The XPB and XPD DNA helicases are components of the p53-mediated apoptosis pathway

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The molecular pathway of p53-dependent apoptosis (programmed cell death) is poorly understood. Because p53 binds to the basal transcription–repair complex TFIIH and modulates its DNA helicase activities, we hypothesized that TFIIH DNA helicases XPB and XPD are members of the p53-mediated apoptotic pathway. Whereas transfer of a wild-type *p53* expression vector by microinjection or retroviral infection into primary normal human fibroblasts resulted in apoptosis, primary fibroblasts from individuals with xeroderma pigmentosum (XP), who are deficient in DNA repair and have germ-line mutations in the *XPB* or *XPD* gene, but not in the *XPA* or *XPC* gene, have a deficiency in the apoptotic response. This deficiency can be rescued by transferring the wild-type *XPB* or *XPD* gene into the corresponding mutant cells. XP-D lymphocytes also have a decreased apoptotic response to DNA damage by adriamycin, indicating a physiologically relevant deficiency. The XP-B or XP-D mutant cells undergo a normal apoptotic response when microinjected with the *Ich-1*$_L$ and *ICE* genes. Analyses of p53 mutants and the effects of microinjected anti-p53 antibody, Pab421, indicate that the carboxyl terminus of p53 may be required for apoptosis. Direct microinjection of the p53 carboxy-terminal-derived peptide (amino acid residues 319–393) resulted in apoptosis of primary normal human fibroblasts. These results disclose a novel pathway of p53-induced apoptosis.

[*Key Words*: p53; TFIIH; XPB; XPD; apoptosis]

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The tumor suppressor gene product p53 safeguards the genomic integrity of mammalian cells (Lane 1992), by monitoring the G_1 checkpoint (Kastan et al. 1992), sensing DNA damage (Lee et al. 1995), assembling the DNA-repair machinery (X.W. Wang et al. 1995b), modulating gene amplification (Livingstone et al. 1992; Yin et al. 1992), or activating the cell death program (apoptosis) to remove damaged cells (Yonish-Rouach et al. 1991; Shaw et al. 1992). Apoptosis is a morphologically defined process that plays an important role in maintaining tissue homeostasis (Kerr et al. 1972). The ability of p53 to induce apoptosis has been increasingly recognized as being important for its tumor suppressor function (Yonish-Rouach et al. 1991; Clarke et al. 1993; Lowe et al. 1993b; Symonds et al. 1994; Graeber et al. 1996). Loss of wild-type p53 function, for example, by mutation (for review, see Hollstein et al. 1991; Levine et al. 1991; Greenblatt et al. 1994), decreases cellular apoptosis induced by environmental and therapeutic agents that cause DNA damage (Lee and Bernstein 1993; Lowe et al. 1993a,b, 1994). Inactivation of p53 function through mutation, therefore, may provide a selective advantage for clonal expansion of preneoplastic and neoplastic cells. Identification of the mechanism(s) underlying the p53-mediated apoptotic pathway may improve our understanding of the etiology, diagnosis, prognosis, and therapy of human cancer.

The precise mechanism(s) for the induction of apoptosis by p53 is unclear, although several activities of p53 have been identified, for example, transcriptional *trans*-activator, that could participate in this process. The protein can be physically and functionally divided into four domains. These include an acidic transcriptional *trans*-activation domain at the amino terminus (Fields and Jang 1990; Kern et al. 1991; Funk et al. 1992; Lin et al. 1994), a sequence-specific DNA-binding domain at the hydrophobic center portion of the protein (Cho et al. 1994; Pietenpol et al. 1994), a region near the carboxyl terminus containing a tetramerization domain that may be needed for the stabilization of DNA-binding activity (Sturzbecher et al. 1992), and an extreme carboxy-termi-

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nal domain (CTD) containing a regulatory element involved in sequence-specific DNA binding (Foord et al. 1991; Hupp et al. 1992; Bayle et al. 1995; Jayaraman and Prives 1995; Wolkowicz et al. 1995). The CTD has been shown to contribute to nonspecific nucleic acid binding (Foord et al. 1991; Bakalkin et al. 1994) as well as modulation of its transcriptional *trans*-activation activity (Hupp et al. 1992; Jayaraman and Prives 1995). This region can also be phosphorylated by several kinases including protein kinase C and casein kinase II (Meek et al. 1990; Hupp and Lane 1994; Takenaka et al. 1995). Deletion or phosphorylation of this region leads to activation of the p53 sequence-specific DNA-binding activity needed for its transcriptional *trans*-activator function (Hupp et al. 1992; Hupp and Lane 1994). Several genes that are important in the modulation of the cell cycle and apoptosis have been shown to be activated by p53, including $p21^{WAF1}$ (El-Deiry et al. 1993), *GADD45* (Kastan et al. 1992), *mdm2* (Perry et al. 1993), *cyclin G* (Okamoto and Beach 1994), and *Bax* (Miyashita and Reed 1995) through p53 binding to promoters or regulatory regions containing p53 consensus binding sites. Other genes important in the regulation of apoptosis including *Fas* and *Bcl-2* also can be regulated positively or negatively by wild-type p53 (Miyashita et al. 1994; Owen-Schaub et al. 1995). An intriguing question remains as to whether induction of apoptosis by p53 requires: 1) the transcriptional activation of downstream genes, such as $p21^{WAF1}$, *GADD45*, *cyclin G*, *Bax*, and *Fas*; 2) direct interaction with already existing cellular proteins (Caelles et al. 1994; X.W. Wang et al. 1994; Thut et al. 1995; X.W. Wang et al. 1995b); or 3) both. Mounting evidence indicates that p53 is a potent inducer of apoptosis. Although these factors may modulate the sensitivity of cells to exogenous inducers of apoptosis, Bax or Fas do not induce apoptosis by themselves (Itoh et al. 1991; Oltvai et al. 1993), and no evidence has been published to date to indicate that $p21^{WAF1}$, GADD45, or cyclin G can induce apoptosis. In addition, previous studies have shown that inhibition of protein synthesis by cycloheximide can induce apoptosis in cultured cells in vitro (Bazar and Deeg 1992; Martin 1993; Raff et al. 1993) and rat pancreatic acinar cells in vivo (Harris et al. 1968), and more recent studies (Caelles et al. 1994; Haupt et al. 1995; Rowan et al. 1996) have suggested that p53-mediated apoptosis did not require the activation of downstream genes. These and other studies indicate that factors needed for apoptosis are constitutively present in cells.

We and others recently showed that p53 selectively binds to (X.W. Wang et al. 1994; Xiao et al. 1994; X.W. Wang et al. 1995b) and inhibits the DNA helicase activities of the TFIIH-based transcription–repair complex by binding to its subunits XPB and XPD (X.W. Wang et al. 1995b), which have dual roles in transcription and nucleotide excision repair (NER) (Feaver et al. 1993; Schaeffer et al. 1993; Cleaver 1994; Drapkin et al. 1994; Schaeffer et al. 1994). Binding is mediated through the CTD of p53 and the helicase motif III of XPB (X.W. Wang et al. 1995b). We propose that in response to DNA damage, p53 binding to TFIIH either modulates DNA-repair efficiency or triggers apoptosis. To investigate this hypothesis, we utilized microinjection and retroviral transfer of the p53 expression vector to initiate the apoptotic process in individual normal or mutant primary human fibroblasts. This enabled us to examine the functional interactions between p53 and TFIIH and to identify the TFIIH-associated factors XPB and XPD as components of the p53-dependent apoptotic pathway.

Results

Wild-type p53 induces apoptosis in primary human fibroblasts

Various oncogene products, including the adenovirus early region 1A (*E1A*) gene, *E2F-1* or c-*myc*, can increase the sensitivity of rodent fibroblasts to undergo apoptosis (Bissonnette et al. 1992; Evan et al. 1992; Debbas and White 1993; Lowe and Ruley 1993; Qin et al. 1994; Wu and Levine 1994). One cellular response that these oncogenes share with DNA damage induced by ultraviolet light or ionizing radiation, is the accumulation of the p53 protein (Kastan et al. 1992; Debbas and White 1993; Lowe and Ruley 1993; Lu and Lane 1993; Hermeking and Eick 1994; Wu and Levine 1994). We chose to use primary human fibroblasts because early passage cells from normal donors and donors with genetic diseases are available. Although these cells are less sensitive to inducers of apoptosis, such as ionizing irradiation, when compared to human lymphocytes, one can induce the apoptotic response by retroviral transfer or microinjection of *p53* expression constructs (X.W. Wang et al. 1995a) (data not shown). We initially used a microinjection technique to deliver an expression vector encoding wild type *p53* under the control of the cytomegalovirus (CMV) early promoter into the nuclei of the normal human primary fibroblasts (GM07532). Increased levels of wild type p53 were observed 24 hr following injection, predominantly in the nucleus or in both the nucleus and cytoplasm (Fig. 1A; Table 1). Among the p53 immunopositive cells at 24 hr, an average of 26% displayed the typical characteristic features of apoptosis, including chromatin condensation, nuclear fragmentation, and apoptotic bodies (Fig. 1B and data not shown). The time-course study indicates that the percent of the p53-positive apoptotic cells remaining attached to the coverslip was increased up to 60% at 72 hr, whereas the total number of p53-positive cells was significantly decreased, indicating the progressive detachment of the apoptotic cells (data not shown). These results suggest that the majority of cells expressing p53 eventually die via apoptosis. As a control, microinjection of the β-galactosidase expression vector did not result in a significant elevation of apoptosis of primary normal human fibroblasts up to 72 hr (data not shown). Similar results were obtained when either fused normal fibroblasts (C5RO) from a different donor (Vermeulen et al. 1994a) or unfused primary human mammary epithelial cells were used (L. Elmore, X.-W. Wang, and C.C. Harris, unpubl.).

To determine whether induction of apoptosis was spe-

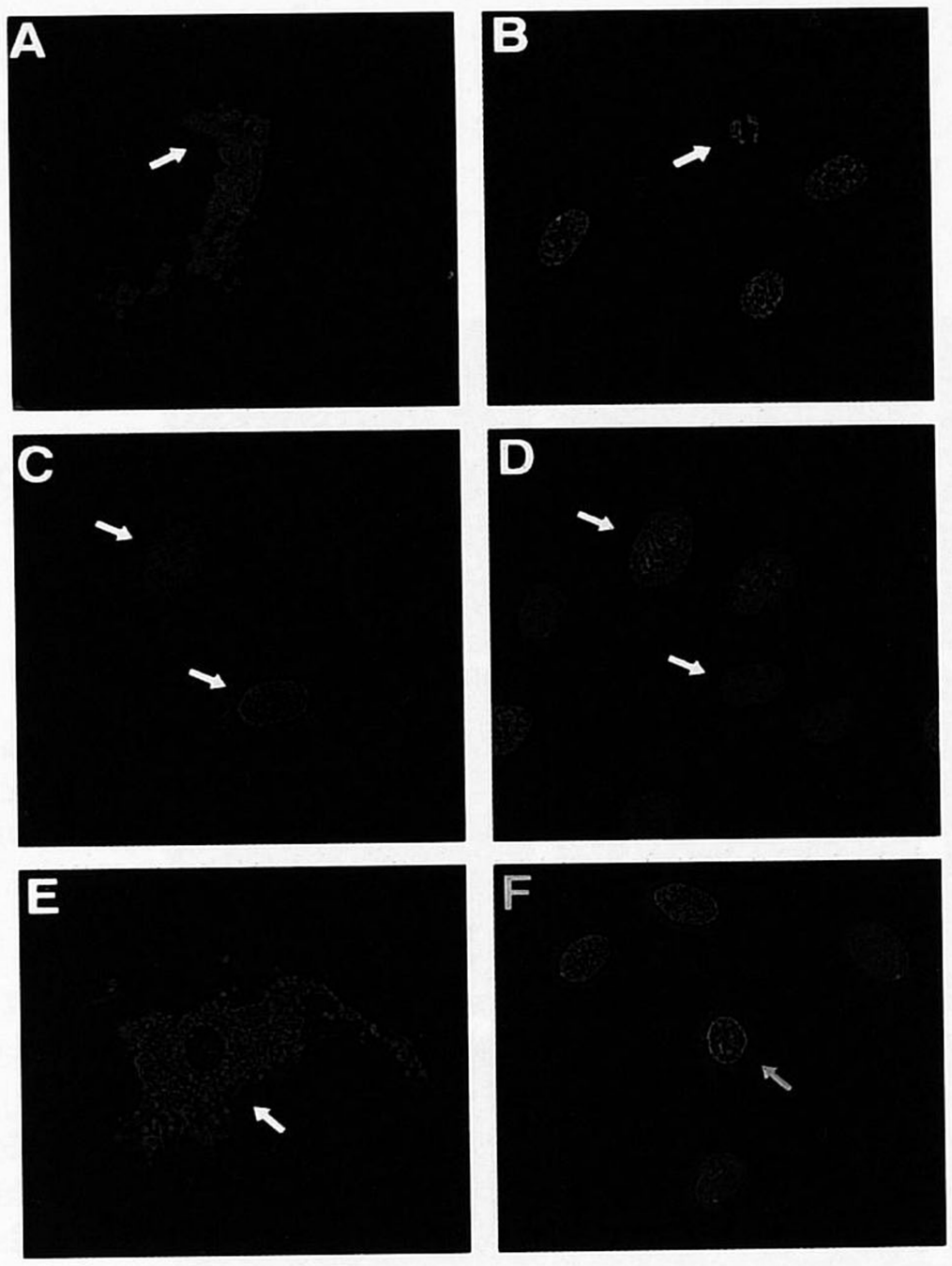

Figure 1. Induction of apoptosis by microinjection of wild-type and various mutant p53 expression vectors in normal primary human fibroblasts (GM07532). Cells were injected with the following p53 expression vectors: wild-type (*A,B*) 143^{ala} (*C,D*), and 249^{ser} (*E,F*), and were incubated for 24 hr prior to fixation. p53 protein was stained with CM-1 antibody (*A,C,E*). Nuclei were stained by DAPI (*B,D,F*).

cifically attributable to an intrinsic activity of wild-type p53 and not the result of nonspecific protein overproduction, *p53* mutants 143^{ala} 175^{his}, 248^{trp} or 249^{ser}, in the same expression vector system, were microinjected. These *p53* mutants lack the sequence-specific transcriptional *trans*-activation activity that is associated with growth suppression (Kern et al. 1992; Crook et al. 1994; Pietenpol et al. 1994). All mutants were expressed at similar or higher protein levels than wild-type p53 (Fig. 1C,E and X.W. Wang. et al., unpubl.). The p53 mutants showed similar subcellular localization as wild type p53, except for the 249^{ser} mutant, that accumulated predominantly in the cytoplasm (Fig. 1C,E and X.W. Wang et al., unpubl.). All mutants lacked the ability to induce apoptosis (Table 1). These data are consistent with previous results (Yonish-Rouach et al. 1991; Clarke et al. 1993; Lowe et al. 1993b) and indicate that an intrinsic property of wild-type p53 expressed in normal fibroblasts is the induction of apoptosis and loss of this ability by the p53 mutants tested.

Mutation of the wild-type *p53* gene can result in a loss of tumor suppressor activity and in some cases, gain of oncogenic properties (Kern et al. 1992; Dittmer et al. 1993). To test whether p53 mutants can have dominant-negative effects, expression vectors encoding both wild-type and mutant *p53* were coinjected into primary fibroblasts. The presence of the mutants 143^{ala}, 175^{his}, 248^{trp} or 249^{ser}, completely abolished the induction of apoptosis by wild-type *p53* (Table 1). When most of the mutants were coexpressed with wild-type *p53*, the subcellular localization patterns were largely unaltered (Wang et al., unpubl.). Interestingly, we observed a fewer number of cells with only cytoplasmic staining when wild-type *p53* was coinjected with the 249^{ser} mutant (20% vs. 77%). These data indicate that although all p53 mutants tested exhibit dominant-negative effects, they may differ in the mechanism of inactivation of wild-type p53 function.

p53-mediated apoptosis is defective in fibroblasts from XP-B or XP-D patients

We recently observed that both wild-type and mutant p53 (135^{tyr}, 249^{ser} or 273^{his}) bind to TFIIH-associated factors, but only wild-type p53 inhibits the TFIIH-associated DNA helicase activity contributed by XPB and XPD (X.W. Wang et al. 1994; X.W. Wang et al. 1995b). If this interaction is an important step in the activation of p53-mediated apoptosis, defects in the *XPD* and *XPB* genes could result in cells resistant to p53-induced apoptosis. Therefore we tested whether wild-type p53, under conditions that induce apoptosis in normal primary fibroblasts, had any effect on primary fibroblasts from xeroderma pigmentosum (XP) donors with XP-B and XP-D germ-line mutation. The cells from patients XPCS2BA and XPCS1BA (sibling of XPCS2BA) contain a missense mutation at codon $99^{phe \rightarrow ser}$ in the *XPB* gene, and cells from patient XP11BE have a frameshift mutation near the carboxyl terminus of the *XPB* gene. These mutations result in a nearly complete inactivation of the NER function of this protein (Weeda et al. 1990; Vermeulen et al. 1994a). Tvhe cells from patient XP6BE contain a defective *XPD* gene that also severely affects its NER activity (Flejter et al. 1992). Microinjection of the wild-type *p53*

Table 1. *Induction of apoptosis by wild-type or mutant p53 in normal primary human fibroblasts*

Expression vectors	Percent apoptotic cells[a] (n[b])	P value
Wild-type p53	26 (231)	
143^{ala}	0 (18)	<0.05
175^{his}	0 (38)	<0.005
248^{trp}	0 (36)	<0.005
249^{ser}	0 (34)	<0.005
Wild type + 143^{ala}	0 (16)	<0.05
Wild type + 175^{his}	0 (17)	<0.05
Wild type + 248^{trp}	0 (24)	<0.01
Wild type + 249^{ser}	0 (20)	<0.05

[a]Cells with condensed and fragmented nuclei as well as the crater-like bodies characteristic of cells undergoing apoptosis at 24 hr following microinjection of p53 expression vectors.
[b](n) Total number of p53 immunopositive cells following microinjection of p53 expression vectors. χ^2 test was used for analyzing the statistical significance between wild-type p53 and mutant p53.

expression vector into these cells resulted in elevated expression of nuclear p53 (Table 2; data not shown). The signal intensity of p53 in these cells was comparable to the levels in normal fibroblasts. Although 26% of the p53-positive normal cells underwent apoptosis, of the p53 immunopositive XP cells scored, only 5% of the XPCS1BA cells, 4% of the XPCS2BA cells, 7% of the XP11BE cells, and none of the XP6BE cells exhibited apoptosis at 24 hr (Table 2). However, p53-mediated apoptosis was normal in primary fibroblasts from TTD1BR cells that have a mutation in a TFIIH-associated factor other than *XPB* or *XPD* gene (Table 2). Then we carried out a time-course study, in which cells were incubated for 6, 24, and 48 hr following microinjection of the wild-type *p53* expression vector (Fig. 2). Although the enhanced signal intensity of p53 is similar in all cell types at 6 hr following microinjection (data not shown), no apoptosis was observed at this time point. At 24 hr, 20% of C5RO cells, but only 4% of XPCS2BA cells, had undergone apoptosis (Table 2, Fig. 2). Apoptosis was not observed in XP6BE cells. At 48 hr, however, 33% of both p53-positive C5RO and XPCS2BA cells and 9% of the p53-positive XP6BE cells had undergone apoptosis (Fig. 2). These data indicate that the p53-associated apoptosis is not completely abolished in these mutant cells, possibly attributable to either the remaining functional XP protein, that is, XPB or XPD, in the TFIIH complex or an alternative TFIIH-independent pathway.

To confirm the microinjection results and to determine whether wild-type p53-induced apoptosis occurs in fibroblasts from individuals with defects in DNA-repair genes other than *XPB* and *XPD*, we infected primary fibroblasts from normal individuals and individuals with XP-A and XP-C germ-line mutations with a retroviral wild-type *p53* expression vector under the control of the CMV promoter. Moderate levels of nuclear p53 protein expression were observed in the infected cell types tested when compared to microinjected cells (Fig. 3; data not shown). The p53 signal intensities are comparable

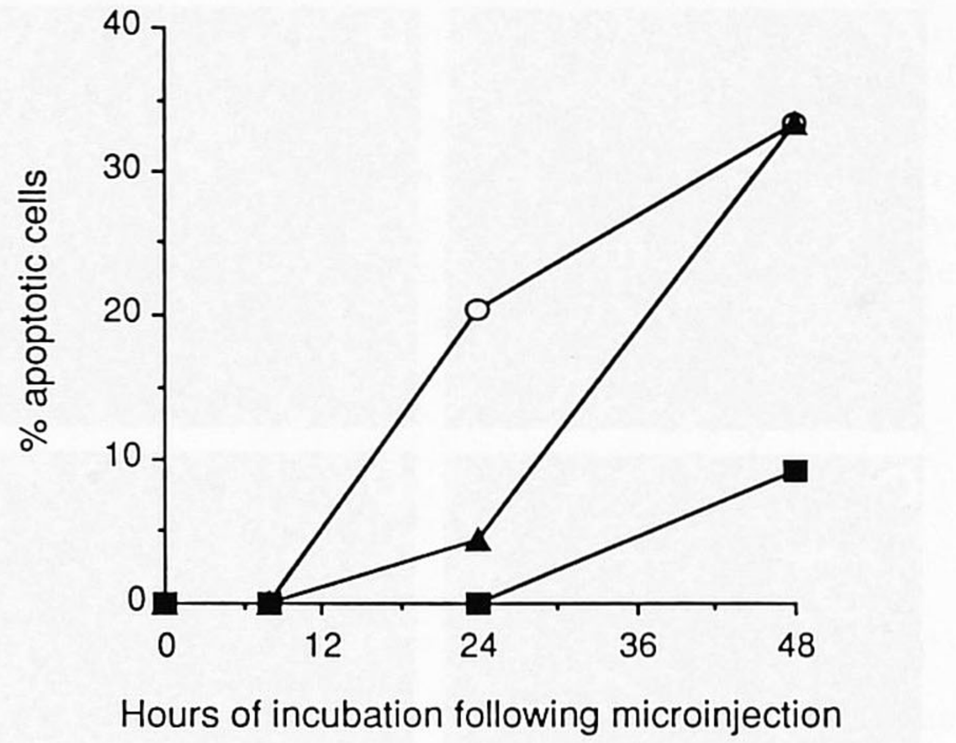

Figure 2. Differential induction of apoptosis between normal primary human fibroblasts (○) and primary fibroblasts from xeroderma pigmentosum [XP-B (▲)and XP-D (■)] donors following microinjection of the wild-type p53 expression vector.

among the infected cells, indicating no significant cell-type differences in protein expression. Among the p53 immunopositive cells, ~8% of normal cells (GM07532), 7% of the XP-C cells (XP1PW), and 6% of the XP-A cells (XP12BE) consistently exhibited apoptosis (Table 3). In contrast, only 1% of the p53-immunopositive XP-B cells (XPCS2BA) and none of the XP-D cells (XP6BE) exhibited the apoptotic phenotype (Table 3), which is consistent with the microinjection data. These results were confirmed in primary fibroblasts from an additional XP-B (XPCS1BA) donor and an XP-D (GM03248) donor (data not shown). Therefore, the decreased sensitivity to wild-type p53-induced apoptosis in XP-B and XP-D cells is not caused by either a general overall defect in the NER pathway or transcriptional defects associated with TFIIH (see Table 2 for the TTD1BR data) but is the result of a mutation in the *XPB* or *XPD* gene.

Wild-type p53 retains its transcriptional trans*-activator activity in XP-B and XP-D cells*

To demonstrate further that the microinjected wild-type *p53* gene was efficiently expressed in XP-B and XP-D cells, and the p53 protein where transiently expressed, retains a wild-type specific function such as sequence-specific transcriptional *trans*-activator activity, we first tested the ability of the wild-type *p53* retroviral vector to induce $p21^{WAF1}$ expression in normal (GM07532), XP-D (XP6BE) and XP-B (XPCS2BA) cells (Fig. 3). Although p53 was undetectable in control vector or uninfected cells by immunocytochemistry, infection of the wild-type *p53* retroviral vector resulted in nuclear colocalization of an elevated p53 protein and $p21^{WAF1}$ protein in all three cell types (Fig. 3). About 5% of cells exhibited $p21^{WAF1}$ immunostaining in uninfected cells or in cells infected with the control vector, indicating p53-independent $p21^{WAF1}$ expression in a subset of the cells. Of the p53-immunopositive cells, 98% of the normal cells, 99% of the XP-D, and 99% of the XP-B cells showed positive for

Table 2. *Differential induction of apoptosis by microinjection of the wild-type p53 gene in primary human fibroblasts from individuals with various mutational defects in the nucleotide excision repair pathway*

Cell strains[a]	Phenotypes	Percent apoptotic cells (*n*)[b]	*P* value
GM07532	normal	26 (231)	
TTD1BR	TTD-A	17 (52)	>0.05
XPCS1BA	XP-B	5 (157)	<0.001
XPCS2BA	XP-B	4 (113)	<0.001
XP11BE	XP-B	7 (87)	<0.001
XP6BE	XP-D	0 (95)	<0.001

[a]All cells are primary fibroblasts that are described in detail elsewhere (Kraemer et al. 1975; Vermeulen et al. 1991, 1994a; Weeda et al. 1990; Stefanini et al. 1993).
[b](*n*) Total number of p53-immunopositive cells 24 hr following microinjection of the wild-type p53 expression vector. χ^2 was used for analyzing the statistical significance between normal cells and cells from XP donors.

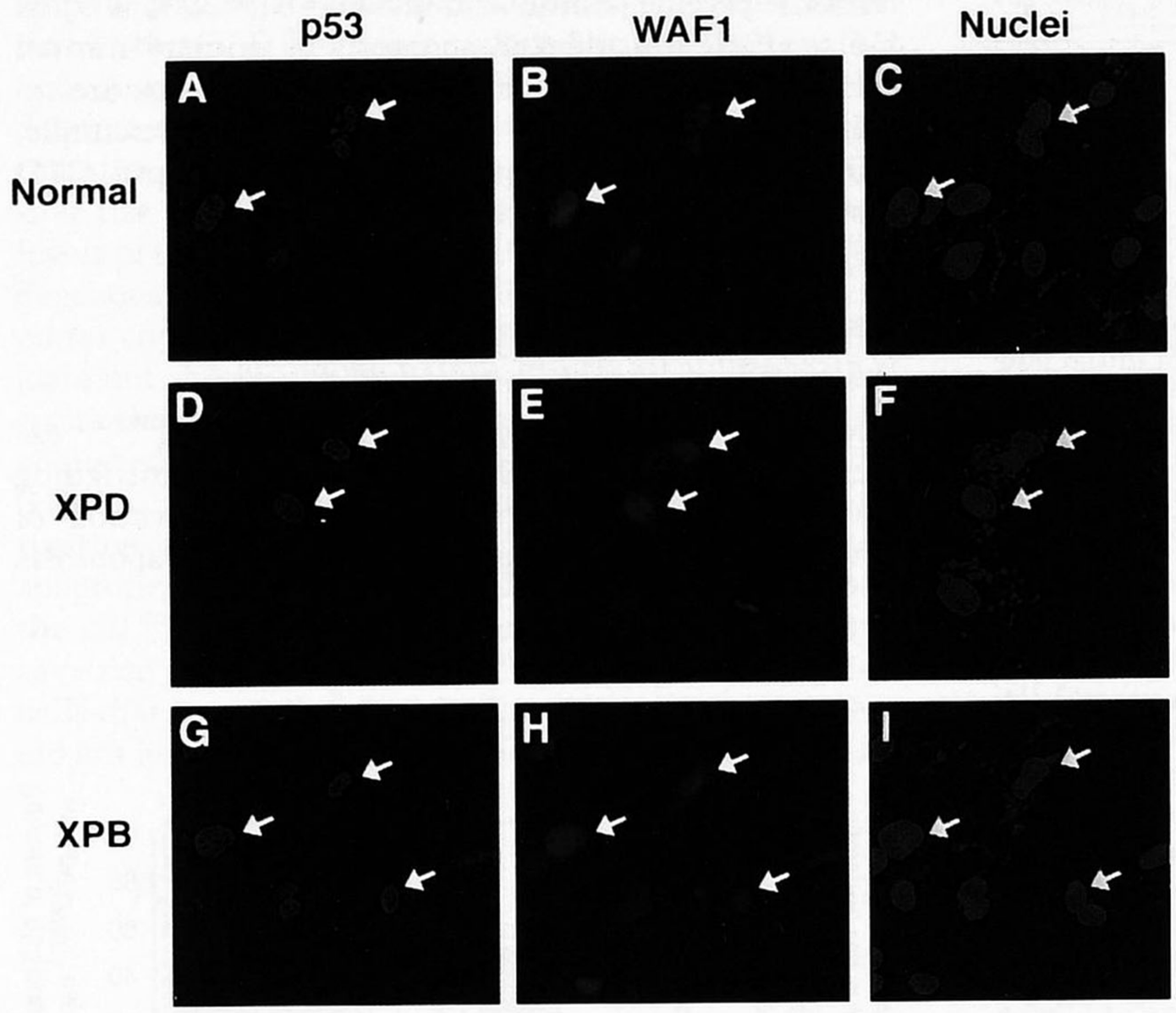

Figure 3. Induction of $p21^{WAF1}$ in normal primary human fibroblasts and in primary fibroblasts from xeroderma pigmentosum (XP-B and XP-D) donors following infection by a retroviral vector encoding wild-type p53. Cells were infected with the wild-type p53 retroviral vector and incubated for 48 hr prior to fixation. For simultaneous demonstration of p53 protein and $p21^{WAF1}$ protein, GM07532 (normal) cells (*A–C*), XP6BE (XP-D) cells (*D–F*) and XPCS2BA (XP-B) cells (*G–I*) were double-labeled with anti-p53 rabbit polyclonal CM-1 antibodies (*A,D,G*) and anti-$p21^{WAF1}$ monoclonal antibody (Oncogene Science) (*B,E,H*), followed by the corresponding secondary antibodies conjugated to FITC for p53 and Texas Red for $p21^{WAF1}$, respectively. Nuclei were stained by DAPI (*C,F,I*).

the $p21^{WAF1}$ staining. The immunocytochemical intensity of $p21^{WAF1}$ is similar among these cells (Fig. 3), indicating a similar level of the wild-type p53 protein is achieved during infection.

Colocalization of the elevated p53 and $p21^{WAF1}$ proteins was also determined in these cells after microinjection of wild-type *p53*. At 6 hr following microinjection of wild-type *p53*, 25% of normal (GM07532) cells, 32% of XP-B (XPCS2BA) cells, and 34% of XP-D (XP6BE) cells showed positive for the $p21^{WAF1}$ staining. At 24 hr, overexpression of p53 resulted in an induction of nuclear $p21^{WAF1}$ in 50% of normal cells, 58% of XP-B cells and 54% of XP-D cells (Fig. 4B). No correlation between cells undergoing p53-mediated apoptosis and cells expressing $p21^{WAF1}$ was observed (data not shown). Although, overall, there were less $p21^{WAF1}$-positive cells observed by microinjection as compared to cells with retroviral infection, there was no significant difference among the various cell types beginning as early as at 6 hr incubation. Therefore, we concluded that wild-type p53 was efficiently expressed and retains its ability to *trans*-activate the $p21^{WAF1}$ gene (El-Deiry et al. 1993) in XP cells.

Deficiency in p53-mediated apoptosis in XP-B and XP-D cells can be rescued by transferring a wild-type XPB *or* XPD *gene*

To test genetically whether the deficiency in p53-mediated apoptosis in XP-B and XP-D cells is a result of a mutation in the *XPB* or *XPD* gene, we coinjected expression vectors for *p53* and wild-type *XPB* or *p53* and *XPD* gene into XP-B (XPCS2BA) or XP-D (XP6BE) cells, respectively. Although coinjection of the *XPB* gene did not alter p53-mediated $p21^{WAF1}$ expression (Fig. 4B), it completely restored p53-mediated apoptosis in XP-B cells (Fig. 4B). Similarly, the *XPD* gene rescued p53-mediated apoptosis in XP-D cells, although the efficiency was lower than in the case of XP-B cells (Fig. 4B). Again, no alteration of p53-mediated $p21^{WAF1}$ expression was observed in these cells (Fig. 4B). As a control, the *XPB* or *XPD* gene alone was injected into XP-B or XP-D cells, respectively. No morphological changes were observed (data not shown). These data strongly indicate that the *XPB* and/or *XPD* gene products are components in the p53-dependent apoptosis pathway.

Carboxyl terminus of p53 contributes to its apoptotic activity

We have shown previously that the CTD of p53 (residues 367–393) is responsible for interaction with XPB and XPD (X.W. Wang et al. 1995b). The Pab421 monoclonal antibody recognizes an epitope in the CTD of p53 within this binding region (Banks et al. 1986) and can disrupt interaction between p53 and XPB in vitro (X.W. Wang et al. 1995b) (data not shown). We hypothesized that if p53

Table 3. *Differential induction of apoptosis by infection of a retroviral vector encoding wild-type p53 in fibroblasts from individuals with various defects in nucleotide excision repair*

Cell name[a]	Phenotypes	Percent apoptosis (mean ± S.D.)	n[b]
1057 (GM07532)	normal	8.3 ± 2.4	402
XP1PW (GM00510)	XP-C	7.0 ± 0.5	497
XP12BE (GM05509B)	XP-A	5.7 ± 1.5	344
XPCS2BA (GM13026)	XP-B	1.0 ± 0.3	533
XP6BE (GM10430)	XP-D	0	448

[a]All cells are primary fibroblasts between passage 10 and 15 obtained from Coriell Institute for Medical Research. The cell names correspond to their local user numbers from initial publications or their catalog numbers. XP-A, XP-B, XP-D, and XP-C designate xeroderma pigmentosum complementation group A, B, D, and C, respectively. Induction of apoptosis was achieved by infection of these cells with a retroviral vector encoding wild-type p53 under the control of the CMV promoter in the presence of Polybrene, followed by an additional 48 hr incubation. The titer of viral stocks used is 1×10^5 CFU/ml, and only one viral stock was used for all experiments to avoid variation. Cells were fixed, stained for p53, and analyzed for apoptosis as described (Vermeulen et al. 1994a). The p53 signal intensities are comparable among the cells tested, indicating no significant difference in protein expression (not shown). Percent apoptosis is calculated by scoring only the p53-immunopositive cells. Data are an average of three independent experiments. Student's *t*-test was used for analyzing the statistical significance between normal cells and cells from XP donors. XP-C and XP-A cells have a P value >0.05, and XP-B and XP-D cells have a P value <0.001 when compared to GM07532 cells.
[b](n) Total number of p53-immunopositive cells scored.

To demonstrate further whether the p53 carboxy-terminal peptide exhibits apoptotic activity, we microinjected peptides into normal primary human fibroblasts. Whereas a control peptide derived from hepatitis B virus X protein had no effect on cell growth, the p53 carboxy-terminal peptide (amino acid residues 319–393) at equal molar efficiently induced apoptosis of primary normal human fibroblasts (Table 4). Additional studies are required to determine the specific regions, for example, tetramerization or epitope of Pab421, in the p53 CTD that mediate the apoptotic response.

Transcriptional trans*-activator activity of p53 is dispensable for p53-mediated apoptosis*

The ability of wild-type p53 to induce the $p21^{WAF1}$ expression in the XP-B or XP-D cells raises an intriguing question as to whether transcriptional activation of downstream genes is required for induction of apoptosis

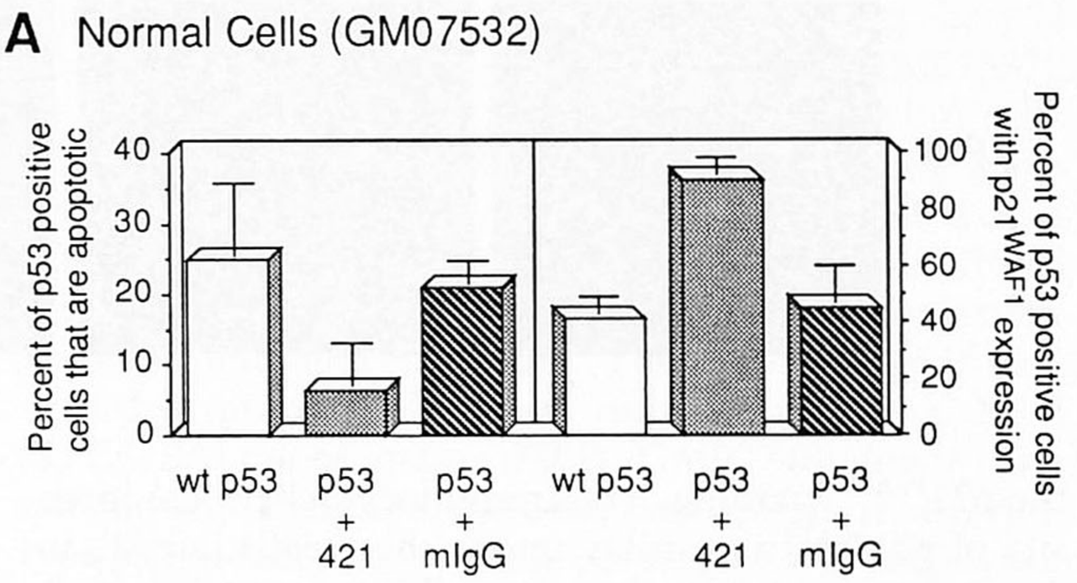

Figure 4. Effects of Pab421 monoclonal antibody and *XPB* or *XPD* genes on p53-mediated apoptosis. Coinjection of p53 with Pab421 antibody (*A*) or a combination of p53 with the *XPB* or *XPD* gene (*B*) was performed to compare the effect of these genes on p53-mediated apoptosis or *trans*-activation activity following a 24-hr incubation. This included microinjection of p53 alone into normal (GM07532), XP-B (XPCS2BA), or XP-D (XP6BE) cells (open bars), coinjection of p53 + Pab421 antibody into normal cells (shaded bars), coinjection of p53 + normal mouse IgG (mIgG) into normal cells (right-hatched bars), coinjection of p53 + XPB into XP-B cells (left-hatched bars), or coinjection of p53 + XPD into XP-D cells (solid bars). Data represent an average of at least three independent experiments that were performed on separate days. Note the difference in scale between the *left* and *right* panels.

induces apoptosis by direct interaction with XPB and/or XPD, coinjection of p53 with Pab421 would block this effect. As shown in Figure 4A, whereas 26% of the *p53*-injected normal cells (GM07532) underwent apoptosis, only 6% of the p53 plus Pab421-injected normal cells exhibited apoptosis ($P<0.05$, as compared to wild-type alone or wild-type plus mIgG). In contrast, the Pab421 antibody enhanced p53-mediated $p21^{WAF1}$ expression in these cells from 45% to 90% (Fig. 4A), which is consistent with our findings that a majority of the p53 expressed in microinjected cells exhibited a transcriptional *trans*-activation-incompetent form, as well as the previously published data that Pab421 can enhance the p53 sequence-specific DNA-binding activity in vitro that is required for its transcriptional *trans*-activation activity in vivo (Hupp et al. 1992). Coinjection of wild-type *p53* and normal mouse IgG (Fig. 4A) or injection of Pab421 alone (data not shown) into GM07532 cells did not result in any significant alteration in the p53-mediated apoptosis and $p21^{WAF1}$ induction. The fact that the Pab421 antibody competes for p53 binding to XPB in vitro and inhibits p53-mediated apoptosis in vivo, argues further that p53-mediated apoptosis in normal primary fibroblasts involves direct interaction of p53 with the XPB or XPD protein.

by p53. To test this hypothesis directly, we microinjected various *p53* mutant expression vectors into normal fibroblasts (GM07532) and compared their transcriptional *trans*-activator and apoptotic activities. *Trans*-activation activity of p53 is not correlated for its apoptotic activity (Fig. 5). For example, a multiple missense mutation at the CTD including codons His 364 Ala, Lys 372 Leu, Arg 379 Ala, and Lys 386 Leu (p53–517), a CTD deletion of the last 50 residues (p53Δ353), or a germ-line mutation found in several types of cancers (p53 181H) gave rise to p53 proteins that retained similar or greater levels of transcriptional *trans*-activation of either the endogenous $p21^{WAF1}$ gene (Fig. 5B) or a heterologous reporter containing multiple p53-binding sites ($PG_{13}CAT$) (data not shown), as compared to wild-type p53. In contrast, these mutants had either reduced (p53–517) or abolished (p53Δ353 and p53 181H) apoptotic activity (Fig. 5A). p53 mutant 273H had no detectable *trans*-activation activity; however, it retained a low degree of apoptotic activity (Fig. 5). In addition, microinjection of the $p21^{WAF1}$ cDNA under the control of CMV promoter gave rise to a high level of $p21^{WAF1}$ protein in GM07532 cells that accumulated predominantly in the nucleus but did not lead to apoptosis (data not shown). These data are consistent with the Pab421 antibody and carboxy-terminal peptide microinjection results (Fig. 4; Table 4) and indicate that the CTD of p53 is required for induction of apoptosis.

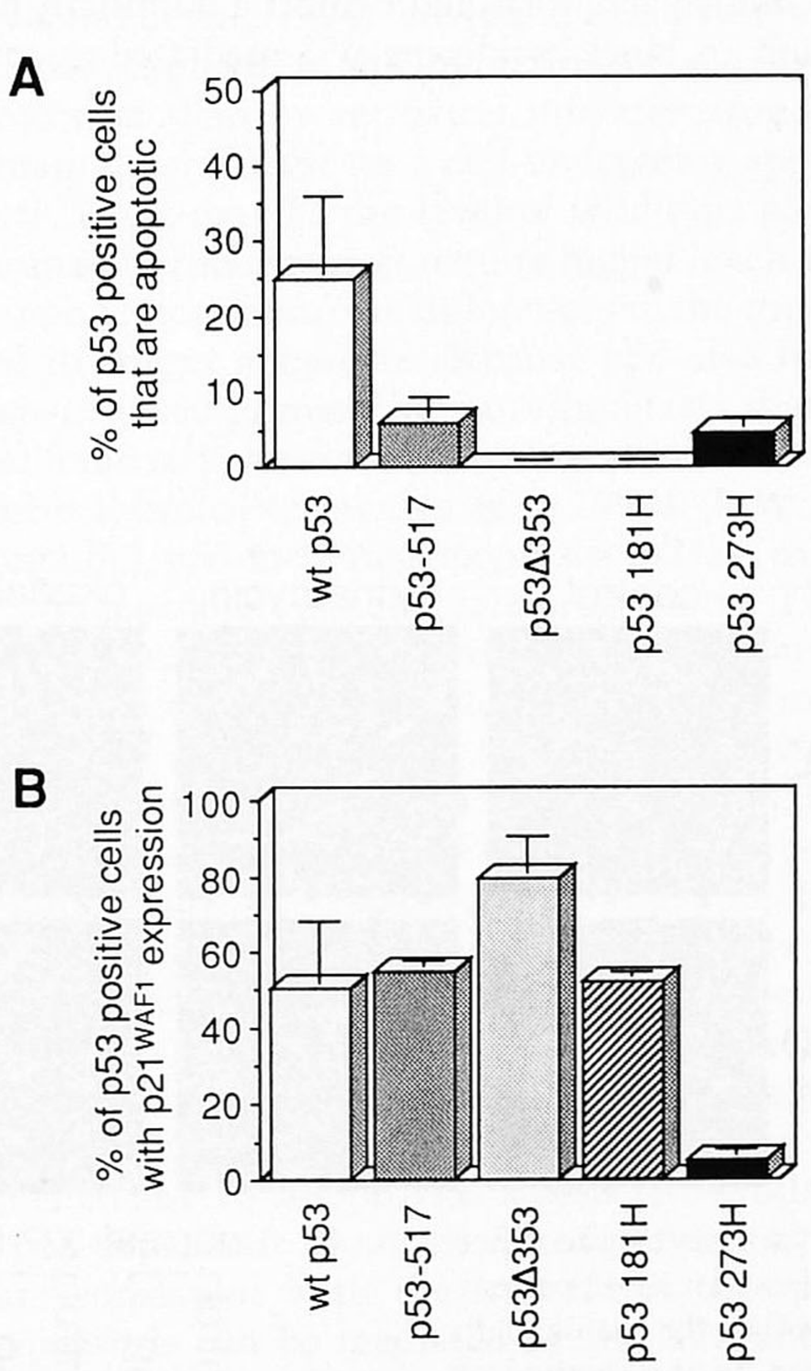

Figure 5. Transcriptional *trans*-activation activity of p53 is not correlated with p53-mediated apoptosis. Various p53 mutants were tested for their ability to induce apoptosis (*A*) and activate the $p21^{WAF1}$ gene expression (*B*) in normal primary human fibroblasts (GM07532) following 24 hr incubation after microinjection. All data are an average of at least three independent experiments.

Table 4. *Induction of apoptosis by microinjection of carboxy-terminal peptide of p53 in normal primary human fibroblasts*

	Percent apoptotic cells		
Peptides[a]	experiment 1	experiment 2	experiment 3
p53	42	43	21
HBx	3	N.D.	0

[a]Peptide p53 corresponding to the amino acid residues 319–393 of the human wild-type p53 protein was provided by Kazuyasu Sakaguchi and Ettore Appella (National Cancer Institute, Bethesda, MD). Peptide HBx corresponding to the amino acid residues 115–131 of the hepatitis B virus X protein was provided by Mark Feitelson (Jefferson Medical College, Philadelphia, PA). Peptides at the concentration of 583 μM were coinjected with the β-galactosidase expression vector (100 μg/ml) into primary human fibroblasts (GM07532). Following 24 hr incubation, cells were fixed, double-stained for p53 and β-galactosidase, and analyzed for apoptosis as described earlier.

Normal induction of apoptosis in normal, XP-B, and XP-D fibroblasts by the Ich-1_L *and* ICE *genes*

To demonstrate further that the XP-B and XP-D cells were efficiently expressing the genes introduced, we microinjected the expression vectors containing the human apoptosis-inducing gene (*Ich-1_L*) or a mammalian *Ced-3* homolog (*ICE* gene) into normal (GM07532), XP-B (GM13026), and XP-D (XP6BE) fibroblasts. These genes were fused to the β-galactosidase gene for detection of their protein expression (L. Wang et al. 1994). Microinjection of the *Ich-1_L* gene resulted in elevated levels of Ich-1_L protein (β-galactosidase activity). Among Ich-1_L-positive cells scored, 49% of normal cells, 48% of XP-B cells, and 57% of XP-D cells underwent apoptosis (Fig. 6) with a typical apoptotic morphology essentially identical to that described previously (L. Wang et al. 1994), and to that induced by p53 (data not shown). It appears then that both XP-B and XP-D cells are normal in Ich-1_L-mediated apoptosis. Similarly, the *ICE* gene also showed a comparable apoptotic activity among these cell types (Fig. 6). This suggests further that the defect in p53-mediated apoptosis in XP-B and XP-D cells is not attributable to subtle changes in transcriptional efficiency, as XP-B and XPD are also the subunits of the basal transcriptional factors that could lead to inefficient transcription of the injected gene in these cells but, rather, a result of a defect in the *XPD* gene in the p53-mediated apoptotic pathway.

Lymphoblasts from XP-D patients have a deficiency in DNA damage-induced apoptosis

To explore further whether XP-D cells are also defective in the DNA damage-induced and p53-dependent apop-

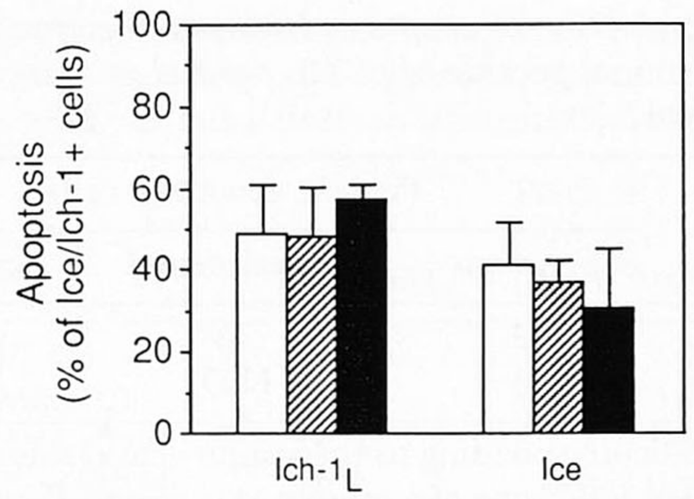

Figure 6. Induction of apoptosis in primary fibroblasts from normal (GM07532) (open bars), XP-B (XPCS2BA) (hatched bars), and XP-D (XP6BE) (solid bars) individuals by *Ced-3/ICE*-related genes. The appearance of apoptosis morphology following microinjection was monitored essentially as described (L. Wang et al. 1994). All data are an average of at least three independent experiments.

totic pathway, we compared the cell-killing effect of either the DNA-damaging agent adriamycin (p53-dependent) or okadaic acid (p53-independent) on lymphoblasts from a normal (GM02184C) and an XP-D donor (GM03249, whose fibroblasts, GM03248, were also deficient in p53-mediated apoptosis) (Fig. 7). Adriamycin or okadaic acid efficiently killed normal lymphoblasts in a time-dependent fashion (Fig. 7) via apoptosis as characterized by nuclear fragmentation. In XP-D lymphoblasts, however, adriamycin exhibited a consistent and statistically significant delay in the cell-killing effect (Fig. 7A). In contrast, both cell types appeared to have a normal response in the DNA damage-induced p53 accumulation (Fig. 7D). These results support the previous findings that adriamycin induced apoptosis through a p53-dependent pathway (Lowe et al. 1993a) and further implicate XP-D as being a component in DNA damage and the p53-dependent apoptosis pathway. Interestingly, okadaic acid appears to have an equally cytotoxic effect in both cells (Fig. 7B). Moreover, XP-D lymphoblasts appeared to have a normal response to Fas-mediated apoptosis induced by a cell-killing anti-Fas antibody (clone CH-11) (data not shown). These data indicate that although XP-D cells are capable of undergoing apoptosis induced by p53-independent pathways, these cells have a deficiency in DNA damage-induced apoptosis under conditions of physiological–relevant levels of p53.

Discussion

We have demonstrated that wild-type p53 can induce apoptosis in normal primary human fibroblasts. Many of the naturally occurring p53 mutants have lost their ability to induce apoptosis and exhibit a dominant-negative function to block wild-type p53-mediated apoptosis, a

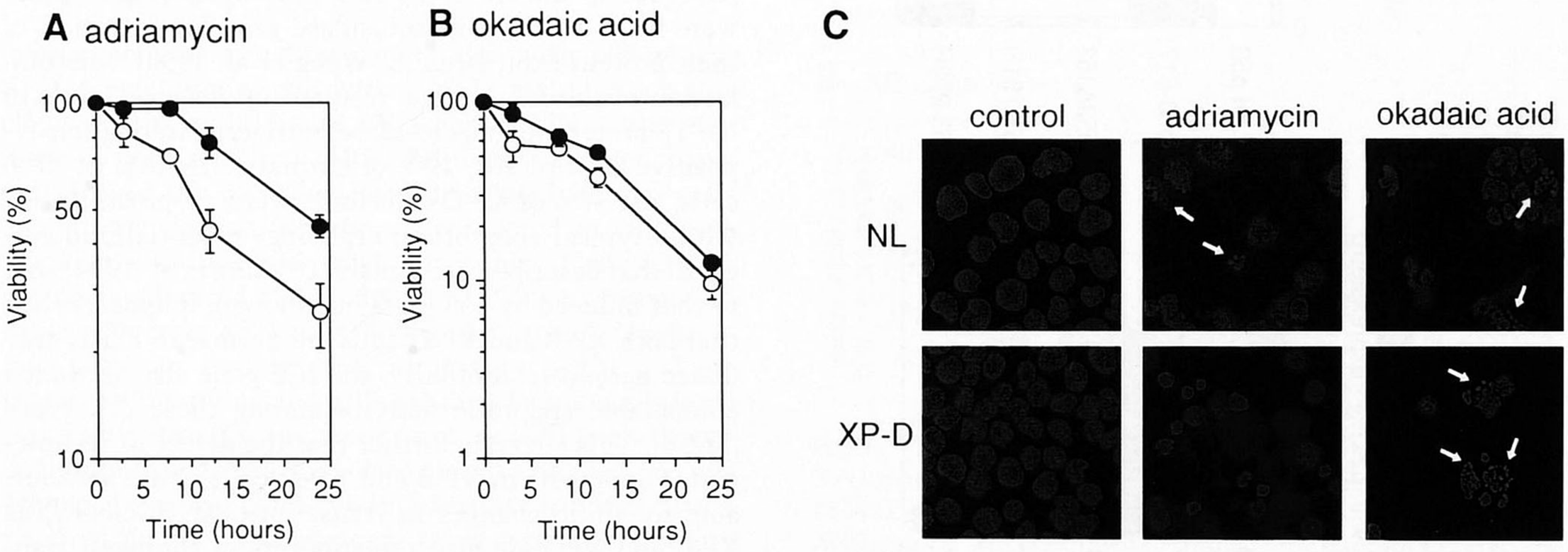

Figure 7. Induction of apoptosis in lymphoblasts from normal and XP-D patients by the other apoptosis inducers. Lymphoblasts from a normal individual (GM02184C; ○) and an XP-D patient (GM03249; ●) were incubated with media containing 17.2 μM adriamycin (*A*) or 0.5 μM okadaic acid (*B*). At various times following incubation, cell viability was measured by the method of trypan blue exclusion. Nuclear morphology (*C*) of normal lymphoblasts (NL, GM02184C) and XP-D lymphoblasts (XP-D, GM03249) treated without (control) or with 17.2 μM adriamycin or 0.5 μM okadaic acid for 24 hr. (*D*) Levels of p53 in lymphoblasts from normal (GM02184C) (lanes *1,2*) and XP-D (GM03249) (lanes *3,4*) individuals were monitored by Western blot analysis following treatment with (lanes *2,4*) or without (lanes *1,3*) 17.2 μM adriamycin for 24 hr. Following adriamycin treatment, the p53 level was increased in both cell types (4.9-fold in normal cells and 6.4-fold in XP-D cells, as determined by densitometer analysis).

process closely associated with its tumor suppressor function (Yonish-Rouach et al. 1991; Clarke et al. 1993; Lowe et al. 1993b; Symonds et al. 1994). Induction of apoptosis by wild-type p53 in fibroblasts is not attributable to nonspecific protein overproduction but is a result of an intrinsic activity of wild-type p53, as many p53 mutants were expressed at much higher levels than wild-type p53, yet, no apoptosis was observed. The fact that these naturally occurring p53 mutants exhibit dominant-negative effects indicates that inactivation of wild-type p53 function is an important step in neoplastic transformation and further emphasizes that regulation of apoptosis by wild-type p53 is an integral part of the organism's defense mechanism against the outgrowth of cells with DNA damage that could lead to mutations in cancer-related genes (Symonds et al. 1994).

It has been a general notion that p53 induces apoptosis primarily in certain types of cells, such as those from the lymphoid system (Yonish-Rouach et al. 1991; Lowe et al. 1993b). In contrast, p53 induces growth arrest in fibroblasts (Kastan et al. 1992). Various oncogene products such as E1A, E2F-1, or c-Myc, however, have been shown to sensitize rodent fibroblasts to undergo p53-dependent apoptosis (Bissonnette et al. 1992; Evan et al. 1992; Debbas and White 1993; Lowe and Ruley 1993; Qin et al. 1994; Wu and Levine 1994). We found a relatively higher level of p53 expression and induction of apoptosis by microinjection than by retroviral infection, suggesting that a main determinant for a cell undergoing apoptosis or growth arrest may be the level of wild-type p53. Primary human fibroblasts may require higher levels of p53 than lymphocytes because of differences in the threshold required to trigger apoptosis. Because p53 also induces apoptosis in fused primary human fibroblasts that have lost proliferative potential and are arrested primarily in G_1 of the cell cycle (Vermeulen et al. 1994a) (X.W. Wang et al., unpubl.), p53-mediated apoptosis, at least in fibroblasts, is independent of cell-cycle progression. This result is also consistent with a recent finding that Myc-mediated apoptosis requires wild-type p53 in a manner independent of cell cycle-arrest (Wagner et al. 1994).

We have shown previously that p53 binds specifically to TFIIH-associated XPB and XPD and inhibits their helicase functions (X.W. Wang et al. 1994; 1995b). In this study we demonstrate that p53-induced apoptosis in primary human fibroblasts is directly associated with the function of the XPB and XPD proteins. The results are consistent with our previous hypothesis that the association of p53 with the TFIIH complex has a functional consequence in vivo and provide a basis for defining targets of p53-dependent apoptosis. The following observations are consistent with the hypothesis that p53-induced apoptosis can be mediated by the XPB and XPD DNA helicases. First, p53-mediated apoptosis is defective in fibroblasts from XP-B or XP-D patients that carry a germ-line mutation in the *XPB* or *XPD* gene, respectively. Second, expression of either wild-type *XPB* or *XPD* gene can rescue p53-mediated apoptosis in XP-B or XP-D cells, respectively, which is further genetic evidence indicating that XPB and XPD are members of the p53-dependent apoptotic pathway. Third, this defect is not caused by an overall abolishment of NER activity because mutations involved in the NER genes other than *XPB* or *XPD* did not affect this pathway. Fourth, although mutations in the *XPB* or *XPD* gene did not completely abolish p53-mediated apoptosis, this partial defect does not appear to be the result of the XP cells being defective in transcription because the signal intensities of p53 and p53-induced $p21^{WAF1}$ were comparable among normal, XP-B, or XP-D cells following introduction of p53. These cells also are normal in responding to Ich-1_L- and ICE-induced apoptosis. In addition, primary fibroblasts from patient TTD1BR with the trichothiodystrophy syndrome (TTD) responded normally to p53-mediated apoptosis, even though this patient carries a germ-line mutation in a TFIIH-associated factor other than the *XPB* or *XPD*, which results in a severe defect in NER (Stefanini et al. 1993) and functional and anatomical defects related to a postulated transcription syndrome (Bootsma and Hoeijmakers 1993; Vermeulen et al. 1994b). Moreover, defects in p53-mediated apoptosis were also observed in XP-B- or XP-D-derived cells carrying mutations at different sites in these NER genes. The partial defect in apoptosis could reflect other signaling pathways (p53-dependent but TFIIH independent) that follow a different time course to trigger apoptosis. Alternatively, it can be explained by a partial deficiency in either the XPB- or XPD-dependent apoptotic pathway in these cells becuase there is always one functional XP protein, that is, XPB or XPD, remaining in the TFIIH complex. We interpret the second possibility as a more likely explanation. Fifth, the Pab421 antibody specifically recognizes the CTD of p53 (amino acid residues 373–381) involved in binding to XPB and XPD. This antibody can compete binding of p53 to XPB in vitro (data not shown). Consistent with this observation is the finding that the microinjected Pab421 also blocked p53-mediated apoptosis in vivo (Fig. 4). In contrast, p53-mediated *trans*-activation activity was either enhanced or unaltered (Fig. 4 and 5). Finally, XP-D lymphoblasts, which are defective in p53-mediated apoptosis, as demonstrated by treatment with a DNA-damaging agent, are still capable of undergoing apoptosis by other cell death inducers including okadaic acid (Fig. 7B) and anti-Fas antibody (data not shown). Taken together, these data are consistent with the model that p53-mediated apoptosis is a result of a direct interaction between p53 and the XPB and XPD proteins in the TFIIH complex.

The *Ich-1_L* and *ICE* gene-induced apoptosis in normal as well as XP-B and XP-D cells, indicate that these *Ced-3* mammalian homologs could use either a separate or a TFIIH downstream pathway to trigger cell death and that XP-B and XP-D cells retain all functional factors needed for the p53-independent or TFIIH downstream apoptotic pathway. Interestingly, the *Ced-3/ICE*-related gene products were shown to be relatively downstream of various apoptotic pathways including Fas-mediated apoptosis (Enari et al. 1995; Los et al. 1995). It will be interesting to identify the functional relationship between TFIIH and *Ced-3/ICE* gene products in the general apoptotic pathway.

p53 is frequently mutated in many types of human cancer (for review, see Hollstein et al. 1991; Levine et al. 1991; Greenblatt et al. 1994). Loss of the wild-type p53 function by mutations appears to be an important step in carcinogenesis. p53 is clearly demonstrated to be involved in transcriptional *trans*-activation, leading to activation of downstream genes, for example, $p21^{WAF1}$ (El-Deiry et al. 1993), or *GADD45* (Kastan et al. 1992; Zhan et al. 1994), contributing to the G_1 cell-cycle checkpoint. Although most of the p53 mutants derived from human cancers appear to be defective in both transcriptional *trans*-activation and tumor suppression (Kern et al. 1991, 1992; El-Deiry et al. 1992; Pietenpol et al. 1994), an intriguing question concerning whether the transcriptional *trans*-activator property of p53 is always required for its tumor suppression function is still under debate (Crook et al. 1994; Rowan et al. 1996). We analyzed a group of p53 mutants and showed a clear correlation between mutants that were still able to *trans*-activate but had diminished apoptotic activity (Fig. 5), which is a function positively correlated with tumor suppressor activity (Lowe et al. 1994; Symonds et al. 1994). In contrast, one p53 mutant, 273^{his} that was shown to retain partial tumor suppressor function (Chen et al. 1991; Slingerland et al. 1993) and some apoptotic activity (Fig. 5), had no detectable *trans*-activation activity in human fibroblasts. Strasser and colleagues (1994) suggest that cell-cycle arrest (possibly by p53-mediated *trans*-activation of the $p21^{WAF1}$ and/or *GADD45* genes) and apoptosis may be independent outcomes following treatment with cytotoxic agents. Whereas p53-induced apoptosis has an important mechanistic role in p53-mediated tumor suppression (Lowe et al. 1994; Symonds et al. 1994), transcriptional *trans*-activation and *trans*-repression by p53 may not be necessary for its tumor suppressor activity (Crook et al. 1994). Moreover, a recent study (Caelles et al. 1994) indicates that protein synthesis is not required for the p53-induced apoptosis. Mice lacking $p21^{WAF1}$ undergo normal development and normal apoptotic response but are defective in G_1 checkpoint control, and these mice (unlike p53−/−) do not have an increased frequency of spontaneous malignancies (Agarwal et al. 1995). Therefore, transcriptional *trans*-activator property of p53 may be dispensable for its tumor suppressor activity, and the data presented here further emphasize the possible importance of protein–protein interactions between p53 and other cellular factors in its apoptotic or tumor suppression pathway. Interestingly, Haupt et al. (1995) recently presented evidence indicating that p53-induced apoptosis is independent of sequence-specific *trans*-activation, Wagner et al. (1994) showed that Myc-mediated apoptosis requires p53 in a manner independent of the ability of p53 to induce $p21^{WAF1}$, and Sabbatini et al. (1995) suggested a requirement of p53-mediated transcription in E1A-induced apoptosis. It is likely that both p53-mediated transcriptional *trans*-activator-dependent and -independent apoptotic pathways exist and are interactive, and this interaction may vary among cell types.

Our studies indicate that the microinjected p53 carboxy-terminal peptide (amino acid residues 319–393) can directly induce apoptosis of primary human fibroblasts, suggesting that this region contains a death domain that is required for binding to XPB and XPD proteins and for induction of apoptosis. Interestingly, a recent report indicates that the amino-terminal half of p53 (amino acid residues 1–214) is sufficient for induction of apoptosis in HeLa cells (Haupt et al. 1995). It is unclear how this amino-terminal half of p53 triggers apoptosis in HeLa cells. However, the p53-mediated transcription activator activity is not required in this system because this mutant is defective in *trans*-activation (Haupt et al. 1995). Nevertheless, although XPB and XPD are important for the CTD of p53-induced apoptosis in our system, alternative pathways may exist that use different targets. The fact that the XP-D or XP-B fibroblasts were not completely defective in p53-mediated apoptosis is consistent with this hypothesis. It will be interesting to test whether the amino-terminal half of p53 can induce apoptosis in normal human cells.

XPB and XPD are part of the TFIIH complex that is involved in basal transcription, NER, and possibly the control of cell cycle via its interaction with a cyclin-dependent kinase (CDK), CDK-activating kinase (CAK) (Schaeffer et al. 1993; Sung et al. 1993; Drapkin et al. 1994; Feaver et al. 1994; Roy et al. 1994a,b; Schaeffer et al. 1994). Our results indicate that TFIIH also plays a role in a p53-dependent apoptotic pathway. It is possible that these four essential cellular processes utilize the same core TFIIH protein complex and have a number of steps in common. A dynamic interaction among these factors may be required in various circumstances to modulate different cellular functions. For example, CAK is not associated with core–TFIIH when the complex is needed for NER (Z. Wang 1994; Svejstrup et al. 1995) but is tightly bound to core–TFIIH when the complex is competent for basal transcription (Feaver et al. 1994; Roy et al. 1994a; Svejstrup et al. 1995). In contrast, core–TFIIH is associated with other repair factors when it is required for NER (Svejstrup et al. 1995). It is plausible that the core–TFIIH complex may also bind to factors involved in the apoptotic pathway, one of the candidates being the p53 tumor suppressor protein. It will be interesting to identify additional factors downstream of the p53–TFIIH complex that are important in this apoptotic pathway.

Cancer therapy may inhibit tumor growth by triggering p53-dependent apoptosis through DNA damage. The results reported here provide a strategy to design new therapeutic agents that would bypass the requirement for p53 and directly target downstream members, that is, XPB and XPD, in this apoptotic pathway.

Materials and methods

Plasmids

pC53SN encodes the human wild-type *p53* cDNA. $p53–143^{ala}$, $p53–175^{his}$, $p53–248^{trp}$, $p53–249^{ser}$, and $p53–273^{his}$ encode a single mutant p53, Val1-43-Ala, Arg-175-His, Arg-248-Trp, Arg-249-Ser, or Arg-273-His, respectively. These constructs were kindly provided by B. Vogelstein and J. Pietenpol (Johns Hop-

kins University). p53–181^{his}, a gift of S. Friend (Fred Hutchinson Cancer Center), encodes a mutant p53 Arg-181-His. p53–517, provided by H.-W. Sturzbacher (Sturzbecher et al. 1992), encodes *p53* cDNA with four missense mutations, His-365-Ala, Lys-372-Leu, Arg-379-Ala, and Lys-386-Leu. p53Δ353, encoding the first 353 amino acids of p53, was kindly provided by J. Pietenpol and B. Vogelstein. pCEPE3 encodes a wild-type *XPD* cDNA inserted into the pCEP4 vector at the *Bam*HI and *Hind*III sites (Invitrogen). pcDNA–XPD encodes a wild-type XPD cDNA inserted into the pCDNA3 vector at the *Eco*RI site (Invitrogen). All genes are under the control of the CMV promoter. *Ich-1*$_L$ and *ICE* genes have been described elsewhere (L. Wang et al. 1994).

Cell strains, culture condition, microinjection, and viral infection

Cell strains, culture conditions, cell hybridization, and microinjection were essentially as described (Vermeulen et al. 1994a). Primary human fibroblasts were grown in Ham's F10 medium supplemented with 10% FBS. Cells were seeded onto coverslips and incubated for an additional 2–3 days prior to microinjection. In some experiments, cells were fused by β-propiolactone-inactivated Sendai virus and the fused cells were used for microinjection. Plasmid cDNA, in a concentration of 100–200 μg/ml suspended in PBS, was injected into the nuclei of cells by use of a glass microcapillary. For each experiment, at least 50 cells were injected. Many of the microinjections were performed in part on the same slide when wild type was compared to various mutants or with the same needle when different types of cells were used to minimize variations caused by differences in immunostaining, processing of the slides, or vector copy numbers, and so forth. For viral infection, a single viral stock was used to infect cells in the presence of 8 μg/ml of polybrene.

Immunocytochemistry analysis

Following incubation, cells were fixed with 2% paraformaldehyde (in PBS) followed by methanol treatment. p53 was visualized by staining cells with the anti-p53 polyclonal CM-1 antibody (Signet Labs) followed by fluorescein-conjugated anti-rabbit IgG (Vector Labs, Burlingame, CA). Nuclei were stained with 4′, 6-diamidino-2-phenylindole (DAPI). For simultaneous demonstration of p53 and p21^{WAF1} proteins, cells were double-labeled with CM-1 antibodies (1:200) and anti-p21^{WAF1} monoclonal antibody (Oncogene Science) (1:100), followed by the corresponding secondary antibodies conjugated to fluorescein isothiocyanate (FITC) and Texas Red, respectively.

Cell viability assay and Western blot analysis.

Lymphoblasts, obtained from Coriell Institute for Medical Research (Camden, NJ), were maintained in RPMI1640 medium containing 20% FBS. Exponentially growing cells were plated at a density of 6.25×10^5 cells per ml. Adriamycin and okadaic acid (Sigma) were used at concentrations of 17.2 and 0.5 μM, respectively. Cell viability was assessed by trypan blue exclusion. For Western blot analysis, lymphoblasts were treated with adriamycin as above for 24 hr. Cells were lysed in RIPA buffer (150mm NaCl, 1% NP40, 0.5% DOC, 0.1% SDS, 50mm Tris-HCl, pH8.0). Total cellular proteins were subjected to immunoprecipitation with anti-p53 DO-1 antibody (Oncogene Science) essentially as described (X.W. Wang et al. 1995b). Immunoprecipitates were separated on 10% SDS-PAGE, transferred to a nitrocellulose membrane, and probed with polyclonal anti-p53 CM-1 antibody by the method of ECL (Amersham, Arlington Heights, IL).

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