infected cells and virus spread. Identification of isoform-specific cellular targets for E1A proteins is important in understanding their roles in regulating cell growth and apoptosis.

p73 has multiple roles ranging from tumor suppression to developmental regulation (35). In addition, several types of tumors, including those of the head and neck, overexpress p73 (33). We found overexpression of p73 in 30% of primary HNSCC tumors and in all seven HNSCC cell lines tested. However, the cause of overexpression and/or the increased stability of p73 in HNSCC tumors is currently unknown. Furthermore, it is not clear whether the activity of p73 protein present in these tumors is impaired. Frasca *et al.* (36) have recently shown that in thyroid tumor cells the overexpressed endogenous p73 was unresponsive to DNA-damaging agents because it failed to elicit a block of the cell cycle or induce an apoptotic response. The p73 overexpressed in HNSCC samples and cell lines might interact with transcriptionally dominant negative variants of p73 (ΔNp73) and/or with the mutant p53 present in all tumor samples tested, resulting in biologically inactive p73 (2, 3). However, the expression of specific p73 isoforms in the HNSCC samples could not be determined because of the lack of high quality, isoform-specific antibodies. In our study we show that the expression of exogenous p73 from adenoviral vectors was able to induce efficient apoptosis in cancer cell lines with p53 aberration. The majority of Ad-p73 expressing cells had an unusual morphology of double nuclei, indicating a possible arrest in mitosis, but the recognition of the precise role of p73 in cell cycle arrest requires further investigation. Interestingly, the HNSCC cell line HN5, which expresses high levels of stabilized mutant p53, was particularly sensitive to the induction of apoptosis by p73, whereas it was resistant to p53-mediated induction of apoptosis. Collectively the present data indicate that aberrations in the p73 pathway are associated with the pathogenesis of HNSCC and suggest that p73 replacement strategies may have important potential for the gene therapy of HNSCC with p53 mutations. This is in agreement with our recent observation showing that intact p73 function is important for head and neck cancer sensitivity to chemotherapy (37).

antibody (Sigma). *F*, SH-SY5Y cells were co-transfected with Noxa luciferase reporter plasmid together with equal molar amounts of plasmids expressing E1A12S, E1A13S, CR1, CR2, and CR3. Luciferase activity was measured at 24 h post-transfection, and the result is shown as fold induction compared with basal luciferase activity level after transfection with empty vector DNA. The *error bars* indicate the standard deviations. *G*, SH-SY5Y cells were co-transfected with Waf-1 luciferase reporter plasmid together with equal molar amounts of plasmids expressing E1A12S, E1A13S, CR1, CR2, and CR3. Luciferase activity was measured at 24 h post-transfection, and the result is shown as fold induction compared with basal luciferase activity level after transfection with empty vector DNA. The *error bars* indicate the standard deviations.

p73 has been shown to be involved in p53-independent apoptosis induced by E2F1, c-Myc, and E1A because expression of these genes resulted in the up-regulation of endogenous p73 proteins and activation of p73 transcription targets in $p53^{-/-}$ tumor cells (6). In contrast, recent reports have shown that E1A expression resulted in the inhibition of p73α- and p73β-mediated transcriptional activation (19, 20). The transcriptional activation function of p73 is specifically targeted by E1A through a mechanism involving p300/CBP proteins but not the pRB family of proteins (20). Our data show that E1A12S and in particular E1A13S activate the p73 promoter, resulting in the induction of p73 mRNA and protein in several p53-deficient cell lines. Furthermore, the presence of intact p300 and pRB binding were required for efficient activation of p73 promoter by E1A. These results parallel our previous observation that intact p300 and pRB binding are required for efficient induction of apoptosis in serum-starved or contact-inhibited normal rat kidney cells (38). The control of the transcriptional activity of p73 requires phosphorylation of p73 followed by Pin-1-mediated prolyl isomerase activity (39), followed by recruitment into the nuclear body onto the PML protein, which allows p300 to acetylate p73 and thus permits its transcriptional activity (29). Our results indicate that E1A is able to interfere with this mechanism. This is further supported by our recent data showing an induction of PML expression and reorganization of PML nuclear bodies in head and neck cancer cell lines expressing functional E1A (16).

The data presented here show that the E1A13S isoform has the ability to highly activate the p53 apoptotic target Noxa in several p53-deficient cell lines. Noxa, a "BH3-only" member of the Bcl-2 family, has been shown to function as a mediator of p53-dependent apoptosis through mitochondrial dysfunction (40). The molecular mechanism by which Noxa causes mitochondrial dysfunction is largely unknown. Noxa is transactivated by p53 in response to genotoxic agents (41). A correlation between the level of p53 and Noxa induction has been shown in chemically induced hepatocellular carcinoma in mice, suggesting that Noxa is a crucial mediator of p53-dependent apoptosis in the liver (42). Furthermore, mouse embryo fibroblasts deficient in Noxa showed notable resistance to oncogene-dependent apoptosis in response to DNA damage, and these mouse embryonic fibroblasts also showed increased sensitivity to oncogene-induced cell transformation *in vitro* (41). Studies using knockout cell lines have indicated that p73, as well as p53 function, is required for the induction of Noxa transcription in response to DNA damage induced by x-ray irradiation (4).

E1A12S was much less effective at inducing Noxa expression than E1A13S. Therefore we analyzed the role of the CR3 region of E1A13S alone on the induction of the Noxa promoter and showed that the expression of CR3 alone or together with CR1 and CR2 did not activate the Noxa promoter. However, these results do not rule out the importance of the CR3 region in E1A13S-mediated activation of p53 target genes. One likely explanation is that CR3 can only perform its function in the framework of the E1A protein. We have recently shown that CR3 expressed on its own is located in the cytoplasm of cancer cells, whereas E1A proteins are mainly located in the nucleus.[2] It is therefore likely that other E1A regions including the nuclear localization domain, which is located outside CR3 in the C-terminal part of E1A, are required for CR3 function. It would be interesting to examine the effect of different E1A domains targeted to the nucleus; such studies are currently in progress.

[2] M. Flinterman, J. S. Mymryk, and M. Tavassdi, manuscript in preparation.

We have demonstrated that induction of Noxa by E1A13S is independent of the p53 status of cells because it occurred in various cell lines in which p53 was inactivated by different mechanisms including mutation (HN5), deletion (Saos-2), or a defect in nuclear localization (SH-SY5Y). E1A expression in these cell lines resulted in the induction of TAp73; it is therefore likely that activation of Noxa is, at least partly, TAp73-mediated. The highest level of induction of Noxa was observed in SH-SY5Y cells, which also showed the maximum E1A13S-mediated activation of the TAp73 promoter. However, p73 expression on its own seems to be insufficient for the activation of the Noxa promoter (Fig. 8*A*). Other groups have shown weak activation of Noxa by p73 (4). The substantial activation of Noxa by E1A observed in our study thus suggests that E1A may cooperate with p73 to induce Noxa. Alternatively, E1A13S could have a direct activation function on the Noxa promoter independent of p53 and p73.

The molecular mechanism through which p73 induces apoptosis is not fully determined. Unexpectedly, TAp73 is able to directly transactivate Scotin and thus elicit endoplasmic reticulum stress, contributing to the killing mechanism (43). More importantly, TAp73 directly transactivates the p53 up-regulated modulator of apoptosis (PUMA), which then allows mitochondrial relocalization of Bax, followed by mitochondrial and apoptosome activation (44). This indicates that TAp73 exerts two distinct actions on Bax: (i) direct transactivation of its promoter, as reported in the present paper, and (ii) indirect (PUMA-mediated) translocation onto the mitochondrial membrane. The present data suggest that Noxa is possibly an additional target of p73 to trigger E1A-induced apoptosis in p53-deficient cells. These data show that p73, like p53, has several different mechanisms to regulate the sensitivity of the individual cell to die. Depending on the pathological stimulus and on the status of the cell, individual cells might use different pathways. This could be relevant, for example, to determine the sensitivity of the cancer cell to chemotherapy.

The activation of Noxa expression by E1A regardless of p53 status is likely to play a key role in the mechanism of apoptosis induction by E1A in some cancers and may provide an avenue for elucidating pathways of apoptosis regulation that could be exploited for tumor therapy, particularly if we consider that, in contrast to p53, p73 is rarely mutated in cancer.

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E1A Activates Transcription of p73 and Noxa to Induce Apoptosis

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