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The mdm-2 gene is induced in response to UV light in a p53-dependent manner

(cell cycle/transcription)

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ABSTRACT **Irradiation of mammalian cells with UV light results in a dose-dependent accumulation of the p53 tumor-suppressor gene product that is evident within 2 hr. UV treatment causes a dramatic increase in p53-specific transcriptional transactivation activity and an increase in expression of the p53-responsive gene mdm-2. UV-stimulated mdm-2 expression is not directly correlated with the level of p53 protein in a cell because mdm-2 induction is delayed at high UV doses even though p53 levels rise almost immediately. Cells lacking p53 protein do not respond to UV by increasing their expression of mdm-2. The delayed induction of mdm-2 at high UV doses suggests that, in addition to p53 protein levels, other factors contribute to the regulation of mdm-2 expression following UV treatment. The time of induction of mdm-2 in cells treated with UV light correlates with recovery of normal rates of DNA synthesis, presumably after DNA repair. These data indicate a possible role for mdm-2 in cell cycle progression.**

Mammalian cells respond to irradiation with UV light by transiently decreasing both RNA and DNA synthesis and by inducing expression of several genes whose products are thought to have protective effects against DNA damage (1). The regulation of these UV response genes appears to be mediated by several transcription factors which function after UV exposure (2). The p53 tumor-suppressor gene product is a transcription factor (3, 4) that also appears to be involved in the response to UV light. The p53 protein levels increase due to the stabilization of this protein in both murine (5, 6) and human (7, 8) cells treated with UV light. Although the role of p53 in the response to UV light has not been fully characterized, this tumor-suppressor protein has been shown to act as a cell cycle checkpoint in the response to γ irradiation (9, 10). γ irradiation induces both a G_1 and a G_2 phase-specific cell cycle block, and expression of wild-type p53 is necessary for the G_1 but not the G_2 block. The specific DNA-binding activity of p53 is increased after γ irradiation and a DNA damage-inducible, growth arrest-specific gene, GADD45, has been shown to contain a p53 response element (10).

Characterization of the role of p53 in the response to γ irradiation led to the hypothesis that p53 acts as a cell cycle checkpoint, causing a delay in the G_1 phase of the cycle during which damage is thought to be repaired (9, 10). It seemed likely that p53 might also act as a checkpoint in the response of cells to UV exposure. In addition, Zhan *et al.* (8) have recently shown that p53 transcriptional transactivation activity is increased in human cells exposed to UV light. Possible targets for p53 transcriptional transactivation activity in the UV response are the GADD45 gene, which was isolated as a UV response gene (11), and the mdm-2 gene. p53 and mdm-2 appear to form a feedback control loop: while p53 can induce mdm-2 transcription (12, 13), high levels of mdm-2 protein inhibit p53 transcriptional transactivation activity (13, 14). The results reported here demonstrate that treatment of murine cells with UV light results in a suppression of DNA synthesis that is independent of the presence of p53. In cells expressing functional wild-type p53, the mdm-2 gene is induced following a lag time that increases with UV dose. This enhanced expression of mdm-2 precedes the appearance of cells entering S phase at normal rates of DNA synthesis. These data are consistent with the idea that mdm-2 may reverse the possible negative regulatory effects of p53 in the G_1 phase of the cell cycle and/or promote the entry of cells into S phase after DNA damage is presumably repaired.

MATERIALS AND METHODS

Cell Culture. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum in an atmosphere of 10% CO_2.

UV Light Treatment. Cells were treated with UV light essentially as described (5). The medium was removed and the cells were irradiated with an 8-W germicidal lamp delivering 1.9 $J \cdot m^{-2} \cdot sec^{-1}$ at a distance of 40 cm. The dose was quantitated with a J-225 short-wave UV meter (Ultraviolet Products, San Gabriel, CA).

Flow Cytometry. Cells were labeled for 30 min with 10 μM BrdUrd at various times following UV irradiation. The cells were trypsinized, fixed in 70% ethanol (−20°C), and later stained with anti-BrdUrd antibodies conjugated to fluorescein isothiocyanate (Becton Dickinson) essentially as recommended by the manufacturer. Samples were analyzed with an EPICS 753 cell sorter (Coulter) with an MDADS II data system. An argon laser (488 nm, 500 mW) was used for the measurement of the logarithm of fluorescein fluorescence at 515–530 nm and linear propidium iodide fluorescence at >610 nm.

Western Analysis. Following incubation of 3 mg of each lysate with the monoclonal antibodies PAb419 (specific for simian virus 40 large T antigen) or PAb421 (specific for p53) or with anti-mdm-2 serum, the immunoprecipitates were separated by SDS/polyacrylamide gel electrophoresis along with high molecular weight prestained markers (Bethesda Research Laboratories) and blotted onto Hybond ECL nitrocellulose (Amersham). The membrane was sliced at the 68-kDa prestained marker and the half of the membrane containing the higher molecular weight proteins was incubated with the anti-mdm-2 serum while the other half was incubated with PAb421. Antibody binding was detected with

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Abbreviations: CAT, chloramphenicol acetyltransferase; MCK, muscle-specific creatine kinase.

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^{125}I-protein A (NEN), and quantitation was on a Molecular Dynamics PhosphorImager.

Chloramphenicol Acetyltransferase (CAT) Assays. The transfection and UV treatment protocol is essentially that of Devary *et al.* (2). A total of 10 μg of DNA (5 μg of CAT construct and 5 μg of salmon sperm carrier DNA) was used, and CAT assays were as described (15). Data were quantitated on a PhosphorImager and numbers quoted in the text are the average of two duplicates. The total conversion of [^{14}C]chloramphenicol to acetylated product was <40% in all reactions.

RESULTS

Wild-Type p53 Protein Increases in a Dose-Dependent Manner in Response to UV Irradiation. Maltzman and Czyzyk (5) showed that p53 levels in BALB/C3T3 cells rose within the first 2 hr after UV treatment. Two different immortal murine cell lines (C127 and 12.1) expressing wild-type p53 protein (16, 17) were treated with increasing doses of UV light and analyzed for changes in p53 levels. Both cell lines showed increasing p53 protein with increasing doses (Fig. 1 and data not shown). At the highest dose used (24.7 J/m^2, 3% colony-forming ability, Fig. 1) C127 cells exhibited a 4-fold increase in the incorporation of [^{35}S]methionine into the p53 protein immediately following UV treatment. The greatest increase in p53 expression in the first 2 hr after UV treatment is in cells sustaining the most damage. Fritsche *et al.* (6) have indicated a possible conformation change in the p53 protein stabilized in response to some types of DNA damage. Monoclonal antibody PAb246, which recognizes the wild-type murine p53 protein (18), reacted with virtually all the p53 present in these cells before and after UV treatment. Antibody PAb240, which recognizes a mutant or denaturation-specific epitope, did not react after UV treatment, and so no evidence for such a conformational change of p53 protein could be demonstrated in this system.

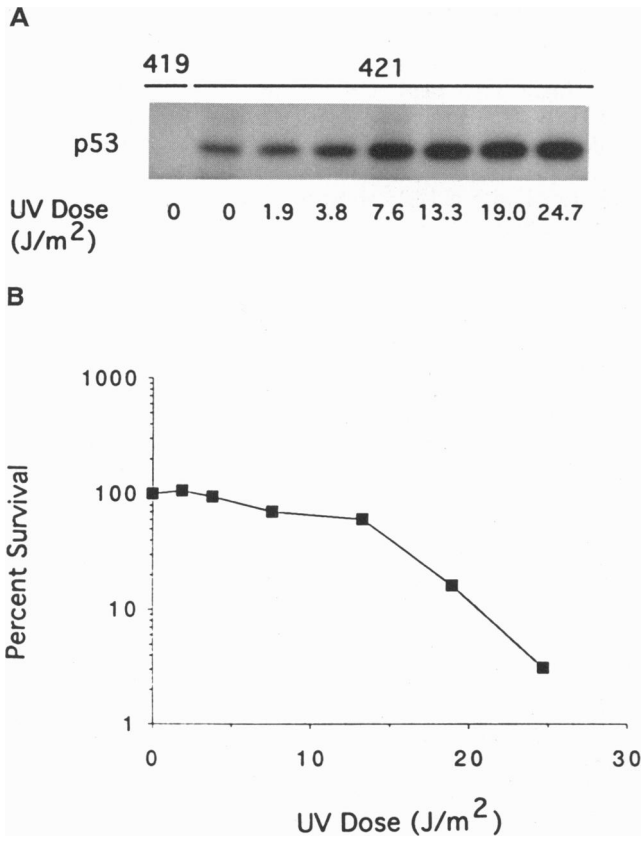

FIG. 1. Increase in p53 as a function of UV dose and inhibition of colony formation ability. (*A*) C127 cells were irradiated with a UV lamp at the indicated doses and then immediately labeled for 2 hr with [^{35}S]methionine. Normalized aliquots of labeled cell extracts were incubated with either PAb421, a p53-specific monoclonal antibody, or PAb419, a negative control. (*B*) C127 cells were plated at a low density, irradiated with various doses of UV light, incubated in growth medium for 1 week, and scored for colony formation.

mdm-2 Protein Increases in Response to UV Irradiation. Since the mdm-2 gene is regulated by the p53 protein (12, 13) and the mdm-2 protein is known to bind to the p53 protein and inhibit p53 transcriptional transactivation (13, 14), it was of interest to determine whether mdm-2 levels increased in response to UV treatment. The steady-state levels of both p53 and mdm-2, as well as the proportion of each bound in complex with one another, were measured by Western blot analysis. C127 cells were treated with either a low (3.8 J/m^2, 94% colony-forming ability) or a high (19 J/m^2, 16% colony-forming ability) UV dose. The cells were harvested at various times after UV treatment, lysed, and incubated with either a p53-specific antibody (PAb421) or an antiserum against mdm-2. The immunoprecipitates were electrophoresed and blotted onto a membrane, the top half of which was incubated with the anti-mdm-2 serum and the bottom half with PAb421. When C127 cells were treated with the low dose of UV, the level of p53 protein rose within 1.5 hr, and mdm-2 protein levels increased and peaked by 2.5 hr following treatment (Fig. 2*A*). Both p53 and mdm-2 proteins increased about 3- to 4-fold in response to the low dose of UV (Fig. 2*A*). However, the proportion of p53 bound to mdm-2 (20–30%) did not change significantly with time, and at no time was a majority of the p53 bound to mdm-2.

When C127 cells were treated with the high dose of UV, p53 levels rose more dramatically: at the low dose of UV (3.8 J/m^2), the maximal increase in p53 levels was about 3-fold, whereas at the high dose (19 J/m^2), p53 levels increased by as much as 60-fold at 8 hr. The steady-state levels (Fig. 2*B*) reflect a larger increase in p53 than the results with labeling with [^{35}S]methionine (Fig. 1), consistent with the demonstration by Maltzman and Czyzyk (5) that the p53 protein is stabilized by UV treatment. The increase in mdm-2 protein was also greater at the high dose and peaked at 13-fold 10 hr after UV treatment (Fig. 2*B*). Thus, the dose of UV affects both the timing and the level of induction of mdm-2. Comparison of the levels of p53 and mdm-2 at 4 hr after treatment clearly shows that the induction of mdm-2 expression at the high dose lags behind the increase in p53 levels. Although p53 levels are higher 1.5 hr after treatment with a high dose than after treatment with a low dose, the induction of mdm-2 expression is delayed after the high dose.

The Western blots (Fig. 2) quantitate the steady-state levels of p53 and mdm-2 in cells treated with UV light. Similar experiments following the incorporation of [^{35}S]methionine into p53 and mdm-2 proteins in these cells demonstrated a lag in increased expression of mdm-2 protein following stabilization of p53 at high UV doses. At high UV doses, p53 levels rose rapidly (Fig. 1*A*), but mdm-2 protein levels increased only after a significant delay (data not shown).

mdm-2 mRNA Is Induced by UV Light in a p53-Dependent Manner. Northern blot analysis of RNA from C127 cells treated with low and high doses of UV and harvested at various times after treatment showed a good correlation between increased mdm-2 message and protein levels (data not shown). At a low dose of UV light (3.8 J/m^2), mdm-2 mRNA increased and peaked 2 hr after treatment, which was the time at which the amount of protein also was maximal (Fig. 2*A*). At higher UV doses, the peak increase in mdm-2 protein was delayed (Fig. 2*B*) and the subsequent increase in mdm-2 protein levels was accompanied by increased mdm-2 mRNA (data not shown). This UV-stimulated increase in

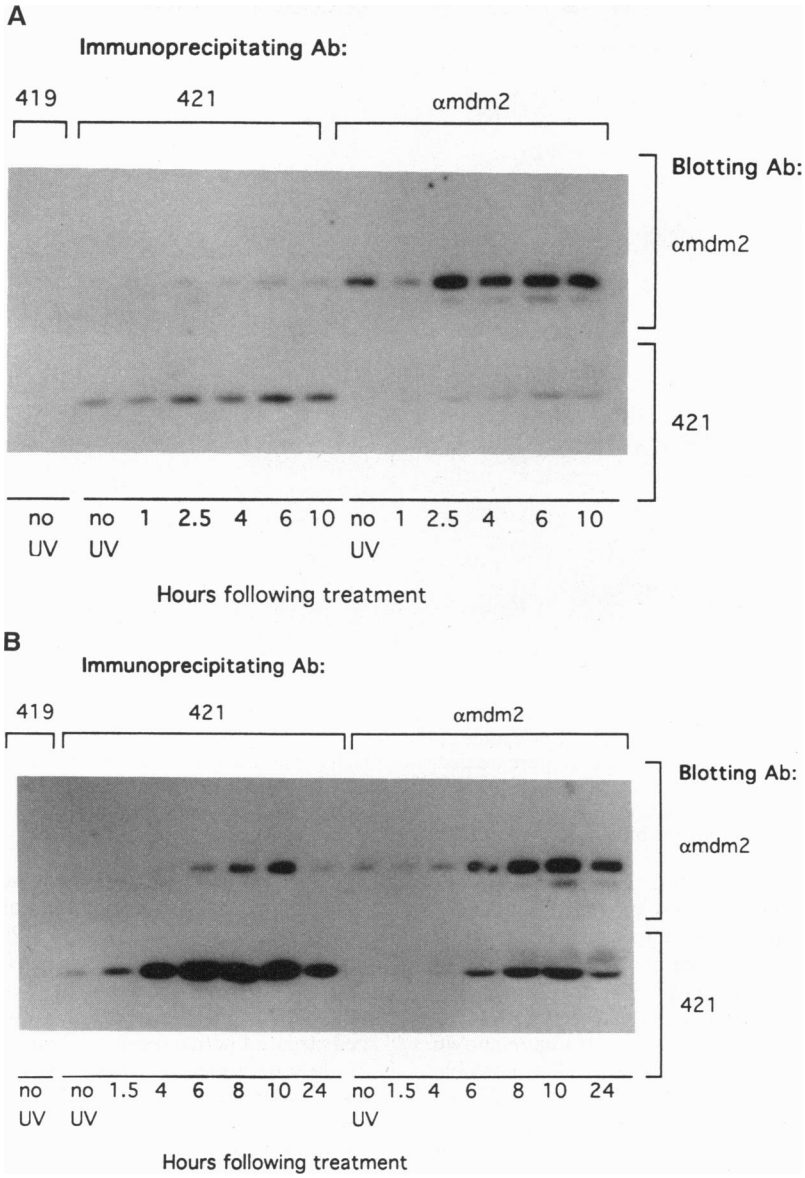

FIG. 2. Levels of p53 and mdm-2 change as a function of dose. (*A*) C127 cells were treated with a UV dose of 3.8 J/m^2, harvested at the indicated times following treatment, and incubated with either PAb419 (negative control), PAb421 (specific for p53), or mdm-2 antiserum (right side of blot). The immunoprecipitates were electrophoresed in a polyacrylamide gel and blotted onto a membrane, the top half of which was incubated with the mdm-2 antiserum while the bottom half was incubated with PAb421. (*B*) C127 cells were exposed to 19 J/m^2 and analyzed as in *A*.

both mdm-2 message and protein was not observed in cells lacking functional p53. Neither (10)3 cells (16), which lack p53 protein, nor (12)1 cells containing T antigen, which express wild-type p53 protein in the presence of the simian virus 40 large T antigen, induce expression of mdm-2 following UV treatment. Therefore, the induction of mdm-2 expression appears to be dependent upon the transcriptional transactivation activity of p53 protein.

p53 Transcriptional Activity in Cells Treated with UV Light. Next, the transcriptional transactivation activity of endogenous murine p53 was measured after treatment of C127 cells with UV light. A construct containing two copies of the consensus p53 DNA binding site from the murine muscle-specific creatine kinase (MCK) promoter linked to a reporter construct containing the CAT gene (15) was induced 39-fold after treatment of C127 cells with 40 J/m^2 (Fig. 3*A*) whereas a similar construct lacking the MCK sequences (1634CAT) was not induced more than 2-fold. Since expression of the mdm-2 gene is regulated by p53 through a p53-binding element in the first intron of the gene (13), three fragments from the first intron of the mdm-2 gene were tested for UV inducibility. Constructs containing the entire intron (COSX1CAT) or an 85-bp fragment including the two imperfect p53 consensus binding sites (BP100CAT) (13) were induced 3.3- and 8.4-fold by a UV dose of 40 J/m^2 (Fig. 3*B*). A third construct, containing 400 bp from the intron excluding the p53 binding sites (HX0.5CAT), was not UV inducible (<10% increase in CAT activity) (Fig. 3*B*). Although the 85-bp fragment conferred UV inducibility on the CAT gene in C127 cells, it did not do so in (10)3 cells, which lack p53 protein (<2-fold induction at 40 J/m^2; data not shown). Because this UV response element is also a p53 response element (13) and it is not induced in cells lacking p53 protein, these experiments indicate that mdm-2 is a p53-inducible UV response gene.

mdm-2 Expression Precedes Normal Entry of Cells into S Phase. Under some circumstances, overexpression of the p53 protein causes cells to arrest in the G_1 phase of the cell cycle

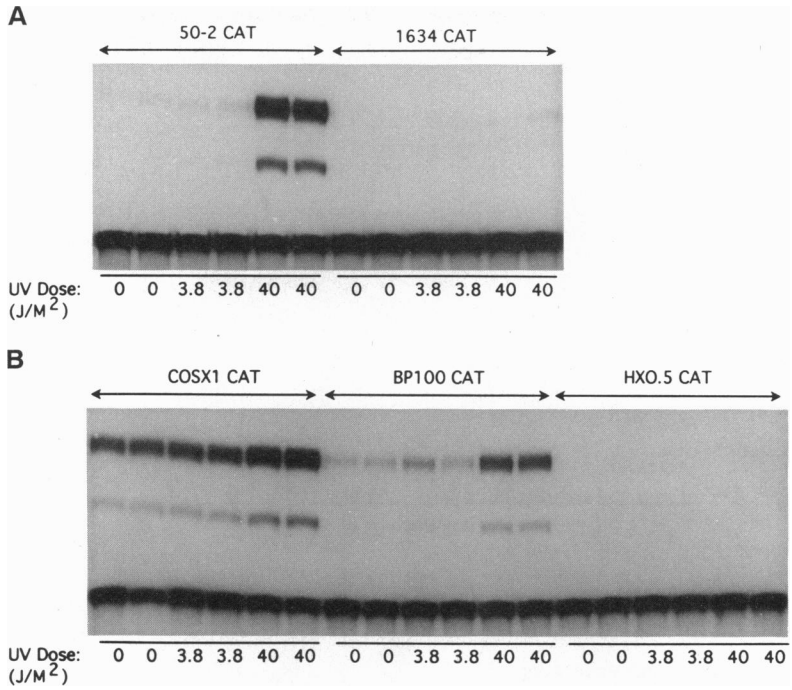

FIG. 3. p53 transcriptional transactivation activity is increased in response to UV light. (*A*) C127 cells were transfected with a DNA construct containing the CAT reporter gene fused to a minimal promoter (1634CAT) or to the p53 response element from the murine MCK promoter (50-2CAT). Twenty-four hours after transfection, cells were treated with a high or a low dose of UV light and after another 24 hr, the cells were harvested and lysed. Equal amounts of cell extracts were assayed for CAT activity. (*B*) Three fragments from the first intron of the mdm-2 gene were tested for UV inducibility in C127 cells by the protocol described in *A*; COSX1CAT contains the entire intron, BP100CAT contains 85 bp that include two imperfect p53 consensus binding sites, and HX0.5CAT contains 400 bp from the first intron excluding the p53 binding sites.

(9, 19). While the function of mdm-2 is not understood, overexpression of the protein has been shown to inhibit p53 transcriptional transactivation activity in transient transfection experiments (13, 14). It has been proposed that mdm-2-mediated inhibition of p53 transcriptional transactivation activity may lead to progression of cells from the G_1 phase to the S phase of the cycle (20). Kastan *et al.* (10) have shown that, after γ irradiation, p53 blocks cells in G_1. It is possible that mdm-2 functions to overcome this G_1 block. To see whether a similar block occurs in UV-treated cells, cells were labeled with the thymidine analogue BrdUrd and analyzed for BrdUrd content by fluorescence-activated cytometry using a fluorescein-conjugated monoclonal antibody specific for BrdUrd. Cells were also stained with propidium iodide, which reflects the total amount of DNA in each cell, whereas BrdUrd incorporation reflects nucleotide incorporation or DNA synthesis in each cell. Flow cytometry profiles of C127 cells treated with a moderately high UV dose of 14 J/m^2 (Fig. 4) showed a clear suppression in BrdUrd incorporation in early, mid, and late S phase by 4 hr after UV treatment. The percentage of UV-treated cells in G_1, S, and G_2 remained fairly constant (propidium iodide). Six to 8 hr after UV

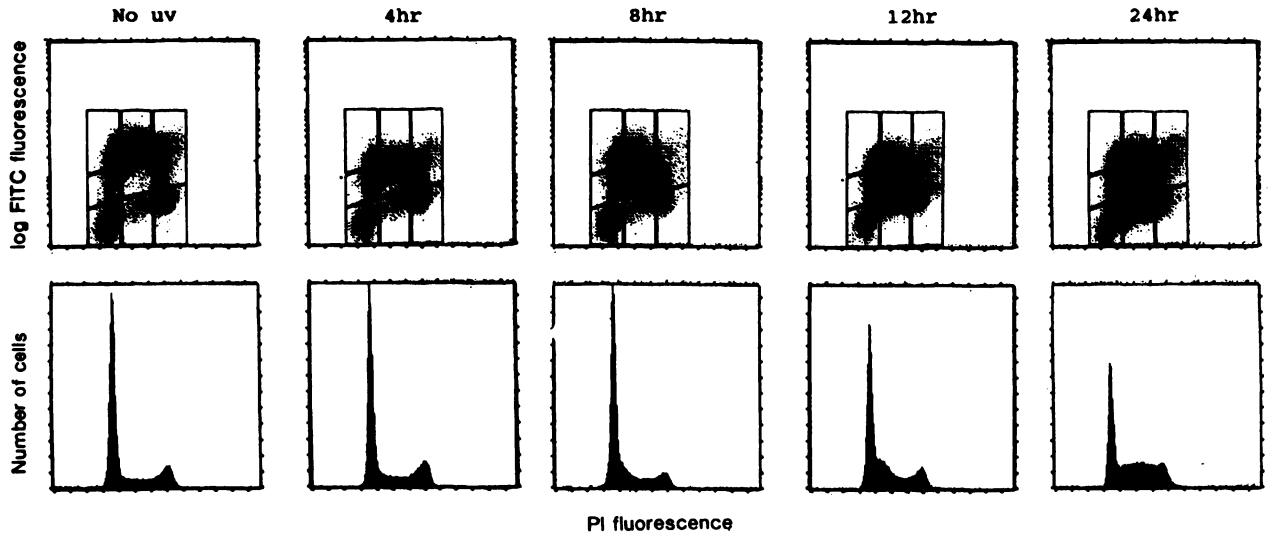

FIG. 4. Expression of mdm-2 precedes the resumption of normal rates of DNA synthesis following UV treatment. C127 cells treated with a UV dose of 14 J/m^2 were labeled with 10 μM BrdUrd for 30 min at the indicated times following treatment. Cells were harvested, processed for fluorescence-activated cytometry, and stained with anti-BrdUrd conjugated to fluorescein isothiocyanate (FITC) and counterstained with propidium iodide (PI). For each time point, the BrdUrd profile is shown above the propidium iodide profile. Fifty thousand cells were analyzed for each time point.

irradiation, mdm-2 expression was maximal (data not shown), and at 8 hr there was a pronounced increase in the incorporation of BrdUrd into cells with a G_1-phase content of DNA (cells in early S phase) as seen by a recovery of high BrdUrd fluorescence (Fig. 4). In fact, there was a greater percentage of cells in early S phase 8 hr after UV treatment than in untreated asynchronous cells. There was also a shoulder on the G_1 peak of cells stained for DNA content (propidium iodide), indicating an increase in the number of cells in early to mid S phase at 8 hr. This trend continued as this wave of cells proceeded through S phase (Fig. 4).

Thus, during the 6- to 8-hr lag before the resumption of normal rates of DNA synthesis, either there must be an accumulation of cells in G_1 which synchronously enter S phase 8 hr following UV treatment or, alternatively, the progression of cells from G_1 to S phase must be accelerated by some signals provided prior to 8 hr following treatment. The wave of cells moving through S phase with normal rates of DNA synthesis might well have resulted from p53 and mdm-2 interactions, since (10)3 cells, which lack p53 protein, also exhibited a decrease in DNA synthesis following UV irradiation but failed to recover from this suppression with a wave of cells incorporating normal levels of BrdUrd (Fig. 4*B*). The (10)3 cells also failed to induce comparable levels of mdm-2 message and protein after UV irradiation (data not shown).

To substantiate the correlation between recovery of normal S-phase DNA synthesis with expression of mdm-2 protein, C127 cells were treated with a UV dose of 3.8 J/m^2. At this low dose, mdm-2 levels peak at 2–2.5 hr following treatment (Fig. 2*A*). A significant increase in cells with a G_1 DNA content incorporating a large amount of BrdUrd appeared 2 hr after UV treatment (data not shown), so mdm-2 expression precedes the resumption of a normal rate of DNA synthesis following UV treatment with either a high or low UV dose. At high doses, enhanced expression of mdm-2 and a return to normal S-phase levels of DNA synthesis were delayed when compared with the response at lower UV doses.

DISCUSSION

Irradiation of mammalian cells with UV light results in a transient inhibition of DNA and RNA synthesis with a coordinate induction of expression of several immediate early genes such as c-*jun* and c-*fos* that in turn appear to regulate expression of other UV response genes (1). It has been proposed that this transient decrease in DNA and RNA synthesis following UV treatment reflects the time during which the UV-induced damage is repaired before the cells continue through the cycle (1, 21). This decrease could be brought about by a checkpoint control mechanism (21), and the p53 tumor-suppressor gene product, which accumulates in response to DNA damage (5–7, 10), acts as a cell cycle checkpoint in the response to γ irradiation (9).

The wild-type p53 protein plays a role in blocking progression through the G_1 phase of the cell cycle after γ irradiation, resulting in a complete inhibition of DNA synthesis in S-phase cells following treatment (9, 10). In contrast, after UV irradiation, there is a decreased rate of DNA synthesis both in cells containing wild-type p53 protein and in cells lacking p53 protein. p53 levels increase within 1–2 hr after UV irradiation and continue to accumulate when cells are treated with a high dose of UV. The high levels of p53 protein, up to 60-fold normal levels, might be expected to block cells from progressing out of G_1 into S phase (19, 22), but there is no direct evidence for a G_1 block in these experiments. The transcriptional transactivation activity of p53 is dramatically increased following UV treatment, and one target of p53 function in the UV response is the mdm-2 gene, which contains a p53 response element in the first intron (13). At low doses of UV irradiation, the responsiveness of this element is not apparent in a transient transfection assay (Fig. 3*B*), but the induction of mdm-2 occurs rapidly (see Fig. 2*A*) and is dependent on the presence of p53, since it does not occur in cells expressing simian virus 40 large T antigen (data not shown) or in cells lacking p53 protein. At higher doses, the synthesis of mdm-2 mRNA and protein are delayed several hours after the stimulation of high p53 protein levels in response to irradiation. Thus, at high UV doses other factors, in addition to p53 protein, are required for mdm-2 induction and these factors are rate-limiting for mdm-2 expression. Presumably, mdm-2 synthesis is delayed to permit repair synthesis to occur before normal rates of scheduled DNA synthesis can resume, and this delay is lengthened with increased amounts of DNA damage.

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