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Participation of p53 Protein in the Cellular Response to DNA Damage[1]

Michael B. Kastan,[2] Onyinye Onyekwere, David Sidransky, Bert Vogelstein, and Ruth W. Craig

Departments of Oncology [M. B. K., O. O., D. S., B. V.] and Physiology [R. W. C.], The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

ABSTRACT

The inhibition of replicative DNA synthesis that follows DNA damage may be critical for avoiding genetic lesions that could contribute to cellular transformation. Exposure of ML-1 myeloblastic leukemia cells to nonlethal doses of the DNA damaging agents, γ-irradiation or actinomycin D, causes a transient inhibition of replicative DNA synthesis via both G_1 and G_2 arrests. Levels of p53 protein in ML-1 cells and in proliferating normal bone marrow myeloid progenitor cells increase and decrease in temporal association with the G_1 arrest. In contrast, the S-phase arrest of ML-1 cells caused by exposure to the anti-metabolite, cytosine arabinoside, which does not directly damage DNA, is not associated with a significant change in p53 protein levels. Caffeine treatment blocks both the G_1 arrest and the induction of p53 protein after γ-irradiation, thus suggesting that blocking the induction of p53 protein may contribute to the previously observed effects of caffeine on cell cycle changes after DNA damage. Unlike ML-1 cells and normal bone marrow myeloid progenitor cells, hematopoietic cells that either lack *p53* gene expression or overexpress a mutant form of the *p53* gene do not exhibit a G_1 arrest after γ-irradiation; however, the G_2 arrest is unaffected by the status of the *p53* gene. These results suggest a role for the wild-type p53 protein in the inhibition of DNA synthesis that follows DNA damage and thus suggest a new mechanism for how the loss of wild-type *p53* might contribute to tumorigenesis.

INTRODUCTION

Exposure to DNA damaging agents probably contributes to the development of many human cancers (1). Therefore, much effort has been focused on understanding how cells respond to DNA damage and restore the linear DNA sequence integrity and chromatin structure. An important component of the cellular response to DNA damage is an inhibition of replicative DNA synthesis (*e.g.*, Refs. 2–6). Presumably, this response allows optimal repair of damage before the cell reinitiates replicative DNA synthesis and/or begins mitosis. If the damage were not repaired before initiation of S-phase, the use of a damaged DNA template during replicative synthesis could "fix" and propagate mutagenic lesions that might contribute to cellular transformation (7). The inhibition of replicative DNA synthesis after DNA damage may be a critical step in avoiding the progressive increase in genomic changes that characterizes neoplastic transformation (8, 9). Furthermore, cells that are inefficient at this inhibitory process may be prone to neoplastic development. We therefore sought to characterize some of the mechanisms in mammalian cells that control the cell cycle changes in response to DNA damage.

The inhibition of DNA synthesis that follows DNA damage could be achieved through inhibition of a positive (stimulatory) regulator pathway of DNA synthesis and/or stimulation of a negative regulator pathway. Since recent data suggest that the wild-type *p53* gene product plays a role in the inhibition of DNA synthesis (10–13), and since this "growth/tumor suppressor" gene is the most commonly mutated gene thus far identified in human cancers (14), with abnormalities of the *p53* gene present in a wide spectrum of cancers, including tumors of the breast, lung, colon, bladder, brain, bone, hematopoietic, and muscle tissues (reviewed in Ref. 15), we examined the possibility that *p53* might be a negative regulator in this cellular response to DNA damage.

Unfortunately, because of its extremely short half-life (16–19) and its growth inhibitory effects (10–12, 20, 21), studies of the roles and mechanisms of action of the normal, wild-type p53 protein have been extremely difficult. We have recently developed a sensitive and specific flow cytometric assay for quantitating relative levels of endogenous p53 protein in human hematopoietic cells (22). We utilized this assay to assess changes in the levels of p53 protein after DNA damage. We also evaluated changes in cell cycle progression after DNA damage in cells with normal or abnormal expression of *p53*.

We observed increases in p53 protein levels in proliferating normal human bone marrow progenitor cells and in ML-1 myeloblastic leukemia cells after DNA damage, apparently occurring via a posttranscriptional mechanism. A similar rise in p53 protein levels had previously been noted in mouse 3T3 fibroblasts after UV irradiation (23). With 2 different DNA damaging agents, this rise in p53 protein levels in ML-1 cells was temporally correlated with a transient G_1 arrest. Both the increase in p53 protein levels and the decrease in DNA synthesis (specifically, the G_1 arrest) after DNA damage were blocked by the protein synthesis inhibitor, cycloheximide, and by the phosphodiesterase inhibitor, caffeine. In contrast to cells with no detectable *p53* gene mutations (ML-1 and normal human bone marrow myeloid progenitors), cells with mutant *p53* genes (Raji and RPMI 8402 lymphoid leukemia cells and KG-1a myeloid leukemia cells) or with no *p53* genes (HL60 myeloid leukemia cells) continued to progress through S-phase after DNA damage. These results suggest that wild-type p53 protein may play a role in the inhibition of DNA synthesis that follows DNA damage.

MATERIALS AND METHODS

Cells and DNA Damage. Cell lines were grown in RPMI 1640 containing 10% serum. Human hematopoietic progenitor cells (CD34+) from normal volunteers were isolated by immunomagnetic adherence as described previously (24). Myeloid progenitor cells from this isolated cell population were grown in a liquid culture system containing HL-1 medium (Ventrex Laboratories, Portland, ME) with 30% fetal calf serum and 7% supernatant from the bladder carcinoma cell line 5637 for maximal growth. After 4 days in culture, these normal bone marrow progenitor cells reached logarithmic phase growth, and cell cycle changes after γ-irradiation were assessed. Cultured cell lines or myeloid bone marrow cells were exposed to a selected dose of γ-irradiation, actinomycin D, or ara-C[3] (continuous exposure over the time course for the chemical agents) and evaluated at various times

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[2] To whom requests for reprints should be addressed, at: Oncology 3-120, Johns Hopkins Hospital, 600 North Wolfe Street, Baltimore, MD 21205.

[3] The abbreviations used are: ara-C, 1-β-d-arabinofuranosylcytosine; IFA, 10 mM 4-(2-hydroxyethyl)-1-peperazinethane sulfonic acid, pH 7.4, 150 mM NaCl, 4% newborn calf serum, 0.1% NaN_3; MFI, mean fluorescence intensity; BrdUrd, bromodeoxyuridine; XRT, γ-irradiation; CHX, cycloheximide.

afterwards for changes in *p53* levels or cell cycle progression. Cell viability was greater than 90% by trypan blue exclusion up to 96 h after DNA damage for virtually all doses and agents used. Cells were irradiated in their flasks in media in a ^{137}Cs γ-irradiator for an appropriate length of time to deliver a preselected dose (usually at ~100 rads/min). When desired, caffeine was added 30 min before irradiation. In each case, control cells were handled in the same way with only the omission of the DNA damaging agent.

Flow Cytometric Assay for *p53* Expression. Hybridoma cells secreting the anti-*p53* antibody, p421, were generously provided by Dr. Arnold Levine. A specific inhibitory peptide (25) was synthesized and purified by The Johns Hopkins University School of Medicine Protein/Peptide Facility. Flow cytometric analysis of p53 protein was carried out as described previously (22). Briefly, cells were fixed by dropwise addition of 70% methanol and incubation for 5 min at -20°C, washed, and then incubated with 0.1 ml p421 hybridoma supernatant [diluted 1:5 in IFA buffer with or without the presence of 0.5 μg of the purified specific inhibitory peptide (previously titrated for maximal inhibition). After another wash, the cells were incubated with goat anti-mouse IgG2a fluorescein isothiocyanate (diluted 1:500 in IFA; Southern Biotechnology Associates, Birmingham, AL) for 30 min, washed in IFA twice, and analyzed on a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, CA). Nonspecific blocking serum (2% goat and human) was present during each antibody incubation. All procedures after fixation were done at 4°C. Raji and HL-60 cells were run in every experiment as positive and negative controls, respectively, for p53 protein expression. Peptide-inhibitable fluorescence is present only in cells expressing p53 protein, and the amount of fluorescence correlates well with the amounts of p53 protein assessed by immunoblot or immunoprecipitation (22). Relative levels of p53 protein were evaluated by determining a "corrected *p53* MFI" as described previously (26). [Corrected MFI equals the difference between the MFI of antibody binding and antibody plus inhibitory peptide; using the p421 antibody, it has previously been shown to accurately reflect the presence of p53 protein (22).]

Northern Blots and Immunoprecipitations. Total cellular RNA was isolated using the guanidine isothiocyanate/cesium chloride procedure (27). Electrophoretic separation, RNA transfer to nylon membranes, hybridization, and autoradiographic identification were done by standard techniques (28). Filters were hybridized successively to *p53* and β-actin probes labeled with [^{32}P]dCTP by the random primer method (29). For immunoprecipitations, cells were incubated in methionine-free RPMI 1640 with 20% dialyzed fetal calf serum for 1 h before labeling cells with 100 μCi/ml [^{35}S]methionine for 2 h. After washing, nuclei were isolated by hypotonic lysis according to the method of Klempnauer and Sippel (30), and *p53* was extracted from the nuclear pellets in 10 mM Tris, pH 7.4, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA, 0.005% leupeptin, 200 units/ml Trasylol, 1 mM phenylmethylsulfonyl fluoride, and immunoprecipitated with either p421 antibody or control antibody, p2037 (a nonspecific IgG2a) bound to protein A-Agarose (Sigma) after preclearing the lysate × 2 with p2037, protein A-Agarose, and sansorbin (Calbiochem, La Jolla, CA). Immunoprecipitated proteins were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the gel was dried and autoradiographed using preflashed film after fluorographic amplification (Amersham).

Cell Cycle/Proliferation Assays. DNA synthesis was assessed by incorporation of BrdUrd and flow cytometric analysis using the method described by Hoy *et al.* (31). Briefly, after incubation with 10 μM BrdUrd for 4 h, cells were fixed in 70% methanol as above, resuspended in 0.1 N HCl/0.7% Triton X-100 for 10 min at 4°C, washed with excess phosphate-buffered saline, and heated to 97°C for 10 min in deionized acidified water. The cells were then chilled in ice water for 10 min and washed twice with IFA buffer containing 0.5% Tween 20. After incubation with 0.1 ml anti-BrdUrd-fluorescein isothiocyanate (1:5 in IFA buffer, 30 min, 4°C; Becton Dickinson), the cells were washed twice and then analyzed by flow cytometry. For simultaneous analysis of DNA synthesis and cell cycle, after BrdUrd staining and washing, the cells were treated with 50 units of RNase (Sigma Chemical Co., St. Louis, MO) at 37°C for 15 min and incubated for at least 1 h with 25 μg/ml propidium iodide (Sigma) in phosphate-buffered saline, pH 7.4.

p53 Gene Sequencing. DNA was isolated from cell lines, and exons 5 through 9 of the *p53* gene were amplified from these DNA samples through the use of the polymerase chain reaction. These products were then subcloned into Lambda Zap (Stratagene) and double-stranded plasmid was obtained. Pooled plasmids were then sequenced and compared with the normal sequence of the *p53* gene as described previously (32).

RESULTS

Alterations in Cell Cycle Progression and *p53* Expression in ML-1 Cells after γ-Irradiation. We have previously demonstrated that the changes in p53 protein expression during proliferation and differentiation of ML-1 myeloid leukemia cells closely parallel those in normal human bone marrow progenitor cells (22). In addition, sequencing of exons 5 through 9 of the ML-1 *p53* gene revealed no mutations (see below). Thus, ML-1 cells appear to be a useful model for investigations of the physiological functions of endogenous, wild-type p53 protein.

Exposure of ML-1 cells to nonlethal doses of XRT results in a dose-dependent, transient decrease in replicative DNA synthesis (Fig. 1). Changes in the cell cycle progression of these cells after DNA damage were assessed by simultaneous flow cytometric analysis of DNA synthesis (by BrdUrd incorporation) and of DNA content (by propidium iodide staining). This analysis can detect a decrease in the number of cells entering S-phase as early as 3 h after XRT (Fig. 1*B*; *see arrows*), even though the change in the percentage of S-phase cells in the total cell population is small at this time (Fig. 1*A*). The decrease in the number of S-phase cells is due to cells arresting in both G_1 and G_2/M (Fig. 1, *B* and *C*). G_1 arrest predominates at low doses of XRT (<100 rad), whereas the G_2 arrest becomes more prominent at higher doses (data not shown). Cells that are already in S-phase at the time of DNA damage appear to continue to progress through to G_2/M, whereas cells in G_1 do not continue to enter S-phase (Fig. 1*B*).

Expression of p53 protein in ML-1 cells after XRT was evaluated by both flow cytometry (Fig. 2, *A* and *C*) and metabolic labeling/immunoprecipitation (Fig. 2*B*). Increases in p53 protein levels were seen within 1 to 2 h after XRT with both techniques. A similar increase in p53 protein levels was seen in proliferating normal bone marrow progenitor cells after XRT (data not shown). We have previously demonstrated the specificity of the p421 antibody and peptide inhibition in the flow cytometric assay for detecting p53 protein (22). The specificity of these assessments of changes in p53 protein levels after XRT is demonstrated here by specific peptide inhibition in the flow cytometric assay [Fig. 2*A*; antibody plus peptide gives identical levels of fluorescence as a control antibody (data not shown)] and the use of a control IgG2a antibody in the immunoprecipitation assay (Fig. 2*B*). In addition, the increase in anti-*p53* antibody binding to ML-1 and bone marrow progenitor cells in the flow cytometric assay could not be attributed to some nonspecific binding simply attributable to DNA damage since no increases in antibody binding were observed in cells with no *p53* expression (HL-60 and KG-1a) or expression of mutant p53 protein (Raji) (Fig. 2*C* and see below).

The increases in p53 protein levels were detectable within 30 min after XRT and returned towards normal levels about 48 to 72 h later (Fig. 3). This time course of increases in p53 protein levels correlated well with the cell cycle changes, which became detectable within 3 h and returned towards normal 48 to 72 h

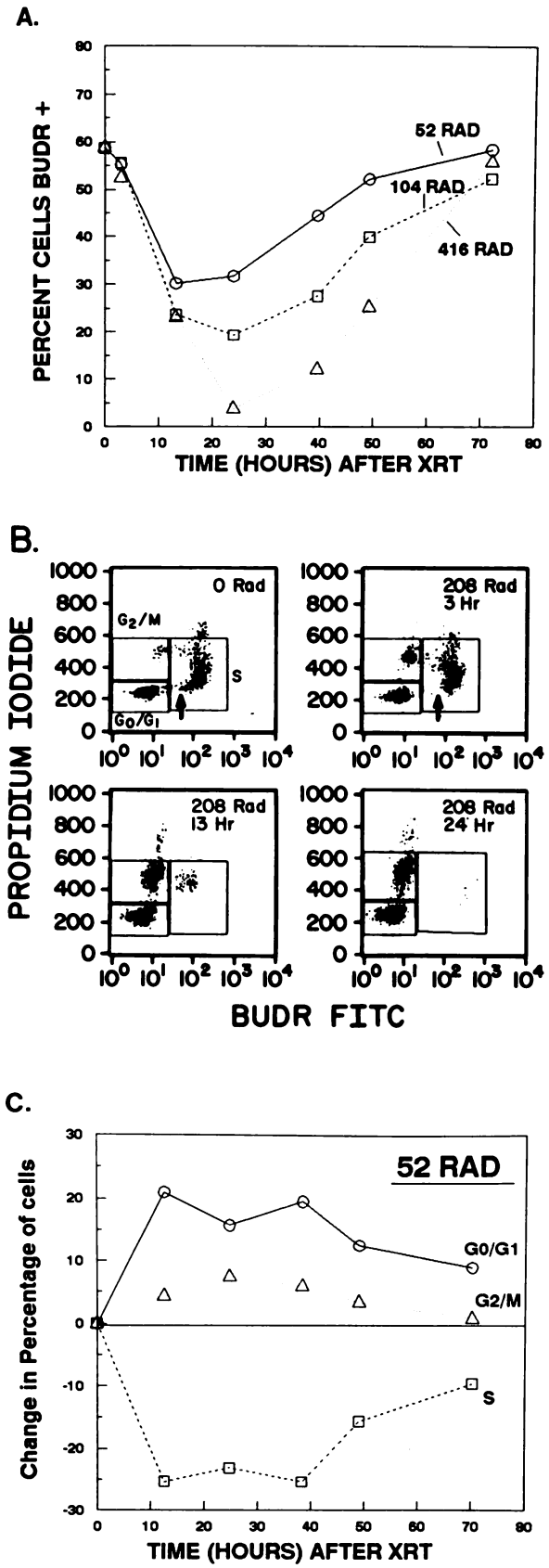


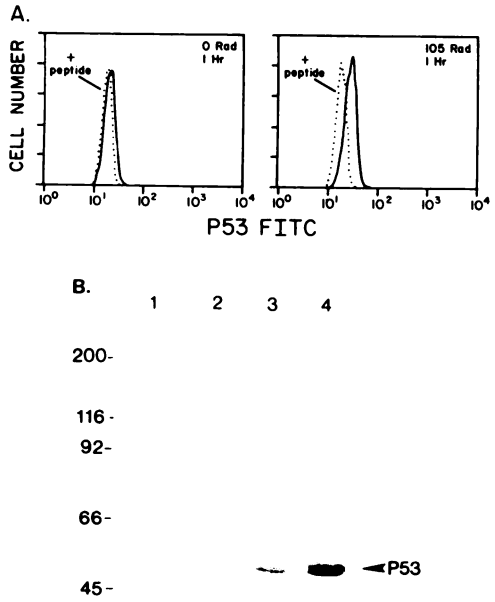
Fig. 1. Time course of cell cycle changes in ML-1 cells exposed to XRT. *A*, DNA synthesis after various doses of XRT was assessed by flow cytometric analysis of BrdUrd (BUDR) incorporation during a 4-h pulse at various times after exposure to 52, 104, or 416 rads. *B*, cell cycle distributions in control cells (0 rad) or cells at 3, 13, and 24 h after exposure to 208 rads. Flow cytometric dot plots display simultaneous analysis of S-phase DNA synthesis (determined after a 4-h pulse with BrdUrd at the various times after XRT) on the *ordinate* and DNA content (determined by staining with propidium iodide) on the *abscissa*. Cell cycle populations are characterized as G_0/G_1 (2N DNA content with no BrdUrd incorporation), S-phase (variable DNA content with BrdUrd incorporation), and G_2/M (4N DNA content with no BrdUrd incorporation during the pulse period). *Arrows*, area containing cells that have recently entered S-phase from G_1. *C*, quantitation of the changes in the percentage of cells in each cell cycle phase (assessed as in *B*) at various times after exposure to 52 rads.

later (Fig. 1). Increases in p53 protein were detectable with doses as low as 52 rad (data not shown) and, in most experiments, 3- to 5-fold increases in the mean fluorescence intensity of p53 protein were noted at doses between 52 and 208 rad. Similarly, densitometer tracings of immunoprecipitated [^{35}S] methionine-labeled p53 protein revealed 3- to 5-fold increases within 1 h after XRT (data not shown). Furthermore, similar to the dose dependence noted for the degree of inhibition of DNA synthesis after XRT (Fig. 1*A*), levels of p53 protein also increased more at higher doses, at least between 52 and 416 rad (data not shown).

To begin to elucidate the mechanism of the induction of p53 protein after DNA damage, total cellular RNA was isolated from ML-1 cells at various times after various doses of γ-irradiation. Northern blot analyses revealed no significant changes in the levels of *p53* mRNA (Fig. 4). This observation suggests that the changes in p53 protein after DNA damage result from post-transcriptional mechanisms and is consistent with the previous report of an increased half-life of p53 protein in 3T3 cells after UV damage (23).

Effects of Other Cytotoxic Agents on Cell Cycle and *p53* Expression in ML-1 Cells. Actinomycin D is a DNA intercalating agent that, like γ-irradiation, induces DNA strand breaks (33, 34). In contrast, ara-C is an S-phase-specific anti-metabolite that does not directly damage DNA (35). Treatment of ML-1 cells with actinomycin D causes a G_1 arrest (36) and induces a significant increase in p50 protein levels (Fig. 5*A*), though the time at which the increase is noted is much later than that seen after γ-irradiation. However, the decrease in replicative DNA synthesis after actinomycin D treatment also occurs later than after XRT (compare Figs. 5*B* and 1*A*), and thus still temporally coincides with the increase in p53 protein. In contrast, treatment with ara-C induces an S-phase arrest [though it does not decrease the number of cells that enter S-phase (Fig. 5*B*)], and no significant changes in p53 protein levels are observed (Fig. 5*A*). Thus, it appears that p53 protein levels increase after exposure to agents that damage DNA and cause a G_1 arrest, such as XRT and actinomycin D.

Effects of Caffeine and Cycloheximide on Cell Cycle Changes and *p53* Expression after DNA Damage. Caffeine treatment potentiates the cytotoxicity of DNA damaging agents by preventing the inhibition of DNA synthesis (2, 37, 38). We found that while exposure of ML-1 cells to caffeine caused an increase in the percentage of undamaged cells in G_1 (Fig. 6*A*, *left column*), caffeine treatment inhibited the usual decrease in DNA synthesis in cells exposed to XRT [Figs. 6*A* (*right column*) and 7*A*]. We reasoned that if p53 protein actively participates in this inhibition of replicative DNA synthesis after DNA damage, then the increases in p53 protein usually seen after DNA damage might also be affected by caffeine treatment. Exposure of cells to 4 mM caffeine, which inhibited the decrease in DNA synthesis after DNA damage (Figs. 6*A* and 7*A*), also blocked the increase in p53 protein levels (Figs. 6*B* and 7*B*). These data suggest that some of the previously described effects of caffeine on cells after DNA damage may be due to a block of p53 protein induction and implicate alterations in cyclic



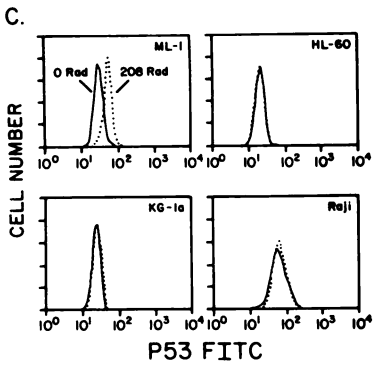


Fig. 2. Levels of p53 protein in ML-1 cells exposed to XRT. *A*, flow cytometric analysis of p53 protein expression 1 h after exposure to 0 or 105 rads. —, Fluorescence histograms after staining with anti-*p53* antibody, p421; · · · ·, control (antibody preincubated with inhibitory peptide) for the same condition. A shift to the right of the histograms in the p421-stained samples indicates the presence of p53 protein. *B*, immunoprecipitation of [^{35}S]methionine-labeled p53 protein 2 h after exposure of ML-1 cells to 0 rad (*Lanes 1* and *3*) or 206 rad (*Lanes 2* and *4*). Nuclear proteins were immunoprecipitated with control antibody, p2037 (*Lanes 1* and *2*), or p421 (*Lanes 3* and *4*). Extracts from equivalent numbers of cells (75 × 10^6 cells/lane) and equivalent numbers of ^{35}S counts (after preclearing of the extracts) were immunoprecipitated for each sample. Molecular weight standards (K_d) are shown on the *left*. *C*, flow cytometric analysis of p53 protein expression (binding of p421 antibody) 2 h after exposure of ML-1, HL-60, KG-1a, or Raji cells to 0 rad (—) or 208 rad (· · · ·). FITC, fluorescein isothiocyanate.

nucleotide levels in this regulatory process.

Brief exposures of ML-1 cells to the protein synthesis inhibitor, CHX, similarly blocked the induction of p53 protein (within 2 h; Fig. 8*B*) and partially inhibited the G_1 arrest measured 15 h after XRT (Fig. 8*A*). [The rapid reversibility of the effect of a pulse of CHX on p53 protein levels (see below) is consistent with the partial inhibition of the G_1 arrest observed in this experiment]. These results demonstrate that the G_1 arrest after DNA damage is an active cellular response dependent on new protein synthesis and/or on a short-lived protein. (The rapid disappearance of all detectable p53 protein after CHX treatment is also consistent with the short half-life of wild-type p53 protein.) The inhibition of p53 protein induction by CHX was reversible, however, since p53 protein levels increased rapidly and the the G_1 arrest was re-initiated after a washout of the CHX from XRT-treated cells (data not shown). Interestingly, as previously noted in yeast (39), the G_2 arrest was not significantly affected by CHX treatment (Fig. 8*A*). These observations with caffeine and cycloheximide further link the changes in p53 protein levels to the inhibition of replicative DNA synthesis after DNA damage. In addition, they indicate that the G_1 arrest is mediated by a physiological control mechanism, rather than by structural constraints of damaged DNA, in agreement with suggestions by Weinert and Hartwell (6).

Cell Cycle Changes after DNA Damage in Cells with Abnormal *p53* Genes. The data presented thus far demonstrate a strong correlation between decreased DNA synthesis and increased p53 protein levels after DNA damage (after XRT and actinomycin D and with caffeine and CHX treatment of irradiated cells). If wild-type p53 protein is a critical participant in the inhibition of replicative DNA synthesis after DNA damage, then DNA synthesis in cells with abnormal p53 proteins should be less inhibited after DNA damage than cells with wild-type *p53*. Therefore, as a first step in addressing the question of a functional linkage between increases of wild-type p53 protein levels and G_1 arrest after DNA damage, we compared the

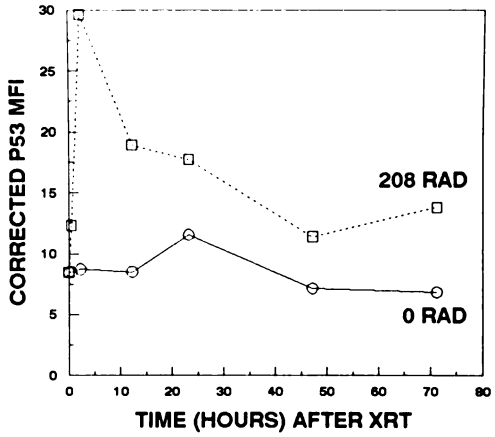


Fig. 3. Time course of changes in p53 protein levels in ML-1 cells exposed to XRT. The corrected *p53* MFI (the MFI of p421 antibody staining minus the MFI of staining with antibody plus inhibitory peptide, as described in "Materials and Methods") was measured in ML-1 cells at various times after exposure to 0 rads (—) or 208 rads (- - - -).

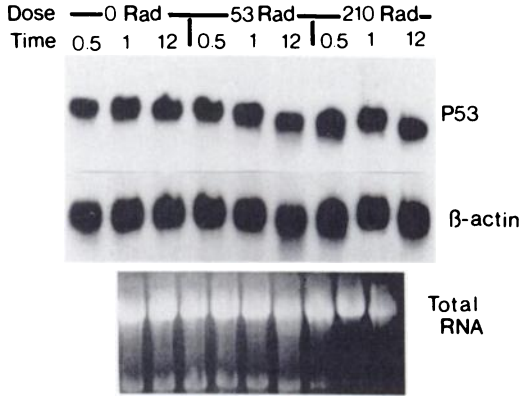


Fig. 4. Levels of *p53* mRNA in ML-1 cells exposed to XRT. Total cellular RNA was isolated from ML-1 cells at 0.5, 1, and 12 h after exposure to 0, 53, or 210 rads XRT. After electrophoresis and transfer, the RNA was hybridized with ^{32}P-labeled probes for *p53* or β-actin. Ethidium bromide staining of the agarose gel is shown as an additional control for the amounts of RNA loaded in each lane (20 μg/*lane*).

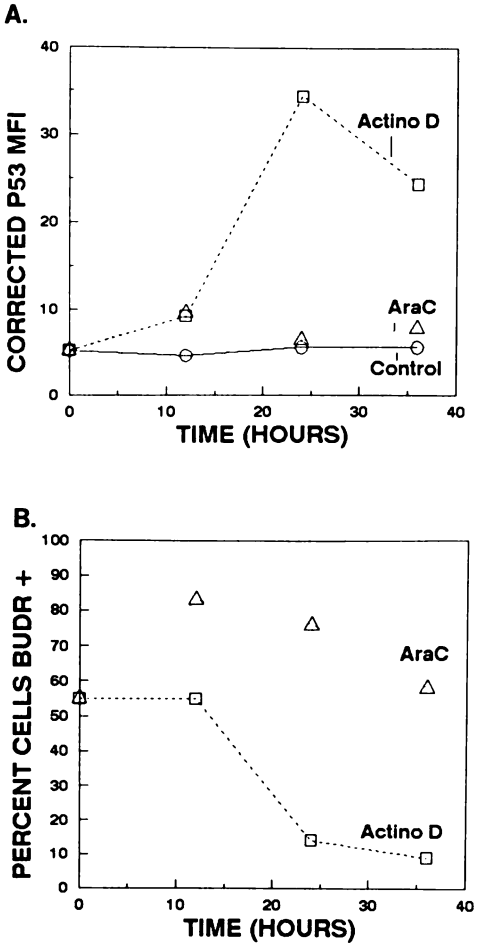


Fig. 5. Alterations in p53 protein levels and DNA synthesis in ML-1 cells exposed to actinomycin D or ara-C. *A*, levels of p53 protein (assessed as in Fig. 3) at various times during continuous exposure to 0.45 nM actinomycin D (Actino D; - - - -), 50 nM ara-C (· · ·), or no cytotoxic agent (—). *B*, percentage of cells in S-phase assessed by flow cytometry after a 4-h pulse of BrdUrd *(BUDR)* incorporation at various times during continous exposure to the same doses of actinomycin D (- - - -) and ara-C (· · · ·) noted in *A*.

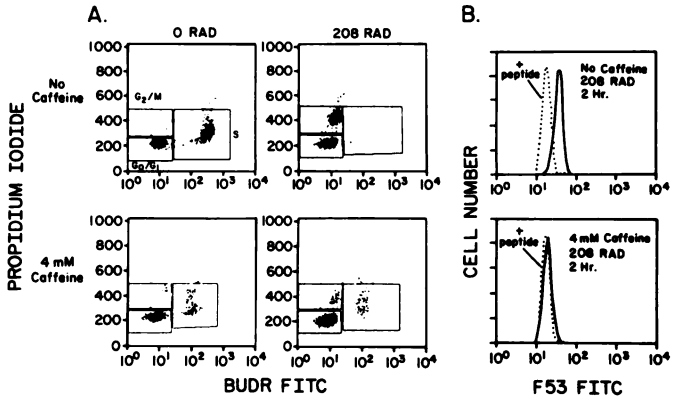


Fig. 6. Effects of caffeine on the alterations in cell cycle progression and p53 protein expression in ML-1 cells exposed to XRT. *A*, ML-1 cells, without (*top panels*) or with (*bottom panels*) 4 mM caffeine (added 30 min before XRT), were exposed to 0 (*left panels*) or 208 (*right panels*) rad. After 18 h, the cells were pulsed for 4 h with BrdUrd *(BUDR)* and the cell cycle populations were analyzed by BrdUrd/propidium iodide staining as described in Fig. 1*B*. *B*, ML-1 cells were analyzed for p53 protein expression (as described in Fig. 2*A*) 2 h after exposure to 208 rad in the absence (*top panel*) or presence (*bottom panel*) of 4 mM caffeine (added 30 min before XRT).

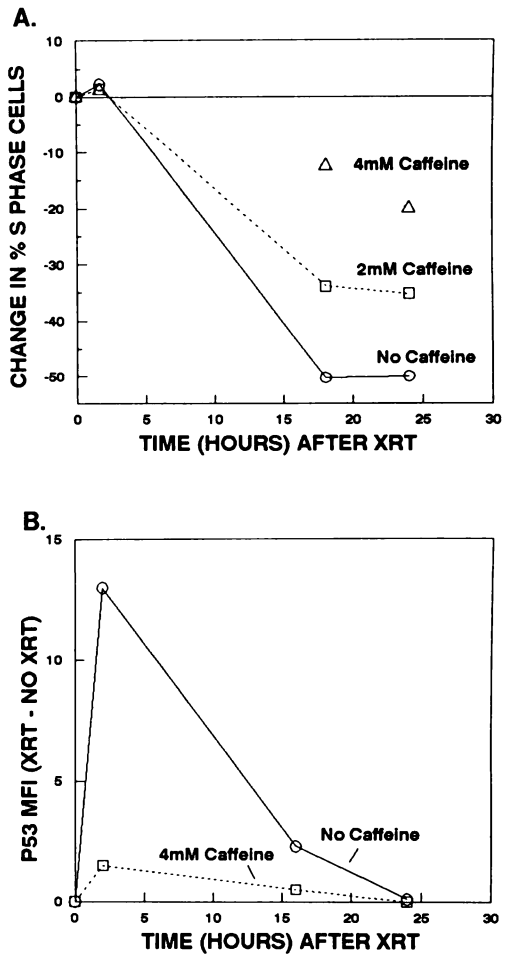


Fig. 7. Time course of the effects of caffeine on DNA synthesis and p53 protein levels in ML-1 cells exposed to XRT. ML-1 cells were exposed to 208 rad in the presence of 0, 2, or 4 mM caffeine and then analyzed for DNA synthesis (BrdUrd incorporation) and for p53 protein expression at various times. *A*, *points*, change in the percentage of cells in S-phase in irradiated compared with unirradiated cells at each time for each dose of caffeine. *B*, *points*, difference between the corrected *p53* MFI (as described in Fig. 3) in irradiated and unirradiated cells at each time for 0 and 4 mM caffeine.

changes in cell cycle progression after DNA damage in cells with altered *p53* genes to cells with normal *p53* genes.

Exons 5 through 9 of the *p53* genes from the myeloid leukemia cell lines, ML-1 and KG-1a, and the lymphoid leukemia cell lines, Raji and RPMI 8402, were amplified, subcloned, and sequenced as described previously (32). No mutations were found in ML-1 cells. KG-1a cells were found to have a mutation at codon 225, resulting in a substitution of isoleucine for valine on one allele, and loss of the other allele. Raji cells were found to have a wild-type *p53* allele and an allele with a G to A transition at codon 213 resulting in a glutamine for arginine substitution. RPMI 8402 cells had one wild-type *p53* allele and one allele with a C to T transition at codon 273 resulting in a cysteine for arginine substitution. In agreement with previous reports (40), HL-60 myeloid leukemia cells did not have an intact *p53* gene on Southern blot analysis (data not shown). Our recent characterizations of *p53* expression in these cell lines had revealed that Raji and RPMI 8402 cells express high p53 protein levels, as seen in other cell lines with *p53* gene mutations. ML-1 cells, on the other hand, had low levels, whereas KG-1a and HL-60 cells lacked both *p53* mRNA and protein expression (22). (It is not yet clear why the *p53* mutation in KG-1a cells results in a virtual absence of *p53* mRNA expression.) Thus, cells with mutant *p53* genes [either express-

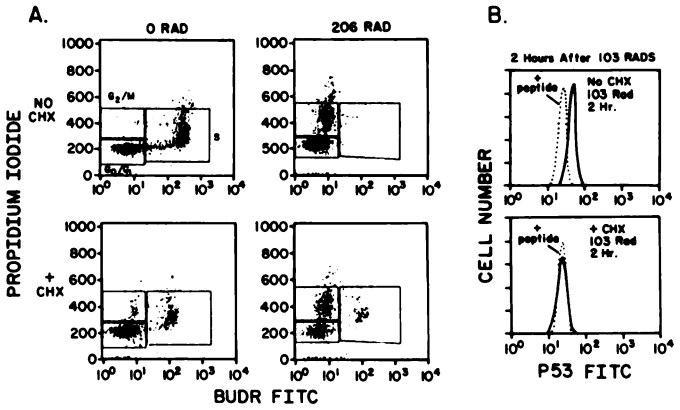


Fig. 8. Effects of cycloheximide on the changes in DNA synthesis and p53 protein levels in ML-1 cells after XRT. *A*, cell cycle populations characterized by BrdUrd (BUDR)/propidium iodide staining 15 h after exposure to 0 rad (*left panels*) or 206 rad (*right panels*) without (*top panels*) or with (*bottom panels*) exposure to 10 μM cycloheximide, which was added 10 min before XRT and washed out 5 h later. *B*, flow cytometric analysis of p53 protein expression (as described above) 2 h after exposure to 103 rad without (*top panel*) or with (*bottom panel*) 10 M cycloheximide added 10 min before irradiation. *FITC*, fluorescein isothiocyanate.

ing p53 protein (Raji and RPMI 8402 cells) or not expressing it (KG-1a)], as well as cells with no intact *p53* genes (HL-60) were available for comparisons to cells with no "hot-spot" *p53* mutations (normal bone marrow myeloid progenitors and ML-1 cells) in studies of the role of p53 protein in the cellular response to DNA damage (Table 1).

If these cells with abnormal or no *p53* genes exhibited arrests of DNA synthesis after DNA damage similar to ML-1 and bone marrow cells, then a significant role for normal p53 protein in this process would effectively be ruled out. However, in contrast to ML-1 cells and normal human bone marrow myeloid progenitor cells, the myeloid leukemia cell lines, HL-60 and KG-1a, and the lymphoid leukemia cell lines, Raji and RPMI 8402, all with altered *p53* genes, continued to progress through S-phase after γ-irradiation (Fig. 9*A*; data not shown for bone marrow and RPMI 8402 cells). Although all of the cell types exhibited an increase in the percentage of cells in G_2 after exposure to γ-irradiation (Fig. 9*A*), only ML-1 cells and normal bone marrow progenitor cells exhibited a G_1 arrest (Fig. 9*B*; data not shown for RPMI 8402). These observations suggest that the wild-type *p53* gene participates in the inhibition of replicative DNA synthesis via a G_1 arrest after DNA damage, but probably does not significantly contribute to the G_2 arrest.

DISCUSSION

Arrest of replicative DNA synthesis after DNA damage is thought to occur to provide ample time for the cell to repair DNA lesions before S-phase (G_1 arrest) and/or mitosis (G_2 arrest). The mechanisms underlying this arrest are poorly understood (6). In this study, we have begun to characterize the cellular mechanisms responsible for this arrest of DNA synthesis in mammalian cells. The *p53* gene is thought to function as an inhibitor of cellular replication, thus allowing the unregulated growth of the tumor cells when it is abnormal (9, 10, 41). Our data confirm that the inhibition of DNA synthesis is an active physiological response to DNA damage in mammalian cells (since it is blocked by both caffeine and CHX) and suggest that one function of the p53 protein is to participate in the cellular response to DNA damage, perhaps by transient inhibition of new replicative DNA synthesis.

The observations that only cells with normal *p53* genes exhibit a rapid increase in the levels of wild-type p53 protein after DNA damage, as well as a temporally correlated G_1 arrest, suggest that wild-type p53 protein is an active participant in the G_1 arrest that follows DNA damage. Interestingly, it appears that either loss of expression of wild-type p53 (a recessive mechanism) or overexpression of a mutant *p53* gene (a dominant mechanism) can result in this abnormal cell cycle response to XRT. In contrast to the G_1 arrest, however, all cell types, regardless of *p53* gene status, exhibited a G_2 arrest after XRT. Consistent with the lack of inhibition by CHX of the G_2 arrest in yeast after XRT (39), brief exposures to CHX blocked the p53 protein induction and inhibited the G_1 arrest, but did not block the G_2 arrest. A role for p53 protein in this G_1 arrest is consistent with other experiments demonstrating that transfection of wild-type *p53* genes into various tumor cell lines induces a G_1 arrest (10–13). In addition, our observation that cells in S-phase continue to progress through to G_2 after XRT (Fig. 1*B*) is also consistent with a role for *p53* in this process, since S-phase cells are immune to the *p53* negative regulation of growth until they enter the next G_1 period (13).

Our results in hematopoietic cells are consistent with, and extend, an earlier observation that treatment of nontransformed mouse fibroblasts with UV irradiation or a UV-mimetic chemical carcinogen caused a rapid increase in the amount of p53 protein (23). Interestingly, as we observed with the *p53* mutant-overexpressing Raji cells, no increase in p53 protein levels was seen in SV40-transformed mouse fibroblasts (which overexpress p53 protein due to binding of T antigen) after DNA damage (23). Thus, p53 protein levels increase after DNA damage in different tissue types with wild-type *p53* genes (hematopoietic cells and fibroblasts) and following different types of DNA damaging agents [XRT, actinomycin D, UV, and UV-mimetics (the latter 2 from Ref. 23)]. In contrast, exposure of cells to cytosine arabinoside, a cytotoxic agent that neither significantly damages DNA nor induces a G_1 arrest, did not cause significant changes in p53 protein levels.

Since there are no detectable changes in the levels of *p53* mRNA after XRT, and since p53 protein levels increase despite

Table 1 *Status of P53 gene, protein, and XRT response in selected cell types*

Cell	Lineage	P53 gene[c]	Mutant codon	A.A. Δ[c]	P53 protein[a]	G_1 arrest[b]
NBMP[c]	Myeloid	WT	–	–	+/–	+
ML-1	Myeloid	WT	–	–	+	+
KG-1a	Myeloid	M	225	VAL→ILE	–	–
HL-60	Myeloid	A	–	–	–	–
Raji	Lymphoid	M/WT	213	ARG→GLN	+++	–
RPMI8402	Lymphoid	M/WT	273	ARG→CYS	+++	–

[a] Relative levels of p53 protein as described here and in Kastan *et al.*, (22); +++, high levels; +, low levels; –, no detectable protein; +/–, proliferative NBMP cells normally have no detectable p53 protein, but express low levels after XRT.

[b] G_1 arrest after XRT.

[c] NBMP, normal bone marrow progenitor cells rapidly growing in liquid culture (only myeloid progenitor cells grow under these conditions); M, mutant p53 gene; WT, wild-type p53 gene; A, absent p53 gene; A.A.Δ, amino acid change.

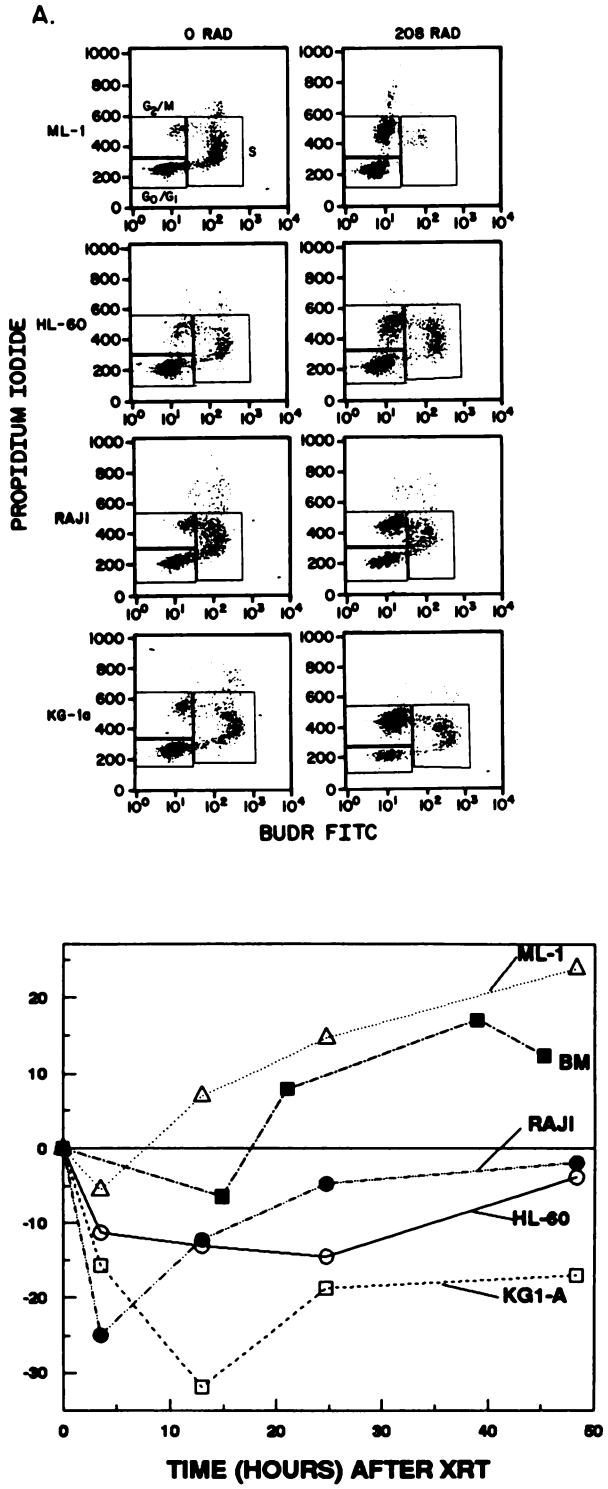


Fig. 9. Cell cycle changes in various cell types after exposure to XRT. *A*, cell cycle populations in ML-1, HL-60, Raji, and KG-1a cells 13 h after exposure to 0 rad (*left panels*) or 208 rad (*right panels*) were characterized by BrdUrd *(BUDR)*/ propidium iodide staining as described in Fig. 1*B*. *B*, quantitation of the changes in the percentage of cells in G_0/G_1 (assessed as in *A*) in ML-1, HL-60, Raji, KG-1a, and normal human bone marrow myeloid progenitor cells (*BM;* isolated and grown in culture as described in "Materials and Methods") at various times after exposure to 208 rads. *FITC*, fluorescein isothiocyanate.

the use of actinomycin D at doses that can significantly inhibit RNA synthesis (36), this increase is likely brought about by a posttranscriptional mechanism. Again, this is consistent with the observation by Maltzman and Czyzyk (23), that the increase in p53 protein in murine fibroblasts after UV damage is due to an increased half-life of the protein. Since we find that the increase in p53 protein is reversibly inhibited by CHX (added before XRT), continued synthesis of p53 protein may be required. However, the synthesis of another gene product involved in the induction or stabilization of p53 protein could also be important.

A posttranscriptional mechanism for the increase in p53 protein after DNA damage would avoid the necessity of transcribing new RNA utilizing a potentially damaged DNA template. If increases in the half-life of p53 protein are occurring after DNA damage, such increases could result from changes in phosphorylation, binding to other proteins, or oligomerization of *p53*, all of which have been reported to occur (19, 42–45). If phosphorylation changes are involved in this process, then $p34^{cdc2}$ may be a logical kinase to investigate, since: (*a*) *p53* has been found to be associated with $p34^{cdc2}$ kinase in transformed cells (46); (*b*) *p53* is a substrate for $p34^{cdc2}$ *in vitro* (44); and (*c*) γ-irradiation rapidly inhibits $p34^{cdc2}$ activity (47). Alternatively, our observations that caffeine exposure inhibits both the arrest of replicative DNA synthesis and the increase in p53 protein levels not only further implicates p53 protein in this process, but also suggests the possible involvement of a cAMP- or cGMP-dependent process (such as a cyclic nucleotide-dependent kinase) in the up-regulation of p53 protein after DNA damage.

Cells with mutant *p53* genes continue to go through S-phase after DNA damage; thus, use of a damaged template for replicative synthesis in these cells might lead to significant mutations, and possibly to genomic instability, in their daughter cells. For example, mutations of the *RAD9* gene in yeast permit cells with unrepaired DNA damage to proceed through the cell cycle, rather than arresting in G_2, and DNA damage of these cells results in increased numbers of cells with chromosomal aberrations (6). Thus, if the cessation of replicative DNA synthesis after DNA damage is a critical cellular response for minimizing the chance of cellular transformation, then our observations suggest a new mechanism for how the loss of wild-type *p53* might contribute to tumorigenesis and to the progressive genetic changes, chromosomal abnormalities, and aneuploidy commonly observed in tumor cells (9, 48). The high frequency of *p53* mutations in a wide variety of tumors, many of which have been linked to carcinogen exposures, underscores the potential significance of this type of mechanism in the development and progression of human cancers.

Patients with the familial cancer-prone syndrome, Li-Fraumeni, have germ line mutations in the *p53* gene (49, 50). Consistent with the possible function of wild-type p53 protein discussed above and the associated physiological consequences of the presence of abnormal *p53* expression and/or function, fibroblasts from patients with Li-Fraumeni syndrome may have an increased incidence of developing aneuploidy in tissue culture compared with fibroblasts from normal individuals (51). In addition, epidemiological data have suggested that an increased susceptibility to carcinogenesis by exposure to environmental carcinogens, such as ionizing radiation and tobacco smoke, may contribute to the array of tumors seen in this syndrome (52, 53). Thus, this type of role for p53 protein in the response to DNA damage may also help to explain why these patients can develop a wide variety of tumor types (this pathway is not tissue-specific) and have a median age of over 30 years for the development of their first tumor.

In conclusion, these experiments demonstrate that p53 pro-

tein levels increase after DNA damage, probably via a posttranscriptional mechanism, and may contribute to a transient inhibition of replicative DNA synthesis. Further characterization of this process should provide additional insights into physiological mechanisms that contribute to radiation- and chemically induced carcinogenesis. It should also provide information about the function of wild-type p53 protein in normal cells and the mechanism(s) whereby alterations in *p53* contribute to tumorigenesis.

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