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Agents that cause DNA double strand breaks lead to p16^{INK4a} enrichment and the premature senescence of normal fibroblasts

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The occurrence of DNA double strand breaks induces cell cycle arrest in mortal and immortal human cells. In normal, mortal fibroblasts this block to proliferation is permanent. It depends on the growth regulator p53 and a protein p53 induces, the cyclin dependent kinase inhibitor, p21. We show here that following DNA damage in mortal fibroblasts, the induction of p21 and p53 is to a large degree shortlived. By 8 days after a brief exposure to DNA strand breaking agents, bleomycin or actinomycin D, p53 protein is at baseline levels, while the p53 transactivation level is only slightly above its baseline. By this time the concentration of p21 protein, which goes up as high as 100-fold shortly after treatment, is down to just 2–4-fold over baseline levels. Following the drop in p21 concentration a large increase in the expression level of the tumor suppressor gene p16^{INK4a} is observed. This scenario, where a transient increase in p21 is followed by a delayed induction of p16^{INK4a}, also happens with the permanent arrest that occurs with cellular senescence. In fact, these cells treated with agents that cause DNA double strand breaks share a number of additional markers with senescent cells. Our findings indicate that these cells are very similar to senescent cells and that they have additional factor(s) beside p21 and p53 that maintain cell cycle arrest.

Keywords: p16^{INK4a}; p21; p53; cellular senescence; DNA damage

Introduction

Mammalian cells have developed a variety of responses to DNA double strand breaks that serve to preserve the overall genomic integrity of a cell population. Gamma irradiation of thymocytes results in apoptosis, while a similar treatment of normal fibroblasts results in a long term, apparently permanent cell cycle arrest (Di Leonardo *et al*., 1994; Li *et al*., 1995; Bates and Vousden, 1996). It is unclear which response to DNA double strand breaks is more representative of most cell types. Both apoptosis and long term cell cycle arrest, have the effect of eliminating cells with genomic lesions from the proliferating cell population. Either strategy may serve as an important safeguard against tumor progression (Di Leonardo *et al*., 1994; Weinberg, 1997). Almost all immortal human cell lines have lost the ability to exhibit an efficient permanent cell cycle arrest as part of their response repertoire to DNA double strand breaks (Li *et al*., 1995; Cox and Lane, 1996).

The tumor suppressor p53 can be induced by agents such as gamma irradiation, bleomycin or actinomycin D that cause DNA double strand breaks (Nelson and Kastan, 1994). This p53 induction contributes to both the G1 arrest and apoptosis that can result (Ko and Prives, 1996). These properties of p53 are thought to play a crucial role in the maintenance of genomic integrity in normal, mortal cells (Hartwell, 1992). Induction of p53 in cells leads to an increase in p21 (El-Deiry *et al*., 1994; Dulic *et al*., 1994). P21 protein in turn binds to cyclin-cyclin dependent kinases at an increased level, resulting in inhibition of these cell cycle regulators and contributing to the block to S phase entry (Xiong *et al*., 1993; Harper *et al*., 1993; Brugarolas *et al*., 1995; Deng *et al*., 1995). In immortal cells which show a transient cell cycle arrest in response to agents that cause DNA double strand breaks, the induction of p53 and p21 is also transient (Cox and Lane, 1996). In contrast, in normal, mortal fibroblasts which show a prolonged arrest in response to DNA double strand breaks, the induction of p53 and p21 is prolonged, lasting for at least 4 days (Di Leonardo *et al*., 1994). It has been proposed that the increase in p21 and p53 is permanent and is responsible for both the initiation and the maintenance of cell cycle arrest (Di Leonardo *et al*., 1994).

Cellular senescence is a genetically programmed irreversible block to proliferation that occurs in a population of cells after a set number of doublings and does not depend on the length of time in culture (Campisi, 1997; Stein and Dulic, 1995; Chiu and Harley, 1997). It is a specific state of differentiation believed to be induced by the gradual telomeric shortening that occurs with cell proliferation. Like DNA damage induced cell cycle arrest, senescence is maximally efficient when p21 and p53 are functional (Stein and Dulic, 1995; Rogan *et al*., 1995; Brown *et al*., 1997; Bond *et al*., 1994). Also, similar to cells with DNA damage, when fibroblasts senesce there are increases in p53 activity and in p21 levels (Bond *et al*., 1996; Atadja *et al*., 1995; Noda *et al*., 1994). However, no prolonged increase in p53 and p21 is observed in senescent cells, though one report suggests a small elevation in p53 levels (Alcorta *et al*., 1996; Afshari *et al*., 1993; Atadja *et al*., 1995; Kulju and Lehman, 1995). In another apparent difference from cells with DNA damage, the CDK4 inhibitor, p16^{INK4a}, is observed to increase substantially after the initial increase of the p21 protein that occurs when senescent cells first start to lose the ability to proliferate (Alcorta *et al*., 1996).

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Expression of p16INK4a is closely associated with cellular senescence in fibroblasts, keratinocytes and uroepithelial cells (Hara *et al*., 1996; Alcorta *et al*., 1996; Loughran *et al*., 1996; Reznikoff *et al*., 1996). There are few other known situations where p16INK4a is induced. P16INK4a induction has not been found to be associated with DNA damage in fibroblasts, although it is rapidly and transiently induced by U.V. irradiation of HeLa cells (Alcorta *et al*., 1996; Wang *et al*., 1996). There is also one example of p16INK4a induction with differentiation, and it is also induced in contact inhibited fibroblasts (Alcorta *et al*., 1996; Lois *et al*., 1995). However, for the most part, early passage cells, whether they are proliferating or not, express very low levels of p16INK4a (Alcorta *et al*., 1996; Zindy *et al*., 1997; Sherr and Roberts, 1995). Curiously, baseline levels of p16INK4a are high in many immortal cell lines, but this is exclusively under conditions where mutations in other proteins (such as the retinoblastoma protein -pRb) or the presence of viral oncoproteins render it ineffective (Sherr and Roberts, 1995; Okamato *et al*., 1994). This has led to a model where p16INK4a transcription is inhibited by active pRb (Li *et al*., 1994; Hara *et al*., 1996; Sherr and Roberts, 1995). Another explanation is simply that p16INK4a is induced after many population doublings (Hara *et al*., 1996).

Our studies were initiated to determine what cell cycle regulatory factors may contribute to the long term maintenance of cell cycle arrest in normal human fibroblasts with DNA double strand breaks. We show that although p21 and p53 may have some role, it is evident that additional factor(s) also contribute. Surprisingly, greater than 96% of the increase in p21 protein level is transient in these cells. By 8 days after drug exposure the p21 levels are less than 2–4-fold above baseline. P53 induction is similarly transient, as is much of the increase in p53 transactivation levels. On the contrary, p16INK4a gene expression increases in these cells sometime between 4 and 30 days after treatment, depending on the fibroblast strain. This scenario of p21 induction first, followed by p16INK4a induction is similar to what is seen in fibroblast senescence (Alcorta *et al*., 1996). These and other findings demonstrate the similarity of these two types of irreversible cell cycle arrest.

Results

Treatment of normal fibroblasts with bleomycin or actinomycin D causes a long term cell cycle arrest

We used cancer chemotherapeutic agents bleomycin and actinomycin D to induce DNA double strand breaks in normal human fibroblasts. Bleomycin is a radiomimetic agent that causes DNA strand breaks directly (Lazo and Chabner, 1996). Like gamma irradiation, at low doses it causes single strand breaks, and at high doses double strand breaks. Actinomycin D works through a different mechanism, it causes DNA single and double strand breaks probably by poisoning the topoisomerase enzymes (Ross and Bradley, 1981). At much higher levels than those used here, actinomycin D efficiently inhibits RNA and DNA synthesis (Zhu *et al*., 1995).

We treated early passage fibroblasts (40–50 population doublings prior to senescence) with these drugs, optimizing the concentrations to cause maximal permanent cell cycle arrest. We monitored cell viability by trypan blue staining and cell counts (data not shown). For bleomycin treated cells no toxicity was detectable nor was there any evidence of apoptosis by the TUNEL assay (Gavrieli *et al*., 1992). Actinomycin D treatment caused <20% cell death. Sparsely plated cells were treated with drug starting at time zero, the drug was left on the cells for 12–24 h depending on the strain and then the drug was washed out. At each timepoint the cells were exposed to tritiated thymidine for 24 h prior to harvesting at 0, 1.5, 4, 8, 12 or 30 days after the drug treatment. Autoradiography was used to determine the number of cells that incorporated radioactive thymidine into nuclear DNA. For the time period assayed, we see that for either treatment (with one exception), less than 5% of the cells traversed S phase during the 24 h labeling period (Table 1). After bleomycin treatment, the block to proliferation to the population lasted at least 30 days. In contrast, by 12 days after actinomcyin D treatment the cells were becoming confluent, indicating some cells had resumed proliferating (data not shown). This is probably because actinomycin D has multiple effects on DNA and cells in addition to causing DNA double strand breaks, and some of these effects are likely reversible (Zhu *et al*., 1995).

For strain NHF-3 we determined the cell cycle distribution of the arrested cells. Cells were treated with the DNA damaging drugs for 12 h and then exposed briefly to the nucleotide analog bromodeoxyuridine (BrdUrd), followed by staining with propidium iodide, and then subjected to bivariate flow cytometric analysis to determine the cell cycle distribution. We found that up till 8 days after treatment, both bleomycin and actinomycin D treated cells had a greater than 95% cell cycle arrest (data not shown) in agreement with the thymidine incorporation data of Table 1. The cells were arrested in both the G1 and G2 phases of the cell cycle. Colony formation assays were done after drug treatment to examine proliferation on an individual cell basis. After bleomycin treatment less than 1% of the cells retained the ability to proliferate versus a control (data not

Table 1 Proportion of cells that traverse S phase after a brief treatment of normal fibroblast strains with bleomycin or actinomycin D

	% ^{3}H-T labeled nuclei						
	0	*1.5*	*4*	*8*	*12*	*30*	*Days*
Bleomycin							
NHF-3	77	4.3	3.6	2.1	2.5	ND	
HDF-2	76	0.7	0.5	1.8	1.5	1.0	
MRC-5	77	12	5.1	3.5	3.5	0	
Actinomycin D							
NHF-3	75	0.2	0.1	1.8			
HDF-2	74	0	0	2.0			
MRC-5	77	0.2	ND	4.5			

At the times indicated after drug treatment, fresh media with 1 uCi/ml [^{3}H]-thymidine is added to the cells for 24 h. The results shown are for at least two independent experiments. There is no data for the 12 days or later timepoints for the actinomycin D treated cells, because the cells were becoming confluent

shown). Actinomycin D was less efficient at maintaining a cell cycle arrest at these late times. About 5%, were capable of forming colonies, indicating that a few cells did not undergo a permanent arrest. We note that at later timepoints these proliferating cells would form an appreciable percentage of the total in a mass population. In sum, these studies show that both treatments cause a cell cycle arrest that can last in the majority of treated cells for at least 3–4 weeks.

The increase in the p53 protein level in normal human fibroblasts treated with bleomycin or actinomycin D is not required for the maintenance of the block to proliferation

The p21 and p53 protein levels in gamma irradiated long term arrested fibroblasts remain high for at least 4 days after treatment (Di Leonardo *et al*., 1994). We asked whether the increases in these two cell cycle regulatory proteins similarly persisted in cells exposed to bleomycin or actinomycin D. Western blots were done for normal human fibroblast strain NHF-3 treated with bleomycin for 12 h (Figure 1a). P53 levels were elevated at 1.5 and 4 days after drug treatment, but they returned to baseline by 8 days after treatment. As others have shown in normal cells treated with DNA damaging agents, p21 induction was quite high, as much as 100-fold by 1.5 days after treatment (Bates *et al*., 1996; Macleod *et al*., 1995; Isaacs *et al*., 1997), but they declined to less than fourfold over baseline by day 8. This experiment has been repeated with strain MRC-5 and with primary antibody to a different p21 epitope with similar results (Figure 1b, data not shown). In bleomycin treated MRC-5, the p21 level was at most twofold above baseline by 8 days after exposure to the drug. Also, note that experiments with a second DNA strand breaking agent, actinomycin D, gave similar results (Figure 1). Because these cells are fully cell cycle arrested at 8 days after bleomycin (and 95% arrested after actinomycin D) treatment, it indicates that prolonged increases in p53 protein levels are not required for maintenance of the block to proliferation. At the same time the increase in the p21 level is minimal.

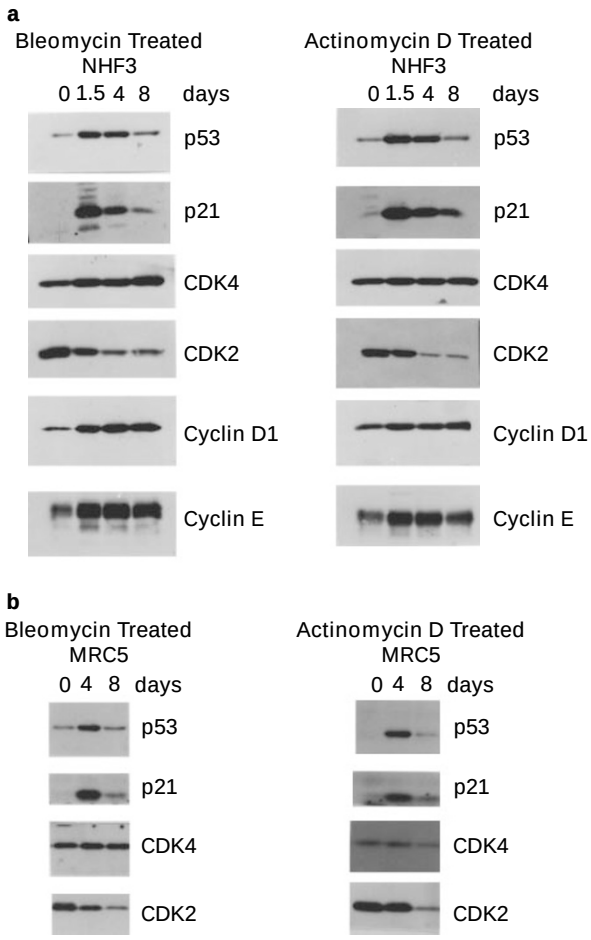

Figure 1 Western analysis of cell cycle regulatory proteins after treatment with bleomycin or actinomycin D. (**a**) Asynchronous NHF-3 were treated with bleomycin or actinomycin D for 12 h as described earlier. At the indicated times cells lysates were prepared and 50 μg lysate was loaded per lane, separated on an SDS polyacrylamide gel, transferred to nitrocellulose and probed with antibodies as labeled (see Materials and methods). Parallel plates were incubated with 1 μCi/ml [^{3}H]thymidine for 24 h prior to harvesting and processed for autoradiography to determine the percentage of cells in S phase during the labeling period (see Table 1). (**b**) Synchronous MRC-5 were similarly treated with drugs that damage DNA and levels of p21, p53, CDK2 and a control, CDK4, were determined

A peak in p53 transactivation activity in normal fibroblasts treated with bleomycin occurs by 4 days after exposure

P53 transactivation levels do not always correspond to the levels of protein (Weinberg *et al*., 1995). We tested mRNA expression levels of the p53 responsive genes mdm-2 and GADD45 in NHF-3 cells treated with this drug (Zauberman *et al*., 1995; Kastan *et al*., 1992). This is just a rough measure of p53 activity as these genes may be regulated by additional mechanisms. We used an RNAse protection assay to quantitate the levels of the p53 induced mRNAs and the controls, $p27^{KIP1}$ and cyclophilin, in cells after bleomycin treatment. As expected mRNA expression levels of the p53 induced genes were found to peak 1.5–4 days after bleomycin treatment and were declining by day 8 (Figure 2). GADD45 and mdm-2 mRNA levels both showed very high induction (50- and 130-fold) at 1.5–4 days, but by day 8 were down to only six- and 16-fold above background, respectively. The data shown was for NHF-3 cells but the experiments have been repeated with HDF-2 and produced similar results. Actinomycin treatment of NHF-3 cells also showed similar effects (data not shown). These data are consistent with a scenario where a short treatment with either agent that causes DNA double strand breaks results in a peak in p53 transactivation activity within 4 days after treatment. These increases are greatly reduced in magnitude by the eighth day after treatment.

To more directly measure p53 transactivation levels after treatment with an agent that causes DNA double strand breaks, we created stable cloned NHF-3 fibroblasts that contain a luciferase reporter gene fused to a simple p53 responsive promoter. This construct contains a p53 binding site from the p21 promoter repeated four times (El-Deiry *et al*., 1993). Control clones have a promoter construct that contains the same repeat with four base changes so it no longer

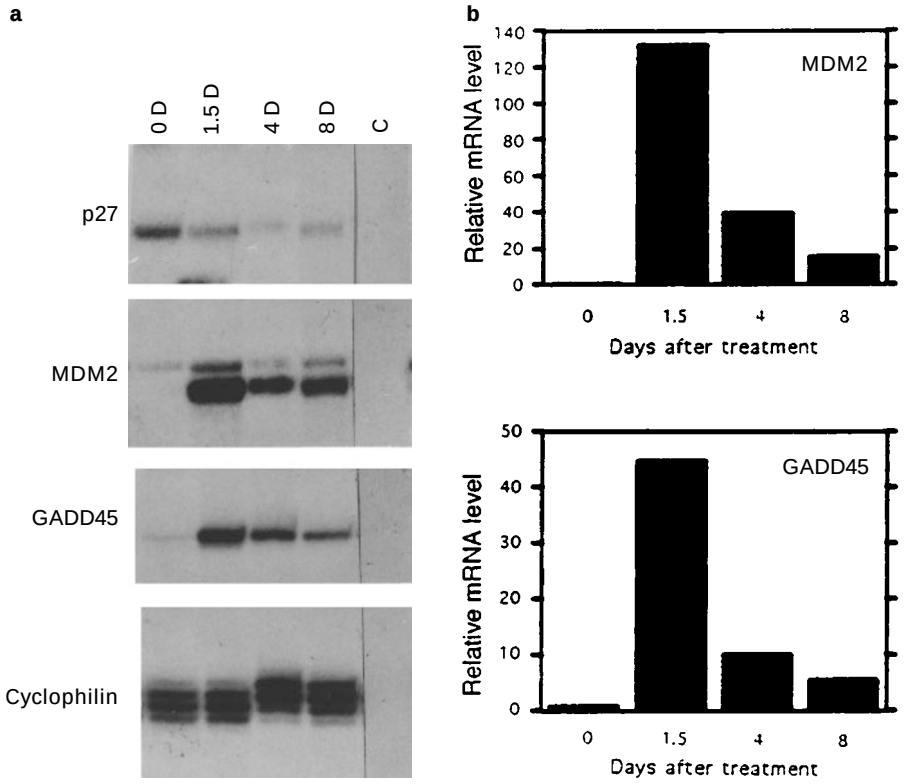

Figure 2 The induction of p53 responsive genes, GADD45 and mdm-2, peaks at 1.5 days after bleomycin treatment. Normal fibroblast strain NHF-3 cells were treated with bleomycin as described and then at the times indicated total RNA was isolated. Shown is an autoradiograph of the gel from the RNAse protection analysis performed to quantitate levels of mdm-2 and GADD45 mRNA. P27 and cyclophilin mRNA levels, which change minimally, were also measured as internal controls. Probes are described in the Materials and methods section. The control reaction, C, contained 25 μg yeast RNA. (**b**) The relative level of the GADD45 and total MDM-2 mRNA is shown after normalization to cyclophilin and p27 mRNA levels. Quantitation was done by scanning multiple exposures of autoradiograms

binds p53 (data not shown). We chose the luciferase gene as a transcriptional reporter as the luciferase protein and RNA have a turnover rate in mammalian cells which facilitates the measurement of rapid changes in promoter activity (Thompson *et al*., 1991). Clones with either the wildtype p53 promoter element or the mutated version, were treated with bleomycin, then at 1.5, 4 and 8 days after treatment, extracts were prepared and tested for luciferase activity. In Figure 3 is a histogram that shows the average induction of activity of the p53 responsive promoter after normalization against the control promoter. With this assay we found p53 transactivation activity peaked 1.5–4 days after drug treatment, showing a threefold induction. By 8 days after treatment the level of p53 activation of the model promoter in the fibroblasts was just above baseline. This experiment indicates that p53 transactivation in these cells was lost by 8 days after the initial exposure to the bleomycin. Cells treated with actinomycin D gave a similar result as shown in Figure 3. We note that this assay system was relatively insensitive and would miss subtle differences in p53 transactivation levels.

Why this simple p53 responsive promoter shows lower maximal induction due to bleomycin treatment than GADD45 and mdm-2 gene expression can be due to many reasons such as the complexity of the promoters of the endogenous genes and post transcriptional regulation. We do note that a simple p53 responsive promoter in a human epithelial cell line shows a similar maximal threefold induction upon high level gamma irradiation (Zhan *et al*., 1993).

The decrease in p53 and p21 levels is followed by a delayed induction of p16^{INK4a} in cells treated with bleomycin

About 96% of the increase in p21 is transient in cells with DNA damage. The levels of p21 that remain are similar to or less than what is seen in cells stimulated to proliferate by growth factor addition (Li *et al*., 1994; Macleod *et al*., 1995, data not shown). For these reasons we reckoned something else may contribute to the maintenance of the cell cycle arrest in these cells. To investigate alternative mechanisms of cell cycle arrest we examined the levels of other cyclin dependent kinase (CDK) inhibitory proteins known to be expressed in fibroblasts. P16^{INK4a} and p27 are CDK inhibitors that contribute to cell cycle arrest in fibroblasts (Sherr and Roberts, 1995; Alcorta *et al*., 1996; Polyak *et al*., 1994). We saw no net change in p27 protein levels at 8 days after bleomycin or actinomycin D treatment in three different cell strains (data not shown).

An increase in the CDK inhibitor p16^{INK4a}, protein has been shown to be involved in the cell cycle arrest seen in senescent fibroblasts (Alcorta *et al*., 1996). Although p16^{INK4a} is not elevated in serum starved cells it is increased reversibly in contact inhibited cells and

has been shown to increase in S phase cells where it is believed to be nonfunctional (Alcorta *et al*., 1996; Sherr and Roberts, 1995). We tested p16^{INK4a} expression in normal human fibroblasts at various times after treatment with bleomycin. We found that p16^{INK4a} is indeed induced by treatment with this drug. However, the time course of induction was very slow. In Figure 4a is the data for one strain, HDF-2. In untreated early passage proliferating cells p16^{INKa} mRNA is almost undetectable. The RNA that is detected is from the overlapping mRNA that encodes the unrelated protein p19^{ARF} (Mao *et al*., 1995; Quelle *et al*., 1995). This is corroborated by a second assay using another probe that hybridizes across exon 1 of the p19^{ARF} message (data not shown). By 12 days after treatment we start to see an increase in p16^{INK4a} mRNA, which by day 30 is 17-fold elevated in this strain (Figure 4b). We saw a similar delayed induction of p16^{INK4a} mRNA after bleomycin treatment of strains MRC-5 and NHF-3 (data not shown). Because p16^{INK4a} can be induced in contact inhibited cells, all cells were at a low density (Alcorta *et al*., 1996).

The increase in p16^{INK4a} expression also occurred on the protein level (Figure 4c). For the three normal fibroblast cell strains, the cells are observed to show an approximate 8–30-fold increase in p16^{INK4a} protein levels 30 days after bleomycin treatment. Other workers have reported that there is no increase in p16^{INK4a} levels in gamma irradiated MRC-5 normal lung fibroblasts, but we speculate these cells were harvested prior to the increase (Alcorta *et al*., 1996). We see no increase in p16^{INK4a} in cells continually passaged for this time period.

We also showed that pRb remained hypophosphorylated in cells with DNA damage. It is known that DNA damage in cells with intact versions of the p53 and p21 genes can lead to a transient accumulation of hypophosphorylated retinoblastoma protein (pRb) (Canman *et al*., 1994a). By 8 days after the initial DNA damage occurs the cyclin D1 level is several fold elevated, cyclin E and CDK4 levels are the same or higher than those in proliferating cells, and p21 levels are minimally elevated (Figure 1). If the increase in p16^{INK4a} works to maintain inhibition of CDK4 and CDK6 in cells with DNA double strand breaks then we would expect pRb to remain hypophosphorylated. Four, 12 and as long as 30 days after bleomycin treatment of normal fibroblasts, the retinoblastoma

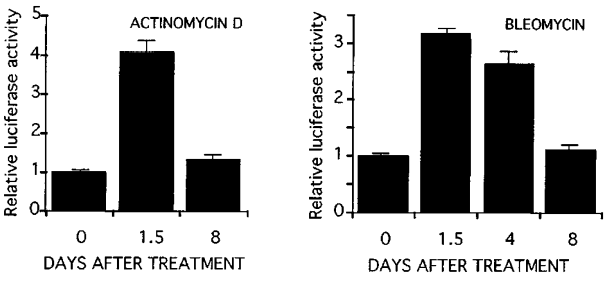

Figure 3 Measurement of p53 transactivation levels in actinomycin D and bleomycin treated human diploid fibroblasts. The p53 responsive reporter plasmid (pGL53) and the control reporter (pGLcon) are described in the Materials and methods section. Shown is the average level of induction of transcription in seven separate stable cell clones containing pGL53 (with the p53 consensus binding element fused to a minimal promoter and the luciferase reporter gene) treated with actinomycin D or bleomycin. The induction level is normalized *versus* the average expression in five similarly treated cell clones containing a control promoter and reporter. Cells were harvested at the times shown after the initiation of a 12 h treatment with bleomycin or actinomycin D. The luciferase activity of each lysate was determined and then normalized to the protein concentration. Results shown are the average of 2–3 experiments done in duplicate

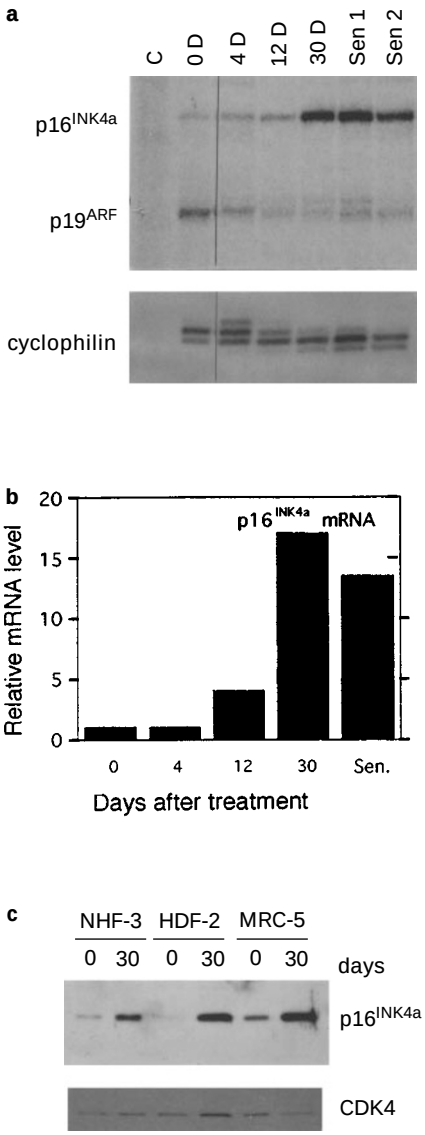

Figure 4 (**a**) An increase in 16^{INK4a} mRNA in normal human fibroblasts after treatment with bleomycin at a level that causes a block to proliferation. Total cellular RNA was harvested from HDF-2 skin fibroblasts at the timepoints indicated after the initiation of a short exposure to bleomycin as described earlier. Equal amounts of total RNA were analysed using an RNAse protection assay with a probe that is antisense to exon 1 and 2 of the mRNA for p16^{INK4a} and exon 2 of p19^{ARF}. Sen 1 and 2 refer to preparations of RNA from senescent HDF-2 at population doubling 69 and 70. (**b**) P16^{INK4a} mRNA levels in strain HDF-2 after treatment with bleomycin. Quantitation is normalized to the level of cyclophilin mRNA. (**c**) Analysis of p16^{INK4a} protein in normal human fibroblasts prior to and 30 days after, treatment with bleomycin. Cell lysates are harvested from NHF-3, HDF-2 and MRC-5 30 days after treatment with bleomycin. 100 μg per lane is separated on 15% SDS polyacrylamide gel, transferred to a polyvinyl difluoride membrane and probed with polyclonal p16^{INK4a} antibody or CDK4 antibody as described in Materials and methods

protein was hypophosphorylated as judged by gel migration (Figure 5). This was similar to what was seen in early passage cells deprived of growth factors and in senescent cells in growth media (Figure 5, lanes marked 'starved' and 'sen'). This is consistent with $p16^{INK4a}$ acting as a CDK4 inhibitor in these cells. Note early passage HDF-2 are at population doubling <28 for these experiments, while senescent cells are at population doubling 69 or 70.

While p21 induction due to DNA double strand breaks was relatively rapid, the increase in the level of $p16^{INK4a}$ was delayed, occurring when p21 was nearly back to the starting level. This indicates that while p21 has a role in initiation of the block to proliferation, $p16^{INK4a}$ may be involved in maintenance of arrest. We also saw that treatment of these cells with actinomycin D led to increases of $p16^{INK4a}$ (data not shown). However, we could not extend these experiments past 8 days due to the fact that actinomycin D does not cause a 100% arrest in these cells and at late times, cells that never arrested, or that temporarily arrested, become a major portion of the cell population.

Specific markers of cellular senescence are expressed in normal human fibroblasts treated with agents that cause DNA double strand breaks

Permanently cell cycle arrested cells treated with DNA damaging agents, such as bleomycin, gamma radiation or ultraviolet radiation, morphologically resemble senescent fibroblasts – they are enlarged and stellate (Di Leonardo *et al*., 1994; Stein and Dulic, 1995; data not shown). Cellular senescence was originally reported to be linked to increases in p21 mRNA (Noda *et al*., 1994). It has since been shown that the increase in the p21 protein level in these cells is shortlived. An increase in $p16^{INK4a}$ expression occurs after the p21 level increases and it is tightly associated with cellular senescence (Alcorta *et al*., 1996). This scenario of p21 induction followed by $p16^{INK4a}$ induction is very similar to what is seen here with cells treated with agents that cause DNA damage. For that reason, we tested for other similarities between senescent normal human fibroblasts and fibroblasts treated with agents that cause DNA double strand breaks.

Collagenase gene expression has been observed to be elevated in senescent cells (Millis *et al*., 1992). We used an RNAse protection assay to quantitate interstitial collagenase mRNA levels in fibroblast strain HDF-2 treated with bleomycin. Controls included reversibly arrested cells that were growth factor deprived or 'starved'. By 12 days after drug treatment collagenase mRNA levels had doubled and by day 30 were up many fold, similar to what was observed in three independent samples of senescent cell RNA (Figure 6). When early passage fibroblasts were growth factor deprived (starved) for 8 days no change in the collagenase mRNA level was observed. We could not starve cells much longer as that would be toxic.

Another biomarker for cellular senescence is the pH 6 β-galactosidase (SA-β-Gal) (Dimri *et al*., 1995). It has been shown to be enriched in senescent cells but not in quiescent cells. We used an *in situ* assay to measure SA-β-Gal activity in the three strains of fibroblasts cell cycle arrested through a variety of mechanisms. Cells were treated with bleomycin or the related compound zeocin, growth factor deprived or continuously treated with mycophenolic acid (Kane *et al*., 1995). Mycophenolic acid is an agent that blocks nucleotide biosynthesis and arrests cells in G1 (Linke *et al*., 1996). When available, senescent cells were also included. Assayed 3 days after the various treatments, none of the early passage cells were positive for SA-β-Gal activity. In contrast, 10 days after drug treatment SA-β-Gal activity was upregulated in cells treated with bleomycin or the related compound zeocin and in the control senescent cells. This was the case for the three different strains of fibroblasts (Figure 7). Two additional drugs that cause DNA double strand breaks, etoposide and camptothecin (Canman *et al*., 1994b), were similarly observed to induce SA-β-Gal activity (data not shown). These data with specific marker genes corroborate earlier findings that ultra-

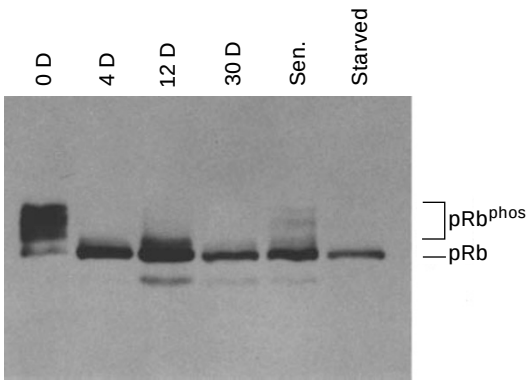

Figure 5 Retinoblastoma protein (pRb) phosphorylation levels remain low in long term arrested fibroblasts. Shown is a Western blot of pRb extracted from HDF-2 before, and at the indicated times after a 24 h treatment with bleomycin. Note that $4\times$ less total protein of the asynchronous cell extract is loaded. As controls, extracts were tested from early passage cells that were serum starved for 6 days (starved) or from late passage (population doubling 70) senescent fibroblasts (sen.)

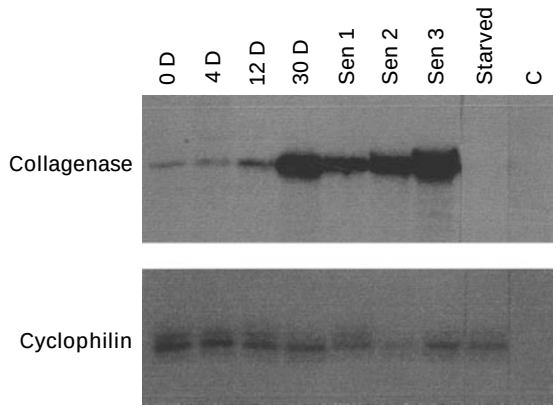

Figure 6 Expression of interstitial collagenase in HDF-2 after treatment with bleomycin or in senesence. Proliferating early passage HDF-2 were treated with bleomycin as described ealier. At the times indicated RNA was harvested from the cells and the levels of the collagenase mRNA in 1.5 μg total RNA were quantitated using the RNAse protection assay as described in the Materials and methods section. Controls included Sen 1, 2 and 3 RNA from senescent HDF-2 that have completed greater than 69 population doublings or 'starved' early passage cells serum deprived for 9 days. A control mRNA was cyclophilin which does not vary with senescence or with DNA damage

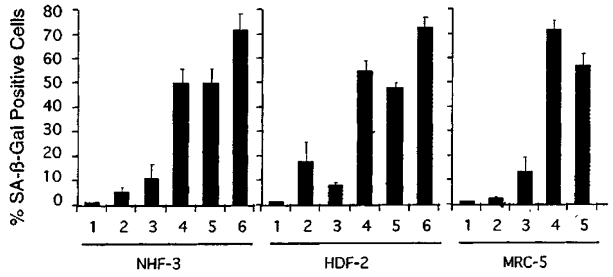

Figure 7 A senescence specific marker (SA-β-Gal) is present in cells treated with agents that cause DNA double strand breaks. Normal fibroblast strains tested are MRC-5, HDF-2 and NHF-3. Early passage proliferating cells were either untreated (lane 1), or blocked from proliferating by: serum starvation (lane 2), continual incubation in 10 μM mycophenolic acid (lane 3), a 12 h treatment with zeocin (lane 4), or a 12–24 h treatment with bleomycin (lane 5). As positive controls senescent NHF-3 and HDF-2 were tested (lane 6). Ten days after treatment with the various agents that block proliferation, cells were processed for the detection of SA-β-gal activity. Cells were verified to be cell cycle arrested by the measurement of tritiated thymidine uptake (data not shown)

violet irradiation produces post mitotic fibroblasts with similar 2-D gel protein patterns to what is seen in senescent fibroblasts (Stein and Dulic, 1995). Our findings show three separate senescence markers are induced by treatment with the DNA damaging agent: the enrichment of $p16^{INK4a}$, collagenase mRNA and SA-β-gal, indicating that the long term cell cycle arrest that is induced by agents that cause DNA double strand breaks phenotypically resembles cellular senescence. Other similarities to the senescent state include the increase in the cyclin D1 level, the decrease in CDK2, and the minimal net changes in the p21 and cyclin E levels (Stein and Dulic, 1995).

Discussion

Normal cells can respond to mutagenic DNA damage such as DNA double strand breaks by containing the damage. One such response is to undergo a permanent cell cycle arrest (Di Leonardo *et al*., 1994; Li *et al*., 1995; Linke *et al*., 1997). It has been proposed that sustained increases in p53 and p21 protein levels are responsible for this cell cycle arrest. We show that much of the increase in the p53 transactivation level in these cells is transient, as is the increase in p21. We find that a delayed increase in the $p16^{INK4a}$ level correlates with the maintenance of a long term cell cycle arrest in these cells.

Many immortal human cell lines with wildtype p53 and p21 undergo a transient cell cycle arrest in response to agents that cause DNA double strand breaks (Canman *et al*., 1994a; El-Deiry *et al*., 1994; Cox and Lane, 1996). In these immortal cell lines, the induction of p53 and therefore p21 is also transient (Cox and Lane, 1996). We show here that in normal human fibroblast strains increases in p53 and p21 levels do last several days, but they are to a large degree not permanent. By 8 days after treatment the levels of these factors are at or near baseline depending on the cell strain. Out of the three strains we tested for an increase in p53 and p21, all three showed no net increase in p53 protein levels by day 8, and at most a 2–4-fold increase in p21 (down from 100-fold) (Figure 1, data not shown). When we tested p53 transactivation levels in one of these strains, they were at or just above baseline 8 days after treatment with bleomycin or actinomycin D (Figure 3). Because the levels of GADD45 and mdm2 (two p53 induced genes) are still somewhat elevated in these cells, it is likely that some residual p53 transactivation activity persists (Figure 2). However, it is much reduced from the level required to initiate a permanent cell cycle arrest across a population of cells (see Figure 4 and data not shown). We conclude that permanent arrest as a response to agents that cause DNA double strand breaks does not correlate well with prolonged increases in p53 or p21. This unexpected finding is similar to what occurs in senescent cells.

Agents that cause DNA double strand breaks cause an irreversible cell cycle arrest in either G1 and G2, just like senescence, though the proportions may not be exactly the same (Di Leonardo *et al*., 1994; Sherwood *et al*., 1988). The cells are also morphologically similar to senescent cells. We have shown that in fibroblasts treated with agents that cause DNA double strand breaks, p21 levels first increase, and then come down while the cells remain permanently arrested, the same scenario that occurs with cellular senescence (Alcorta *et al*., 1996). In addition, there is no net change in the p53 protein level in both states. Paradoxically, the cell cycle stimulatory proteins cyclin D1 and E are enriched in both types of cell arrest (Stein and Dulic, 1995). Also, in both situations the $p16^{INK4a}$ protein is seen to increase after the decrease in p21 occurs. And atypically, in both cases the $p16^{INK4a}$ elevation occurs in the presence of active hypophosphorylated pRB (Sherr and Roberts, 1995). Finally, we have shown that the cells treated with agents that cause DNA double strand breaks also express additional markers of cellular senescence in a delayed fashion, the collagenase gene and the SA-β-galactosidase (Hara *et al*., 1996; Dimri *et al*., 1995; Millis *et al*., 1992). By all these criteria we were not able to distinguish senescent cells from early passage cells that had been treated weeks earlier with agents that cause DNA double strand breaks.

Cellular senescence *in vitro* correlates with telomeric shortening (Harley *et al*., 1990; Chiu and Harley, 1997; Stein and Dulic, 1995; Campisi, 1997). Most somatic cells, but not germ cells or immortal cells, show a gradual loss of telomere length with proliferation resulting in senescent cells having short telomeres (Hastie *et al*., 1990; Harley *et al*., 1990; Chiu and Harley, 1997; Campisi, 1997). It has also been shown that a mechanism for telomere restoration becomes active in immortal cells (Chiu and Harley, 1997; Rogan *et al*., 1995). Finally, artificial telomere lengthening increases the doubling potential of cell hybrids (Wright *et al*., 1996). The indication is that telomere shortening is a counting mechanism that leads to replicative senescence in human cells.

The three models for how shortening of telomeres is converted into a signal to induce the senescent state have recently been reviewed (Campisi, 1997). The first model is the transcriptional silencing model. It is proposed that long telomeres sequester transcription factors required for expression of senescence inducing genes. It is based on experiments with the RAP1 gene

in yeast (Marcand *et al*., 1995; Smeal *et al*., 1996), but there is no evidence for this phenomenon in mammalian cells. The second model, also based on experiments in yeast, indicates that genes required for senescence are early on shut off due to their proximity to telomeres (Wright and Shay, 1992; Shore, 1995). When telomeres shorten this heterochromatin loosens up and the genes are expressed. Again, there is little evidence for this model in mammalian cells. The third model depends on shortened telomeres being recognized as irreparable DNA damage. In support of this idea, shortened telomeres observed in senescent cells are unstable and recombinogenic just like DNA strand breaks (Harley *et al*., 1990 and refs. within). Also, in yeast there is much indirect evidence to support the idea that a RAD9 DNA damage response is induced when telomeres are allowed to shorten artificially (McEachern and Blackburn, 1996 and refs. within). While in yeast this leads to cell death, in mammalian cells it is proposed to cause cellular senescence. Two problems with this model are that the DNA damage response protein, p53, is not enriched in senescent cells, and though cells with DNA damage look senescent, they are thought to express different genes then senescent cells (Afshari *et al*., 1993; Atadaj *et al*., 1995; Campisi, 1997).

We focused on the delayed effects of DNA double strand breaks and were able to show the similarities of the DNA damage induced cell cycle arrest and cellular senescence. This new evidence presented here, suggests, but by no means proves, that these two states may be manifestations of the same effect: DNA damage. It also supports the premise that diseases such as Werner Syndrome, which cause increased levels of DNA recombination, may elicit premature senescence of cells *in vitro* directly by causing DNA double strand breaks within the genome (Yu *et al*., 1996; Lombard and Guarante, 1996; Friedberg *et al*., 1995). In addition, hyperoxia which induces macromolecular damage may also accelerate senescence rates by inducing DNA damage in some cells and causing a premature senescence. We speculate that the resulting loss of some proliferating cells would cause the total population to senesce after a reduced number of population doublings (indicated by an accelerated telomere loss) (Von Zglinicki *et al*., 1995). Our data support the idea that irreparable DNA strand breaks anywhere in a chromosome can rapidly induce premature senescence on an individual cell basis.

Other agents have been shown to cause irreversible growth arrest in normal fibroblasts and to induce at least some similarities to the senescent state, such as treatment with histone deacetylating agents and the expression of oncogenic *ras* (Ogryzko *et al*., 1996; Serrano *et al*., 1997; Chen *et al*., 1995). In addition, work with an immortal epithelial cell line has shown that transient p53 expression itself is sufficient in that background to induce a senescent like state (Sugrue *et al*., 1997). One might speculate that the pathways to a premature senescent state initiated by oncogenic *ras*, histone deacetylase inhibitors treatment or DNA damage, may also share overlapping elements. Although it is possible that these types of premature senescence are induced by very different pathways.

Many days after DNA double strand breaks occur, the p53 level is at baseline. P21 is slightly enriched but is at levels low enough to be conducive to S phase entry (Li *et al*., 1994; Macleod *et al*., 1995, data not shown). This study supports the premise that a short term induction of p53 and p21 may be responsible for the initiation of cell cycle arrest in response to DNA damage, but that other factors are responsible for the maintenance of arrest (Sugrue *et al*., 1997). Our data suggest one candidate for maintenance of cell cycle arrest is $p16^{INK4a}$. There are high levels of cyclin D1 and CDK4 in cells with DNA damage, yet pRb is in the hypophosphorylated active form. Also, $p16^{INK4a}$ is absent or nonfunctional in immortal human cell lines, and these immortal cells seldom if ever respond to DNA damage by undergoing a permanent cell cycle arrest (Sherr and Roberts, 1995; Okamato *et al*., 1994; Li *et al*., 1995; Cox and Lane, 1996). These observations are consistent with $p16^{INK4a}$ contributing to the maintenance of cell cycle arrest in normal fibroblasts treated with DNA strand breaking agents. It should be noted that because of the timing of the $p16^{INK4a}$ induction (much of the increase occurs long after the peak of p21 expression), it is likely that other possibly redundant factors are involved in maintaining the cell cycle arrest (Weinberg, 1997; Terada *et al*., 1995; Sanchez *et al*., 1997; Peng *et al*., 1997). Regulation of CDK4 (and CDK2, CDK6 and CDK1) dephosphorylation by factors such as cdc25a-c may contribute to the G1 (and G2) cell cycle arrest (Weinberg, 1997; Terada *et al*., 1995; Sanchez *et al*., 1997; Peng *et al*., 1997). In addition, increases in concentrations of additional cell cycle inhibitory proteins may also contribute, though we have not detected prolonged increases in p27 protein and $p19^{ARF}$ mRNA levels are decreased. Finally, the reduction of CDK2 may cause arrest, though similar low levels of CDK2 do not prevent the cell cycle re-entry that occurs in serum starved cells after refeeding (Dulic *et al*., 1993).

Materials and methods

Cell culture and drug treatment

HDF-2 and NHF-3 are human foreskin fibroblasts, while MRC-5 are lung fibroblasts obtained from the ATCC. Early passage cells were used between population doubling 18 and 30 for HDF-2 and NHF-3 and 30 and 35 for MRC-5. Senescent cells (at population doubling greater than 69 for both HDF-2 and NHF-3) showed less than a 5% labeling index after a 24 h incubation with 1 μCi/ml ^{3}H-thymidine. Cells were grown in Dulbecco's modified Eagle medium supplemented with 10% FBS. Unless otherwise noted, cells were plated at least 40 h prior to treatment to insure that they would be in log growth. Serum starved cells were plated in growth media and then 6–8 h later the cells were washed with PBS three times followed by the addition of new media with 0.1% fetal bovine serum.

For drug treatments, cells were plated at approximately 2×10^3 cells/cm^2, 48 h before the addition of 0.04 μg/ml actinomycin D (Sigma), 0.06 units/ml bleomycin sulfate (Mead Johnson Oncology Products) or 0.8 μg/ml Zeocin (Invitrogen). The cells were incubated with the drug for 12 h, then the plates were washed three times with PBS and fed. Treatment of HDF-2 with bleomycin was for 24 h. If necessary all cells were replated 3–4 days prior to harvesting to insure that they would be less than 50% confluent when harvested. For timepoints longer than 4 days, drug treated cells were routinely fed weekly and fed or split 2–4 days prior to harvesting. Mycophenolic acid was used at 20 μM

and was replenished at least every 6 days. This causes greater than 95% cell cycle arrest as measured by [^{3}H]thymidine incorporation over a 24 h period (Linke *et al*., 1996, data not shown).

Cell cycle analysis

FACS analysis was performed as described earlier, except cells were labeled with BrdUrd for 4 h prior to harvesting (Walker *et al*., 1995).

Colony formation assay

Cells were treated for 12 h with either DNA damaging drug, the drug was removed, the cells were washed with PBS and fed. Four days after drug treatment approximately 100% of the adherent cells were viable by trypan blue staining. The cells were harvested and approximately 100 viable cells were replated on 10 cm plates. Cells were fed weekly for 3 weeks and then stained with Cresyl Violet to determine the number of colonies.

Senescence-associated β-galactosidase

Cells were plated and then treated with drugs or serum starved as described above. Eight days later fresh media (with 0.1% serum or with mycophenolic acid, where applicable, was added), 2 days prior to detection of SA-β-gal activity. SA-β-gal activity was measured as described (Dimri *et al*., 1995).

Western blot analysis

Cell lysates were prepared and characterized essentially as described (Di Leonardo *et al*., 1994; Walker *et al*., 1995). Protein concentrations were determined by a modified method of Bradford (Bio-Rad). We used 12% SDS–PAGE for the p53, p21, CDK2, and cyclin E immunoblots and 15% SDS–PAGE for p16^{INK4a} and p21. Membranes from both percentage gels were immunoblotted with anti-CDK4, and anti-cyclin D1 as controls. The protein was electrotransferred to nitrocellulose (Schleicher & Schuell) or for p16^{INK4a}, to an Immobilon P nylon membrane (Millipore). Nitrocellulose membranes were stained with Ponceau S to verify transfer and equal loading. Detection was performed using an enhanced chemiluminescence detection system (ECL, Amersham) according to manufacturer's recommendations. Rabbit polyclonal anti-CDK4 was purchased from Upstate Biotechnology Incorporated and anti-CDK2(M2) from Santa Cruz Biotechnology. The mouse monoclonals anti-p53 Ab-2 (PAb 1801), anti-53 Ab-3 (PAb 240), anti-p53 Ab-6 (DO-1), anti-p21 Ab-1 and Ab-6, anti-cyclin D1 Ab-3 and anti-cyclin E Ab-1 were purchased from Oncogene Research Products while anti-pRb (G3-245) was from Pharmingen. Only the westerns with DO-1 are shown for p53 and Ab-1 for p21.

p53 Trans-activation assays

Plasmids pGL53 and pGLcon were constructed from the pGL3-promoter vector (Promega). In brief, a neomycin resistance gene was inserted into the *Bam*HI site and the SV40 promoter was replaced by a minimal promoter from the polyomavirus genome as used in previous p53 transactivation studies (Zhan *et al*., 1993). Four repeats of the sequence 5′-GAACATGTCCCAACATGTTG-3′ matching the p53 responsive element in the p21 promoter were inserted in an engineered *Hind*III site upstream of the polyomavirus promoter for pGL53 and four repeats of the mutated p53 response element 5′-GACGAGGTCC-CAACCTCGTG-3′ were similarly inserted in pGLcon. First, we verified that the reporter construct was inducible by p53, by using cotransfections with a p53 expression plasmid (data not shown). Next, calcium phosphate transfections with this plasmid were used to obtain stable clones which were selected using G418 selection. For these transfections, NHF-3 with few population doublings (less than 15) were used. Seven pGL53 clones that showed the highest actinomycin D induction of activity and five control pGLcon clones were selected for further studies. These clones are mortal cell strains at approximately population doubling 37–42 for these experiments. The level of induction shown is the average for the seven PGL53 clones *versus* the induction in the control clones. The luciferase activity of stable clones was normalized to total protein in the cell lysates. Luciferase activity was determined using the Luciferase Assay System from Promega and scintillation counting in the single-photon-counting mode.

RNAse protection analysis

Total RNA was isolated using guanidium isothiocyanate extraction. The production of stable RNA was scored using internally ^{32}P-labeled antisense RNA probes. Hybridizations to cellular RNA, followed by T2 digestion and separation of protected products on denaturing PAGE were as described earlier (Walker *et al*., 1995). Templates for the probes contain sequences from the cDNAs: GADD45, nt 298–583 from pHu145B2 (Papathanasiou *et al*., 1991); MDM2 contains the last 14 bases of exon 1 and the first 273 bases of exon 2 (Zauberman *et al*., 1995); CDK4, nt. 192–643 from clone pcD-PSKJ3 (Hanks, 1987); p27, nt. 1–339 from the p27KIP1 cDNA (Polyak *et al*., 1994); p21, nt. 1–322 from the p21 cDNA (Harper *et al*., 1993); Collagenase I, nt. 284–605 (Millis *et al*., 1992); Cyclophilin is from Ambion and gives a protected fragment of 103 bases. The probe for p16^{INK4a} and p19^{ARF} contains the last 39 bases of exon one of p16^{INK4A} and the first 236 bases of the shared exon two and is from a full length p16^{INK4A} clone. In the RNAse protection assay it produces a protected fragment of 236 bases for p19^{ARF} mRNA and one of 275 bases for p16^{INK4A} mRNA (Mao *et al*., 1995; Merlo *et al*., 1995; Quelle *et al*., 1995; Serrano *et al*., 1993). All assays were done simultaneously with the cyclophilin probe (Oncogene) as an internal control, because the cellular level of cyclophilin mRNA was not effected by any of the treatments used.

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References

Afshari CA, Vojta PJ, Annab LA, Futreal PA, Willard TB and Barrett JC. (1993). *Exp. Cell Res.,* **209,** 231–237.

Alcorta DA, Xiong Y, Phelps D, Hannon G, Beach D and Barrett JC. (1996). *Proc. Natl. Acad. Sci. USA,* **93,** 13742–13747.

Atadja P, Wong H, Garkavtsev I, Veillette C and Riabowol K. (1995). *Proc. Natl. Acad. Sci. USA,* **92,** 8348–8352.

Bates S and Vousden KH. (1996). *Curr. Opin. Genet. Dev.,* **6,** 12–19.

Bates S, Rowan S and Vousden KH. (1996). *Oncogene,* **13,** 1103–1109.

Bond JA, Wyllie FS and Wynford-Thomas D. (1994). *Oncogene,* **9,** 1885–1889.

Bond J, Haughton M, Blaydes J, Gire V, Wynford-Thomas D and Wyllie F. (1996). *Oncogene,* **14,** 2097–2104.

Brown JP, Wei W and Sedivy JM. (1997). *Science,* **277,** 831–834.

Brugarolas J, Chandrasekaran C, Gordon JI, Beach D, Jacks T and Hannon GJ. (1995). *Nature,* **377,** 552–557.

Canman CE, Chen C-Y, Lee M-H and Kastan MB. (1994). *Cold Spring Harbor Symp. Quant. Biol.,* **LIX,** 277–285.

Canman CE, Wolff AC, Chen C-Y, Fornace AJ and Kastan MB. (1994). *Cancer Res.,* **54,** 5054–5058.

Campisi J. (1997). *Eur. J. Cancer,* **33,** 703–709.

Chen Q, Fiosher A, Reagan JD, Yan L-J and Ames BN. (1995). *Proc. Natl. Acad. Sci. USA,* **92,** 4130–4134.

Chiu C-P and Harley CB. (1997). *Proc. Soc. Exp. Biol. Med.,* **214,** 99–106.

Cox LS and Lane DP. (1996). *Bioessays,* **17,** 501–508.

Deng C, Zhang P, Harper JW, Elledge SJ and Leder P. (1995). *Cell,* **82,** 675–684.

Di Leonardo A, Linke SP, Clarkin K and Wahl GM. (1994). *Genes & Dev.,* **8,** 2540–2551.

Dimri GP, Lee X, Basile G, Acosta M, Scott G, Roskelley C, Medrano EE, Linskens M, Rubelj I, Pereira-Smith O, Peacocke M and Campisi J. (1995). *Proc. Natl. Acad. Sci. USA,* **92,** 9363–9367.

Dulic V, Drullinger LF, Lees E, Reed SI and Stein GH. (1993). *Proc. Natl. Acad. Sci. USA,* **90,** 11034–11038.

Dulic V, Kaufmann WK, Wilson SJ, Tisty TD, Lees E, Harper JW, Elledge SJ and Reed SI. (1994). *Cell,* **76,** 1013–1023.

El-Deiry WS, Harper JW, O'Connor PM, Velculescu VE, Canman CE, Jackman J, Pietenpol JA, Burrell M, Hill DE, Wang Y, Wiman KG, Mercer WE, Kastan MB, Kohn KW, Elledge SJ, Kinzler KW and Vogelstein B. (1994). *Cancer Res.,* **54,** 1169–1174.

El-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW and Vogelstein B. (1993). *Cell,* **75,** 817–825.

Friedberg EC, Walker GC and Siede W. (1995). *DNA Repair and Mutagenesis,* ASM Press: Washington, DC.

Gavrieli Y, Sherman Y and Ben-Sasson SA. (1992). *J. Cell Biol.,* **119,** 493–501.

Hanks SK. (1987). *Proc. Natl. Acad. Sci. USA,* **84,** 388–392.

Hara E, Smith R, Parry D, Tahara H, Stone S and Peters G. (1996). *Mol. Cell Biol.,* **16,** 859–867.

Harley CB, Futcher AB and Greider CW. (1990). *Nature,* **345,** 458–460.

Harper JW, Adami GR, Wei N, Keyomarsi K and Elledge SJ. (1993). *Cell,* **75,** 805–816.

Hartwell L. (1992). *Cell,* **71,** 543–546.

Hastie ND, Dempster M, Dunlop MG, Thompson AM, Green DK and Allshire RC. (1990). *Nature,* **346,** 866–868.

Isaacs JS, Chiao C, Merrick BA, Selkirk JK, Barrett JC and Weissman BE. (1997). *Cancer Res.,* **57,** 2986–2992.

Kane SA, Hecht SM, Sun J-S, Garestier T and Helene C. (1995). *Biochemistry,* **34,** 16715–16724.

Kastan MB, Zhan Q, El-Deiry WS, Carrier F, Jacks T, Walsh WV, Plunkett BS, Vogelstein B and Fornace AJ. (1992). *Cell,* **71,** 587–597.

Ko LJ and Prives C. (1996). *Genes & Dev.,* **10,** 587–597.

Kulju KS and Lehman JM. (1995). *Exp. Cell Res.,* **217,** 336–345.

Lazo JS and Chabner BA. (1996). *Cancer Chemotherapy and Biotherapy*. Chabner BA and Longo DL (ed.). Lippincott-Raven: Philadelphia, pp. 379–393.

Li C-Y, Nagasawa H, Dahlberg WK and Little JB. (1995). *Oncogene,* **13,** 1885–1892.

Li Y, Nichols MA, Shay JW and Xiong Y. (1994). *Cancer Res.,* **54,** 6078–6082.

Linke SP, Clarkin KC, Di Leonardo A, Tsou A and Wahl GM. (1996). *Genes & Dev.,* **10,** 934–947.

Linke SP, Clarkin KC and Wahl GM. (1997). *Cancer Res.,* **57,** 1171–1179.

Lois AF, Cooper LT, Geng Y, Nobori T and Carson D. (1995). *Cancer Res.,* **55,** 4010–4013.

Lombard DB and Guarente L. (1996). *Trends Genet.,* **12,** 283–286.

Loughran O, Malliri A, Owens D, Gallimore PH, Stanley MA, Ozanne B, Frame MC and Parkinson EK. (1996). *Oncogene,* **13,** 561–568.

Macleod KF, Sherry N, Hannon G, Beach D, Tokinao T, Kinzler K, Vogelstein B and Jacks T. (1995). *Genes & Dev.,* **9,** 935–944.

Mao L,Merlo A, Bedi G, Shapiro GI, Edwards CD, Rollins BJ and Sidransky D. (1995). *Cancer Res.,* **55,** 2995–2997.

Marcand S, Buck SW, Moretti P, Gilson E and Shore D. (1996). *Genes Dev.,* **10,** 1297–1309.

McEachern MJ and Blackburn EH. (1996). *Genes & Dev.,* **10,** 1822–1834.

Merlo A, Herman JG, Mao L, Lee DJ, Gabrielson E, Burger PC, Baylin SB and Sidransky D. (1995). *Nature Med.,* **1,** 692–695.

Millis AJT, Hoyle M, McCue HM and Martini H. (1992). *Exp. Cell Res.,* **201,** 373–379.

Nelson GN and Kastan MB. (1994). *Mol. Cell. Biol.,* **14,** 1815–1823.

Noda A, Ning Y, Venable SF, Pereira-Smith OM and Smith JR. (1994). *Exp. Cell Res.,* **211,** 90–98.

Ogryzko VV, Hurai TH, Russanova VR, Barbie DA and Howard BH. (1996). *Mol. Cell. Biol.,* **14,** 5210–5218.

Okamato A, Demetrick DJ, Spillare EA, Hagiwara K, Hussain SP, Bennett WP, Forrester K, Gerwin B, Serrano M, Beach DH and Harris CC. (1994). *Proc. Natl. Acad. Sci. USA,* **91,** 11045–11049.

Papathanasiou MA, Kerr NC, Robbins JH, McBride OW, Alamo Jr I, Barrett SF, Hickson ID and Fornace Jr AJ. (1991). *Mol. Cell. Biol.,* **11,** 1009–1016.

Peng C-Y, Graves PR, Thoma RS, Wu Z, Shaw AS and Piwinica-Worms H. (1997). *Science,* **277,** 1501–1505.

Polyak K, Lee M-H, Erdjument-Bromag H, Tempst P and Massague J. (1994). *Cell,* **78,** 59–66.

Quelle DE, Zindy F, Ashmun RA and Sherr CJ. (1995). *Cell,* **83,** 993–1000.

Reznikoff CA, Yeager TR, Belair CD, Savelieva E, Puthenveettil JA and Stadler WM. (1996). *Cancer Res.,* **56,** 2886–2890.

Rogan EM, Bryan TM, Hukku B, MacLean K, Chang AC-M, Moy EL, Englezou A, Warneford SG, Dalla-pozza L and Reddel RR. (1995). *Mol. Cell. Biol.,* **15,** 4745–4753.

Ross WE and Bradley MO. (1981). *Biochim. Biophys. Acta,* **654,** 129–134.

Sanchez Y, Wong C, Thoma RS, Richman R, Wu Z, Piwinica-Worms H and Elledge SJ. (1997). *Science,* **277,** 1497 – 1501.
Serrano M, Hannon CJ and Beach D. (1993). *Nature,* **366,** 704 – 707.
Serrano M, Lin AW, McCurrach ME, Beach D and Lowe SW. (1997). *Cell,* **88,** 693 – 702.
Sherr CJ and Roberts JM. (1995). *Genes & Dev.,* **9,** 1149 – 1163.
Sherwood SW, Rush D, Ellsworth JL and Schimke RT. (1988). *Proc. Natl. Acad. Sci. USA,* **85,** 9086 – 9090.
Shore D. (1995). *Telomeres*. EH Backburn and CW Greider (eds). Cold Spring Harbor Press, pp. 247 – 263.
Smeal T, Claus J, Kennedy B, Cole F and Guarente L. (1996). *Cell,* **84,** 633 – 642.
Stein GH and Dulic V. (1995). *Bioessays,* **17,** 537 – 543.
Sugrue MM, Shin DY, Lee SW and Aaronson SA. (1997). *Proc. Natl. Acad. Sci. USA,* **94,** 9648 – 9653.
Terada Y, Tatsuka M, Jinno S and Okayama H. (1995). *Nature,* **376,** 358 – 362.
Thompson JF, Hayes LS and Lloyd DB. (1991). *Gene,* **103,** 171 – 177.
Von Zglinicki T, Saretzki G, Docke W and Latze C. (1995). *Exp. Cell Res.,* **220,** 186 – 193.
Walker DH, Adami GR, Dold KM and Babiss LE. (1995). *Cell Growth & Diff.,* **6,** 1053 – 1061.
Wang XQ, Gabrielli BG, Milligan A, Dickinson JL, Antalis TM and Ellem KAO. (1996). *Cancer Res.,* **56,** 2510 – 2514.
Weinberg RA. (1997). *Cell,* **88,** 573 – 575.
Weinberg WC, Azzoli CG, Chapman K, Levine AJ and Yuspa SH. (1995). *Oncogene,* **10,** 2271 – 2279.
Wright WE, Brasiskyte D, Piatyszek M and Shay JW. (1996). *EMBO J.,* **15,** 1734 – 1741.
Wright WE and Shay JW. (1992). *TIG,* **8,** 193 – 197.
Xiong Y, Hannon G, Zhang H, Casso D, Kobayashi R and Beach D. (1993). *Nature,* **366,** 701 – 704.
Yu C, Oshima J, Fu Y-H, Wijsman EM, Hisama F, Alisch R, Matthews S, Nakura J, Miki T, Ouais S, Martin GM, Mulligan J and Schellenberg GD. (1996). *Science,* **272,** 258 – 262.
Zauberman A, Flusberg D, Haupt Y, Barak Y and Oren M. (1995). *Nucleic Acids Res.,* **23,** 2584 – 2592.
Zhan Q, Carrier F and Fornace AJ. (1993). *Mol. Cell. Biol.,* **13,** 4242 – 4250.
Zhu L, Harlow E and Dynlacht BD. (1995). *Genes & Dev.,* **9,** 1740 – 1752.
Zindy F, Quelle DE, Roussel MF and Sherr CJ. (1997). *Oncogene,* **17,** 203 – 211.