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Differential Upregulation of p53-Responsive Genes by Genotoxic Stress in Hematopoietic Cells Containing Wild-Type and Mutant p53

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Cells respond to genotoxic stress by activation of many genes, including the tumor suppressor p53. p53 activates transcriptionally target genes, such as $p21^{waf1}$ and gadd45, which can lead to cell cycle arrest, or bax, which can lead to cell death. We examined the response to genotoxic stress in two hematopoietic cell lines that harbor either wild-type (MOLT-4) or a mutant p53 with a codon 161 mutation (U266). We adapted a multiprobe RNase protection assay (RPA) to determine the steady-state RNA levels, and in combination with nuclear runoff assays, transcriptional rates of multiple stress-induced genes. We found a differential activation of growth arrest and cell death-specific p53 target genes in cells with wild-type or mutant p53. Our results show that genotoxic stress can activate the $p21^{waf1}$ and gadd45 genes in both cell lines. However, the bax gene was not induced in U266 cells. Bax and gadd45 gene induction could be efficiently blocked by pretreating the cells with the antioxidant compound pyrrolidine dithiocarbamate, suggesting that oxidative stress was involved in these responses. Induction of all three genes in MOLT-4 cells was clearly at the transcriptional level, because we detected transcriptional activity by nuclear runoff RPA assays, and transfection with a consensus p53 binding sequence. U266 cells did not activate the same reporter, in spite of the upregulation of $p21^{waf1}$ and gadd45 RNA levels. However, the $p21^{waf1}$-reporter constructs containing 0.9 to 2.4 kb of the native p21 promoter were potently activated in U266 cells. These results indicate a differential regulation of p53 target genes in cells containing wild-type or codon 161 mutant p53.

RNase protection Ionizing radiation p21/waf1 gadd45 bax Cell cycle

THE response of eukaryotic cells to genotoxic agents includes activation of genes participating in growth arrest or apoptosis, depending on the cell type. This includes the tumor suppressor p53, a transcription factor that is mutated in the majority of tumors and tumor cell lines (15). Following treatment with genotoxic agents, p53 has been shown to act as a transcription factor responsible for transactivating multiple genes that contain p53-responsive elements in their promoter (1,12).

Among the key p53 transcriptional targets that lead to growth arrest are $p21^{waf1/cip1}$ (from here on called p21) and the growth arrest and DNA damage-inducible gene gadd45. p21 is the protoypic cyclin-dependent kinase inhibitor (9,18,30,35). Treatments of cells with a variety of DNA-damaging agents, such as ionizing radiation or topoisomerase inhibitors, induce a p53-dependent G_1 arrest (12,21–23) and p21 upregulation (7–9,35), and thus p21 is thought to be an essential part of the p53-mediated growth arrest pathway. p21 lies downstream of p53 in the DNA damage response and thus forms a critical bridge between p53 and Rb in cell cycle control (2,34). It is also implicated in the control of DNA replication and repair through its interaction with PCNA (33). p21 can be regulated independently of p53 under physio-

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logical conditions, such as during normal tissue development, following serum stimulation, or during cellular differentiation (20,24,31,36). However, numerous studies have shown that induction of p21 by DNA-damaging agents is dependent on functional p53 (7–9).

Growth arrest and DNA damage-inducible (gadd) genes were isolated after UV treatment of Chinese hamster cells (38). They respond to a wide variety of DNA-damaging agents in a p53-independent manner. As an exception, gadd45 is frequently induced in response to ionizing radiation in a p53-dependent manner. Gadd45 has a p53 consensus binding site within its third intron (19). In addition, p53 can participate in transcriptional induction of the gadd45 promoter in the absence of direct DNA binding (39). Expression of gadd45 has been correlated with a strong growth arrest. Bax, on the other hand, is a promoter of cell death, which is activated in some types of cells following treatment with ionizing radiation, chemotherapeutic drugs, and other forms of genotoxic stress in a p53-dependent manner (26,40).

In this study we examined the expression of genotoxic stress-induced genes, known to be critical for growth arrest or apoptosis. We adapted a multiprobe RNase protection assay to determine the steady-state RNA levels, and in combination with runoff assays, transcriptional rates of multiple stress-induced genes. We found a differential activation of growth arrest and cell death-specific p53 target genes in cells with wild-type compared to those with mutant p53.

MATERIALS AND METHODS

Cell Culture and Treatments

The human T lymphoblastic MOLT-4 cells and multiple myeloma U266 cells were obtained from the American Type Culture Collection (Rockville, MD) and grown in RPMI-1640 with 10% (v/v) heat-inactivated fetal bovine serum, 50 U/ml penicillin, and 50 mg/ml streptomycin (Life Technologies Inc). Exponentially growing cells were adjusted to a density of $2–3 \times 10^5$ cells/ml the day before the experiment was performed. γ-Irradiation (2–20 Gy) was performed at room temperature using a ^{137}Cs source, as described previously (13).

The cells were also treated with 1 or 10 μM etoposide (VP16), 2.5 μM camptothecin, 0.1 mM hydrogen peroxide (H_2O_2), and 0.1 mM pyrrolidine dithiocarbamate (PDTC). The antioxidant PDTC was added 1 h prior to irradiation and remained in the medium until the time of RNA isolation. Unless otherwise noted, all chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

RNase Protection Assay

Total RNA was isolated from cells at various intervals postirradiation using the acid guanidine isothiocyanate-phenol-chloroform extraction method and the Trizol reagent (Life Technologies, Inc.). To determine the steady-state levels of RNA, we used the RiboQuant system (Pharmingen) for RNase protection assay (RPA) with a Multi-Probe Template set, as described (14). Briefly, the hStress-1 template Set was used for the T7 polymerase directed synthesis of high specific activity ^{32}P-labeled antisense RNA probes, including p53, gadd45, c-*fos*, p21, and bax, as well as two housekeeping gene products, L32 and GAPDH. Probes (4×10^5 cpm) were synthesized using T7 polymerase labeled with [^{32}P]UTP and hybridized to 10 μg of total RNA overnight at 56°C. RNA hybrids were digested with RNAse A and T1, purified, and resolved on 6% denaturing polyacrylamide gels (PAGE). The level of each RNA species was determined by PhosphorImage analysis based on signal intensities given by the appropriately sized, protected probe fragments, which were also normalized to expression levels of the L32 housekeeping gene.

Nuclear Runoff-RNase Protection Assay

Nuclear runoff transcription was performed essentially as described previously (4) with certain modifications. Briefly, 3×10^7 cells were washed twice in ice-cold PBS, cells incubated for 5 min on ice in NP-40 lysis buffer A, centrifuged for 5 min at $500 \times g$ at 4°C, and then the supernatant removed. The procedure was repeated and the nuclei were resuspended in 100 μl glycerol storage buffer and frozen in liquid nitrogen. To perform nuclear runoff transcription, 150 μl of frozen nuclei was used together with 40 μl of 5× reaction buffer with nucleotides and 100 μCi [α-^{32}P]UTP. Incubation was continued for 30 min at 30°C, then ^{32}P-labeled RNA was purified using the Trizol reagent (Life Technologies, Inc.). The major modification of the procedure is that we examined simultaneously expression of multiple genes using the hStress-1 template for the T7 polymerase directed synthesis, to hybridize labeled cDNA. The RNase protection assay was performed as described above.

p53 Functional Assays

To determine promoter activity, three p53-responsive promoters were used. pG13-Luc (9) contains 13 copies of a p53 binding site. 0-Luc, 2-Luc, and 4-Luc contain 2.4, 1.5, and 0.9 kb DNA fragments, respectively, of the natural p21 promoter DNA sequence (37). MOLT-4 and U266 cells were transfected using the DMRIE-C reagent, a lipofectine derivative using

the manufacturer's instructions (Life Technologies Inc). Briefly, to each well of a 24-well plate, 0.1 ml OPTI-MEM I Reduced Serum Medium and 3 μl DMRIE-C Reagent were added. After 10-min incubation at room temperature, 0.1 ml of OPTI-MEM I containing 2–5 μg of luciferase reporter plasmid was added to the wells containing the lipid reagent and incubated for 30 min at room temperature to allow formation of lipid–DNA complexes. To each well containing the lipid–DNA complexes, 40 μl of a cell suspension containing 4×10^5 cells in OPTI-MEM I was added. Cells were incubated for 4 h at 37°C, after which they were supplemented with 0.4 ml growth medium containing 15% FBS. For MOLT-4 cells, PHA-L was added to the medium at a final concentration of 1 μg/ml to enhance promoter activity and gene expression. At 23 h following transfection, the cells were divided equally, half being used as control and half being irradiated. Luciferase activity was measured 48 h after initiating the transfection in lysates from untreated cells or those that had been irradiated, using the reporter lysis system (RLS, Promega) and a Bio-orbit 1253 luminometer. The assays were normalized for protein content determined using the BioRad Protein Assay.

Cell Cycle Assays

For cell cycle analyses, 5×10^5 control and irradiated MOLT-4 and U266 cells were washed twice in PBS then fixed in 75% ethanol in PBS. Flow cytometric measurements were performed on these cells as described (3,32), following treatment for 30 min at 37°C with 100 μg/ml RNase A and 40 μg/ml propidium iodide (PI), by bivariate flow cytometry using a FACScan. Data were analyzed with the CellQuest software (Becton Dickinson, San Jose, CA) from the cell population from which debris were gated out.

RESULTS AND DISCUSSION

Response to Genotoxic Stress in MOLT-4 Cells

An important outcome of the exposure of mammalian cells to genotoxic agents, such as ionizing radiation, is cell cycle arrest or apoptosis (12). The present work examined gene expression activated by genotoxic stress, which could lead to arrest or apoptosis in two hematopoietic cell lines that greatly differ in their survival to radiation exposure (B. Gong and A. Almasan, unpublished). To investigate changes in gene expression following genotoxic stress, we chose to use the RNase protection assay and a multiprobe template set to examine total steady-state RNA levels. This method allows a quantitative assessment of the expression levels of multiple stress-response genes in one experiment. The human stress template set we used included p53, its transcriptional target genes involved in growth arrest (p21, gadd45), and cell death (bax), as well as six other genes. We first examined the steady-state mRNA levels of these stress-response genes in exponentially growing MOLT-4 T cell lymphoma cells, as a model hematopoietic cell line we have previously studied with respect to the mechanism of cell death induced by ionizing radiation, a prototypic genotoxic agent (14). We found low basal steady-state mRNA levels for gadd45, and somewhat higher levels for bax (Fig. 1A). In contrast, there were no detectable p21 transcripts present. Analyses of the kinetics of induction following 4 Gy γ-irradiation indicated that, in addition to bax (14), the levels of gadd45 and p21 were also upregulated, reaching a 6- and 11-fold increase over control cells at 8 h. In contrast, the expression levels of L32, an internal control gene, remained unchanged (Fig. 1A). Interestingly, while both growth arrest-causing (p21 and gadd45) and cell death-inducing (bax) genes were upregulated in MOLT-4 cells, the kinetics of their induction was quite different. The most notable difference was between the regulation of p21 and bax. At all doses tested, p21 induction was earlier and stronger than that of bax. In fact, at the lower doses (2 and 4 Gy), bax upregulation was not detected until 4 h following irradiation, while a robust increase of p21 levels was seen by 2 h.

The lethal effect of ionizing radiation is thought to be mediated by formation of DNA double-strand breaks. In addition, many of the DNA-damaging effects of ionizing radiation are caused indirectly by reactive free radicals (17). Therefore, we examined the effects of typical oxidants and DNA-damaging agents on gene expression in both cell lines. Exposure of MOLT-4 cells for 4 h to the oxidant hydrogen peroxide and the topoisomerase II inhibitor etoposide induced all three genes (Fig. 1B). Moreover, pretreatment with a typical antioxidant, PDTC, abrogated the bax and gadd45 gene induction by ionizing radiation, indicating the critical role of oxidants in this process either directly, or through secondary DNA damage. This indicates that ionizing radiation signals in these cells through both DNA damage and oxidant pathways.

Transcriptional Activation of p21 and gadd45

We next examined the mechanism of RNA upregulation following irradiation. To determine the mechanism responsible for accumulation of the p21, gadd45, and bax transcripts following treatment with genotoxic agents such as γ-irradiation, we next per-

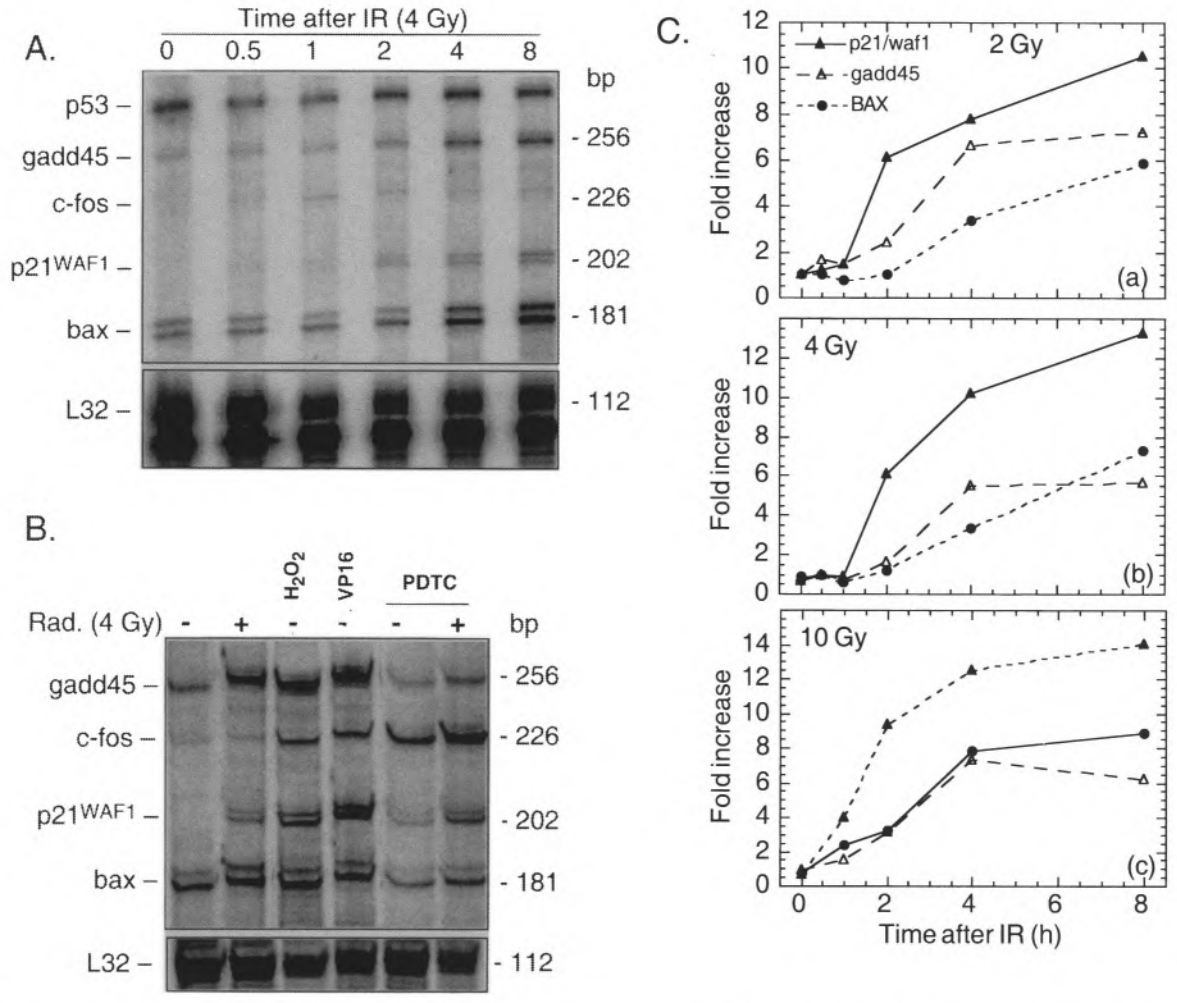

FIG. 1. Upregulation of stress-responsive genes in MOLT-4 cells. Exponentially growing MOLT-4 cells were treated with genotoxic agents and the steady-state RNA expression was analyzed by the multiprobe RNase protection assay, at the indicated times, as described in Materials and Methods. (A) Ionizing radiation-induced gene expression. RNase protection was used to examine the cellular response to 4 Gy γ-irradiation. All data are from a single gel, with only representative portions of the gel shown. (B) Effect of oxidants and DNA-damaging agents. RNase protection was used to examine the cellular response to radiation, the oxidant hydrogen peroxide (H_2O_2; 0.1 mM) and etoposide (VP16; 1 μM). The effect of the antioxidant PDTC was examined in the absence or presence of ionizing radiation, with cells being pretreated with 0.1 mM PDTC 1 h before radiation exposure. (C) Kinetics of radiation-induced gene expression. Transcripts were quantitated as described in Materials and Methods by PhosphorImager analyses. The ordinate indicates fold induction of RNA levels, representing values obtained by normalizing levels of RNA to those of untreated cells and that of the housekeeping L32 mRNA. A dose–response is shown with RNA levels determined following 2, 4, or 10 Gy.

formed transcriptional runoff assays. However, instead of examining a single gene, we developed a novel assay for simultaneously determining newly transcribed RNA specific to multiple genes by combining nuclear runoff transcription with RNase protection, a method we named runoff-RPA. Results of the runoff-RPA assay (Fig. 2A) indicate that, in cells subjected to γ-irradiation, the transcriptional rate of p21, gadd45, and bax genes increased 4.5-, 5.6-, and 5.6-fold at 2 h and 16-, 4.7-, and 8.9-fold, respectively, when measured at 4 h following irradiation (Fig. 2B).

Next, to examine postranscriptional regulation in irradiated cells, untreated or 4 Gy-irradiated cells were cultured for 4 h before actinomycin D (5 μg/ml) was added to the culture. Cells were then harvested at the indicated times, RNA extracted, and its levels examined by RNase protection. Based on this experiment, the half-life of p21, gadd45, and bax in irradiated cells was of 1, 2, and more than 2 h, respectively. To determine the contribution of postranslational mechanisms to mRNA stability, cells were treated with actinomycin D (5 μg/ml) and cycloheximide (10 μg/ml) (Fig. 2C). There was no difference in gene expression when cycloheximide was present. These results indicate a predominantly transcriptional control responsible for the γ-radiation-induced upregulation of the p21, gadd45, and bax genes in these cells.

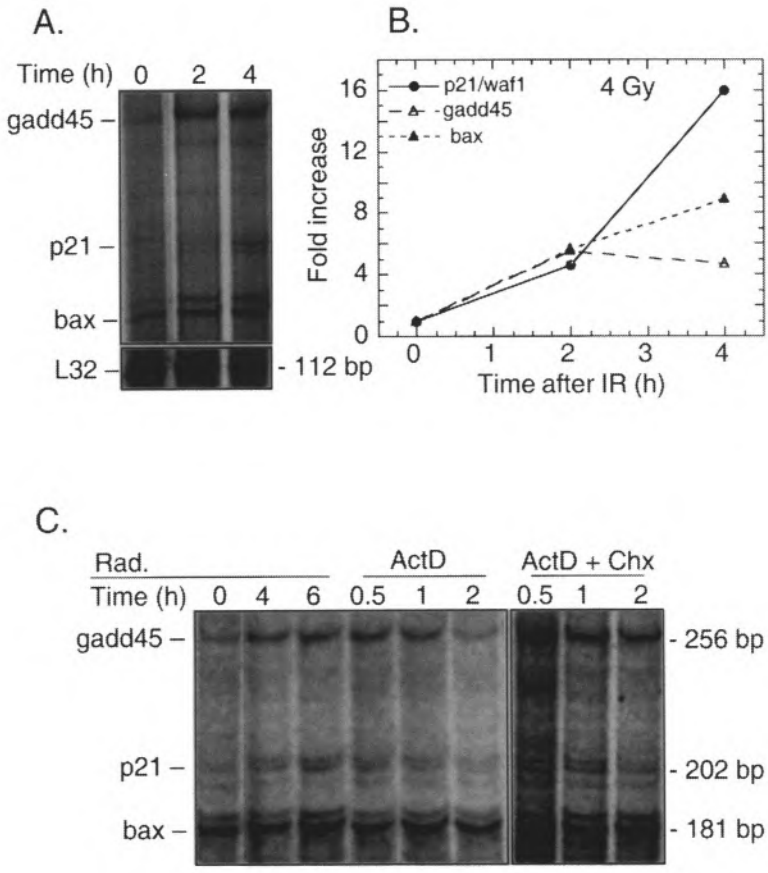

FIG 2. Transcriptional regulation of p21 and gadd45 RNA in MOLT-4 cells. (A) Nuclear runoff-RPA. Nuclei were isolated from control or irradiated (4 Gy) MOLT-4 cells and incubated as described in Materials and Methods. RNA was isolated 2 or 4 h after irradiation and examined as described for RNase protection. The kinetics of RNA synthesis is also shown (B). (C) Half-life. RNA was isolated from MOLT-4 cells irradiated in the absence or presence of an inhibitor of transcription (actinomycin D, 5 μg/ml) or translation (cycloheximide, 10 μg/ml). The inhibitors were added 4 h after irradiation and RNA isolated at the indicated times following the addition of inhibitors.

Radiation Induces Upregulation of p21 and GADD45 But Not of bax in U266 Cells

We found a differential cytotoxic effect of radiation treatment between MOLT-4 and U266, a multiple myeloma cell line (B. Gong and A. Almasan, unpublished). To investigate the nature of this difference in cell survival, we sought to further examine steady-state mRNA levels of p53-responsive genes. Differential radiation doses were used for the two cell lines, because MOLT-4 cells are radiation sensitive, U266 cells being more radiation resistant. Examination of gene expression following genotoxic stress in exponentially grown U266 cells indicated that the gadd45 and p21 transcripts were at moderate levels in untreated U266 cells and were further upregulated following 20 Gy ionizing radiation (Fig. 3A). Kinetic analyses showed that p21 was induced 4.5-, 5-, and 7-fold at 2, 4, and 8 h after IR (Fig. 3B). Similarly, the gadd45 gene was also induced 3-, 4.3-, and 3.7-fold at 2, 4, and 8 h after IR. In contrast, constitutive levels of bax were low compared to those in MOLT-4 cells. Moreover, ionizing radiation did not alter bax expression in these cells. The expression of the internal control gene L32 also remained unchanged, further confirming the specificity of the changes in expression levels of p21 and gadd45, but not bax. Similarly, treatment with etoposide or campthotecin also potently induced expression levels of p21 and gadd45. These findings were surprising, because U266 cells have been shown to harbor a p53 mutation at codon 161 (Ala to Thr) on one allele, with a loss of heterozygosity reported for the second p53 allele (25). However, our data clearly show that cells harboring this p53 mutation upregulated differentially the p53 target genes. The low levels of bax and a lack of induction following radiation may explain the relative radioresistance of these cells.

Transcriptional Activation of p53-Responsive Promoters

The transcriptional activation of p21 following treatment with ionizing radiation is believed to be

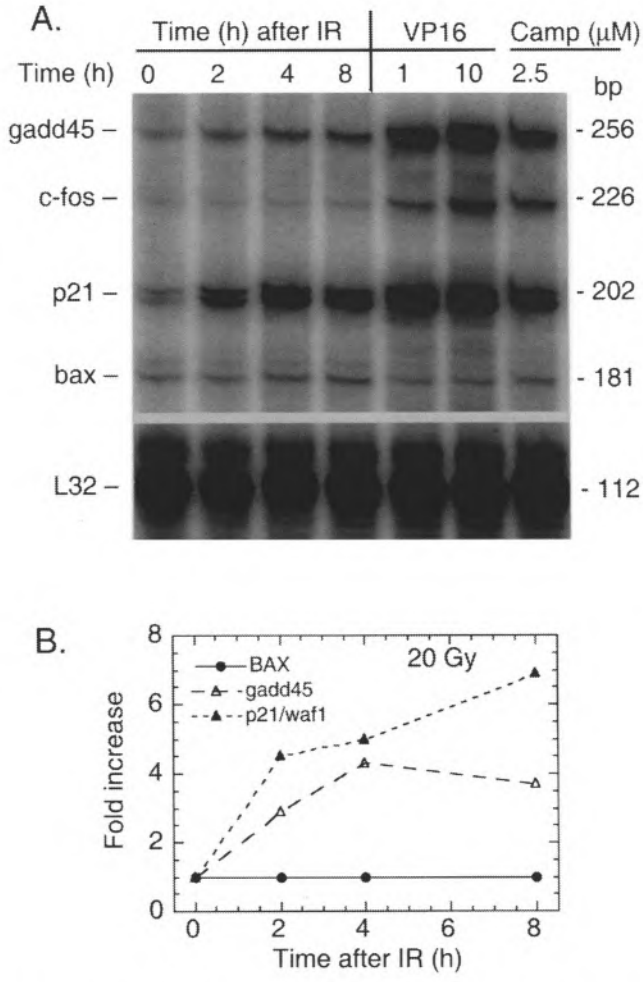

FIG. 3. Induction of gadd45 and p21, but not bax, RNA in U266 cells. Exponentially growing U266 cells were irradiated or treated with two doses of etoposide (VP16; 1–10 μM) or campthotecin (Cam; 2.5 μM). The steady-state RNA expression was analyzed at different times following irradiation or 4 h following treatment with VP16 or Cam by the multiprobe RNase protection assay as described in Materials and Methods. (B) Kinetic analysis of radiation-induced gene expression. Transcripts were quantitated as described in Materials and Methods by PhosphorImager analyses. The ordinate indicates fold induction of RNA levels, representing values obtained by normalizing levels of RNA to those of untreated cells and that of the housekeeping L32 gene. All data are from a single gel, with only representative portions of the gel shown.

mediated by the tumor suppressor protein p53. We have previously reported that p53 levels and activity are increased following irradiation of MOLT-4 cells (14). To determine p53 function in these cells, we measured the transcriptional activity of a deletion series of a p53-responsive reporter construct PG13-Luc (9), which contains a synthetic p53-responsive consensus sequence. After transfection of the PG13-Luc construct, MOLT-4 and U266 cells were exposed to 4 and 20 Gy ionizing radiation, respectively. As shown in Fig. 4A, a twofold induction of luciferase activity was seen in MOLT-4 cells treated with 4 Gy γ-irradiation. In contrast, no induction was detected in U266 cells with 20 Gy (data not shown). These results are consistent with a mutant p53 in U266 cells, which does not have transcriptional activity. The p53 mutation at codon 161 resides in the DNA binding domain of p53 and is expected to abolish its transcriptional activity.

To further examine the specific regulation of p21 by p53 in U266 cells, we employed three additional p53-responsive luciferase reporter constructs. These contain 2.4, 1.5, and 0.9 kb DNA fragments, respectively, of the p21 promoter, harboring two, one, or none of the p53 binding sites located 2.2 kb and 1.3 kb upstream of the major transcription start site (10,37). Transient transfections followed by ionizing radiation showed that there was an equivalent, 11–12-fold induction of promoter activity, regardless of the presence or absence of p53 binding sites (Fig. 4B). These results indicate that, while radiation could not activate a consensus p53-responsive reporter, it

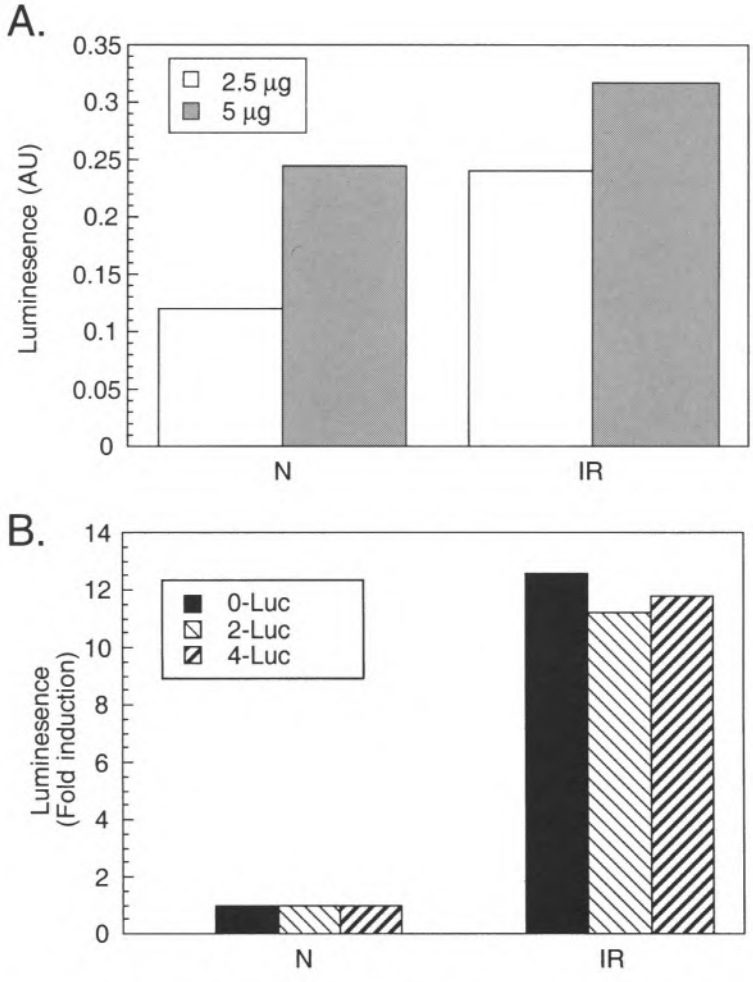

FIG. 4. Reporter assays for transcriptional activation. MOLT-4 (A) or U266 (B) cells were transfected with the p53-responsive consensus promoter PG13-Luc or the p21 promoter containing 0-Luc, 2-Luc, and 4-Luc reporter constructs. Following transfection, using the DMRIE-C reagent, cells were irradiated and then the luciferase activity was measured 24 h after irradiation. The assays were normalized for protein content. For MOLT-4 cells, PHA-L was added to the medium at a final concentration of 1 µg/ml to enhance promoter activity and gene expression.

could effectively activate the native p21 promoter in U266 cells and that this activation seems to be independent of p53. Taken together, the results described above indicate a differential regulation of p53-responsive genes in U266 cells subjected to γ-irradiation.

The main inducer of p21 in DNA-damaged cells has been considered to be p53 (7–9). However, some reports have shown that p21 can be induced independently of p53 in certain cell backgrounds following oxidative and genotoxic stress (6,16,29). Our results would suggest that it is likely that another factor might be facilitating p21 induction. A number of transcriptional factors such as E2F1, Ap2, SP1, and BRCA1 have been shown to regulate transcription of the p21 gene for which consensus binding sequences are located in the 0.9 kb DNA fragment [see (11) for a recent review]. Binding and activation of p21 but not bax or bax-like sequences could have implications for the inducibility of other p53-responsive genes that have been implicated in cell death following an extensive screen for p53 target genes (27). In support of this possibility, we show that the kinetics of induction of p21 and bax was in fact quite different even in cells with wild-type p53, such as MOLT-4. The most notable difference was observed between the regulation of p21 and bax. At all doses tested, p21 induction was earlier and stronger compared to that of bax. In fact, at the lower doses (2 and 4 Gy), bax upregulation was not detected until 4 h following irradiation, while a robust activation of p21 was seen by 2 h. A similar difference in timing of activation of p21 and bax has been reported with rat embryo fibroblasts subjected to UV irradiation (28). More-

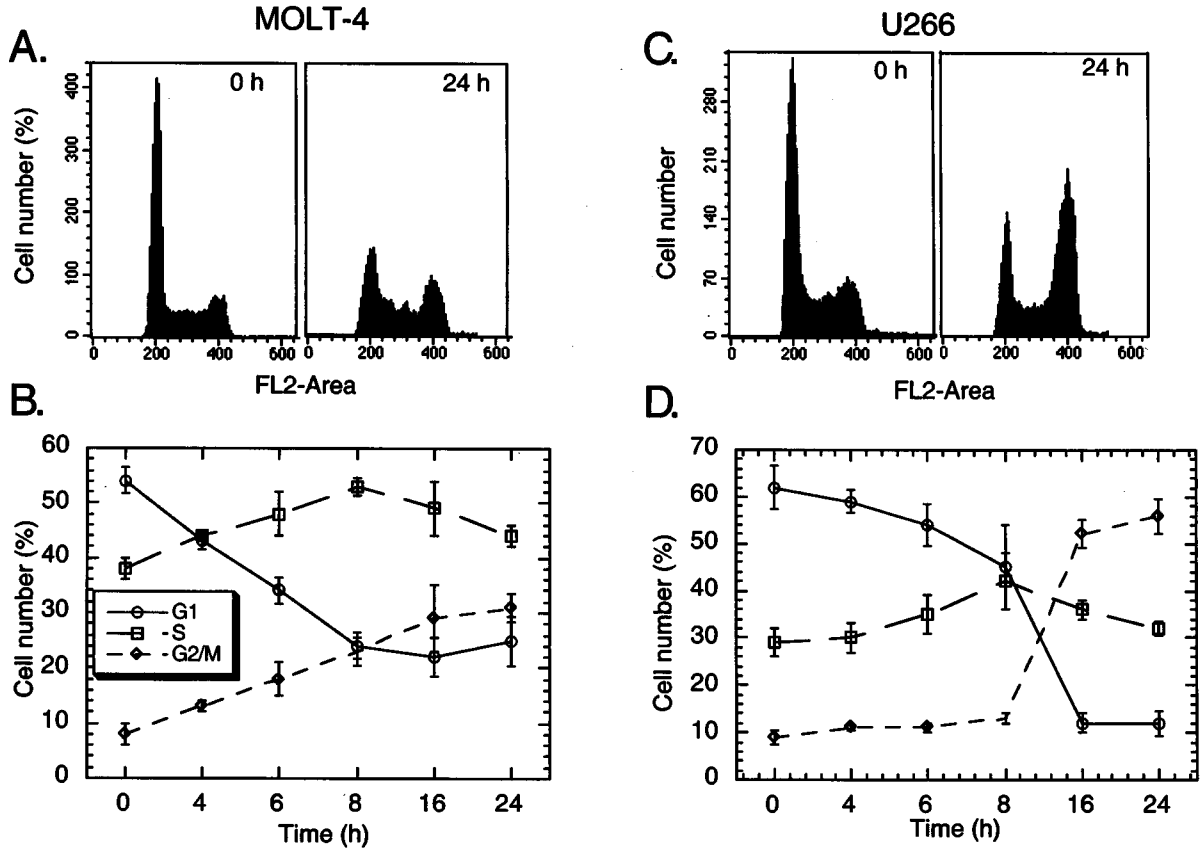

Fig. 5. Effects of irradiation on the cell cycle response of MOLT-4 and U266 cells. MOLT-4 (A, B) and U266 (C, D) cells were irradiated and their cell cycle distribution assessed by propidium iodide staining and flow cytometry as described in Materials and Methods. At the indicated times following ionizing radiation, cells were washed, pelleted, resuspended in 75% cold ethanol, and stored at –20°C overnight. Cells were pelleted, resuspended in PBS, incubated for 30 min at 37°C with 100 μg/ml RNase A and 40 μg/ml propidium iodide, and then analyzed by flow cytometry. The histograms are shown for control and 24-h time points (A, C). The kinetics of cell cycle changes are also shown (B, D).

over, cell types in which apoptosis is not characteristic showed little bax responsiveness even when wild-type p53 was present (40). Taken together, these results show that induction of genes with a critical role in apoptosis, such as bax, differs considerably from expression of genes involved in growth arrest, such as p21.

Cell Cycle Progression Following Irradiation

We next sought to determine the possible effect of the changes in gene expression following ionizing radiation on cell cycle progression. It is known that radiation primarily affects cell cycle checkpoints by halting cellular progression through the cell cycle at the G_1 to S phase or the G_2 to M phase transitions, with DNA complements of 2n or 4n, respectively (5,12,21–23). We examined the cellular response to ionizing radiation of MOLT-4 and U266 cells. Even though cells with wild-type p53 are expected to arrest in the G_1/S phase, and p53 was induced in MOLT-4 cells (14), we found that these cells arrested only in G_2/M phase (Fig. 5). This arrest in G_2/M phase was in fact similar to that seen with U266 cells, which harbor a mutated p53.

Irradiation caused mainly an increase in the proportion of cells in the G_2/M phase, from 10% to 30% in MOLT-4 and from 10% to 50% in U266 cells. The proportion of cells in G_1 did not increase, in fact decreased steadily, indicating the lack of G_1 arrest in these cells. As cells exited G_1, they first accumulated in S phase and at later time in G_2/M phase. A more effective and prolonged G_2/M arrest in U266 cells might be responsible for the radiation resistance of these cells. It has been reported that, following DNA damage, many cells appear to enter a sustained arrest in the G_2 phase of the cell cycle, and that this arrest could be sustained only when p53 was present in the cell and capable of transcriptionally activating the cyclin-dependent kinase inhibitor p21 (5). It is un-

clear, however, why cells harboring a codon 161 mutation experience a more potent G_2/M arrest, even though p53 and p21 are expected to be essential for maintaining the G_2 checkpoint only in human cells with wild-type p53.

In summary, the experiments presented in this report explored the effect of genotoxic stress on the induction of p53-responsive genes. Using an approach based on RNase protection, we showed that p21 and gadd45 genes were induced in two hematopoietic cells, regardless of whether p53 was wild-type or contained a codon 161 mutation. However, Bax levels were unchanged following irradiation of U266 cells, in contrast to the substantial changes observed in MOLT-4 cells. This finding is consistent with previous studies which found that ionizing radiation induced an increase in bax mRNA in several leukemia and lymphoma cell lines that contain wild-type p53 but failed to induce bax expression in most solid tumor lines, or in leukemia and lymphoma cell lines that lacked p53, or those that contained mutant p53 (40).

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