

Identification of Direct p73 Target Genes Combining DNA Microarray and Chromatin Immunoprecipitation Analyses*

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The newly discovered p53 family member, p73, has a striking homology to p53 in both sequence and modular structure. Ectopic expression of p73 promotes transcription of p53 target genes and recapitulates the most characterized p53 biological effects such as growth arrest, apoptosis, and differentiation. Unlike p53-deficient mice that develop normally but are subject to spontaneous tumor formation, p73-deficient mice exhibit severe defects in the development of central nervous system and suffer from inflammation but are not prone to tumor development. These phenotypes suggest different biological activities mediated by p53 and p73 that might reflect activation of specific sets of target genes. Here, we have analyzed the gene expression profile of H1299 cells after p73 α or p53 activation using oligonucleotide microarrays capable of detecting ~11,000 mRNA species. Our results indicate that p73 α and p53 activate both common and distinct groups of genes. We found 141 and 320 genes whose expression is modulated by p73 α and p53, respectively. p73 α up-regulates 85 genes, whereas p53 induces 153 genes, of which 27 are in common with p73 α . Functional classification of these genes reveals that they are involved in many aspects of cell function ranging from cell cycle and apoptosis to DNA repair. Furthermore, we report that some of the up-regulated genes are directly activated by p73 α or p53.

but, upon different types of stress, becomes modified and activated (3) and exerts its biological activities, including growth arrest, apoptosis, and differentiation (4–6).

The recently discovered p53 family member, p73, shares a remarkable homology in DNA sequence and protein structure with p53. Indeed, p73 can be roughly divided into three domains, (a) the N-terminal transactivation domain, which shares 29% homology with the N-terminal part of p53, (b) the sequence-specific DNA binding domain, which shares 63% of homology with the corresponding p53 domain, and (c) the tetramerization domain, which shares 42% of homology with the oligomerization domain of p53 (7). Unlike p53, p73 is subject to alternative splicing, giving rise to a family of proteins whose individual function has yet to be elucidated (1, 8–11). p73 is not inactivated by viral oncoproteins such as SV40 large T antigen, HPV E6, and Ad E1Bp55, well known inactivators of p53 (12–15). Furthermore, although p53 is stabilized and activated by diverse types of stress including DNA-damaging agents, radiation, oncogenes, hypoxia, and ribonucleotide depletion, to date p73 is known to be stabilized only in response to cisplatin and γ -radiation (3, 4, 16–18). It has recently been shown that p73 can be acetylated in response to doxorubicin and selectively directed to activate specific target genes (19). More recent work has reported that p73 is required for p53-dependent apoptosis in response to DNA damage (20).

Ectopic expression of p73 in p53^{-/-}, and p53^{+/+} cells causes growth arrest, apoptosis, and differentiation, as does p53 (7, 21–25). These effects are achieved mainly through the activation of a plethora of specific target genes. Several reports show that p73 binds to p53 binding sites *in vitro* and *in vivo* and, consequently, activates p53 target genes (7, 22). Thus, transcriptional activation or repression of specific sets of target genes mediates the biological effects of both p53 and p73. The importance of functional and physical integrity of the transcriptional activation domain for p53 activity has been clearly demonstrated by the findings that mice carrying a p53 mutated in the N-terminal transactivation domain are prone to develop tumors, as do p53-deficient mice (26). Major differences between p53 and p73 have been revealed by *in vivo* ablation of the genes. Thus, p53- and p73-deficient mice exhibit quite different phenotypes; p53-deficient mice develop normally but undergo spontaneous tumor formation (mainly sarcoma and lymphomas), whereas the p73 counterparts exhibit severe defects in the development of the central nervous system and suffer from inflammations (27, 28). Such differences are likely to depend also on activation or repression of different sets of target genes that need to be identified. Indirect support for such hypothesis has been provided by the recent findings that the potent tran-

The p53 tumor suppressor gene is the most frequent target for genetic alterations in human cancers (1, 2). The wild type (wt)¹ p53 protein is apparently latent under normal conditions

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¹ The abbreviations used are: wt, wild type; RT, reverse transcription; TGF, transforming growth factor.

scriptional co-activator yes-associated protein (YAP) binds to the long forms of p73 and p63 but not to p53 (29).

The recent development of DNA microarrays has allowed global analysis of the pattern of activated or repressed genes in response to different types of stimuli, including p53 activation (30–32). Taking advantage of this approach, we compared the gene expression profiles upon ponasterone A induction of p73 α or p53 in the same cellular context, H1299. We found that p73 α or wt-p53 expression modifies 141 and 320 genes, respectively. p73 α up-regulates 85 genes, of which 25 are specific and 27 are in common with p53 regulation, whereas p53 induces 153 genes, of which 63 are specific. We will focus here on the p73 activated genes in response to ponasterone induction. Functional classification of these genes reveals that they are involved in many aspects of cell function ranging from cell cycle and apoptosis to DNA repair, including also several brain-specific genes involved in synaptic regulation. Furthermore, we report that p73 α or p53 are recruited directly to some of the activated genes. Our findings indicate that, upon ectopic expression in the same cellular context, p73 α promotes a specific transcriptional gene profile that only partially overlaps with that of p53.

EXPERIMENTAL PROCEDURES

Cell Lines—The H1299 cell line is derived from a human non-small cell lung carcinoma. H1299 cells were maintained in RPMI medium supplemented with 10% fetal calf serum. Before transfection, the culture medium was changed to Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. H1299-pVgRXR cells were maintained in the same medium containing zeocin (100 μ g/ml). The H1299-p73 α and H1299-wt-p53 cell lines were maintained in medium containing zeocin and G418 (400 μ g/ml). To induce expression of p73 α or p53, ponasterone A, a synthetic analogue of ecdysone, was added to the medium (final concentration, 2.5 μ M).

Plasmids and Transfections—Plasmids pVgRXR and pIND were from Invitrogen. pIND/p73 α was prepared by cloning the BamHI/EcoRV fragment of human p73 α , generated by PCR (the sequence of the oligonucleotides is available upon request), into pIND. pIND/wt-p53 was prepared by cloning the HindIII/XbaI fragment of human p53 (a kind gift of Dr. T. Unger) into pIND (33). H1299 cells were transfected with each plasmid using the calcium phosphate method. Clone selection was done with zeocin (100 μ g/ml) for pVgRXR and with G418 (400 μ g/ml) for pIND/p73 α and pIND/wt-p53. Stable clones obtained by double selection were screened by immunoblotting using antibody Ab4 for p73 (Neomarker) and DO1 for p53.

Immunoblot Analysis—Total cell lysates were prepared as previously described (34), and protein content was determined with the Bio-Rad protein assay kit (Bio-Rad). Samples containing 50 μ g of total protein were resolved by SDS-10% PAGE and transferred to nitrocellulose membranes (Bio-Rad). For p73 detection, a p73 monoclonal antibody was used at a 1:200 dilution; for p53 detection, a mixture of p53 monoclonal antibodies DO1 and 1801 was used at a 1:40 dilution; for p21^{waf1} detection, a p21 polyclonal antibody (C19, Santa Cruz) was used at a 1:200 dilution.

Cell Cycle Analysis—Cells (3×10^5) were seeded on 60-mm dishes, and 12 h later the medium was replaced by medium containing ponasterone A (2.5 μ M final concentration). 24 h after ponasterone A addition, floating cells and attached cells were collected, washed in phosphate-buffered saline, resuspended in 1 ml of phosphate-buffered saline, and fixed in 5 ml of cold methanol for 30 min at -20°C . After centrifugation and a further wash in phosphate-buffered saline, cells were resuspended in 1 ml of phosphate-buffered saline containing 50 μ g/ml RNase and 50 μ g/ml propidium iodide (Sigma) and analyzed by cytofluorimetry with an Epics-XL analyzer (Coulter Corp.). Data were analyzed using the Cellfit program.

RNA Extraction and Reverse Transcriptase Reaction—Cells from H1299-pIND clone 1, H1299-p73 α clones 9 and 11, and H1299-p53 clones 23 and 16 were harvested in TRIzol reagent (Invitrogen) at specific time points (0, 5, 9, 12, 24 h) after ponasterone A addition, and total RNA was isolated as per the manufacturer's instructions. Five micrograms of total RNA were reverse-transcribed at 37°C for 45 min in the presence of random hexamers and Moloney murine leukemia virus reverse transcriptase (Invitrogen). PCR analyses were carried out by using oligonucleotides specific for the following genes: CaN19 (down,

5'-CTCTGAATTCGCCACAGATCCATGATGTGC; up, 5'-CTCTGCGG-CCGCCAACAGACAAAAAAGTTTAT TGAATACAAAAC); CaN19 (down, 5'-GTAAGGGGAAATGAAGGAAC TTCT; up, 5'-ACAAAAC-CAAAGGCATCAACAGTC); 14-3-3 σ (down, 5'-TCTCAGTAGCCTATA-AGAACGTGGTG; up, 5'-ATCTCGTAGTGGAAAG ACGGAAAAGT); PIG3 (down, 5'-CCGAAAACCTCTACGTGA; up, 5'-CTCTGCGGATA-GGCATGAGGA); α_1 -antitrypsin (down, 5'-TTCTTCTCC CCAGTGAG-CAT; up, 5'-GTGTCCCCGAAGTTGACAGT); p21^{waf1} (down, 5'-CCTC-TTCGCCCCGGTGGAC; up, 5'-CCGTTTTTCGACCCTGAGAG); PTGF- β (down, 5'-GAGCTGGGAAGATTGCAACA; up, 5'-AGA TTCTGCCAGC-AGTTGGT); JAG2 (down, 5'-CCTTAAGGAGTACCAGGCCAA; up, 5'-AAGTGGCGGTGTAGTAGTTCTCGT). The housekeeping aldolase A mRNA, used as an internal control, was amplified from each cDNA reaction mixture using the following specific primers: down, 5'-CGCA-GAAGGGTCTCTGGTGA; up, 5'-CAGCTCCTTCTCTGCTGCG. Amplified PCR products were electrophoresed on a 2% agarose gel containing ethidium bromide (0.5 μ g/ml) and visualized under UV light.

Preparation of Labeled cRNA and Hybridization of Microarrays—Total RNA was isolated from H1299-PIND clone 1, H1299-p73 α clone 9, and H1299-p53 clone 23 cells after the addition of ponasterone A for 0, 5, 9, 12, and 24 h. Biotin-labeled cRNA was synthesized and hybridized as described (35) to the Genechip HuGene FL array (Affymetrix, Santa Clara, CA), which contains probes for $\sim 11,000$ mRNA species, and one chip was hybridized to cRNA from each time point. Scanned output files were visually inspected for hybridization artifacts. Arrays lacking significant artifacts were analyzed using Genechip 3.3 software (Affymetrix). Arrays were scaled to an average intensity of 1200/gene and analyzed independently. The expression value for each gene was determined by calculating the average of differences (perfect match intensity minus mismatch intensity) of the probe pairs in use for this gene. Ratios were determined by dividing the average difference of H1299-p73 α or H1299-p53 for each time point with those of the 0-h time point.

Clustering Analysis—Clustering analysis was performed by the super-paramagnetic clustering method (36) on the 211 genes that were up-regulated more than 2-fold in at least 3 time points by the induction of p53 (153 genes) or p73 α (85 genes), where 27 genes were common to both p73 and p53. The gene had to be "Present" in the Present/Absent call provided by Affymetrix software at least at one time point.

Each gene was represented by eight components representing the ratio of the average difference value provided by the Affymetrix software at each time point divided by that of 0-h time point (before the addition of ponasterone). The 0-h time point was the average of the 0 h of the two cell lines and the eight components containing four from the p73 α and four from the p53 induced cells, each at the indicated time point (5, 9, 12, and 24 h). Before clustering we normalized each row such that its mean vanishes, and its norm is one (35) as follows. A_{ij} represents the ratios of the expression of gene i (where $i = 1-211$), measured at experiment (and time point), $j = 1-8$. We subtract from A_{ij} its average $\langle A_i \rangle$ (see Equation 1) and divide the difference by σ_i , its S.D. for gene i (see Equation 2); the resulting 8-component vector represents gene i , and the resulting normalized matrix is denoted by B_{ij} (see Equation 3).

$$\langle A_i \rangle = \frac{1}{8} \sum_{j=1}^8 A_{ij} \quad (\text{Eq. 1})$$

$$\sigma_i^2 = \frac{1}{8} \sum_{j=1}^8 (A_{ij} - \langle A_i \rangle)^2 \quad (\text{Eq. 2})$$

$$B_{ij} = \frac{(A_{ij} - \langle A_i \rangle)}{\sigma_i} \quad (\text{Eq. 3})$$

The clustering algorithm measured the distance between the genes using the regular Euclidean distance between their normalized values. Genes with similar expression profiles (over the eight time points) are represented by two nearby vectors and are placed in the same cluster.

Formaldehyde Cross-linking and Chromatin Immunoprecipitation—H1299-p73 α clone 9 and H1299-wt-p53 clone 23 cells were treated with ponasterone A to induce the expression of p73 α and wt-p53 for 24 h. DNA and proteins were cross-linked by the addition of formaldehyde (1% final concentration) 10 min before harvesting, and cross-linking was stopped by the addition of glycine pH 2.5 (125 μ M final concentration) for 5 min at room temperature. Cells were scraped off the plates, resuspended in hypotonic buffer, and passed through a 26-gauge needle. Nuclei were spun down, resuspended in 300 μ l of SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8, and a protease inhibitor

mixture), and sonicated to generate 500–2000-bp fragments. After centrifugation, the cleared supernatant was diluted 10-fold with immunoprecipitation buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40). The cell lysate was precleared by incubation at 4 °C with 15 μ l of protein G beads preadsorbed with sonicated single-stranded DNA and bovine serum albumin. The cleared lysates were incubated overnight with a mixture of anti-p73 polyclonal antibodies (C17 and C19) (Santa Cruz), anti-green fluorescent protein polyclonal antibody (Invitrogen), or anti-p53 (Ab7) or without any antibody. Immune complexes were precipitated with 30 μ l of protein G beads preadsorbed with sonicated single-stranded DNA and bovine serum albumin. After centrifugation the beads were washed, and the antigen was eluted with 1% SDS, 100 mM sodium carbonate. DNA-protein cross-links were reversed by heating at 65 °C for 4–5 h, and DNA was phenol-extracted and ethanol-precipitated. Levels of CaN19, p21^{waf1}, 14-3-3 σ , PIG3, α_1 -antitrypsin, JAG2, and PTGF- β DNAs were determined by PCR using oligonucleotides spanning the p53/p73 binding sites. The following specific oligonucleotides were used: CaN19-up site (down, 5'-GTGTTCAAAGCCGTGACACCTAATT; up, 5'-TGGATCATAGCTCACTGTAAATCTCG); CaN19-down site (down, 5'-AAGTAGCTGGGACTACAAGCGTATG; up, 5'-GGGATAGAAAAGCCAGCTAAGATA); p21^{waf1}-up site (down, 5'-CTATTTGGGACTCCCAGTCTCTT; up, 5'-GGTTACTCTGGGGTCTTTAGAGGTC); p21^{waf1}-down site (down, 5'-ATGTATAGGAGCGAAGGTGCA GAC; up, 5'-CCTCCTTCTGTGCCTGAAACA); 14-3-3 σ (down, 5'-CTGTACTTCAGCCTGGACATCAGAG; up, 5'-CCGACCTAATAGTTGAGCCAG GAT); PIG3 (down, 5'-CAGGACTGTACAGGAGCGCAGTATAAGG; up, 5'-GTGCGATTCTAGCTCTCACTCAAGCAGAGG); JAG2 (down, 5'-ACTGCTGCCTTCTGGAAACTC; up, 5'-CAAGTGGTGAACAAGGGAGACT). Oligonucleotides specific for thymidine kinase promoter (down, 5'-GTGAACCTCCCGAGGCGCAA; up, 5'-GCCCTTTAAACTTGGTGGGC) were used as negative control.

RESULTS

Generation of Stable Cell Lines Expressing Inducible p73 α or p53—Inducible cell lines overexpressing p73 α or p53 were generated in two steps. First, H1299 cells were transfected with pVgRXR followed by zeocin selection (33, 34). The resulting clones were transiently transfected with pIND/p73 α or with pIND/wt-p53 plasmid and maintained in the presence of ponasterone A. The highest expressor of either p73 α or wt-p53 was chosen and stably transfected with the above-mentioned plasmids or the pIND control vector followed by G418 selection. Western blot and immunostaining with an anti-p73 monoclonal antibody or with a mixture of anti-p53 DO1 and 1801 monoclonal antibodies were performed to screen for p73 α - and p53-inducible expression and intracellular localization. As seen in Fig. 1, A and B, upper panels), for 2 representative clones

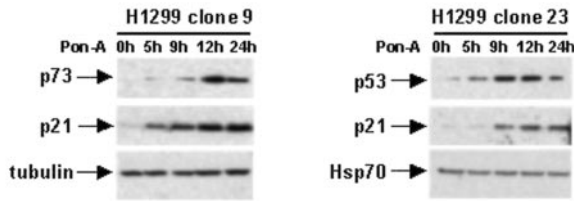


FIG. 1. Ponasterone A induced expression of p73 α , p53, and p21^{waf1} in H1299 cells. Total cell lysates (100 μ g/lane) derived from H1299-p73 α clone 9 and H1299-p53 clone 23 cells, respectively, were lysed at the indicated time points after the addition of 2.5 μ M ponasterone A. Levels of p73 α , p53, and p21^{waf1} were detected by probing the nitrocellulose filter with a monoclonal anti-p73 antibody (Ab4) with a mixture of anti-p53 monoclonal antibodies DO1 and 1801 and with an anti-p21^{waf1} polyclonal serum, respectively. Equal loading of protein amount for each line was determined by probing with anti-tubulin or anti-Hsp70 antibodies.

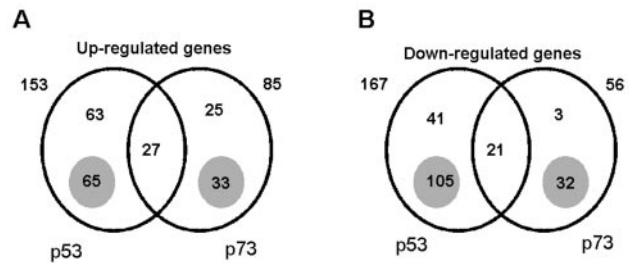


FIG. 3. Venn diagram of genes regulated by p53 and p73 α . Only genes that showed at least 2-fold up (A) or down (B) regulation at a minimum of 3 time points as compared with the control were included in the analysis. The diagram shows that 27 and 21 genes were up- and down-regulated, respectively, by both p53 and p73 α . These genes are denoted “common” genes. The number of genes regulated, respectively, by either p53 or p73 α are shown in the upper part of the circle, whereas genes that passed the cutoff (2-fold overexpression at 1 or 2 time points only) for one of the transcription factors (p73 or p53) are shown in the shaded small circles.

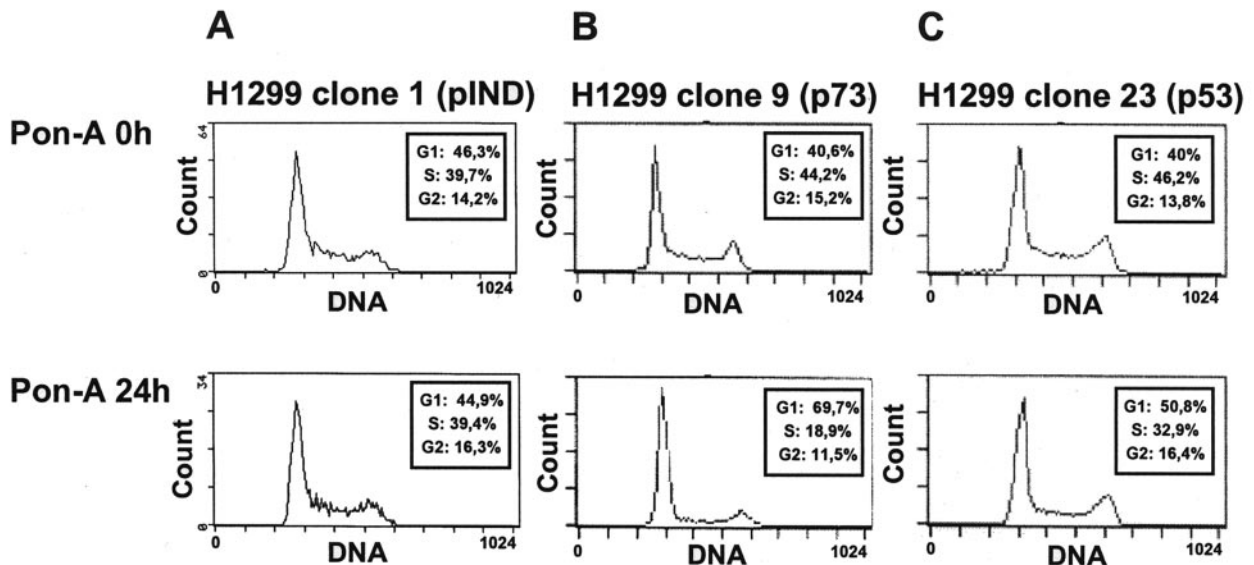


FIG. 2. Ectopic expression of either p73 α or p53 promotes cell cycle modification in H1299 cells. Shown is cell cycle analysis of H1299-pIND clone 1, H1299-p73 α clone 9, and H1299-p53 clone 23 cells by propidium iodide staining. To induce p73 α and p53, cells were stimulated for 24 h with 2.5 μ M ponasterone A (Pon-A). At that time floating and adherent cells were pooled, fixed, and stained as described under “Experimental Procedures.” The cytofluorimetric analysis was performed with the aid of an Epics-XL analyzer (Coulter). Data were analyzed using the Cellfit program.

TABLE I
Target genes modulated by p73 α and p53

The data were derived from affymetrix chip analysis and show fold change of expression at the indicated time points for 85 genes (see Fig. 3). The letter C indicates genes that were clustered together with the p73-only genes (10 genes, see "Results"). The asterisks (p73*) indicates genes that were regulated by p73 and partially regulated by p53 (33 genes, Fig. 3A) but only once or twice above 2-fold. HIV, human immunodeficiency virus; MDR, multidrug resistance; PML, promyelocytic leukemia.

Inducer	Accession No.	Symbol	Gene function	p73			p53				
				5	9	12	24	5	9	12	24
Cell cycle											
p73	AF001436	CEP2	Cdc42 effector protein 2	3.6	2.4	2.7	2.2	1.0	1.5	1.4	1.6
p73	X57348	SFN	Stratifin (14-3-3 sigma)	1.2	2.9	2.5	8.2	1.0	0.8	1.0	0.9
p73	M87068	CAN19	CaN19	2.0	3.5	3.4	7.9	1.5	1.2	1.2	1.4
p73*	W28729	MCM3AP	MCM3-associated protein	2.6	3.0	1.6	2.4	3.3	0.9	2.0	2.1
p73*	U47414	CCNG2	cyclin G2	2.1	2.3	2.3	3.8	1.6	1.9	3.8	1.8
p73/p53	AI304854	CDKN1B	p27, Kip1	2.9	2.3	2.7	2.7	3.4	2.9	6.4	5.4
p73/p53	U03106	CDKN1A	p21 WAF1	1.6	2.9	2.9	3.3	1.9	3.2	3.0	4.3
DNA repair											
p73	AL039458	LIG1	DNA ligase	2.5	2.3	2.6	2.3	0.7	0.7	0.7	0.7
p73*	CAC004770	FEN1/RAD2	Endonuclease involved in base excision repair	1.2	3.2	4.2	7.9	2.4	0.6	2.3	1.2
p73/p53	D42045	SNM1	DNA-cross-link repair gene <i>SNM1</i>	2.5	1.2	2.1	2.2	2.0	3.2	2.0	2.3
p73/p53	AF076838	RAD17	DNA damage G ₂ checkpoint	2.7	1.7	4.6	4.1	2.0	2.1	3.4	7.4
Tumor suppressor/apoptosis											
p73	AF010309	PIG3	Quinone oxidoreductase	1.1	1.8	2	2.2	1.2	1.5	1.5	1.1
p73	U60060	FEZ1	Lung cancer tumor suppressor	0.6	2.1	2.1	5.5	0.6	1.8	0.8	1.5
p73*	AL049783	CG005	Hypothetical tumor suppressor from BRCA2 region	2.5	1.3	2.1	2.7	1.8	1.3	2.5	1.7
p73/p53	D86958	RB1CC1	Rb transcription regulator	2.7	2.0	2.6	2.7	2.2	2.0	3.1	2.6
p73/p53	AF045581	BAP1	BRCA1-associated ubiquitin hydrolase	3.8	1.9	3.5	4.2	3.8	2.0	4.9	3.4
p73/p53	U13897	DLG1	Homolog to <i>Drosophila</i> tumor suppressor	2.2	1.8	2.5	3.8	2.5	2.1	3.3	3.5
p73/p53	AI813532	TNFRSF1B	Tumor necrosis factor receptor superfamily	2.4	2.8	1.7	2.5	1.6	2.8	2.7	2.6
Growth factor/growth regulator											
p73	U20982	IGFBP4	Insulin-like growth factor-binding protein 4	2.7	2.7	2.4	2.4	0.5	0.6	0.5	0.5
p73/p53	AB000584	TGF- β	TGF- β superfamily protein MIC1	1.4	5.1	5.3	11.7	3.4	8.7	8.8	5.3
p73/p53	M29053	IL-7	Interleukin 7	2.6	2.3	2.7	2.8	3.1	3.1	3.4	3.0
p73/p53	M34057	LTBP1	TGF- β -binding protein	1.3	2.2	2.0	2.4	2.9	2.3	2.1	2.5
Receptor											
p73	X52425	IL-4R	Interleukin 4 receptor	1.9	2.4	2.2	2.4	0.9	0.8	0.7	0.8
p73	AF065388	TSPAN-1	Tetraspan 1 cell adhesion differentiation	2.0	3.4	2.4	2.2	1.7	1.6	1.2	1.4
p73*	CAF022853	ABCC1	MDR-associated protein	2.4	2.2	2.3	3.0	1.4	1.5	2.5	1.6
p73/p53	CU83993	P2RX4	Purinergic receptor ligand gated, ion channel	6.4	7.9	9.6	9.1	2.4	3.5	4.7	3.3
Signaling											
p73	X99076	NRGN	Neurogranin (protein kinase C substrate, RC3)	2.1	2.2	2.4	1.2	0.6	0.4	0.4	1.1
p73	AF023917	DUSP11	Dual specificity phosphatase 11	2.1	1.7	2.0	2.6	1.3	1.2	1.8	2.0
p73	AF029778	JAG2	Jagged 2 Notch ligand, signaling development	1.0	2.9	2.5	2.2	1.0	0.9	0.6	0.6
p73	L35545	PROCR	Protein C receptor, endothelial (EPCR)	1.5	2.4	2.6	2.9	1.0	1.2	1.3	1.0
p73	M64572	PTPN3	Protein-tyrosine phosphatase type 3	1.0	4.9	3.6	2.9	1.4	1.0	1.0	1.0
p73	L15388	GPRK5	G protein-coupled receptor kinase 5	2.2	2.1	2.3	1.3	1.9	1.7	1.1	1.3
p73*	CAF059617	SNK	Serum-inducible kinase	2.7	2.7	2.7	5.3	1.3	1.4	2.0	1.5
p73*	CL22342	SP110	Interferon-induced protein nuclear, PML-associated	3.1	2.6	4.4	3.8	2.0	2.1	1.8	1.7
p73*	AB011143	GAB2	GRB2-associated-binding protein 2	2.8	1.4	2.1	2.5	1.6	1.7	2.8	1.7
p73*	AF068179	CAMLG	Calcium-signaling modulating ligand	2.4	2.4	1.8	3.6	1.4	1.6	4.1	2.0
p73/p53	AA485440	SPHK2	Sphingosine kinase type 2 isoform	3.7	3.4	2.3	3.7	2.7	3.1	2.3	1.2
p73/p53	W29065	PTP4A3	Protein-tyrosin phosphatase type Iva	1.9	5.6	3.1	3.4	4.1	4.3	2.8	4.7
p73/p53	W26524	PPP4R2	Protein phosphatase 4 regulatory subunit 2	2.0	2.6	2.5	4.2	3.5	3.1	4.1	1.7
p73/p53	CAA457029	INPP4A	Inositol polyphosphate-4-phosphatase	2.4	5.9	2.7	5.0	3.2	2.1	2.2	1.6
Matrix/protease inhibitors											
p73	X01683	SERPINA1	α_1 -Antitrypsin	1.0	4.1	3.9	8.4	0.9	1.8	1.9	0.4
p73	Y14690	COL5A2	Collagen, type V, α 2	0.9	2.1	2.4	2.2	1.7	1.5	1.5	1.4
p73*	AF001691	PPL	Periplakin	0.9	2.4	2.0	5.3	1.9	1.3	4.8	2.5
p73*	AA142964	ADAM17	TNF α ; converting metalloproteinase	2.0	1.9	2.5	2.4	1.3	1.5	2.3	2.1
Metabolism/protein degradation											
p73	U18543	TRIM32	Putative F3-ubiquitin ligase	2.3	2.2	1.6	2.1	1.2	1.4	1.3	1.1
p73	J00139	DHFR	Dihydrofolate reductase	2.3	3.3	2.6	1.4	0.9	1.9	0.9	1.3
p73	AF061741	SDR1	Short chain dehydrogenase/reductase 1	1.6	2.7	2.6	2.6	1.5	1.1	1.1	0.7
p73*	CS52028	CTH	Cystathionase	2.0	3.5	4.0	3.7	1.0	1.4	2.6	1.4
p73*	CAL050290	SAT	Spermine N1-acetyltransferase	2.7	2.2	3.0	5.1	1.3	1.2	2.2	1.7
p73*	U25165	FXR1	Translation inhibition/RNA binding	2.4	2.2	1.1	2.7	1.8	1.7	3.8	2.4
p73*	AL080177	UBL3	Ubiquitin-like 3	3.3	3.3	1.3	2.4	2.3	1.9	2.0	1.3
p73*	X69433	IDH2	Isocitrate dehydrogenase 2	3.4	2.8	2.9	1.6	1.3	1.4	5.6	1.6
p73/p53	AF020761	UBE2D1	Ubiquitin-conjugating enzyme E2D 1	3.1	1.0	2.2	3.1	2.9	1.7	2.9	3.3
Transcription factor											
p73	M99701	TCEAL1	Transcription elongation factor A	2.3	2.6	3.7	3.6	1.6	1.5	1.4	1.4
p73*	AF044288	ARNTL	Aryl hydrocarbon receptor nuclear translocator-like	2.7	1.3	2.9	2.3	0.9	1.9	2.7	1.8
p73*	X51435	HIVP1	HIV virus type I enhancer-binding protein 1	3.0	1.9	2.7	3.1	1.7	1.5	3.1	2.3
p73*	U03105	B4-2	Proline-rich protein with nuclear-targeting signal	1.4	2.0	2.1	3.1	1.2	1.5	3.1	1.6
p73/p53	U48213	DBP	Albumin promoter-binding protein	2.7	2.5	2.6	3.5	3.3	2.8	2.3	0.7

TABLE I—continued

Inducer	Accession No.	Symbol	Gene function	p73				p53			
				5	9	12	24	5	9	12	24
Redox/metalloproteins											
p73	W27419	?	Similar to metallothionein 1 E	1.8	4.4	3.4	3.1	0.5	1.1	0.9	0.4
p73*	M13485	MT1B	Metallothionein antioxidant	1.9	2.1	2.7	2.6	1.8	1.8	2.3	2.7
p73/p53	A224832	MT1L	Metallothionein 1L	1.8	4.1	3.3	6.5	2.2	2.2	5.3	4.0
Others/unknown											
p73	D50370	NAP1L3	Nucleosome assembly protein 1-like 3	2.1	2.1	2.2	2.5	0.8	0.9	1.1	0.6
p73	AI655015	?	cDNA DKFZp586F2224	1.7	2.2	2.0	2.5	1.1	1.1	1.2	1.5
p73	AB007969	KIAA0500	KIAA0500 protein	2.0	3.3	4.6	6.8	0.7	1.0	1.7	1.3
p73*	CF038202	?	Clone 23570 mRNA	2.8	2.0	2.4	2.8	1.3	1.4	2.0	1.8
p73*	CAL080133	SYNE-2	Synaptic nuclei expressed gene 2	2.2	1.5	2.3	2.9	1.4	1.7	2.3	1.5
p73*	F070569	MGC14376	Hypothetical protein MGC14376	2.4	1.1	2.1	2.1	1.4	1.2	1.7	2.5
p73*	AL049404	MFAP3	Microfibrillar-associated protein 3	2.3	0.9	2.9	2.4	3.0	1.1	1.9	4.6
p73*	W87858	HSA9761	Putative dimethyladenosine transferase	2.1	0.8	2.0	2.3	2.0	2.1	1.5	1.8
p73*	X79888	AUH	AU RNA-binding protein	2.3	1.3	2.3	2.5	1.8	1.8	2.9	3.0
p73*	AA398463	?	cDNA DKFZp564C1563	2.5	2.1	2.2	1.9	2.1	1.1	1.1	1.7
p73*	AI857458	UCN	Urocorin, secreted	2.5	2.2	2.6	1.1	2.3	1.5	3.3	1.9
p73*	AB007938	KIAA0469	KIAA0469 protein	2.8	1.7	2.1	2.1	1.8	1.4	1.7	2.1
p73*	AF038202	?	Clone 23570 mRNA sequence	2.8	2.0	2.4	2.8	1.3	1.4	2.0	1.8
p73*	AL050172	?	cDNA DKFZp586F1322	2.9	2.8	2.2	2.6	2.2	0.5	1.9	1.2
p73*	AL031447	?	?	1.8	3.2	2.7	2.7	2.0	2.8	1.5	0.7
p73*	AF042181	TSPYL	TSPY-like	3.8	3.4	1.0	5.1	4.5	1.3	1.1	1.5
p73/p53	X13839	ACTA2	Actin, α 2, smooth muscle, aorta	1.9	2.3	3.3	7.5	2.1	4.9	14.8	7.6
p73/p53	D43636	KIAA0096	KIAA0096 protein	3.7	1.5	2.5	2.5	2.2	2.1	4.4	4.4
p73/p53	D50419	ZNF175	Zinc finger protein 175	2.7	2.4	2.7	2.5	3.9	2.3	3.2	4.3
p73/p53	D38037	FKBP1B	FK506-binding protein	1.4	2.8	2.2	6.3	2.0	2.8	4.0	2.2
p73/p53	AA149637	TBL1	Transducin (β)-like 1	3.1	2.4	1.7	2.9	2.7	1.1	2.0	3.1
p73/p53	U79751	BLZF1	Basic leucine zipper nuclear factor 1 (JEM-1)	3.1	3.2	2.5	3.2	2.6	2.1	2.8	2.2
p73/p53	M29386	PRL	Prolactin	2.6	3.3	1.8	2.8	2.6	1.1	2.1	2.1
p73/p53	U07364	KCNJ4	Potassium inwardly-rectifying channel	2.0	2.4	0.7	2.1	2.6	2.1	2.0	2.3

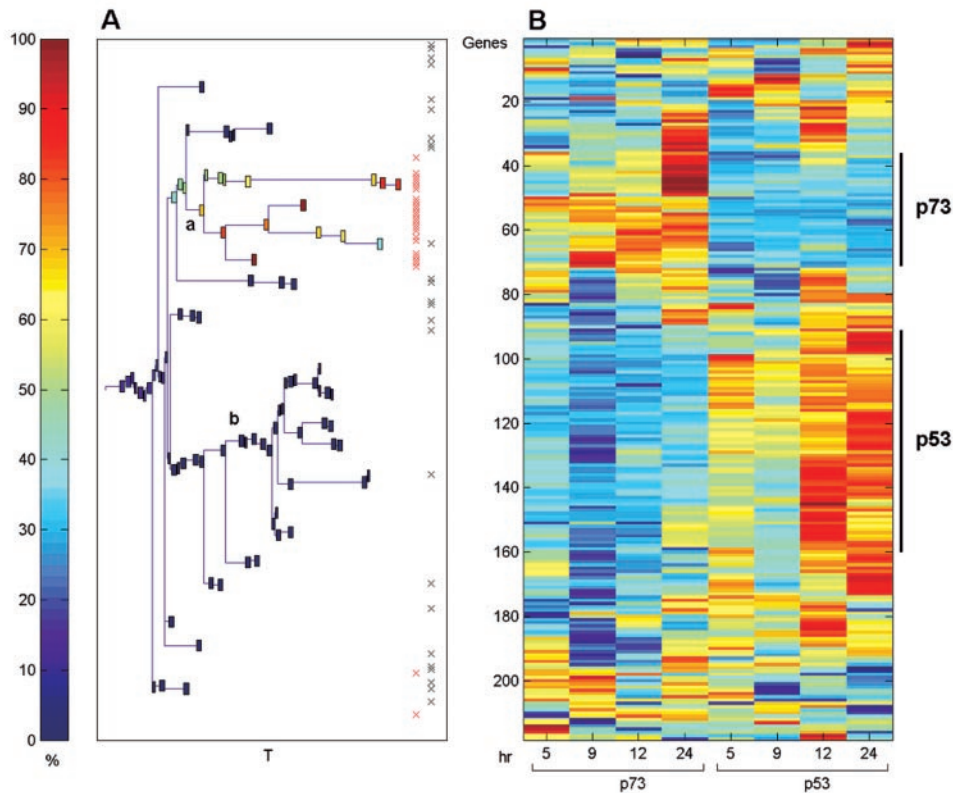


FIG. 4. Clustering results using the super-paramagnetic clustering algorithm for the 211 genes that were up-regulated at 3 or more time points upon ponasterone A activation of p73 α or p53. A, dendrogram of the genes that includes clusters of size 5 and larger. Each cluster is represented by a box colored according to the percent of p73-only target genes (25 genes) contained in the cluster. The distribution of these 25 genes is marked by red 'x's at the right. The distribution of the common genes (27 genes up-regulated by both p73 α and p53) is shown by the black 'x's. The clusters marked by a and b were used to plot the expression profile of the genes in that cluster (see Fig. 5). B, the expression matrix of the genes according to the dendrogram on the left. The color represents induction (red) or repression (blue). T is a parameter of the super-paramagnetic clustering algorithm that controls the resolution at which the cluster is found (36). The genes activated by either p73 α or p53 as defined by the clustering procedure are shown on the right-hand side of the expression matrix.

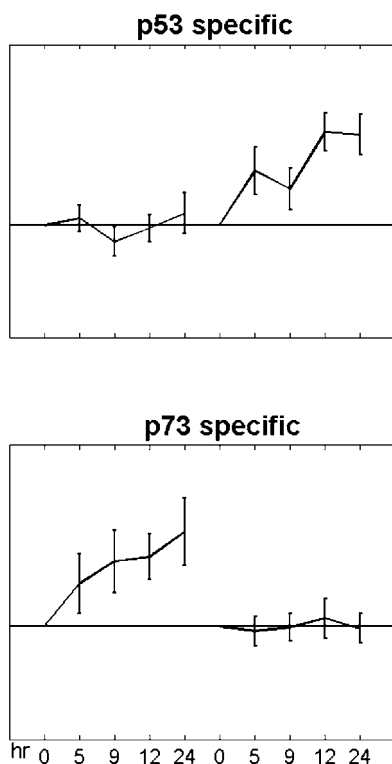


FIG. 5. Average expression profile of genes in clusters *a* and *b* of Fig. 4A. The expression profile of each gene in the cluster was normalized as described under "Experimental Procedures." The profiles provide representative examples of the expression profiles of the groups of genes defined as p73-only (*a*) and p53-only (*b*) obtained by the clustering shown in Fig. 4A.

(H1299-p73 α clone 9 and H1299-wt-p53 clone 23) expression of both p73 α and p53 was induced by ponasterone A (2.5 μ M). To verify whether the induced proteins were transcriptionally active we checked the expression of p21^{waf1} and found it induced by both p73 α and p53 (Fig. 1, A and B, middle and lower panels). In addition, both of these proteins were predominantly nuclear, as expected (data not shown). Identical analyses performed in H1299-pIND cells revealed no p53 protein, since H1299 cells are p53 null, and no p21^{waf1} induction was shown upon ponasterone A (data not shown). With regard to endogenous p73 α , H1299 cells express very low protein levels, undetectable by direct immunoblot (data not shown) and detectable only in p73 immunoprecipitates followed by Western blot with specific anti-p73 antibodies (34).

To further verify whether transcriptionally active p73 α and p53 were capable of altering the growth of H1299 cells, we analyzed the cell cycle profile of each clone upon ponasterone A induction. We found that p73 α or p53 expression causes an increase in the G₁ population accompanied by a decrease in the S phase population (Fig. 2, B and C). No alterations in the cell cycle profile of H1299-pIND clone 1 cells were found (Fig. 2A).

Identification of p73 α or p53 Target Genes by DNA Microarray Analysis—H1299-pIND clone 1, H1299-p73 α clone 9, and H1299-wt-p53 clone 23 cells were stimulated with ponasterone A and harvested in TRIzol reagent after 0, 5, 9, 12, and 24 h (Fig. 1). To eliminate background noise in the analysis of the hybridization experiments, we chose a very stringent filter and considered only those genes that showed more than 2-fold induction or repression at 3 or more time points in the p73 α or p53 clones over the average of the 0-h time points of the cell lines.

By this criteria, 153 genes were found up-regulated by p53 and 167 genes were found down-regulated. In the p73 α -ex-

pressing cells, 85 and 56 genes fulfilled the threshold criteria for up and down regulation, respectively. To characterize the global gene expression due to p53 or p73 α activation we employed a scatter plot analysis of these genes at 5 and 24 h compared with the 0-h time point (before ponasterone A addition). We found that the expression level of this collection of genes changed very little in the control cell line (H1299-pIND clone 1) but showed extensive changes in the p53- and p73-expressing cell lines (data not shown). It also appears that in the p53 cell line there may be leaky expression of p53 since some of the genes showed increased expression even at 0 h (data not shown). Indeed, low level expression of p53 at 0 h, before induction, is detectable in Fig. 1.

Analysis of the genes whose expression was altered in both cell lines identified a group of common genes that were activated by both p53 and p73 and a group of genes that responded only to p53 or p73 (Fig. 3). For example, 27 and 21 genes were up- and down-regulated, respectively, by both proteins using the filter of 2-fold change at three or more time points. These were defined as "common genes." Fig. 3 shows that 65 genes (shaded circles) passed this filter for p53 but were up-regulated by p73 over 2-fold only once or twice (but not three times). Similarly 33 genes (shaded circles, Fig. 3A) passed the filter for up-regulation by p73 and were also up-regulated more than 2-fold by p53 once or twice. These groups of genes therefore showed preference in their response to activation by either p73 or p53. Last, two groups of genes were up-regulated more than 2-fold at least at three time points by only one of the transcription factors and not even once by the other. p73 induced 25 such genes (denoted "p73", see Table I and Fig. 3A), and p53 induced 63 such genes (denoted "p53"; see Fig. 3A).

Identifying the common, p53-only, and p73-only Groups of Genes by Clustering Analysis—The grouping of induced genes (Fig. 3) was based on an arbitrary cutoff, and it seemed conceivable that clustering the genes according to their correlated expression profiles may yield a better understanding of their functional role. Cluster analysis (super-paramagnetic clustering) was done on the 211 genes up-regulated by p53 or p73 or both (see Fig. 3). Each of the 211 genes is represented by 8 components, which represent the expression ratio at each time point over that of 0 h (before the addition of the inducer).

The results are summarized in the dendrogram of Fig. 4A. The parameter *T* controls the resolution at which the data are viewed. At *T* = 0 all the 211 genes are in a single cluster; as *T* increases, large groups split into smaller ones. When we ordered the genes according to their position in the dendrogram, i.e. rearrange the rows of the expression data matrix according to the order imposed by the clustering process, the color-code matrix of Fig. 4B is obtained. In Fig. 4A, the boxes indicate clusters that contain at least five genes, and each box is colored according to its "purity," the percentage of the members of a given group (e.g. the 25 genes of the p73-only group, see Fig. 3A) among the genes contained in the corresponding cluster. The cluster of the p73-only genes (cluster a) contains 23 (92%) of the 25 genes identified in Fig. 3. The position of the members of this group is marked by red \times s, and their change of expression is shown in the matrix of Fig. 4B in the area marked p73. Similar results were also obtained for the p53-only genes. Of the 63 genes identified in Fig. 3 as p53-only, 52 (83%) are contained in cluster b (Fig. 4A).

An illustration of the advantage of clustering in pointing out genes that show correlated expression in contrast to the grouping by arbitrary filtering (Fig. 3A) is shown in Table I and Fig. 4. The cluster that contains most of the p73-specific genes (23 of 25) also includes an additional 10 genes that show a similar expression profile (marked by "C" in column 2 of Table I). Only

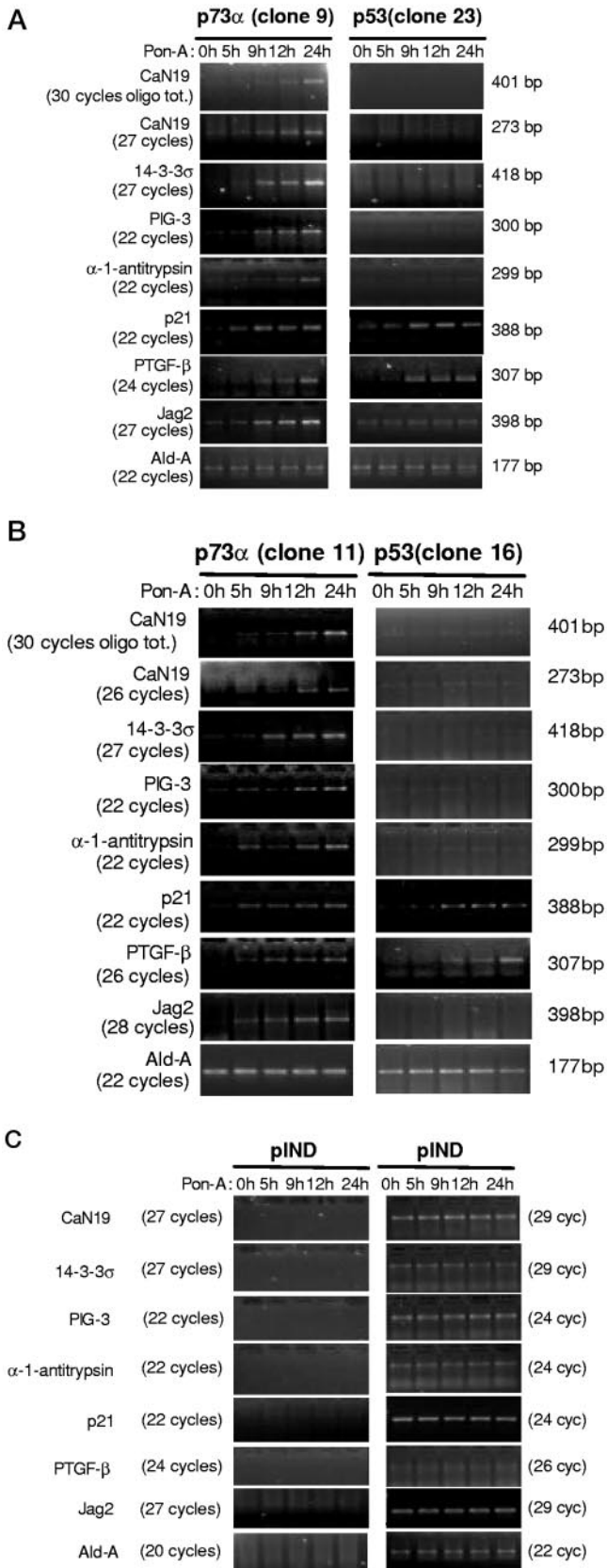


FIG. 6. Analysis of induced genes upon ponasterone A-induced expression of p73 α or p53. RNA was extracted from the indicated cell lines at the specific time points after the addition of ponasterone A and subjected to a RT/PCR reaction as indicated under "Experimental Procedures." *A*, RT/PCR analysis of an aliquot of the RNA from H1299-p73 α clone 9 and H1299-p53 clone 23 used in the microarray analysis. *B*, RT/PCR analysis of RNA from additional p73- and p53-expressing cells, H1299-p73 α clone 11, and H1299-p53 clone 16. *C*, RT/PCR anal-

2 of these 10 genes are common, whereas the other 8 can be considered p73-specific, although they also show once or twice expression levels that are above 2-fold over the 0-h time point in the p53 induced cell line. Two of the p73-specific genes defined in Fig. 3 diverge from cluster a (Fig. 4B) since their profile is different (these are COL5A2 and GPPK5, see Table I). Hence the clustering may group together co-regulated genes that may escape the grouping by the use of the filter threshold. This clustering procedure expanded the group of p73-only by recruiting genes that are co-regulated and may be p73-specific.

We selected representative clusters from Fig. 4A to analyze the profile of their expression patterns. Fig. 5 shows the expression profile of clusters a (p73-only) and b (p53-only). The average expression profiles of these clusters correlate with their definitions as shown in Fig. 5.

The group of genes designated common (indicated by black \times 's in Fig. 4A) is clearly not homogeneous and segregates into several clusters (Fig. 4B). It is possible that such subdivision spreading represents different affinities of the target sites of these genes for either p53 or p73. This indicates that the filtering of genes according to an arbitrary threshold is not always sufficiently informative with regard to the profile and level of their expression, whereas the clustering analysis allows a better classification of the response of the target genes.

Classification of the Genes Up-regulated by p53 and p73—The functional classification and expression data for the 85 genes up-regulated in the ponasterone-induced cell lines (see Fig. 3A) is presented in Table I as genes activated by p73 α or both by p53 and p73 α . The p73-induced genes, which also show partial response to p53 (*i.e.* only once or twice more than 2-fold over control) are indicated by an asterisk (p73*) in Table I. The list does not contain the genes activated by p53 except the 27 common genes (Fig. 3). The full list of the 211 genes (Fig. 3) used for the clustering analysis in the numerical order shown in Fig. 4B, with their fold expression, is available upon request.

It has been previously shown that the cell line used in this study, H1299, is resistant to p53-induced apoptosis (Ref. 29 and Fig. 1C). Cell cycle analysis shows that p73 also does not lead to programmed cell death in H1299 cells (Fig. 2) and, indeed, there is no induction of a significant number of genes known to be related to apoptosis. On the other hand, p21^{waf1} is highly expressed in response to both p53 and p73 induction and may be responsible for the growth arrest observed after ponasterone A addition (Fig. 2).

We compared the results of Table I to those previously reported on p73-induced genes by Vikhanskaya *et al.* (37). Of 16 genes that were found to be up-regulated by p73, only PIG 3 was found to be common to our work. Such discrepancy may be related to the different cell line (ovarian) and p73-overexpressing system (stably transfected clones) used in that work (37).

From the list in Table I and from Figs. 4 and 5 it is clear that there are common and distinctive genes activated by p73 α and p53. The function of these distinct genes may be one of the reasons for the different phenotypes of p73 and p53 knock-outs in mice.

To confirm the microarray data, reverse transcriptase analyses (RT/PCR) were performed on a pool of activated genes with an aliquot of the RNA used in the DNA chip analysis. As shown in Fig. 6A, the transcripts of the genes CaN19, 14-3-3 σ , PIG-3, α -1-antitrypsin, and Jag2 are specifically induced by p73 α but not by p53, whereas the p21^{waf1} and PTGF- β (indicated as "TGF- β superfamily protein" in Table I) transcripts were acti-

ysis of RNA from H1299-pIND clone 1. The number of the PCR cycles employed for each gene is indicated on the *left side*. The length of the amplified fragments is indicated on the *right side*.

TABLE II
Location and sequence of p73/p53 binding sites in the regulatory regions of the indicated genes are reported

Gene	GenBank Accession No.	Position	Sequence
Human CaN19 (S100A2)	NT_004441	679658 (intron 1) 680200 (intron 1)	cacCATGcctggccaatttt (+) cacCATGcctggccaatttt (-)
Human 14-3-3 α	AF029081	6753 (promoter)	ggaCATGtctgggctaATGc (-)
Human PIG3	AF010317	5331 (promoter)	aggCATGgccaggcttttgg (-)
Human p21	U24170	2293 (promoter) 3189 (promoter)	gaaCATGtcccaaCATGtt (+) agaCATGcccagtcttcttc (-)
Human PTGF- β	AF008303	985 (exon 1)	gggCATGgctgtgCAgGttg (-)
Human α_1 -antitrypsin	AL117259 (BAC from Chr14)	4861 (intron 1)	tccCATGgctaggCATGaga (-)
Human Jagged2	AF111170	103158 (promoter)	accCATGcccgtcCAcGctc (+)

vated by both proteins. These results overlap significantly with those obtained by microarray analysis (Table I). The expression profile of the above-mentioned genes was also analyzed by RT/PCR using RNA from additional p73 α (H1299-p73 α clone 11) or p53 (H1299-p53 clone 16) clones and from the control H1299-pIND clone 1. We found (Fig. 6B) that the CaN19, 14-3-3 σ , PIG-3, α_1 -antitrypsin, and JAG2 transcripts as well as those of p21^{waf1} and PTGF- β were induced in H1299-p73 α clone 11 and H1299-p53 clone 16 with a similar kinetic and specificity as those reported in Fig. 6A. No induction of any of these transcripts was detected in H1299-pIND clone 1 cells (Fig. 6C). These results together with those of the microarray analysis indicate that overexpression of p73 α or p53 in the same cellular context promotes distinct and partially overlapping gene expression profiles.

p73 Is Recruited Directly onto Its Target Genes—Using MatInspector Professional software (genomatrix.gsf.de) to analyze TRANSFAC 5.0 data base (transfac.gbf.de/TRANSFAC) we examined whether p53/p73 consensus sites were contained within the promoter region or the first intron of the pool of activated genes analyzed in RT-PCR assays. As shown in Table II, CaN19, 14-3-3 σ , PIG-3, α_1 -antitrypsin, p21^{waf1}, PTGF- β , and JAG2 genes contain some putative or already characterized p53/p73 binding sites within their promoter or first intron regions. To verify whether p73 α and p53 are able to directly bind their consensus sequences *in vivo*, we performed chromatin immunoprecipitations. Cross-linked chromatin from H1299-p73 α clone 9 or H1299-p53 clone 23 was immunoprecipitated with the indicated antibodies (Fig. 7). We found that p73 α specifically binds the regulatory regions of CaN19 (up and down), 14-3-3 σ , p21^{waf1} (up and down), α_1 -antitrypsin, PTGF- β , PIG-3, and JAG2 (Fig. 7). No specific binding to any of the analyzed regulatory regions was revealed in the chromatin immunoprecipitates with anti-green fluorescent protein serum (Fig. 7). Unlike p73 α , p53 binds only the two consensus motifs on the p21^{waf1} promoter (Fig. 7). The thymidine kinase (*TK*) promoter, which does not contain any p53/p73 consensus site, was used as negative control, and indeed, p73 α and p53 were not recruited to such a promoter (Fig. 7, lower panels). Of note, PTGF- β whose transcript was induced by p73 α or p53, does not directly recruit p53, at least in our cell system and experimental conditions. However, it is not excluded that the transcriptional activation could occur through some other p53/p73 binding sites present along PTGF- β gene, since our search for consensus sequences was confined to the promoter and first intron regions. These results partially overlap with those derived from the microarray analysis and strongly contribute to the identification of genes that are direct targets of p73.

DISCUSSION

The recent discovery of two p53 homologues, p73 and p63, has established a new family of transcription factors. Furthermore, p73 and p63 are subject to alternative splicing, giving rise to a complex family of proteins that might exert distinct as well as overlapping functions (38, 39). This obviously adds a

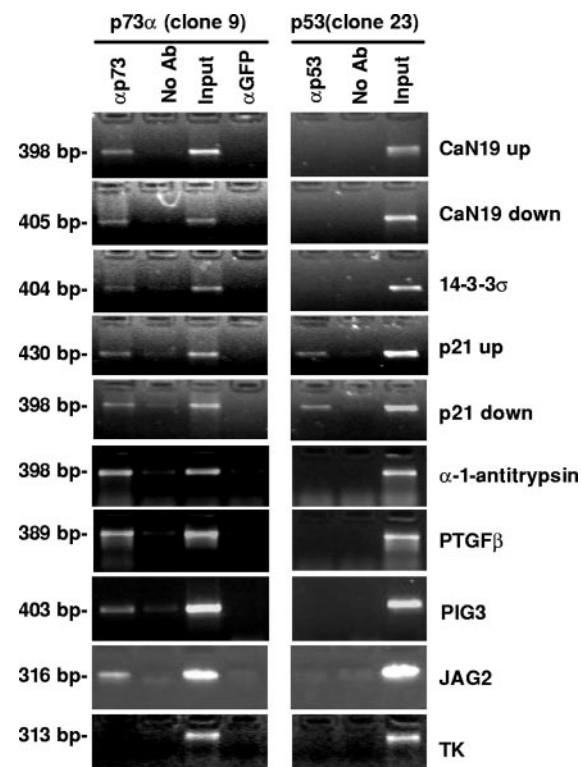


FIG. 7. *In vivo* recruitment of p73 α and p53 to their target genes. Cross-linked chromatin was extracted either from H1299-p73 α clone 9 or H1299-p53 clone 23 upon induction with ponasterone A (2.5 μ M) and subjected to immunoprecipitation with the indicated antibodies. The length of the amplified fragment is indicated on the left side. TK, thymidine kinase.

level of complexity to p53 signaling in normal and cancer cells. In the present study, we have compared the gene expression profiles promoted by ectopic expression of p73 α or p53 in the same cellular context, H1299 cells. We found that overexpression of p73 α and p53 modifies the expression of 141 and 320 genes, respectively. A further analysis of these genes revealed that p73 α up-regulates 85 genes, of which 25 are specific and 27 are in common with p53 regulation. Our results, together with the already described p53 target genes, contribute to the identification of a large repertoire of genes that serve as transcriptional mediators of the p53 family activities. We further demonstrate that in p53-null cells, p53 target genes such as 14-3-3 σ , p21^{waf1}, PIG3, and PTGF- β (40–43) can directly recruit and be activated by p73 α .

Although a certain level of knowledge regarding protein-protein interactions among the p53 family members in cancer cells has been provided by *in vitro* and *in vivo* studies, there is still a lack of basic information on the transcriptional cross-talk among p53 and its close relatives (44–48). A recent report has clearly shown that DNA damage resulting in p53-dependent apoptosis requires p73 and p63 activities (20, 49) and that in

p73^{-/-} and p63^{-/-} cells, p53 is not recruited to apoptotic target genes such as *Bax*, *PERP*, and *NOXA*. It has also been reported that DNA damage-induced acetylation of p73 selectively alters the choice of target genes of this protein (19).

Here we report the identification of novel p73 target genes such as CaN19, α_1 -antitrypsin, and JAG2. These genes are not up-regulated by p53, at least under our experimental conditions, and indeed, unlike p73, p53 was not found to occupy their regulatory regions. The gene product of CaN19 is a member of the S100 family proteins and was originally isolated from primary human keratinocytes by subtractive hybridization (50); it seems to be involved in skin and regenerative differentiation and may also play a role in suppressing tumor cell growth (51, 52). To date no sufficient information is available for a complete understanding of the molecular mechanisms underlying its biological activities. We are currently investigating the functional link between p73, p63, and CaN19 during keratinocytic differentiation.

α_1 -Antitrypsin is the major serine proteinase inhibitor (serpin A1) in human plasma. Its target proteinase is neutrophil elastase, and its main physiological function is the protection of the lower respiratory tract from the destructive effects of neutrophil elastase during an inflammatory response (53–55). p73-deficient mice suffer from inflammation, but very little is known on the pathogenesis of such process. A rather speculative hypothesis would suggest that p73-mediated anti-inflammatory effects might include the induction of α_1 -antitrypsin.

The finding that JAG2 gene is a target of p73 α correlates with a recent study showing that JAG1 and JAG2 are specific p63- and p73-responsive genes (56). JAG1 and JAG2 genes encode transmembrane proteins that serve as ligands for Notch receptors (57). Our findings together with those of Sasaki *et al.* (56) indicate that p73 α , p73 β , and p63 γ but not wt-p53 up-regulate JAG-1 and JAG-2 genes. Mutations in the Notch ligands cause developmental defects. Indeed, JAG2-deficient mice exhibit defects of limb and craniofacial development that closely resemble the abnormalities of ectrodactyly ectodermal dysplasia patients carrying p63 mutations (58, 59). Thus, JAG1 and JAG2 are direct transcriptional targets of either p73 or p63, a finding that suggests a direct involvement of these activators in Notch signaling pathways.

Among the genes induced by both p73 α and p53, PTGF- β is quite peculiar. Although the PTGF- β transcript is induced by both p73 α and p53 (Fig. 6, A and B), at least under our experimental conditions only p73 α was recruited directly to the gene (Fig. 7). Up-regulation of the PTGF- β transcript has been previously reported and linked to p53-mediated growth suppression through an autocrine as well as paracrine mechanism (40, 42). It has also been reported that induction of PTGF- β can occur through a p53-independent mechanism that, in agreement with our findings, might be induced by p73. TGF- β is a family of secreted factors that play pivotal functions during embryonic development and adult tissue homeostasis (60). Despite the heterogeneity of TGF- β mediated cellular responses, these cytokines signal to the nucleus through a quite simple mechanism. Ligand activation of TGF- β receptors results in the nuclear translocation of SMAD family proteins that control target gene expression (61). A certain level of specificity of the effects mediated by TGF- β cytokines might be dictated by different transcriptional activators. Thus, p53, p73, and probably p63 induction of PTGF- β might result in quite distinct effects ranging from growth arrest to the regulation of development and homeostasis.

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