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p53 Transactivation and Protein Accumulation Are Independently Regulated by UV Light in Different Phases of the Cell Cycle

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DNA damage-induced activation of the p53 tumor suppressor gene is suggested to be central in the cellular damage response pathway. In this study, we analyzed the responses of p53 to UVC radiation in synchronized mouse fibroblasts in terms of p53 accumulation, transcriptional activation, and sequence-specific DNA-binding activity. UVC was found to induce accumulation of p53 cell cycle dependently in G_1/S- and S-phase cells but not in G_0 or G_1 cells. In contrast, p53 transcriptional activity and its target genes, *p21* and *GADD45*, were stimulated by UVC in G_0 and G_1 cells in the absence of detectable p53 protein. The accumulation of p53 and increased *p21* and *GADD45* expression were replication dependent in S-phase cells. Interestingly, sequence-specific p53 DNA-binding activity was stimulated also replication independently in S phase, though the effect was not conveyed to stimulation of p53 target genes, suggesting that additional events are required for p53-stimulated gene expression. The results show that opposed to the cell cycle dependence of p53 accumulation, the UVC-mediated transactivation by p53 is independent of the cell cycle phase and protein stabilization.

The ability of cells to respond to genetic insults either by repairing DNA lesions or by cell suicide through apoptosis ensures the removal of genetically altered cells from the organism. The p53 tumor suppressor gene, based on its ability to control the cell cycle and mediate apoptosis, has been suggested to be a key controller of the integrity of the genome (31, 47). Growth arrest and apoptosis upon DNA damage are circumvented in tumor cell lines harboring p53 mutations (12, 26, 27, 30, 32) and in p53 knockout animals and cell lines derived therefrom (7, 35). DNA damage inflicted by a variety of treatments such as ionizing or UV radiation or exposure to alkylating agents or DNA nucleases causes nuclear accumulation of p53 and is associated with enhanced transcription of p53-responsive genes (14, 37, 43, 54). As a possible means of action in DNA damage, p53 has been suggested to bind to single-stranded DNA ends (2, 44) and DNA lesions (33) mediated through its C terminus, which results in protein stabilization and increased DNA-binding activity (25). Furthermore, latent p53 is directly activated with C-terminal peptides or with an antibody directed to the C terminus, suggesting that the activation involves a conformational change of the protein that relieves the interaction between the basic C terminus and the core domain (24). The ability of p53 to regulate genomic integrity has also been associated with transcriptional activation of its target genes, of which *p21*$^{CIP\text{-}1/WAF\text{-}1}$ (13, 14), *GADD45* (26), and *bax* (42) have been envisioned to participate in growth arrest, DNA repair, and apoptosis. However, depending on the cell type and inducing agent, apoptosis can proceed in the absence of functional p53 (4, 7, 35) or p53 transcriptional activity (5, 51).

The signals that initiate p53 action following environmental DNA damage of cells are not well known. First, the types of DNA damage, due to either direct DNA strand breaks, base damage, DNA cross-links, or agents that intercalate with DNA, vary considerably in their mode of action and in the time it takes the lesion to form. A precondition for p53 accumulation appears to be the presence of DNA strand breaks (37, 43). This fits well with the ability of p53 to bind DNA lesions and single-stranded DNA ends (2, 33, 44). For example, γ irradiation of cells, which generates direct DNA strand breaks, provokes a G_1 checkpoint arrest of cells, followed by rapid accumulation of high levels of p53 (26, 30). Similarly, drugs that induce direct DNA strand breaks and topoisomerase-targeted chemotherapeutic agents induce p53 accumulation with rapid kinetics (43). At least the action of camphotecin, a topoisomerase I inhibitor, has been shown to be DNA replication dependent, whereas γ radiation, DNA nucleases, or other agents creating targeted DNA lesions are not, suggesting that DNA strand breaks, either direct or exposed during replication, are required for p53 accumulation. UV damage of cells, on the other hand, causes the formation of pyrimidine dimers and (6-4) photoproducts and leads to p53 accumulation with significantly slower kinetics than γ radiation (37). DNA strand breaks in UV-damaged cells occur during excision repair processes or during DNA replication. If DNA strand breaks are a precondition for p53 accumulation and thereby its action, it could be assumed that in UV-damaged cells, its action would be largely replication dependent, in contrast to its apparent G_1 checkpoint function.

To study the action of p53 in UV-damaged cells, we have irradiated synchronized mouse fibroblasts and compared the cell cycle responses and p53 action. In a previous study, we observed a disjunction of p53 accumulation and G_1 arrest of the cells (21). UV irradiation of G_1-synchronized mouse fibroblasts caused an initial 12-h G_1 arrest of the cells followed by entry of the cells into S phase. However, p53 accumulation was observed only 12 h after the DNA damage, which correlated with a shift of the cells to S phase and the start of DNA replication. Second, the accumulation of p53 in G_1-synchronized cells occurred with significantly delayed kinetics compared to unsynchronous cells (12 h in G_1-treated cells and 6 h in asynchronous cells). The results thus suggested that the timing of p53 accumulation was discordant with the G_1 arrest

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(21). We therefore wanted to explore in detail the responses of synchronized cells to UVC radiation with respect to p53 accumulation, its transcriptional activity, and its DNA-binding activity. We show here that upon UVC damage of synchronized cells, the transcriptional activity of p53 precedes, and is dissociated from, its stabilization. While UVC stimulated transactivation by p53 in G_1- and S-phase cells, protein stabilization was detected only in cells at the G_1/S border and in S phase. Moreover, accumulation of p53, but not its DNA-binding activity, in S-phase cells was replication dependent. The results demonstrate that UV radiation-triggered p53 transcriptional activity is independent of events required for its stabilization, suggesting different, put perhaps overlapping, pathways in initiating p53-mediated DNA damage control of cells.

MATERIALS AND METHODS

Cell culture, synchronization, and UV treatment. NIH 3T3 (ATCC CRL 1658) cells were cultured in Dulbecco modified Eagle medium in the presence of 10% newborn fetal calf serum (NBCS; Gibco) at 37°C in a humidified atmosphere containing 5% CO_2. The cells were synchronized to G_0/G_1 by serum starvation for 8 h in the presence of 0.2% NBCS and released by replating in culture medium containing 10% NBCS as described previously (21). Cells were synchronized to G_1/S by initial serum starvation as described above, followed by replating of the cells in fresh medium in the presence of 0.25 mM hydroxyurea (HU; Sigma), and were incubated for 16 h (see Fig. 2A). HU alone did not affect p53 levels or p53 DNA-binding activity (see Fig. 3 and 6 and references 39 and 43). Cell cycle distribution was analyzed by flow cytometry analysis (FACScan; Becton Dickinson) of fixed and propidium iodide-stained cells (21). The percentage of DNA replicating cells was assayed by 5-bromo-2′-deoxyuridine (5-BrdU; Sigma) (50 μM) incorporation followed by immunostaining (21). 5-BrdU did not affect p53 accumulation (data not shown and reference 17). UV treatment at 254 nm was carried out with a Stratalinker 1800 (Stratagene) at a dose of 50 J/m^2. As shown in reference 21, over 50% of cells treated in G_1 with UVC at 50 J/m^2 entered the subsequent S phase, indicating that the cells tolerated the dosage well.

Immunoblotting and immunostaining analysis. Immunoblotting assays were carried out as described earlier (21). Briefly, cells were lysed with 25 mM Tris-HCl buffer (pH 8.0) containing 120 mM NaCl, 0.5% Nonidet P-40 (NP-40), 4 mM NaF, 100 μM Na_3VO_4, 100 kallikrein-inhibiting units of aprotinin per ml, 1 mM phenylmethylsulfonyl fluoride, and 10 μg of leupeptin per ml. Protein concentrations were determined by Bradford analysis. Lysates (300 μg) were analyzed by sodium dodecyl sulfate–12.5% polyacrylamide gel electrophoresis followed by transfer of proteins to membranes, probing with monoclonal antibody PAb 240 against p53 (Pharmingen) and polyclonal antibody M-19 against p21 (Santa Cruz Biotechnology, Santa Cruz, Calif.), and detection with enhanced chemiluminescence (Amersham). Equal loading was verified by staining of parts of the gel by Coomassie brilliant blue R and immunoblotting membranes by Ponceau S. For immunostaining, the cells were grown on glass coverslips, fixed with ice-cold methanol, permeabilized with 0.5% NP-40, and incubated with p53 antibody PAb 122 (Pharmingen) followed by rhodamine-conjugated rabbit anti-mouse antibody (Dako) (21).

Northern (RNA) blot analysis. Poly(A)$^+$ RNA isolation and Northern analysis were carried out as described in reference 29. RNA was detected by probing with p21 (13) or *GADD45* (16) cDNA inserts (kindly provided by B. Vogelstein and A. J. Fornace, Jr., respectively) labeled with [α-^{32}P]dCTP by random priming. Quantitations of the autoradiograms were carried out with a Fujifilm BAS-1500 image analyzer and the MacBAS 2.1 program. Fold inductions were calculated by normalizing the mRNA levels to the level of *GAPDH* and comparing the mRNAs of UV-treated and control cells.

Cell transfections and CAT analysis. NIH 3T3 cells were cotransfected with the PG13-CAT or MG15-CAT construct (kindly provided by B. Vogelstein) (28) and the pcDneo neomycin resistance construct (6) by calcium phosphate precipitation and were grown in the presence of 0.6 mg of G418 (Gibco) per ml for 2 weeks. Stable cell colonies from both transfections were trypsinized and pooled. For measurement of chloramphenicol acetyltransferase (CAT) activity, the cells were pelleted and lysed, and equal concentrations of protein were incubated with acetyl coenzyme A (Pharmacia) and 0.4 μCi of [^{14}C]chloramphenicol (Amersham) followed by separation of the acetylated chloramphenicol by thin-layer chromatography as described previously (19). The signals of the [^{14}C]chloramphenicol acetylated forms were quantitated by phosphorimage analysis as described above.

Electrophoretic gel mobility shift assays. Nuclear extracts were prepared as described previously (1). Oligonucleotides representing the consensus p53 binding site in the *p21$^{CIP-1/WAF-1}$* gene promoter (13), 5′-AATTCTCGAGGAACA TGTCCCAACATGTTGCTCGG-3′, were synthesized, annealed into a double-stranded form, and labeled with ^{32}P. Mutant oligonucleotide 5′-GAATTCTCG AGGAAAATTTCCCAAAATTTGCTCGAG-3′ was additionally used. Binding reaction mixtures contained 10 μg of nuclear extract, 10 μl of 2× binding buffer (40 mM HEPES-KOH [pH 7.9], 50 mM KCl, 0.2 mM EDTA, 20% glycerol, 4 mM MgCl, 1 mM dithiothreitol, 0.05% NP-40, 4 mM spermidine [Sigma]), 100 ng of poly(dI-dC) (Pharmacia), and, if indicated, 500 ng of monoclonal antibody in a final volume of 20 μl. Binding reaction mixtures were incubated at room temperature for 20 min, 0.2 ng of labeled oligonucleotide probe was added, and incubation was continued for an additional 20 min at room temperature. Reaction products were separated on a 4% nondenaturing polyacrylamide gel with 5% glycerol in 0.25× Tris-borate-EDTA buffer at 4°C. The gel was dried and exposed to X-ray film.

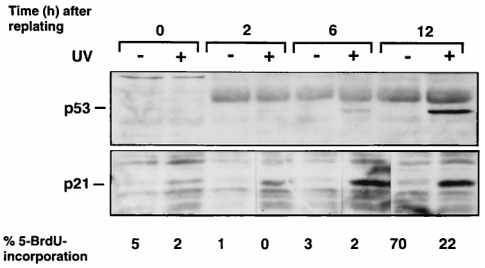

FIG. 1. UVC responses of G_0/G_1-synchronized NIH 3T3 fibroblasts. NIH 3T3 cells were synchronized by serum starvation and released by replating and addition of growth medium. Cells were treated with UVC (50 J/m^2) at the indicated times and were incubated for 6 h. Lysates of control cells (−) and UV-treated cells (+) were analyzed by sodium dodecyl sulfate–12.5% polyacrylamide gel electrophoresis followed by immunoblotting with p53 and p21 antibodies. The percentage of cells incorporating 5-BrdU for the last hour of incubation was determined by immunostaining and is shown below each lane.

RESULTS

Cell cycle-dependent accumulation of p53 induced by UV radiation. UV radiation is a potent inducer of accumulation of p53 (17, 22, 37, 39, 54). The accumulation is largely posttranslationally regulated, leading to an increase in protein half-life (17, 39). We have previously shown that UVC-irradiation of G_1-synchronized mouse fibroblasts causes a transient 10- to 12-h arrest of cells in G_1 and that p53 accumulates as the cells enter into S phase (21). In this study, the UVC-induced responses of G_1- and S-synchronized cells were explored further in terms of p53 stabilization and function. The cells were radiated with UVC (50 J/m^2) and incubated for 6 h, the dose and kinetics being optimal for detection of p53 in unsynchronized NIH 3T3 cells (21). In contrast to results obtained previously with unsynchronized cells, immunoblotting analyses of lysates from cells synchronized to G_0/G_1, UVC treated in G_0 or early to mid-G_1 (0 to 6 h after release), and incubated for 6 h indicated that p53 levels were low or undetectable (Fig. 1). Flow cytometry analyses (not shown) and 5-BrdU immunostainings showed that the cells were in G_1, with less than 5% of cells replicating DNA at the time of radiation or during the subsequent incubation (Fig. 1). However, high levels of p53 were found in cells UVC treated at the G_1/S border (12 h after release), with 70% of cells replicating DNA at the time of irradiation (Fig. 1). In long exposures of the immunoblotting chemiluminescence signals, p53 levels became detectable also in cells UVC treated 2 and 6 h after release, though the level of p53 in cells treated 12 h after the release was over 10- to 20-fold higher (Fig. 1). The synchronized UVC-treated cells were therefore examined by p53 immunostaining to determine the levels in individual G_1 cells. As shown in Table 1, the number of p53-positive cells increased significantly, from 6 to 50% in cells UVC treated 6 and 12 h after G_0 release, respectively, which correlated with the number of cells replicating DNA at the given time as measured by 5-BrdU incorporation (Table 1). This result is in accordance with our previous results indicating that p53 is detected only in UV-damaged

TABLE 1. DNA replication and p53 expression in G_0/G_1 UVC-treated cells

Time[a] (h)	% Immunopositive cells	
	5-BrdU	p53
Controls		
0	5	0
2	1	0
6	3	0
12	70	0
UVC treated		
0	2	2
2	1	6
6	3	6
12	38	50

[a] G_0-synchronized cells were restimulated and treated with UVC at the indicated time and incubated for 6 h.

cells in S or entering S after a G_1 arrest (21). Interestingly, as shown by immunoblotting, the cyclin kinase inhibitor $p21^{CIP\text{-}1/WAF\text{-}1}$ was induced by UVC in G_1 cells at a time when p53 levels were low or undetectable (Fig. 1, 2 and 6 h after release).

UVC-induced accumulation of p53 during S phase is dependent on DNA replication. The apparent accumulation of p53 only in G_1/S irradiated cells or cells entering S after a G_1 insult (Fig. 1 and reference 21) prompted us to study in detail the effects of UVC during the S phase. Cells were synchronized to G_1/S border by serum starvation and then replated in fresh medium in the presence of HU (Fig. 2A). As determined by flow cytometry analyses and 5-BrdU immunostainings, >80% of the cells released from the HU block progressed into S within 2 h, approached G_2/M by 6 h, and were in G_1 10 h after the release (Fig. 2B and C). Subsequently, cells were treated with UVC at the G_1/S border, in early S phase, and in mid-late S phase (times 0, 2, and 4 h) and monitored for 6 h. Comparison of the flow cytometry profiles of control and UV-treated cells indicated that all UV-treated cells were able to slowly progress further in S phase (Fig. 2B, UV).

To assess the rate and changes in DNA replication, UV-treated cells were labeled with 5-BrdU either for the whole incubation time (6 h) or for the last hour of incubation (1 h). Results shown in Fig. 2C indicated that 5-BrdU incorporation decreased from over 80 to 35% in cells UVC treated at time zero (G_1/S) and from 55 to <1% in cells UVC treated at time 4 h (mid-late S), suggesting that cells initially replicating DNA subsequently arrested in S. Addition of HU to the cells after

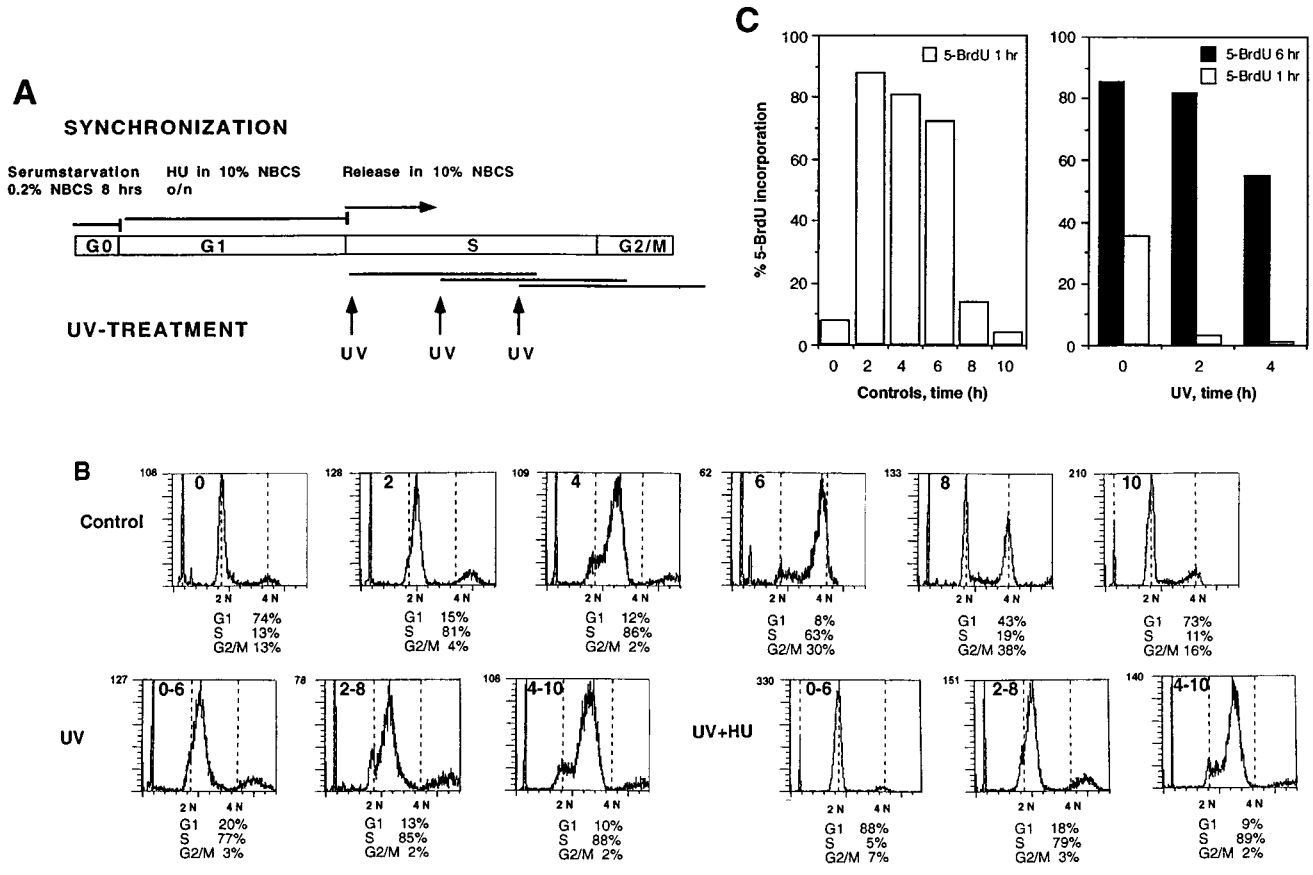

FIG. 2. Effect of UVC treatment on cell cycle parameters of G_1/S- and S-phase fibroblasts. Cells were initially arrested to G_0 by starvation in 0.2% NBCS for 8 h. To enrich cells at the G_1/S border, cells were stimulated with 10% NBCS in the presence of 0.25 mM HU. Subsequently, cells were released from G_1/S arrest and analyzed at the indicated times (controls) or were treated with UVC (50 J/m^2) 0, 2, or 4 h after the release and incubated for 6 h in the presence (UV+HU) or absence (UV) of HU (0.25 mM) as indicated. (A) Schematic representation of cell synchronization and UV treatment. (B) Flow cytometry profiles of fixed and propidium iodide-stained cells. The cell cycle distribution is indicated below each time point. *x* axis, 2N and 4N DNA contents of cells; *y* axis, number of events. (C) 5-BrdU incorporation. Cells were labeled with 5-BrdU for the last hour of incubation (1 h) or for the whole incubation time (6 h), and 5-BrdU incorporation was detected by immunostaining. The percentage of DNA-replicating cells in the presence of HU was negligible and is not shown.

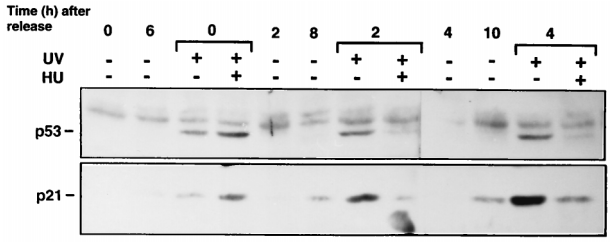

FIG. 3. Accumulation of p53 and p21 in UVC-treated G_1/S- and S-phase cells. p53 and p21 were detected by immunoblotting from lysates of cells synchronized to G_1/S, treated with UVC (50 J/m^2) (+) at the indicated times after the release in the presence (+) or absence (−) of HU, and incubated for 6 h. Lysates from unirradiated control cells were prepared at the indicated times.

UVC treatment caused an arrest of the cells to the prevailing position of the cycle, as judged by flow cytometry and 5-BrdU immunostaining (Fig. 2B and C, UV+HU).

Immunoblotting analyses of cells synchronized to G_1/S and S and treated with UVC as described above showed accumulation of p53 irrespective of time of irradiation (Fig. 3). However, inhibition of DNA replication by addition of HU to S-phase cells immediately after UVC treatment prevented the accumulation of p53 (Fig. 3, 2 and 4 h, UV+HU). Treatment of control cells with HU did not affect p53 levels. Interestingly, p53 was found in cells UVC treated at the G_1/S boundary and incubated with HU (Fig. 3, 0 h, UV+HU). Similarly, although basal levels of p21 in the control cultures increased toward the end of S and early G_1 as detected by immunoblotting (Fig. 3, 8 and 10 h), the induction of p21 in the UVC-treated samples was constant at all time points and was abrogated by HU, except at G_1/S (Fig. 3, quantitations not shown). This finding suggested that active DNA replication is required for p53 accumulation and p21 induction for cells residing in S phase during the insult, whereas the G_1/S-phase cells have retained the capacity to stabilize p53 and induce p21 despite inhibition of DNA replication.

Induction of p53 transcriptional activity by UVC in G_1- and S-phase cells. The apparent discrepancy of induction of p21, a transcriptionally regulated target of p53 (13), in both the presence and absence of high levels of p53 prompted us to analyze p53-mediated gene regulation and transcription. Northern analysis of mRNAs of UVC-treated synchronized cells indicated that *p21* and *GADD45* mRNAs were induced in G_1 (up to eight- and threefold, respectively), and in S (two- to threefold) (Fig. 4A and B). There was no induction of *p21* and *GADD45* mRNAs in the presence of HU in the S-phase UVC-treated cells (Fig. 4C), which, except in the case of the G_1/S-phase UVC-treated cells, correlated well with the p21 protein analyses (Fig. 3). The discrepancy with respect to the induction of p21 protein in G_1/S UV-treated cells in the presence of HU in the absence of an increase in mRNA levels was found in four independent experiments and is suggestive of regulatory events independent of p53 transcriptional stimulation.

The transcriptional activity of p53 was further assayed by using p53-responsive reporter plasmid construct PG13-CAT and as a control MG15-CAT with mutated p53 binding sites (28). NIH 3T3 cells stably transfected with the PG13-CAT or MG15-CAT construct were synchronized as described above and treated with UVC at different points in the cycle. As the increase in CAT protein synthesis was slow, incubations longer (10 h) than those used in the foregoing experiments were required (kinetics of induction studied in detail in asynchronous cells [not shown]). Further, since basal CAT activity in S-phase-treated PG13-CAT cells was increased, the S-phase cells were incubated for 18 h. UVC treatment of G_1-synchronized PG13-CAT cells was found to increase the CAT activity up to sixfold (Fig. 5A). In G_1/S- and S-phase-synchronized PG13-CAT cells, the CAT activity was increased threefold in both the presence and absence of HU (Fig. 5B). The CAT activities of MG15-CAT cells were less than 2% of that in PG13-CAT cells and are not shown.

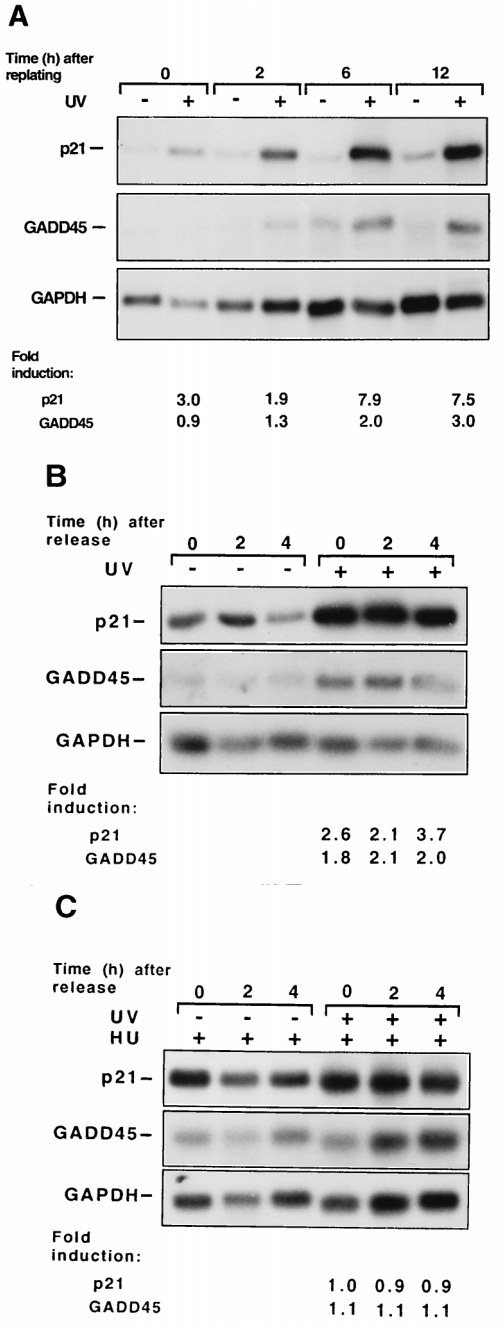

FIG. 4. Northern analysis of UVC-treated synchronized fibroblasts. Cells were synchronized to G_0 (A) or to G_1/S (B and C) and treated with UVC (50 J/m^2) at the indicated times after release from the arrest. HU was added to the cells after UV treatment as indicated (C, +). All UVC-treated cells were incubated for 6 h; then poly(A)$^+$ RNA was isolated and analyzed by Northern blotting and probed with *p21*, *GADD45*, and *GAPDH* cDNA inserts. The fold inductions by UVC, as measured by phosphorimage analysis, are shown at the bottom.

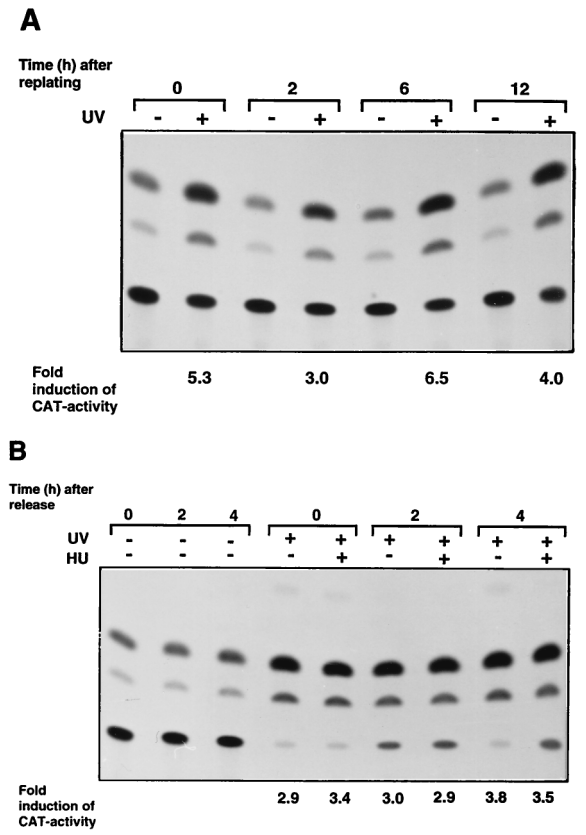

FIG. 5. Transcriptional activation of p53 in UVC-treated synchronized cells stably expressing the PG13-CAT construct. Cells synchronized to G_0/G_1 (A) or to G_1/S and S (B) were treated with UVC (50 J/m^2) at the indicated times after the release and were incubated for 10 h (A) or 18 h (B). Cells were lysed and assayed for CAT activity as described in Materials and Methods. The fold inductions by UVC shown below representative lanes are means of results from two experiments.

To detect changes in the capacity of p53 to bind sequence-specific motifs, gel electrophoretic mobility shift assays of synchronized UVC-treated cells were carried out with an oligonucleotide containing the *p21* p53-binding site (13). As shown in Fig. 6A and B, the mobility shift assays indicated that UVC radiation increased the DNA-binding capacity of G_0-, G_1-, G_1/S-, and S-phase cells and generated in each case a major and a lesser DNA-protein complex (dark arrows). An additional, slower-migrating complex was detected in S-phase UVC-treated cells (Fig. 6B, lanes 2 and 5, open arrow), which was visible also in longer exposures of G_1/S UVC-treated cells (not shown). In S-phase cells, the p53 DNA-binding capacity was independent of DNA replication, as it was not inhibited by HU (Fig. 6B, lane 5). The UVC-induced DNA-binding activity was shown to be specific for p53, as the complex was supershifted with p53 antibodies PAb 122 and 421 (Fig. 6B, lanes 6 and 7), was competed with excess unlabeled oligonucleotide (Fig. 6B, lanes 8 and 9), and was unreactive to a p21 oligonucleotide containing a mutant p53-binding site (Fig. 6C, lanes 6 to 9). Similarly, the UVC-induced p53 DNA-complex was not supershifted with a control antibody against retinoblastoma protein (Fig. 6C, lanes 4 and 5). Taken together, the results suggest that transcriptional activation and DNA-binding activity of p53 induced by UVC are independent of the cell cycle phase and DNA replication.

DISCUSSION

We show here that in synchronized murine fibroblasts, the UV-induced accumulation of p53 is a cell cycle-specific event which is detectable in G_1/S-phase cells and cells actively replicating DNA. Further, p53 DNA-binding activity is dissociated from the protein accumulation, as it is observed in G_1- and S-phase UV-treated cells in a replication-independent manner. The timing of the genetic insult leading to p53 stabilization is inconsequential, as UV treatment of both G_1- and S-phase cells leads to p53 accumulation. However, in G_1 UV-treated cells, p53 becomes detectable only when the cells overcome the

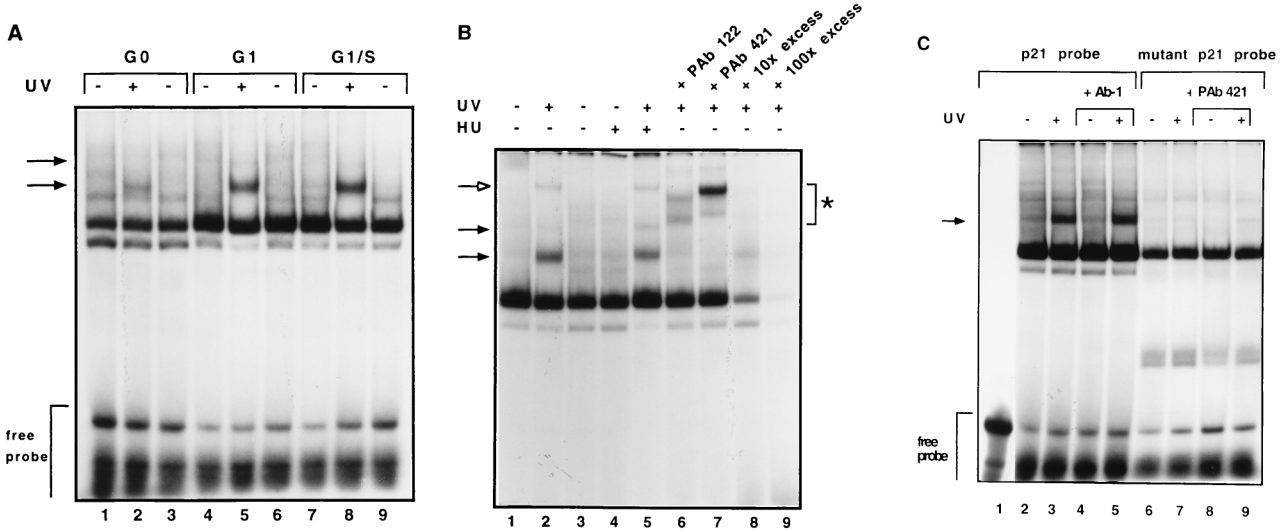

FIG. 6. Gel mobility shift assays of synchronized UVC-treated cells, using *p21* p53-binding oligonucleotides. Synchronized cells were treated with UVC at the indicated point in the cycle (+) and incubated for 6 h. DNA-binding assays were performed with labeled p21 oligonucleotides. (A) Cells synchronized to G_0, G_1, and G_1/S; (B) cells synchronized to G_1/S and treated 2 h after release with UVC treatment in the absence (lanes 2 and 6 to 9) or presence (lane 5) of HU. Antibodies PAb 122 (lane 6) and PAb 421 (lane 7) or excess unlabeled oligonucleotides (lanes 8 and 9) were added to the reactions as indicated. (C) Gel mobility shift assay of cells synchronized to G_1/S, using *p21* oligonucleotides (lanes 1 to 5) and mutant *p21* oligonucleotides (lanes 6 to 9). Monoclonal antibody against human retinoblastoma protein (Ab-1; Oncogene Science) (lanes 4 and 5) and PAb 421 (lanes 8 and 9) were added to the reactions as indicated. Lane 1, free probe alone. Solid and open arrows, p53-DNA binding complexes; asterisk, supershift of p53-DNA binding complexes with antibodies.

UV-induced G_1 arrest and enter S phase (21). As shown in Fig. 3, inhibition of p53 stabilization by HU indicates that active DNA replication is required. The replication dependence of p53 accumulation by UV is in agreement with the findings of Nelson and Kastan (43) but specifically extends these findings to a cell cycle-dependent regulation of p53 accumulation. However, we cannot exclude the possibility that p53 would accumulate also in G_0 or early G_1 cells, albeit with considerably slower kinetics. Nevertheless, this would not account for the rapid activation of p53 observed within 6 h. Similarly, discordance between accumulation of p53 and its transcriptional activity has been previously linked to UV dosage of cells, as low amounts of UVC (10 J/m^2) cause p53 transactivation in the absence of detectable protein (36, 37).

The cell cycle-dependent stabilization of p53 by UVC differs from the faster kinetics and apparent cell cycle independence elicited by γ radiation. Whereas γ radiation induces DNA strand breaks, UV causes pyrimidine dimer formation, and DNA strand breaks occur in a replication-associated manner or during nucleotide excision repair. The appearance of single- or double-strand DNA breaks upon DNA replication may serve as a major signal for p53 accumulation in UV-treated cells during S phase. Interestingly, p53 accumulation was detected at the G_1/S border even in the absence of DNA replication. This finding suggests that alternatively, at G_1/S, UVC stimulates stabilization of p53 independently of DNA replication, possibly through interactions of p53 with other cellular proteins, or that during initiation of DNA replication, damaged DNA strands are exposed sufficiently for p53 to bind and stabilize. However, since HU does not block initiation of replication which occurs throughout the S phase at multiple origins, stabilization of p53 should be observed also at subsequent points in S phase. Since p53 stabilization is mainly posttranslational and is prevented by cycloheximide (17, 27), it seems plausible that UV-stimulated protein synthesis at the G_1/S border is required for p53 stabilization in the absence of DNA replication.

In the absence of detectable p53 protein, the activation of p53 DNA-binding activity occurs in G_1- and S-phase cells replication independently. The activation is therefore caused directly by UV and is independent of protein stabilization. In consequence, although the sequence-specific DNA-binding activity of p53 is reported to be increased by single- or double-stranded DNA lesions (25, 33), it appears that p53 binding to DNA strand breaks generated during DNA replication of damaged templates is not a sole signal for its activation. Alternative, UV-stimulated events such as activation of p53 by protein phosphorylation or possibly protein-protein interactions may take place. Indeed, p53 is phosphorylated by multiple kinases, casein kinase II (23), JNK-1-like kinase (41), protein kinase C (49), cyclin-dependent kinase cdk2 (52), and DNA-dependent protein kinase (34), of which at least JNK-1 is activated in the UV response pathway (10). p53 can be transcriptionally active also in situations where DNA damage does not exist or protein synthesis is prevented through p53 conformational changes (24, 25), phosphorylation by S- and G_2/M-phase cyclin-kinase complexes (52), and stimulation of quiescent fibroblasts with serum in the presence of cycloheximide (38).

Induction of $p21^{CIP\text{-}1/WAF\text{-}1}$ by UVC may participate in both the G_1- and S-phase arrest of the cells, either by inhibiting cyclin-cdk activities required for cell cycle progression or by binding to proliferating cell nuclear antigen and thus preventing DNA replication (15, 50). Interestingly, p21 protein levels were enhanced by UVC during S phase only in DNA-replicating cells. Even though p53 DNA-binding activity was stimulated replication independently in cells expressing p53-responsive constructs, using gel mobility shift assays, the *p21* and *GADD45* mRNA levels were not induced by UVC in the presence of HU. It therefore appears that though p53 DNA-binding activity is stimulated in cells in which replication is prevented, an additional modification, perhaps by stabilization, is required for p53 to effectively induce *p21* and *GADD45* mRNA levels. Alternatively, it can be speculated that p53 affects also posttranscriptional events or that part of the UV response of these genes is p53 independent. Discrepancies in the mRNA and protein levels of p21 have been noted previously in γ-irradiated epithelial cells (20), during differentiation of murine erythroleukemia cells, and in serum-stimulated growth-arrested fibroblasts (38), where a sustained increase of p21 protein was found in the absence of increased mRNA. Therefore, induction of p21 protein in the UV-damaged S-phase cells may require protein-protein interactions with, e.g., replication-associated proteins like proliferating cell nuclear antigen (53). Furthermore, p21 is transcriptionally regulated also in a p53-independent manner by growth factors, differentiation, cell senescence, and tumor promoters (46).

Despite the highly suggestive presence of p21 both in G_1- and S-phase cells and its capacity to hinder cell cycle progress, we cannot firmly ascribe the UV-mediated arrest in either G_1 or S phase to p53. First, the DNA insult during G_1 leads to an approximately 12-h arrest of the cells (21), while no p53 protein is detected. However, at this point, p53 is transcriptionally active and may elicit G_1 arrest through regulation of cell cycle genes. Other pathways leading to growth arrest of DNA-damaged cells may also be in use. Indeed, we have previously shown that the retinoblastoma tumor suppressor protein is converted to its underphosphorylated form by UVC parallel to growth arrest of several cell types, including p53 mutant cells (21). Similarly, p21 induction and subsequent decreases of cdk activities by UVB in keratinocytes have been associated with the G_1- and S-phase arrests (45). Second, cells with mutant p53 alleles, cells expressing large T antigen sequestering p53 and prohibiting its transcriptional functions, and $p53^{-/-}$ fibroblasts (reference 21 and data not shown) arrest upon UV radiation. Detailed analysis using synchronized cells should, however, be carried out to determine at which point in the cycle the arrest occurs. A deficiency in the ability of cells to replicate DNA upon UV irradiation was found by Lu and Lane (37) and was attributed to a switch from active DNA replication to repair synthesis. While p53 is a checkpoint controller of G_1, G_2, and M (3, 8, 11, 18, 40, 48), there is no direct evidence that its presence or function is required for arrest of UV-damaged cells. However, a damage control function for p53 is suggested (7, 12, 26, 27, 30–32, 35, 37, 47). Based on the cell cycle-dependent differences in p53 regulation by UV, showing dissociation of p53 activation from its stabilization, multiple, perhaps complementing functions for p53 can be suggested. The rapid transactivation of p53-responsive genes such as *p21* and *GADD45* probably initiates a DNA damage response pathway possibly involving growth arrest or DNA repair of the cells. The observed replication-dependent stabilization of p53 may reflect its binding to damaged DNA strand ends, signifying irreparable DNA damage with potential initiation of the apoptotic pathway.

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