

# p53 levels, functional domains, and DNA damage determine the extent of the apoptotic response of tumor cells

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It is well established that induction of the p53 tumor suppressor protein in cells can lead to either cell cycle arrest or apoptosis. To further understand features of p53 that contribute to these cell responses several p53-null Saos2 and H1299 cell lines were generated that express wild-type or mutant forms of p53, or the cyclin-dependent kinase inhibitor p21/WAF1, under a tetracycline-regulated promoter. Our results show that the cellular level of p53 can dictate the response of the cell such that lower levels of p53 result in arrest whereas higher levels result in apoptosis; nevertheless, DNA damage can heighten the apoptotic response to p53 without altering the protein level of p53 in cells. We also demonstrate that arrest and apoptosis are two genetically separable functions of p53 because a transcriptionally incompetent p53 can induce apoptosis but not arrest, whereas induction of p21/WAF1, which is a major transcriptional target of p53, can induce arrest but not apoptosis. Finally, we show that a full apoptotic response to p53 requires both its amino and carboxyl terminus, and our data suggest that there is synergism between transcription-dependent and -independent functions of p53 in apoptosis. Thus, there are multiple independent cellular responses to p53 that together may account for the extraordinarily high frequency of p53 mutations in diverse types of human tumors. The implications of these results are discussed and a model is proposed.

[Key Words: p53; p21/WAF1; cell-cycle arrest; apoptosis; DNA damage]

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Following genotoxic stress such as that emanating from damaged DNA or conditions of hypoxia, induction of p53 results in cell-cycle arrest or apoptosis (for review, see Levine 1993; Gottlieb and Oren 1996; Ko and Prives 1996). It is not fully understood which of these two responses to p53 is chosen in a given cell. Cell type appears to be one of the factors involved in this process (for review, see Gottlieb and Oren 1996) and the microenvironment of a cell can also influence the response (Boudreau et al. 1995, 1996). In addition, several cellular and viral proteins contribute to the propensity of cells to undergo either response (for review, see Fisher 1994; White 1996).

It is also not fully understood what functions of p53 are required for cell cycle arrest or apoptosis. p53 is a sequence-specific transcriptional activator of genes containing p53 response elements (for review, see Vogelstein and Kinzler 1992). A number of transcriptional targets of p53 have been identified. One of these is the cyclin-de-

pendent kinase inhibitor p21 (El-Deiry et al. 1993; Harper et al. 1993; Xiong et al. 1993), which inhibits the protein kinase activities of G<sub>1</sub> cyclin/CDK complexes, thereby preventing phosphorylation of the retinoblastoma (RB) protein (Slobos et al. 1994). p21 is thus an excellent candidate for mediating p53-induced cell-cycle arrest. It is not known whether activation of additional targets of p53 is required for arrest as well.

It is less clear how p53 induces apoptosis. There are, however, at least two candidate genes that play roles in apoptosis that can be transactivated in response to p53 induction. In murine cells, p53 upregulates expression of the *bax* gene (Miyashita and Reed 1995), whose product dimerizes with Bcl-2 and prevents the ability of Bcl-2 to block apoptosis (Oltvai et al. 1993). It is possible, therefore, that transcriptional activation of the *bax* gene by p53 induces apoptosis. A second p53 target that might influence apoptosis is IGFBP-3 (Buckbinder et al. 1995), which is an antagonist of insulin-like growth factor-1 (IGF-1) (Baserga 1994). Down-regulation of IGF-1 or the IGF-1 receptor is correlated with the apoptotic response (Baserga 1994). Several recent studies have provided evidence, however, that p53 may have a transcription-independent function in apoptosis (Caelles et al. 1994;

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Wagner et al. 1994). Furthermore, different reports have provided essentially contradictory results as to the requirement for the sequence-specific transactivation function of p53 for induction of apoptosis (Haupt et al. 1995; Sabbitini et al. 1995). If indeed there is an alternative function of p53, then it will be important to identify the domains or regions of the protein responsible for this function.

p53 has been subjected to extensive analysis of its functional domains (Gottlieb and Oren 1996; Ko and Prives 1996). The p53 polypeptide consists of an activation domain located within the amino-terminal 43 amino acids, a sequence-specific DNA binding domain located within the central, conserved portion of the protein, and, within the carboxyl terminus resides a tetramerization domain as well as a regulatory region that controls the ability of the protein to allosterically switch from a latent form to one that is active for sequence-specific DNA binding. Either naturally occurring or experimentally produced mutations within these regions of p53 have identified residues that are important for their function. Mutation of two residues within the p53 activation region, leu22 and trp23, abrogates transactivation by p53 (Lin et al. 1994). This is presumably the case because these residues are required for the interaction of the activation domain with TATA-box binding protein associated factors (TAFs) (Lu and Levine 1995; Thut et al. 1995). The vast majority of the missense mutations that have been detected in tumors of cancer patients map to the central DNA-binding domain of p53 (Hollstein et al. 1991). Among these are a number of mutational hot spots that occur with unusually high frequency and together make up ~40% of all tumor-derived p53 mutations. Most mutations within the central DNA-binding domain impair or abolish sequence-specific DNA binding by p53 (Vogelstein and Kinzler 1992). Finally, mutational analysis of the carboxyl terminus has revealed residues that are important for oligomerization of the p53 protein (Sturzbecher et al. 1992). Moreover, deletion of the carboxy-terminal 30 amino acids of p53 has striking stimulatory effects on the ability of the protein to bind to DNA *in vitro* (Hupp et al. 1992; Halazonetis and Kandil 1993).

To further understand how p53 might regulate arrest versus apoptosis we have used the p53 null cell lines Saos2 and H1299 to generate tetracycline-regulated cell lines inducibly expressing either wild-type or mutant forms of p53 or wild-type p21. This system has been utilized previously by others for a variety of goals (Buckbinder et al. 1994; Van Meir et al. 1994; Agarwal et al. 1995; Chen et al. 1995). The lines we have generated have allowed us to (1) identify a quantitative response to different amounts of p53, (2) examine a number of p53 variants in clonally derived cell populations to derive information about the influence of functional domains of p53 on cell arrest and apoptosis, and (3) determine the effect of DNA damage on p53 in this context. Our results have provided new insight into p53 and should provide a paradigm for this approach to studying p53 structure and function.

## Results

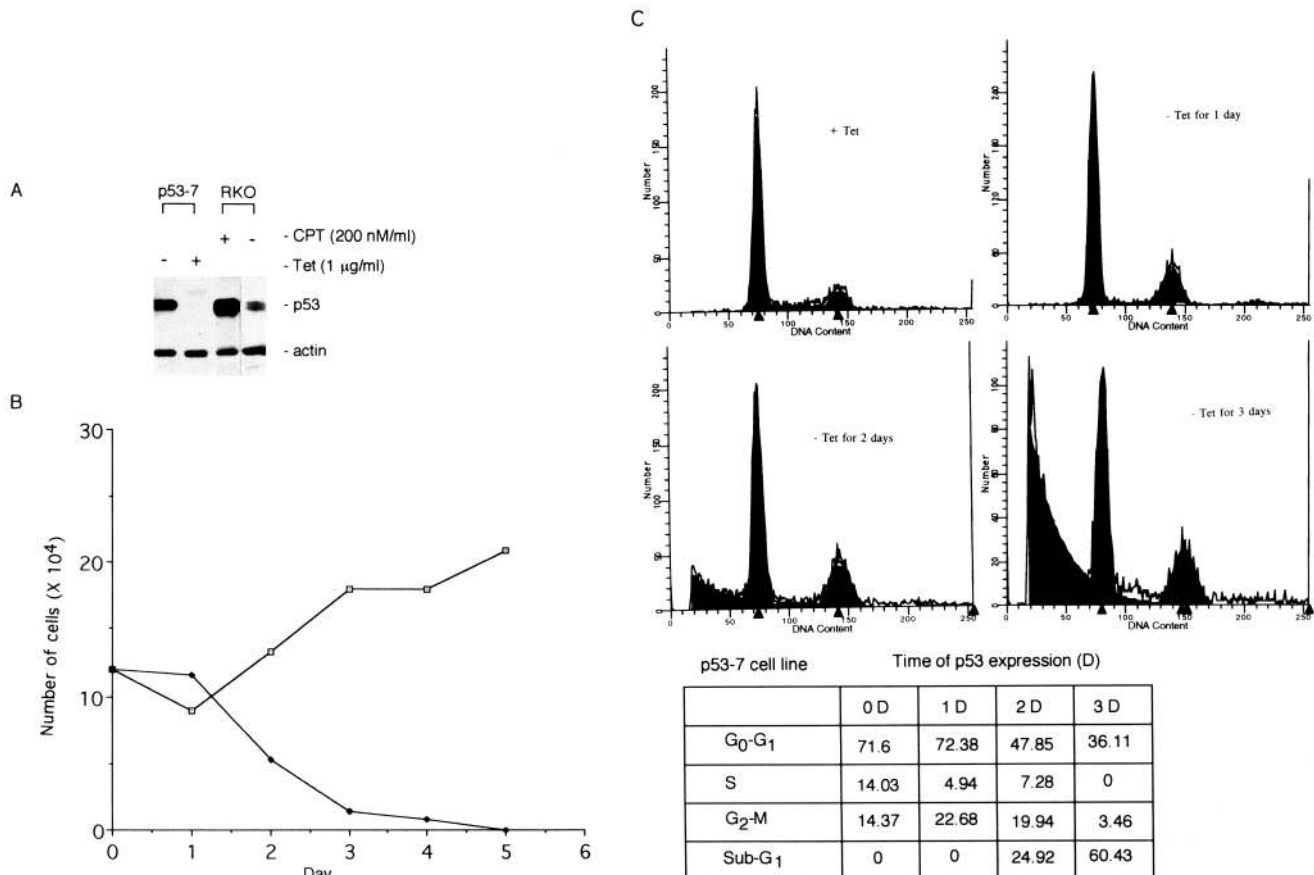
### *The level of p53 within Saos2 cells determines cell death or arrest*

To generate the first series of inducible cell lines, we chose the human osteosarcoma cell line Saos2 because they are null for p53, are easily transfected, and have been shown previously to arrest upon overexpression of wild-type p53 (Chen et al. 1990). Each individual cell line used for this study was chosen from a number of clonal lines based on relative levels of protein expressed after induction.

Of the two wild-type p53 inducible cell lines obtained, one (p53-7) expressed p53 protein at relatively high levels upon withdrawal of tetracycline and will be referred to as the "high p53 producer" line (Fig. 1A). p53 protein was detected within 8 hr and reached maximal levels at 24 hr post-induction (data not shown). Note that the amount of detectable induced p53 protein in p53-7 cells, however, was substantively less than that induced in DNA damaged human RKO cells that contain wild-type p53 (Kastan et al. 1992), demonstrating that the level of p53 expressed in the Saos2 cell line is within the physiological range. When the growth curve of induced and uninduced p53-7 cells was examined, there was a dramatic difference in cell viability between the two states (Figure 1B). The uninduced cells continued to grow with a doubling time of ~48 hr, whereas the p53 expressing cells started to die within 2 days (as determined by reduced cell count), and by 3 days, only 10% of the cells survived. By 5 days after induction there were virtually no visible cells remaining on the plate (data not shown). DNA histogram analysis of induced p53-7 cells (Fig. 1C) showed that 1 day after tetracycline withdrawal the percentage of cells in S phase was reduced from 14% to 5%, and the percentage of cells in G<sub>2</sub> was increased from 14% to 23%, suggesting that a transient G<sub>2</sub> arrest had occurred in at least a fraction of the cells; at this time point little or no sub-G<sub>1</sub> content cells were scored. By 2 days, however, 25% of the cells had a sub-G<sub>1</sub> DNA content and at 3 days after induction >60% of the cells had sub-G<sub>1</sub> DNA content indicating apoptotic death, with the remaining cells primarily arrested in G<sub>1</sub>. Virtually all cells die after induction of p53 in p53-7 cells, thus, the arrest must be transient because the cells are not protected from eventually entering the apoptotic pathway. It should be mentioned that upon continued passage of p53-7 cells, the apoptotic phenotype of the cells was diminished and eventually lost. However, the levels of p53 were still high and the cells still exhibited the cell-cycle arrest component of the response.

When the second p53 cell line (p53-13) was analyzed, upon withdrawal of tetracycline, the amount of p53 within p53-13 cells was ~25–50% of that detected in p53-7 cells (Fig. 2A). Although p53-13 cells showed substantially slowed cell growth, there was in contrast with p53-7 cells, no reduction in cell number (Fig. 2B) nor did cells with sub-G<sub>1</sub> DNA content appear (Figure 2C). DNA histogram analysis showed that the percentage of S phase p53-13 cells was reduced from 31% to 15% within

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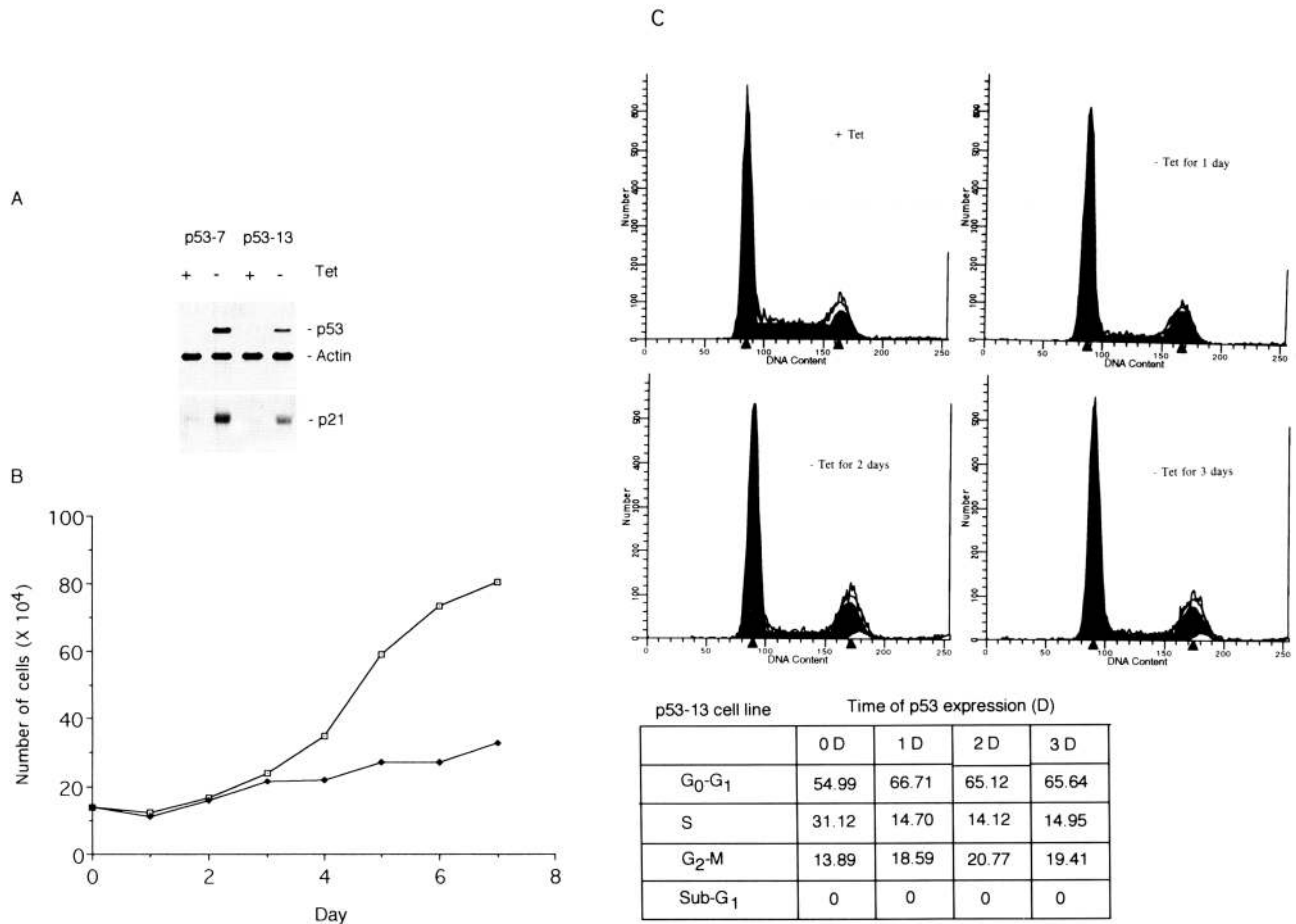


**Figure 1.** Induction of apoptosis by high levels of p53 in p53-7 cells. (A) Inducible expression of p53 and levels of actin in p53-7 cells in the presence or absence of tetracycline (1  $\mu$ g/ml) for 24 hr, and in RKO cells treated with or without camptothecin (200 nM/ml) were assayed by Western blot analysis. The blot was probed with p53 monoclonal PAb1801 and actin polyclonal antibodies. (B) Growth rates of p53-7 cells in the presence ( $\square$ ) or absence ( $\blacklozenge$ ) of tetracycline were measured as described in Materials and Methods. Note that in this and other experiments there was a slight decrease in cell number at day 1 which is most likely due to trypsinization. (C) DNA contents were quantitated by propidium iodide staining of fixed cells at 0, 1, 2 and 3 days following withdrawal of tetracycline as described in Materials and Methods. (Bottom) Percentage of cells in the G<sub>0</sub>-G<sub>1</sub>, S, G<sub>2</sub>-M, and sub-G<sub>1</sub> phases of cell cycle at 0, 1, 2, and 3 days following withdrawal of tetracycline was calculated from flow cytometric measurements of DNA content.

1 day following p53 induction and after 2 to 3 days the proportion of S phase cells remained at that low level (14%) (Fig. 2C). p53-13 cells, therefore, exhibit a significant arrest in both G<sub>1</sub> and G<sub>2</sub>, but no apoptosis. The induction of p53 in both p53-7 and p53-13 cells, notably, was accompanied by a marked increase in the amount of detectable p21 (Fig. 2A).

Because the only obvious difference between the p53-7 and p53-13 cells was the amount of p53 detected after induction, we tested whether the level of p53 was, in fact, capable of regulating the apoptotic versus arrest response. This was done by varying the amounts of tetracycline in the culture medium of the high producer p53-7 cells. The results showed that low concentrations of tetracycline allowed for a partial expression of p53, whereas more complete withdrawal of tetracycline caused greater amounts of p53 to accumulate (Fig. 3A).

Cells were counted at day 0, 1, or 3 following induction of p53 to different extents. The number of surviving cells was generally inversely proportional to the amount of p53 expressed and directly proportional to the amount of tetracycline present in the culture media (Fig. 3B). With the conditions resulting in the two lowest amounts of p53 induced (that is, 40 and 20 ng/ml of tetracycline), however, there was evidence of some increase in cell number, albeit significantly less than with no induction. Thus, intermediate levels of p53 caused slowed cell growth and cell arrest but not a significant amount of cell death, whereas levels close to or the same as those seen with full induction caused loss of cells comparable to that shown in Figure 1. We conclude from these data that the level of p53 in the p53-7 Saos2 cell line can determine whether the cells undergo growth arrest or apoptosis.



**Figure 2.** Induction of cell-cycle arrest by low levels of p53 in p53-13 cells. The experiments were performed in an identical manner to those in Fig. 1.

*DNA damage can sensitize cells to p53-mediated apoptosis without affecting the level of p53 protein*

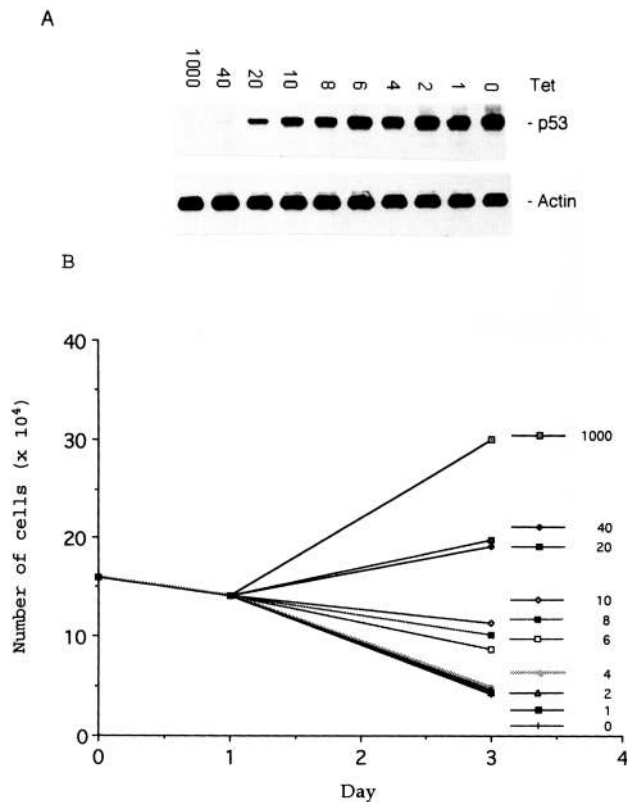
Effectors of DNA damage have been shown to increase the amount of p53 in cells by a post-transcriptional mechanism (Maltzman and Czyzyk 1984; Kastan et al. 1992; Lu and Lane 1993). Because the quantity of p53 induced was clearly a determinant of the switch between arrest and apoptosis in p53-7 cells, we wished to test whether the levels of p53 in the low producer cell line, p53-13, could be augmented after DNA damage, and whether the cells would now undergo apoptosis. Camptothecin (CPT), a topoisomerase inhibitor and cancer therapy drug, has been shown to induce DNA damage in cells (Nelson and Kastan 1994). Moreover, as shown in Figure 1A, we have confirmed that treatment of RKO cells with CPT results in a significant induction of p53 protein levels. When p53-13 cells were treated with increasing amounts of CPT in the presence or absence of tetracycline we observed that even without p53 induction there was a modest apoptotic response to CPT, sug-

gesting that Saos2 cells can undergo DNA damage-associated apoptosis in a p53-independent manner (Fig. 4B). Unexpectedly, however, when p53 was induced in CPT-treated p53-13 cells there was a significant increase in the number of apoptotic cells (Fig. 4B), and yet no discernable increase of p53 protein levels (Fig. 4A). Thus, p53 and CPT cooperate in Saos2 cells to cause a strong apoptotic response, and this occurs in a manner that is independent of p53 protein accumulation.

*p21 induction in Saos2 cells leads to arrest but not apoptosis*

The cyclin-dependent kinase inhibitor p21 (WAF1), a potential mediator of p53 tumor suppression, has been shown in a number of studies to be strongly induced by p53. Consistent with these results p21 expression was markedly increased in both p53-13 and p53-7 cells after removal of tetracycline (Fig. 2A). Because the low p53 producer cells (p53-13) did not undergo apoptosis this

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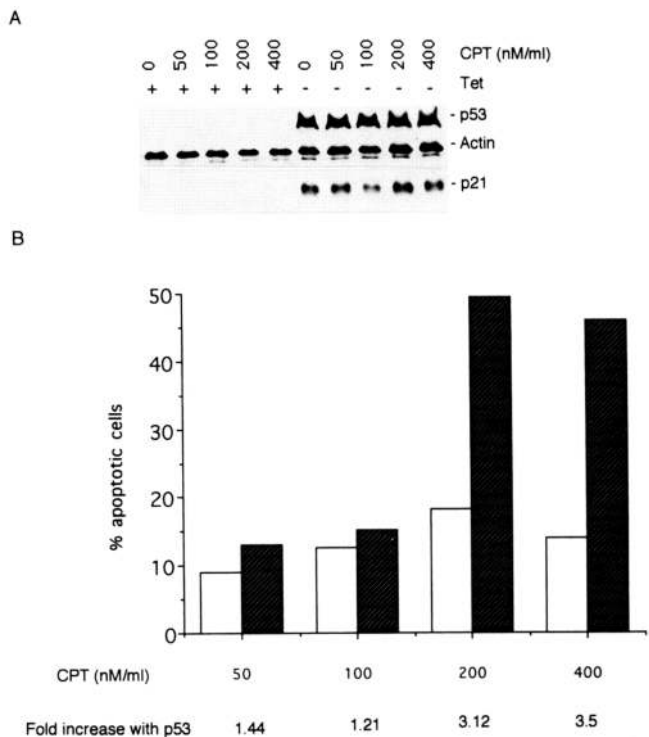
**Figure 3.** The level of p53 determines cell death or arrest. (A) Inducible expression of p53, and levels of actin in p53-7 cells in the presence of 1000, 40, 20, 10, 8, 6, 4, 2, 1, and 0 ng/ml of tetracycline as indicated were assayed by Western blot analysis. The blots were probed with a mixture of p53 monoclonal PAb1801 and actin polyclonal antibodies. (B) The growth rates of p53-7 cells in the presence of varying concentrations of tetracycline were measured as described in Materials and Methods.

suggested that p21 induction alone is not sufficient for apoptosis. To directly analyze the role of p21 in cell death or arrest, we examined the cellular response to induction of p21 in the absence of p53. One of the p21-inducible cell lines (p21S4) expressed p21 protein to an even greater extent than in induced p53-7 cells (Fig. 5A). However, upon maximal p21 induction, p21S4 cells underwent cell-cycle arrest with no evidence of apoptosis (Fig. 5B). DNA histogram analysis showed that in the p21S4 line S-phase cells were reduced from 32% to 14% following 3 days of p21 induction and that cells were arrested in both G<sub>1</sub> and G<sub>2</sub> (Fig. 5C). It is notable that the extent of arrest at day 1 after induction of p53-7 cells was greater than that in p21 cells (cf. Fig. 2B and Fig. 5B), suggesting that p21 may not be the sole mediator of arrest after p53 induction. This observation is consistent with the results of Brugarolas et al. (1995) and Deng et al. (1995) indicating that the absence of p21 led to only a partial defect in G<sub>1</sub> arrest in response to radiation. Our

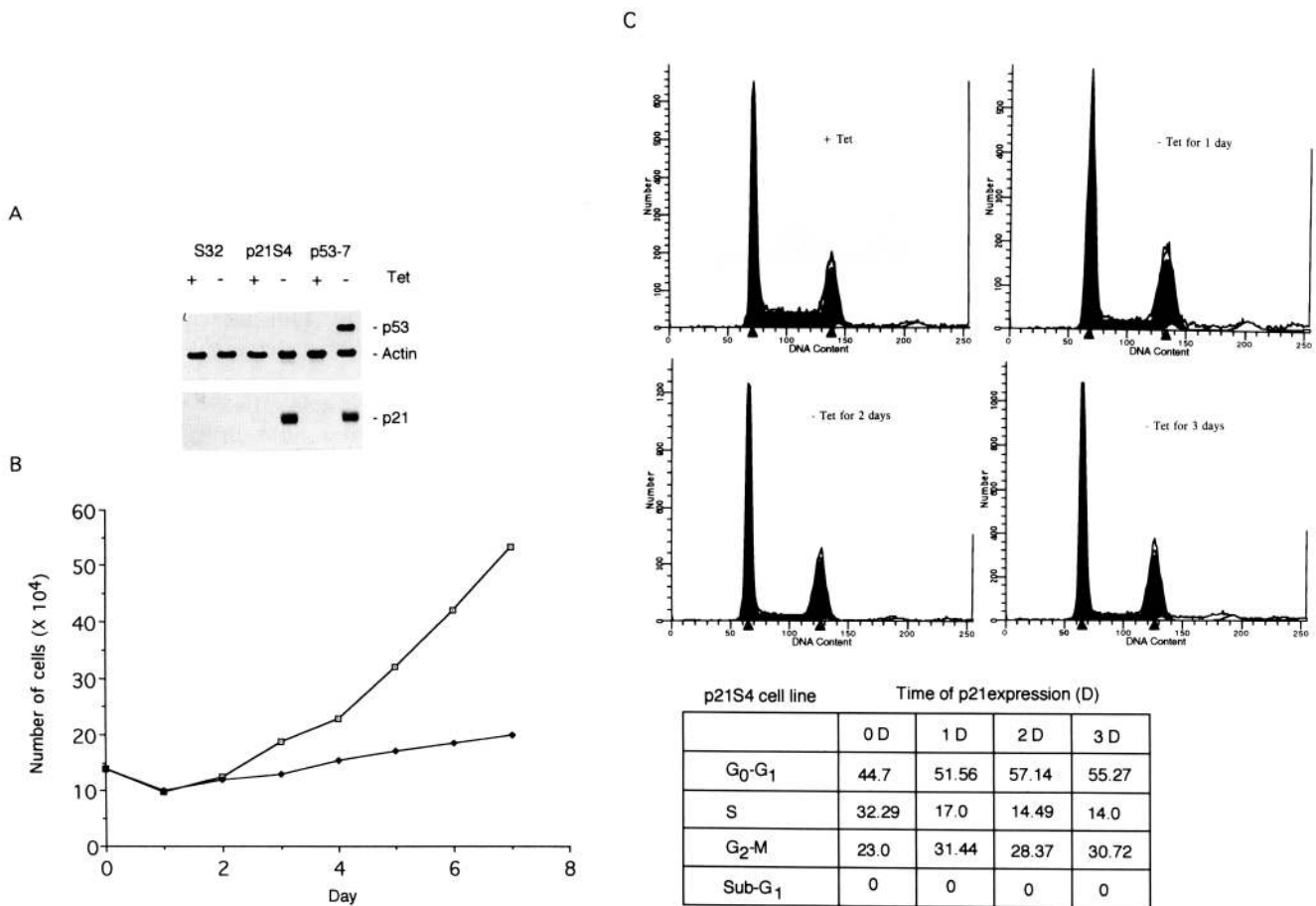
data thus strongly suggest that p21 on its own is unable to produce an apoptotic response.

*A weak and delayed apoptotic response is induced by a transactivation-deficient mutant form of p53*

A p53 double mutant p53(gln22/ser23) was shown to be defective in transactivation (Lin et al. 1993) presumably because of the inability of this mutant to bind to TAFs that are critical for p53-mediated activation (Lu and Levine 1995; Thut et al. 1995). This mutant, however, has produced contradictory results as to whether transcriptional activation is necessary for p53-mediated apoptosis (Haupt et al. 1995; Sabbitini et al. 1995). To determine whether apoptosis can occur through a p53 transactivation-independent pathway in Saos2 cells, a cell line, 22/23-4, that expresses high levels of inducible transactivation deficient p53(gln22/ser23) was used. Consistent with evidence from in vitro studies (Lin et al. 1994) and transient transfections (Lin et al. 1995), this mutant form of p53 was transcriptionally inert because endogenous p21 was not induced even by high levels of



**Figure 4.** p53 sensitizes cells to undergo apoptosis by CPT-induced DNA damage. (A) Levels of p53, p21, and actin were assayed by Western blot analysis in p53-13 cells in the presence or absence of tetracycline for 24 hr, followed by treatment with CPT at concentrations of 0, 50, 100, 200, and 400 nM/ml for another 24 hr. (B) Number of apoptotic cells of the untreated or CPT-treated p53-13 cells in the presence (open bars) or absence (solid bars) of tetracycline.



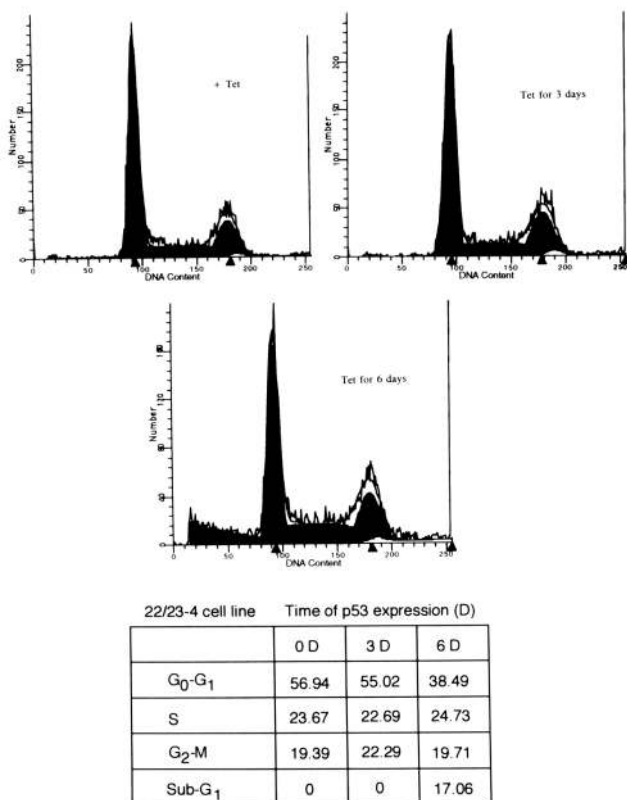
**Figure 5.** p21 induction leads to cell cycle arrest but not apoptosis. The experiments were performed as those in Fig. 1.

p53(gln22/ser23) (data not shown). The amount of p53 in 22/23-4 cells was approximately equivalent to that detected in p53-7 cells. Consistent with the lack of induction of p21 in 22/23-4 cells, no detectable cell-cycle arrest was observed after induction of mutant p53 as observed by DNA histogram analysis (Fig. 6). Nevertheless, in these cells p53(gln22/ser23) reproducibly induced cell death (Fig. 6), although to a lesser extent and with delayed kinetics as compared with wild-type p53. Thus, those cells that did not undergo apoptosis contained a normal S-phase DNA content and presumably kept cycling. The apoptosis induced by p53(gln22/ser23), although reduced, was significantly greater than either the background levels of cell death that occur in the presence of tetracycline, or than in cells expressing mutant p53 completely defective in apoptosis (see Fig. 8, below, for comparison). Our data confirm and extend observations by Oren and colleagues (Haupt et al. 1995), who showed that apoptosis can be brought about by p53 mutants such as p53(gln22/ser23) that are defective in sequence-specific transactivation in transiently transfected HeLa cells. These data also provide clear evidence that the abilities of p53 to induce cell-cycle arrest and apoptosis are genetically separable.

#### *The p53 carboxyl terminus is necessary for efficient apoptosis*

p53 contains an autoinhibitory region within the last 30 amino acids of the protein. Deletion of this region generates a p53 protein that is activated for DNA binding in vitro (Hupp et al. 1992) and that is comparable to full-length p53 in activating transcription in transient transfection assays in cells (Halazonetis and Kandil 1993; L. Ko, unpubl.). To determine the cellular response to p53 lacking the carboxy-terminal 30 amino acids p53( $\Delta$ C30), cell lines expressing this p53 variant were isolated. One of these lines, p53( $\Delta$ C30)-6, contained at least twofold more p53 than the high producer cell line p53-7 when normalized to the cellular actin protein levels (Fig. 7A), although still in the range of p53 induced in DNA-damaged RKO cells. Consistent with observations that the  $\Delta$ C30 mutant is functional in transactivation, p21 was induced in p53( $\Delta$ C30) cells to a similar extent as in wild-type p53 cells (Fig. 7B). Although the growth of p53( $\Delta$ C30)-6 cells was completely arrested upon induction, the cell number did not decrease detectably throughout the time course of the experiment (Fig. 7C). Because very slow but detectable cell growth had been

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**Figure 6.** Induction of a delayed apoptosis but not arrest by p53 transactivation defective mutant p53(gln22/ser23). DNA contents were quantitated by flow cytometric analysis in 22/23-4 cells at day 0, 3, and 6 following withdrawal of tetracycline. The percentage of cells in various phases of cell cycle at day 0, 3, and 6 after withdrawal of tetracycline.

observed for both the low p53 producer cell line p53-13 (Fig. 2B) and high p21 producer cell line p21S4 (Fig. 5B), and both cell lines do not undergo apoptosis, the flat growth curve of p53( $\Delta$ C30)-6 cells suggested the possibility that a minor proportion of p53( $\Delta$ C30)-6 cells underwent apoptosis. Consistent with this idea, DNA histogram analysis of p53( $\Delta$ C30)-6 cells taken over 4 days showed that upon induction of p53( $\Delta$ C30), a weak apoptotic response with delayed kinetics ensued (Fig. 7D). Therefore, in this cell line and in others (data not shown), p53( $\Delta$ C30) can induce apoptosis, but far more weakly than full-length p53, suggesting that the carboxy terminal 30 amino acids of p53 are required for efficient apoptotic activity.

*A full apoptotic response to p53 in tumor cells requires both sequence-specific transactivation and carboxy-terminal regulatory domains of p53*

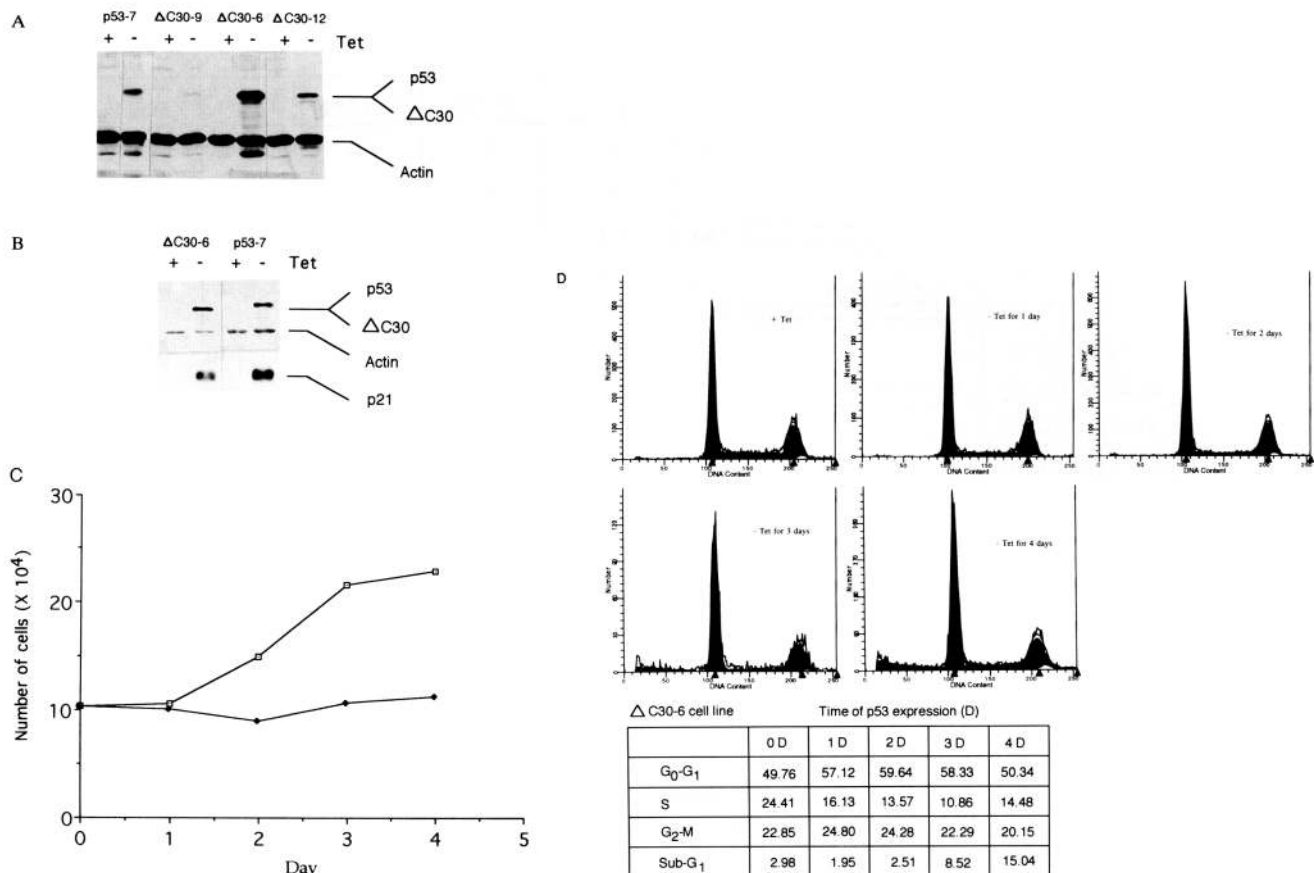
To gain further information as to whether the carboxy-domain of p53 would itself be sufficient to induce apoptosis in the absence of the amino-terminus, a Saos2 cell

line p53( $\Delta$ N96)-5 expressing amino acids 97 to 393 [p53( $\Delta$ N96)] was used. p53( $\Delta$ N96)-5, which lacks the entire transactivation domain, is capable of sequence-specific DNA binding (L. Jayaraman and C. Prives, unpubl.) but is completely defective in transactivation (Pietenpol et al. 1994). Upon withdrawal of tetracycline, p53( $\Delta$ N96)-5 cells expressed markedly higher levels of truncated p53 than in the high producer wild-type p53 cell line, p53-7 (data not shown), yet induction of p53( $\Delta$ N96) had no measurable effect on cell growth or arrest (Table 1 and Fig. 8). This indicates that neither DNA binding nor the carboxy-terminal domain are sufficient for apoptosis.

Our results show that p53 alleles with mutations within the amino- and carboxyl termini were inefficient but not completely inert in inducing apoptosis in Saos2 cells. Tumor-derived mutant forms of p53 contain intact amino and carboxyl termini, but are incapable of binding specifically to p53 responsive elements and of transactivation (for review, see Gottlieb and Oren 1996; Ko and Prives 1996). To determine if such mutants would display any apoptotic activity in Saos2 cells, cell lines which contained inducible mutant forms of p53 [p53(ser249) or p53(his175)] were generated. Upon induction of high levels of either p53(ser249) or p53(his175), essentially no changes in growth or survival were detected in the Saos2 cells as compared with either the uninduced state or the parental cell from which they were derived (Table 1 and Fig. 8). These results confirm that tumor-derived mutant forms of p53 are inert for inducing the apoptotic response. Note that although we show here only data for hot-spot mutant p53 proteins p53(his175) and p53(ser249), we have observed that several additional tumor derived mutants are unable to induce apoptosis as well (Friedlander et al. 1996; data not shown).

*H1299 cells with inducible wild-type and mutant forms of p53 confirm and extend results in Saos2 cells*

During the course of the experiments described above with Saos2 cells we decided to use the same strategy to generate a number of additional inducible cell lines in another p53-null human cell background. We chose H1299 cells because, although like Saos2 osteosarcoma cells they are easily transfectable and can undergo p53-mediated apoptosis, they are of a different cellular origin (small cell lung carcinoma) and, importantly, in contrast to Saos2 cells, they express the RB tumor suppressor protein (data not shown). Because a relationship between RB and p53 has been established in many experimental models (for review, see White 1996) it was of interest to determine the response of these cells to the different forms of p53 that were tested in inducible Saos2 cells. The results of our experiments are summarized in Table 1. As was observed with Saos2 cells, one high p53 producer H1299 cell line underwent apoptosis after induction while a low producer H1299 cell line underwent



**Figure 7.** The carboxyl terminus of p53 is required for full apoptosis. (A) Inducible expression of wild-type p53 and p53( $\Delta$ C30), and levels of actin in p53-7, p53( $\Delta$ C30)-9, p53( $\Delta$ C30)-6, and p53( $\Delta$ C30)-12 cell lines in the presence or absence of tetracycline (1  $\mu$ g/ml) for 24 hr. The blot was reacted with p53 monoclonal PAb1801 and actin polyclonal antibodies. (B) The inducible expression of wild-type p53, p53 ( $\Delta$ C30) and p21, and levels of actin in p53-7 and p53( $\Delta$ C30)-6 cells in the presence or absence of tetracycline (1  $\mu$ g/ml) for 24 hr. The blot was reacted with a mixture of p53 monoclonal PAb1801 and actin polyclonal antibodies, and p21 monoclonal antibody, respectively. (C) and (D) The experiments were performed in an identical manner to those in Fig. 1B and 1C.

arrest. However, the high producer H1299 cells displayed a more rapid and extensive apoptotic response than seen with the Saos2 high producer cell line. The increased kinetics seen with this cell line may be caused by the faster doubling time of H1299 cells (e.g., 24 hr) as compared with Saos2 cells (~48 hr). Interestingly, a cell line with inducible p53 lacking the first 22 amino acids was as potent in inducing apoptosis as wild-type p53. Since p21 accumulated after induction in these cells, this suggests that p53( $\Delta$ N22) is transcriptionally active, although whether this truncated p53 is fully comparable to wild-type p53 is not yet established.

Consistent with results observed in Saos2 cells, H1299 lines expressing ser249, his275, or  $\Delta$ N96 mutant forms of p53 were completely unable to induce apoptosis (Table 1). Moreover, cells expressing the carboxy-terminally truncated mutant p53( $\Delta$ C30) or the *trans*-activation-defective mutant p53(gln22/ser23) underwent apoptosis with reduced kinetics and extent over the time course examined. Importantly, a p53 variant that contained both the amino-terminal double mutation at residues 22 and 23 but that also lacked the carboxy-terminal 30

amino acids when expressed at high levels in H1299 cells, was completely inert in inducing apoptosis or growth arrest. This finding provided the strongest evidence that both the amino- and carboxyl terminus of p53 are required for apoptosis in tumor cells.

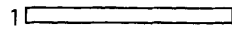
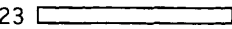
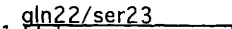
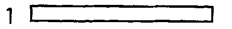
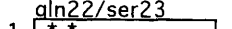

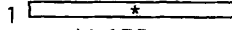
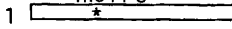
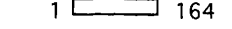
## Discussion

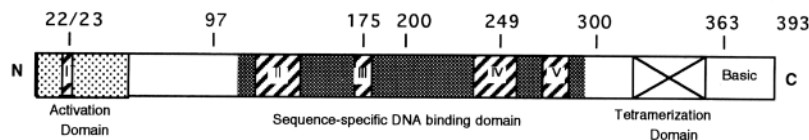
### *Inducible cell lines provide insight into p53 responses in tumor cells*

The cell lines described above have provided several novel observations about the cellular response to p53. We show for the first time that within a given clonal cell line the level of p53 can determine whether cells arrest or die. We also demonstrate that although DNA damage can cooperate with p53 to elicit an apoptotic response, this occurs without detectable alteration in the amount of the p53 protein. Furthermore, our results show that the arrest and apoptotic response are genetically separable activities of p53. Finally, our data suggest that the p53 protein has multiple domains that function in in-



**Table 1.** Cell lines expressing inducible p53 or p21

Protein expressed <sup>a</sup>		Saos-2 <sup>b</sup>	H1299 <sup>b</sup>	Apoptosis <sup>c</sup>	Arrest <sup>c</sup>	Cycling <sup>c</sup>
Wild-type p53 (H)	1  393	1	2	+++	+ <sup>d</sup>	-
Wild-type p53 (L)		1	5	-	+	-
p53 (ΔN22) (H)	23  393	nd	2	+++	+ <sup>d</sup>	-
p53 (ΔN22) (L)		nd	2	-	+	-
p53 (22/23) (H)	1  393	3	2	+	-	-
p53 (22/23) (L)		3	3	-	-	+
p53 (ΔC30) (H)	1  363	2	2	+	+	-
p53 (ΔC30) (L)		3	2	-	+	-
p53 (22/23ΔC30) (H)	1  363	nd	1	-	-	+
p53 (ΔN96) (H)	97  393	4	1	-	-	+
p53 (ser249) (H)	1  393	4	4	-	-	+
p53 (his175) (H)	1  393	4	3	-	-	+
p21 (H)	1  164	3	3	-	+	-



<sup>a</sup>Proteins were detected by Western blotting with p53 specific monoclonal antibodies PAb1801 or PAb421, or p21 specific monoclonal antibody AB-1 (Oncogene Science). Clones were divided where possible into high (H) and low (L) producers when protein levels differed by at least twofold.

<sup>b</sup>Number of individual clones of Saos-2 or H1299 cells expressing inducible p53 or p21.

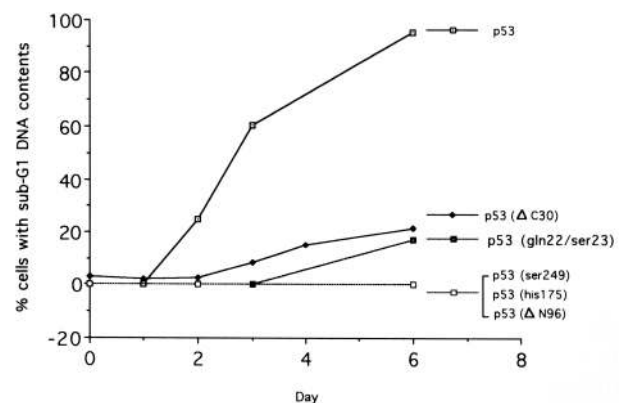
<sup>c</sup>Apoptosis, arrest, or cycling states of cells were determined by growth curves and FACScan analysis.

<sup>d</sup>In cells that underwent massive apoptosis, cell arrest was transient.

ducing cell death and that these domains cooperate to produce a full apoptotic response.

#### The role of p53 as a transcriptional regulator in arrest and apoptosis

The response of Saos2 cells to the induction of p53 was dictated by the quantities of protein produced. When levels of p53 were lower, cells showed slowed or arrested growth, whereas at higher levels of p53, cell death ensued. As a transcriptional regulator, p53 binds to its cognate sites in p53 responsive genes and activates transcription of those genes. Cooperation between two separate p53 binding sites in the p21 promoter (El-Deiry et al. 1995) may allow for its activation by relatively little p53. Indeed, our preliminary results suggest that even with very low levels of p53 in p53-7 cells, p21 shows maximal activation (data not shown), and, thus, very lit-



**Figure 8.** A full apoptotic response requires intact wild-type p53. Summary of the percentage of cells with sub-G<sub>1</sub> DNA contents that express inducible wild-type p53, p53(ΔC30), p53(gln22/ser23), p53(ΔN96), p53(his175), and p53(ser249) at various times following withdrawal of tetracycline.

tle p53 is required to drive expression of p21 and consequently to effect growth arrest.

It is likely that p53 transactivation also contributes to the apoptotic response because the cell death induced in response to the transactivation-defective p53 p53(gln22/ser23) occurs with delayed and reduced kinetics as compared with that seen with wild-type p53. p53( $\Delta$ C30), which lacks the carboxy-terminal 30-amino-acid regulatory domain but has comparable ability to activate transcription, also induces a weak and delayed apoptosis. However, the doubly altered mutant p53(gln22/ser23 $\Delta$ C30) is inert for such activity (Table 1). Therefore, our data imply that although the ability of p53(gln22/ser23) to induce apoptosis is *trans*-activation-independent, that of p53( $\Delta$ C30) is *trans*-activation-dependent, highlighting the fact that p53 acts to induce apoptosis by at least two discrete pathways.

Because p21 induction is not correlated with p53-mediated apoptosis in the cell lines examined, there may be alternate p53 target genes involved in apoptosis that might be bound relatively weakly by p53 and would thus require more p53 protein to ensure sufficient site occupancy for transcriptional activation. Interestingly, the tumor-derived mutant forms p53(ala143) (Friedlander et al. 1996) and p53(pro175) (Ludwig et al. 1996) are defective in inducing apoptosis but can induce transcription from a limited subset of p53 responsive elements. These mutants can activate transcription from promoters with responsive elements from p21, mdm-2, and cyclin G, but not Bax or IGFBP3 genes whose cognate sites, notably, are bound relatively poorly by p53. Although the Bax gene is an obvious candidate for an apoptotic p53 target gene, induction of Bax RNA or protein was not observed in Saos2 cells (data not shown), implying that other p53 responsive genes are activated in these cells. We are currently examining whether other candidate target genes such as IGFBP3 are activated by p53 in these inducible cell lines.

#### *The role of p21 in arrest and apoptosis*

Cell lines expressing p21 underwent arrest but not apoptosis in the absence of p53. The growth curves and FACS profiles of cells expressing p21 were similar to those expressing lower levels of p53 (cf. Figs. 2 and 5). In each case a dramatically reduced growth rate was accompanied by arrest in both G<sub>1</sub> and G<sub>2</sub>. Although we cannot rule out that other targets of p53 may also be involved, these data imply that the arrest response of Saos2 and H1299 cells to moderate levels of p53 is caused primarily by induction of p21. It is also clear that p21 induction in these cells is insufficient to induce apoptosis. This conclusion is derived from the following results: (1) High levels of p21 expressed either with or without p53 did not cause apoptosis in Saos2 cells; (2) the p53( $\Delta$ C30) cell line, which shows a very reduced apoptotic response, is as effective as wild-type p53 in inducing p21 and cell cycle arrest; and (3) the transcriptionally defective p53 mutant p53(gln22/ser23) can not induce p21 (nor can it

effect a cell-cycle arrest) and yet it can induce apoptosis, albeit to a lesser extent.

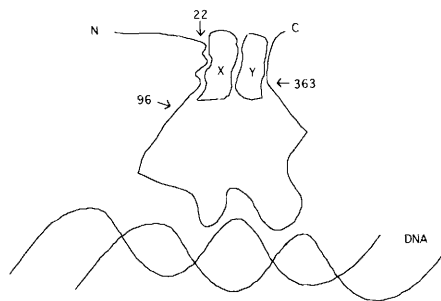
Despite the fact that p21 was induced to comparable levels in p21- and p53-inducible cell lines, the high producer p53-7 cells underwent both arrest and apoptosis. The fact that apoptosis was the eventual fate of virtually all p53-7 cells indicates that the p21-mediated arrest in these cells is not sufficient to protect the cells from the death response.

#### *Speculation on a transcription-independent role for p53 in apoptosis*

Given data from previous studies as well as the data presented here, it is clear that p53 can induce apoptosis in Saos2 and H1299 cells without transcriptional activation. Clearly, however, in some cases transcriptional activation is required (Sabbatini et al. 1995; Attardi et al. 1996). Although there is as yet a lack of full understanding of the reason for the differences in requirements noted, it can be speculated that species, cell type, and immortalization status differences may be involved. The magnitude of the apoptotic response varied dramatically with the p53 mutant that was induced (Fig. 8 and Table 1). Because the extent and kinetics of apoptosis induced by intact wild-type p53 are far greater than those by either of p53( $\Delta$ C30) or p53(gln22/ser23), we propose that transcription-dependent and -independent apoptotic pathways induced by these p53 variants, respectively, cooperate to induce a full apoptotic response. Cells expressing p53( $\Delta$ N96), which lacks the amino-terminal 96 amino acids but has an intact carboxyl terminus, can not induce apoptosis. Taken together with the results of the p53( $\Delta$ C30) mutant we conclude that both amino- and carboxyl termini must be intact to produce a strong p53 apoptotic response. Again, the fact that a mutant p53 with both a mutated amino terminus and a truncated carboxyl terminus [p53(gln22/ser23 $\Delta$ C30)] is absolutely inert for both apoptosis and arrest in H1299 cells underscores this conclusion. Paradoxically, however, tumor-derived mutants that contain intact amino and carboxyl termini are also completely inert for inducing apoptosis. It is well established that the one feature common to the tumor-derived p53 mutations is a defect in sequence-specific p53 DNA binding. Therefore, our results suggest that p53 might need to be bound to cognate sites in DNA but not necessarily activating transcription for it to be in the correct conformation for its role in apoptosis. Alternatively, the identification of the cellular proteins 53BP1 and 53BP2 that can bind to the central core region of wild-type but not mutant p53 (Iwabuchi et al. 1994) provides the possibility that there may be cellular proteins rather than DNA with which this region of p53 must associate.

To explain our results, we propose the following model (Fig. 9): Interactions with a bi- or multicomponent factor would be required to associate with regions both at amino and carboxyl termini of p53, when it is bound to DNA, to cause apoptosis. Whereas the amino-termi-

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**Figure 9.** A model of p53-dependent apoptosis.

nal 22 amino acids of p53 would be dispensable for its interaction with the hypothetical factor at both ends [e.g., p53( $\Delta$ N22)], deletion of the entire amino terminus [e.g., p53( $\Delta$ N96)] would completely abrogate its interaction with the factor at the amino terminus, rendering this truncation of p53 incapable of inducing apoptosis. By contrast, mutations affecting part, but not all, of the amino- and carboxyl termini, such as p53(gln22/ser23) and p53 $\Delta$ C30, would diminish but not completely destabilize interaction with the putative multiprotein complex, resulting in a lesser degree of apoptosis. Finally, amino- and carboxy-terminal double mutations p53 [e.g., p53(gln22/ser23 $\Delta$ C30)] would completely disrupt complex formation so as to preclude an apoptotic response. In this model, massive over-expression of either the amino terminus alone or carboxyl terminus alone would be predicted to induce apoptosis to some extent, a suggestion that is borne out by results showing that over-expressed p53 amino-terminal fragment 1–214 (Haupt et al. 1995) or carboxy-terminal fragment 319–393 (Wang et al. 1996) can induce some degree of cell death.

A number of proteins have been reported to interact with p53 *in vitro* and *in vivo*. Of particular relevance to the function of p53 as a transcriptional regulator is its interaction with the basal transcription factors TFIID (Liu et al. 1993; Martin et al. 1993; Seto et al. 1992; Lu and Lane 1995; Thut et al. 1995) and TFIIF (Xiao et al. 1994; Wang et al. 1995), both of which contain more than one polypeptide component that interacts with p53. In each case these interactions involve both amino- and carboxyl termini of p53 (Xiao et al. 1994; Horikoshi et al. 1995; Lu and Levine 1995; Thut et al. 1995; Wang et al. 1995; Leveillard et al. 1996). Interaction of p53 with TFIID or TFIIF would most likely be facilitated when p53 is bound to DNA. Indeed, previously we reported cooperative interaction between p53 and TFIID when both were bound to DNA (Chen et al. 1993). It is also possible that other cellular factors might be involved because a considerable number of cellular proteins have been reported to interact with p53 (for review, see Ko and Prives 1996). Any of these and other as yet unidentified proteins may be involved in the initiation of the pathway toward apoptosis in response to p53.

#### *p53 expressing cells are more sensitive to apoptosis when their DNA is damaged*

Even though a low level of p53 is not sufficient to induce

apoptosis in Saos2 cells, it can sensitize cells to undergo apoptosis following CPT-induced DNA damage. Because calcium phosphate-mediated DNA transfection of cells can induce p53 and an ensuing growth arrest (Renzing and Lane 1995), this procedure might also cooperate with expressed p53 to amplify the apoptotic response in transient transfection assays. This may explain why our experiments show a much weaker and delayed cell death for the p53(gln22/ser23) cell lines than previously reported when transient transfection assays were employed (Haupt et al. 1995). How these two agents, DNA damage and p53, cooperate is a matter of great interest. Additionally, it will be important to determine whether this cooperation occurs in cells other than Saos2. Although there is considerable evidence that p53 is stabilized after DNA damage, it has been speculated that DNA damage might also convert p53 to a more active DNA binding state (Lu and Lane 1994). This intriguing possibility is currently under investigation. We are excited by the potential of using low p53 producer cells to screen a variety of cancer therapy drugs that may cooperate with p53 to induce apoptosis. Thus, it is hoped that eventually more effective chemotherapeutic drugs can be identified.

## Materials and methods

### *Plasmids*

The pUHD15-1 neo plasmid contains the tTA transactivator gene and the neomycin gene as described in Resnitzky et al. (1994). Genes of interest were cloned into the 10-3 plasmid to allow their conditional expression as described in Gossen and Bujard (1992). The pBabe plasmid containing the puromycin resistance gene was used for secondary selection. To construct tetracycline regulated expression vectors, the following cDNA fragments were cloned into the 10-3 vector: wild-type p53, tumor-derived mutant forms of p53 (his175 and ser249), p53(gln22/ser23) (Lin et al. 1993), p53( $\Delta$ N22), p53( $\Delta$ N96), p53( $\Delta$ C30), p53(gln22/ser23 $\Delta$ C30), or p21 (El-Deiry et al. 1993). To generate p53(gln22/ser23 $\Delta$ C30), the carboxyl terminus of the p53(gln22/ser23) cDNA beginning at amino acid 144 at the *PvuII* site was replaced by the carboxyl terminus of the p53( $\Delta$ C30) cDNA.

### *Cell lines and transfection, and selection procedures*

The Saos2 and H1299 cells were purchased from the American Type Culture Collection. RKO cells were obtained from M.B. Kastan, Johns Hopkins University, Baltimore, MD (Kastan et al. 1992). All cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum in a 37°C incubator with 5% CO<sub>2</sub>. Transfections were performed using the calcium chloride method (Chen and Okayama 1987). To generate cell lines expressing inducible proteins of interest, a two-step procedure was used (Resnitzky et al. 1994). First, low passage Saos2 or H1299 cells were transfected with pUHD15-1 neo and clones were selected and maintained in 400 and 250  $\mu$ g/ml of G418 (Geneticin, Gibco), respectively. To test for cloned cell lines capable of inducing expression from the tetracycline promoter, the 10-3(p53) plasmid was transiently transfected in the presence of tetracycline (1  $\mu$ g/ml) for 10–18 hr and the transfected cells were then split 1:2 and grown in the pres-

ence or absence of tetracycline. Cells were extracted 24–48 hr later, and expression of the p53 protein was determined by Western blot analysis. Two of the 15 clonal Saos2 cell lines (S32 and S2) and two of the 30 clonal H1299 cell lines (H15 and H24) were found to induce p53 expression upon withdrawal of tetracycline. Both S32 and H24 cell lines were used as parental cell lines for subsequent generation of inducible cell lines on the basis of their lower basal (leaky) expression of the tTA transactivation. Second, various 10-3 plasmids containing cDNAs encoding either wild-type or mutant forms of p53 or p21 were cotransfected with the puromycin selectable pBabe plasmid into either S32 or H24 cells. Clones were selected and maintained in the presence of 2  $\mu$ g and 1  $\mu$ g of puromycin (Sigma) per ml, respectively. Individual clones were screened for inducible expression of the p53 and p21 proteins by Western blot analysis using monoclonal antibodies against p53 and p21 as described below.

#### Immunoblot analysis

Cells were collected from plates in PBS, resuspended with 1  $\times$  sample buffer, and boiled for 5 min. For immunoblot analysis, a standard procedure was followed as described previously (Chen et al. 1995). Monoclonal antibodies PAb1801 and PAb421 were used to detect p53. The affinity-purified monoclonal antibodies against p21 (Ab-1) and Bcl-2 (Ab-1) were purchased from Oncogene Science (Uniondale, NY) and affinity-purified monoclonal antibodies against Bax (P-19) and antiactin polyclonal antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and Sigma (St. Louis, MO), respectively.

#### Growth rate and cell-cycle analyses

To determine the rate of cell growth,  $1 \times 10^5$  cells were seeded per 60-mm plate with or without tetracycline. The medium was replaced with fresh medium with or without tetracycline every 48 hr. At indicated times, cells were trypsinized and collected individually from at least two plates. Cells from each plate were counted with a hemacytometer at least twice. The average number of cells from at least two plates were used for growth rate determination.

For cell-cycle analysis,  $2.5 \times 10^5$  cells were seeded per 90-mm plate with or without tetracycline. The medium was replaced every 48 hr as needed with fresh medium with or without tetracycline. At the indicated times, cell were trypsinized and fixed with 2 ml of 70% ethanol for at least 30 min. For FACS analysis, the fixed cells were centrifuged and resuspended in 1 ml of PBS solution containing 50  $\mu$ g/ml each of RNase A (Sigma) and propidium iodide (PI) (Sigma). The stained cells were analyzed in a fluorescence-activated cell sorter (FACSCaliber, Becton Dickinson) within 4 hr. The percentage of cells in various cell cycle phases was determined by using the CellFit program.

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