Differential Regulation of the p21/WAF-1 and *mdm2* Genes after High-Dose UV Irradiation: p53-Dependent and p53-Independent Regulation of the *mdm2* Gene

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ABSTRACT

Background: DNA damage in mammalian cells stabilizes the p53 protein which then functions as a cell cycle checkpoint by leading to growth arrest or apoptosis. p53 is a transcription factor and positively regulates the expression of the p21/WAF-1 gene and the *mdm2* gene. After high-dose UV irradiation, p53 increases the expression of the p21/WAF-1 gene immediately (2 to 5 hours after irradiation) while the induction of the *mdm2* gene is delayed (8 to 12 hours after irradiation). Experiments presented here explore this differential expression of two different p53-regulated genes.

Materials and Methods: IP-Western (protein) and Northern (mRNA) blot experiments are used to follow *mdm2* and p21/WAF-1 expression in primary rat or mouse cells after a low-dose (4 J/m^2) or a high-dose (20 J/M^2) of UV irradiation. Northern blot and nuclear run-on experiments are employed to study mRNA stability as well as transcription rates of selected genes.

Results: After high-dose UV irradiation, p53 is rapidly stabilized and the expression of p21/WAF1 is immediately increased. By contrast, both protein and mRNA levels of *mdm2* first decrease in a p53-independent manner, and later increase in a p53-dependent manner. The initial decline of *mdm2* expression following high-dose UV irradiation is UV-dosage dependent and regulated at the level of transcription.

Conclusion: p53 regulates two genes, p21/WAF1 (blocks cell cycle progression) and *mdm2* (reverses p53 activity), that mediate opposite actions. This process is regulated in a temporal fashion after high-dose UV irradiation, so that cell cycle progression can be halted while DNA repair continues prior to reversal of p53-mediated arrest by *mdm2*.

INTRODUCTION

The *mdm2* gene was originally identified as a gene that was amplified and overexpressed in a spontaneously transformed mouse 3T3 cell line (1). Two lines of evidence suggest that *mdm2* has oncogenic potential. First, *mdm2* is amplified in a significant percentage of human sarcomas (2–4). It is also overexpressed in a variety of other types of tumors, including leukemias (5), breast carcinomas (6), lymphomas (7), and malignant gliomas (8). Second, overexpression of *mdm2* immortalizes primary cultures of rodent fibroblasts and cooperates with an activated *ras* gene to transform these cells (9,10). The oncogenic properties of Mdm2 have been attributed to its ability to complex with p53 and inhibit p53's tumor suppression functions (2,10–13). p53 is a transcription factor which regulates the expression of a number of genes involved in cell cycle control and the apoptotic pathways. For instance, p53 activates expression of the cyclin-dependent kinase inhibitor p21/WAF1 (14), the growth inhibitory protein GADD45 (15) and the apoptosis-promoting protein Bax (16). In addition, it was recently shown that p53 represses the expression

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of the microtubule-associated protein MAP4 (17). It is believed that these types of transcriptional regulation by p53 are directly responsible for its tumor suppression function. Mdm2 negatively regulates p53 function by binding to p53 and blocking its transcriptional activities. Interestingly, the *mdm2* gene itself can be induced by p53 (18,19). This creates a feedback loop in which p53 induces *mdm2* expression, and Mdm2 binds to p53 and inhibits its transactivation activity. This loop results in the autoregulation of *mdm2* expression as well as modulating p53's transcriptional activity which is essential for controlling cell cycle progression (18,20). In fact, lack of p53 regulation by Mdm2 can be lethal; mice deficient in Mdm2 die in early stages of embryonic development and this embryonic lethality can be rescued or suppressed by the absence of p53 (21,22).

Following exposure to DNA-damaging agents such as UV or γ irradiation, the cellular levels of p53 rise due to posttranslational stabilization of the protein (15,23,24). The elevated p53 levels induce expression of downstream genes such as p21/WAF1 that at least in part results in cell cycle arrest, presumably to allow DNA repair to occur in the absence of replication (14,15,24). In certain cell types, the elevated p53 protein induces apoptosis after DNA damage, possibly by increasing expression of *bax* (16). Expression of *mdm2* is also induced in response to DNA damage in a p53-dependent manner (25,26). The role for *mdm2* in response to UV or γ irradiation is thought to function in a "feedback loop" mechanism inhibiting the transcriptional activity of p53 when it is no longer needed after repair is complete. In fact, the timing of *mdm2* induction is critical; it was previously demonstrated in a tumor cell line that the induction of *mdm2* expression was delayed when compared to the rapid increase of p53 protein levels, and *mdm2* expression was best correlated with recovery of normal rates of DNA synthesis, presumably after DNA repair (25,26).

To understand the mechanism by which the *mdm2* expression is regulated following irradiation, the expression profiles of the *mdm2* gene were examined using primary rat embryo fibroblasts (REF) and mouse embryo fibroblasts (MEF). After a high dose of UV irradiation (20 J/M^2) the induction of *mdm2* mRNA and protein was delayed. Surprisingly, the expression of *mdm2* protein and mRNA actully declined immediately after UV treatment. This initial decrease in *mdm2* expression was dependent upon the UV dosage. Unlike high doses of UV irradiation, lower doses of UV irradiation (4 J/M^2) induce the *mdm2* gene shortly after treatment with no initial decrease in *mdm2* protein and mRNA levels. A high-dose UV treatment of the MEF derived from a p53 knockout mouse (with no p53) failed to cause an increase of *mdm2* mRNA or protein levels; yet it resulted in the initial decrease of *mdm2* expression. Therefore, the expression of *mdm2* is initially down-regulated in a p53-independent fashion and then induced in a p53-dependent fashion in response to a high dose of UV irradiation (20 J/M^2). Furthermore, it is shown that UV irradiation has little effect on the stability of the *mdm2* mRNA and the initial down-regulation of *mdm2* was controlled at the level of transcriptional initiation. These results suggest that after high-dose UV irradiation, Mdm2 levels first decrease, allowing for the maximal transcriptional activity of p53 which is essential for p53 to function as a cell cycle checkpoint; *mdm2* is later induced by p53, inhibiting p53 transcriptional activity after a sufficient period of time for DNA repair. These data indicate that *mdm2* gene expression is regulated in both p53-independent and p53-dependent manner, and that both could contribute to the regulation of cell cycle progression in response to high-dose UV irradiation.

MATERIALS AND METHODS

Cell Culture and UV Treatment

All cells except SP2/0 were grown at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) in a humidified 5% CO_2 atmosphere. SP2/0 cells were grown in suspension in Rosewell Park Memorial Institute 1640 media (RPMI) with 10% FBS under the same conditions.

Cells were exposed to UV light essentially as described (25). The medium was removed and the cells were irradiated with an 8-W germicidal lamp delivering 2 J/M^2/sec at a distance of 38.5 cm. The dose was calibrated with a J-225 shortwave UV meter (Ultraviolet Products, San Gabriel, CA).

IP-Western Analysis

The IP-Western experiments were performed as described (25). Briefly, 2 to 3 mg of each lysate

were incubated with monoclonal antibody pAb2A10 (specific for mdm2) or monoclonal antibody pAb421 (specific for p53), the immunoprecipitates were separated on a SDS-polyacrylamide gel, and blotted onto Immobilon-P membrane (Millipore, Bedford, MA). The membranes were incubated with anti-mdm2 serum or pAb421. Antibody binding was detected with ^{125}I-protein A (NEN), and quantitation was done with a Molecular Dynamics Phospho-Imager.

Northern Analysis

Total cellular RNA was isolated with TRIzol reagent (BRL). 20 μg of total RNA was used for Northern analysis on 1.2% agarose gel containing 6.7% formaldehyde. The RNA was transferred to GeneScreen (Dupont, Boston, MA) by capillary transfer in 20× SSC, and then crosslinked by exposure to UV followed by baking at 80°C for 2 to 4 hours. Blots were prehybridized for several hours at 65°C in Church buffer containing 0.5M $NaHPO_4$ pH 7.0, 1% BSA, 7% SDS and 1 mM EDTA. Hybridizations were for 16 to 24 hours at 65°C in fresh Church buffer with 10^8 cpm of a randomly primed ^{32}P-labeled DNA probe. The filters were washed twice in a solution of 1× SSC and 0.1% SDS for 20 min at 65°C, and further washed twice in a solution of 0.2× SSC and 0.1% SDS for 20 min at 65°C. The washed filters were exposed to a Molecular Dynamics Phospho-Imager for quantification and to Kodak films for photography. Equal loading of RNA was confirmed by staining with ethidium bromide and hybridizing with cDNA probes of the GAPDH (glyceraldehyde-3-phosphate dehydrogenase) or β-actin genes.

Nuclear Run-on Analysis

Nuclear run-on analysis was performed basically as described (27). Briefly, the nuclei were isolated from the SP2/0 cells using NP-40 lysis buffer (10 mM Tris · HCl pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$ and 0.5% NP-40). These SP2/0 cells were either untreated, or were harvested at 6 hours after a treatment of 20 J/M^2 UV irradiation. Run-on transcription assays were done with 2×10^7 nuclei of each sample for 15 minutes at 30°C in the reaction buffer containing 5 mM Tris · HCl, 2.5 mM $MgCl_2$, 0.15 M KCl, 0.5 mM of each of ATP, CTP, and GTP, and 15 μCi of [α-^{32}P]UTP (NEN). The newly transcribed ^{32}P-labeled RNA molecules were extracted, denatured, and precipitated. The labeled RNA pellets were resuspended in TES solution (10 mM TES, 10 mM EDTA and 0.2% SDS) and a small aliquot of each sample was counted. Equal amounts (greater than 5×10^6 cpm) of the labeled RNA of each sample were hybridized with a nylon filter bound by the various Bluescript plasmids containing cDNAs of mdm2, p21/WAF1, GAPDH and β-actin. The filters were washed twice in the solution of 2× SSC at 65°C for 1 hour, and once in the solution of 1× SSC and 10 μg/ml RNase at 37°C. The washed filters were exposed to a Molecular Dynamics Phospho-Imager for quantification and to Kodak films for photography.

RESULTS

Mdm2 Protein Levels Change in Response to UV Irradiation

It was previously reported that Mdm2 protein levels increased in a p53-dependent manner following UV irradiation in C127 tumor cells. However, the *mdm2* induction was delayed when the cells were treated with a high dose of UV irradiation (20 J/M^2) and this delayed induction of Mdm2 was correlated with the recovery of cells to produce a normal rate of DNA synthesis or entry into S-phase (25). These observations indicated that there might be multiple mechanisms regulating the *mdm2* gene expression in response to UV irradiation. In this study, primary rat embryo fibroblasts (REF) were used for examining Mdm2 levels in response to UV irradiation. Following UV treatment at a dose of 20 J/M^2, the primary REF cells were harvested at 2.5, 5, 8, 12, and 24 hours posttreatment. The cellular levels of the *mdm2* protein were then detected by an immunoprecipitation—Western blot analysis, in which the soluble *mdm2* proteins were immunoprecipitated, separated on a SDS-polyacrylamide gel, and transferred onto a blot for Western analysis. After UV irradiation, the levels of the *mdm2* protein first decreased and then increased starting at 8 to 12 hours post irradiation (Fig. 1A). The levels of Mdm2 and p53 at each time point after UV irradiation were compared to the untreated level (zero time level) respectively, and the relative protein levels versus time after UV irradiation were plotted (Fig. 1B). Consistently with the previous report in C127 cells (25), the p53 protein began accumulating immediately (2.5 hours after treatment), increased to more than 30-fold higher than the original level by 8 hours and stayed at that level even at 24 hours

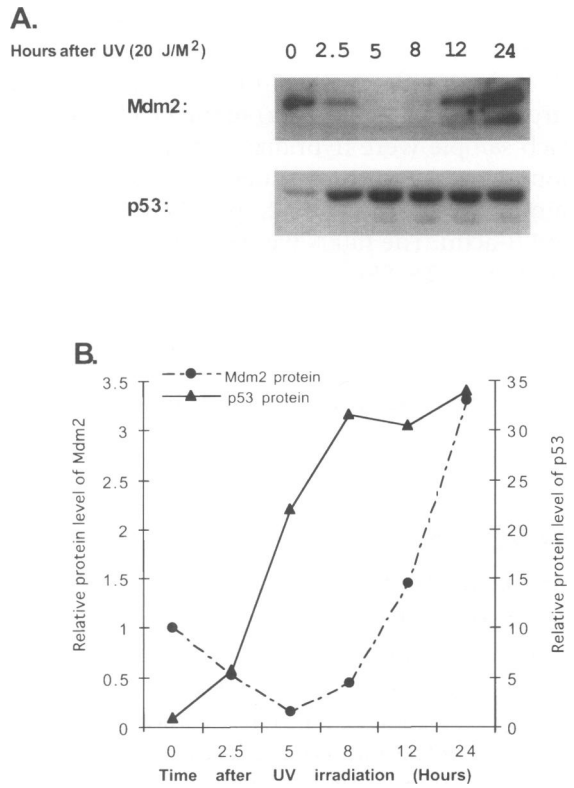

FIG. 1. Mdm2 protein levels change in response to a high dose of UV irradiation

(A) IP-Western analysis of Mdm2 and p53. Primary Rat Embryo Fibroblasts (REF) were irradiated with a UV dose of 20 J/M^2 and harvested at the indicated times following treatment. Two to three mg of cell lysate was incubated with monoclonal antibody pAb2A10 (specific for Mdm2) or monoclonal antibody pAb421 (specific for p53). The immunoprecipitates were electrophoresed in a SDS-polyacrylamide gel and blotted onto a membrane. Western analysis was then performed with anti-Mdm2 serum for Mdm2 or pAb421 for p53 and ^{125}I-protein A. (B) Quantitation of Mdm2 and p53 protein levels. The antibody-bound ^{125}I-protein A was quantified with a Phospho-Imager. The relative protein level was calculated by dividing the amount of protein at each time following UV treatment by the original level with no treatment. The relative protein levels of Mdm2 and p53 were plotted in different scales as indicated.

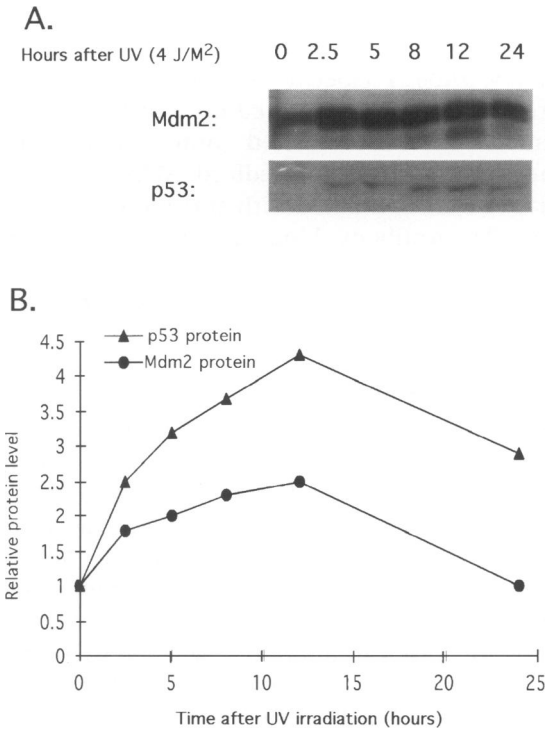

FIG. 2. Mdm2 protein levels increase in response to a low dose of UV irradiation

REF were irradiated with a UV dose of 4 J/M^2, analyzed and quantified as in Figure 1. (A) IP-Western analysis of Mdm2 and p53. (B) Quantitation of Mdm2 and p53 protein levels.

after UV treatment. In contrast, the level of the *mdm2* protein first decreased at 2.5 to 5 hours following UV treatment to a level less than 10% of the untreated level. At 8 hours after UV treatment, Mdm2 protein began to increase. By 12 hours, the level of Mdm2 was 1.5-fold higher than the original level and continued to increase until 24 hours after UV treatment. Therefore, the induction of the *mdm2* protein was delayed as compared to the rapidly increasing level of p53.

The initial decrease of the *mdm2* protein levels was not seen when the REF cells were irradiated with a low dose of UV light (Fig. 2). After REF cells were irradiated with a dose of 4 J/M^2 UV, the cellular levels of Mdm2 started to increase at 2.5 hours after treatment and stayed about 2-fold higher until 12 hours after UV treatment; meanwhile, the level of p53 was also increased but only up to 4-fold. Primary MEF cells were irradiated with high-dose or low-dose UV light, and changes in the levels of Mdm2 were similar to those observed in REF cells (data not shown).

The Initial Decrease of the *mdm2* Protein After High-Dose UV Irradiation is Regulated at the mRNA Level

It has been demonstrated that the induction of the *mdm2* protein in response to both high- and low-dose UV irradiation depends upon the transcriptional activation activity of p53 (25). To evaluate whether the initial decrease of Mdm2

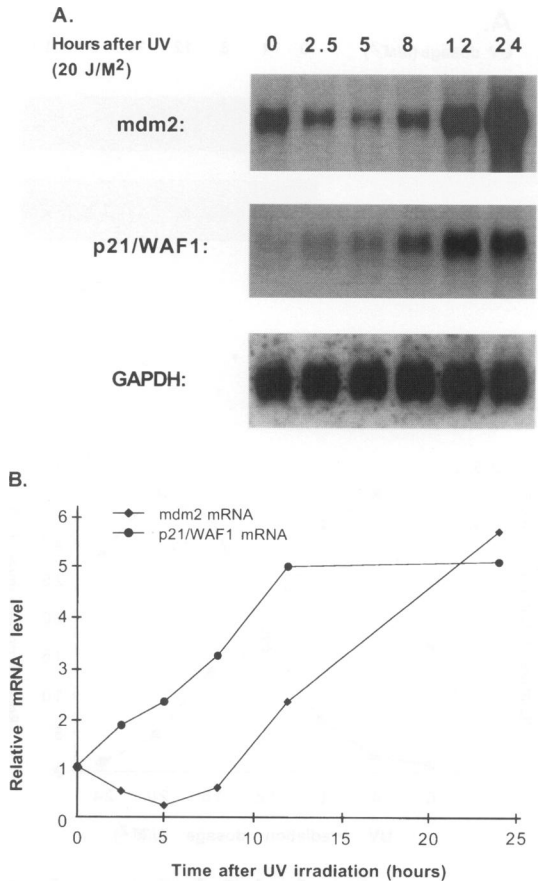

FIG. 3. Expression of Mdm2 is initially decreased and later increased in response to a high dose of UV irradiation

(A) Northern analysis. Primary MEF were treated with a UV dose of 20 J/M^2, and then harvested at each indicated time following treatment. Total cellular RNA was isolated and 20 μg of each was used in Northern analysis. The mRNA levels of mdm2, p21/WAF1 and GAPDH were examined with each cDNA probe as indicated. (B) Quantitation of the mRNA levels of mdm2 and p21/WAF1. The amount of ^{32}P-labeled probe bound to the Northern blots was quantified on a Phospho-Imager. The relative mRNA level was calculated by dividing the amount of mRNA at each time following UV treatment by the original level with no treatment. The relative mRNA levels of mdm2 and p21/WAF1 at each time after UV were plotted as indicated. The GAPDH signal was used as a loading control.

protein was in fact due to the alterations of the *mdm2* gene expression following high-dose UV irradiation, Northern analysis was performed to examine the mRNA level of *mdm2* (Fig. 3). Total cellular RNA was extracted from the MEF cells that had been irradiated with a high dose (20 J/M^2) of UV light and harvested at various times after that treatment. Northern analysis using these total RNA pools showed that the *mdm2* mRNA level was reduced by about 80% within 5 hours. It then recovered at 8 hours, and kept increasing to 5 to 6 fold over the original level by 24 hours after treatment. This result indicated that the initial decrease of *mdm2* following high-dose UV irradiation was regulated at the mRNA level. The same blot was probed for the mRNA level of p21/WAF1. This showed that the p21/WAF1 mRNA level increased immediately after UV treatment. The immediate increase in the p21/WAF1 mRNA level suggested that p53 is transcriptionally active after UV irradiation although the level of *mdm2* mRNA was down-regulated. This result implied that the decrease in *mdm2* expression could be p53-independent. By 12 hours after UV irradiation, the induction of *mdm2* expression started while at this time the mRNA level of p21/WAF1 stopped increasing and reached its maximum level. This result is consistent with the possibility that the newly produced *mdm2* protein could prevent further transactivation of p21/WAF1 by binding to p53 and blocking its transactivation activity for that gene but not the *mdm2* gene. The mRNA level of GAPDH did not decrease after UV treatment indicating that the initial decrease of *mdm2* expression is not due to a general inhibition of RNA polymerase II by UV irradiation.

The Decrease of *mdm2* Expression by UV Irradiation Is p53-Independent

To test whether the initial decrease in *mdm2* mRNA in response to high-dose UV treatment could be mediated by p53, the expression of *mdm2* in cells that lack endogenous p53 was examined (Fig. 4). MEF cells from the p53 knockout mice (MEFKO p53−/−) and the cells from two p53−/− human cell lines, H1299 and Saos-2, were treated with 20 J/M^2 UV and then harvested at various times after treatment. In all three types of cells, Mdm2 protein levels initially decreased following UV irradiation (Fig. 4A). In the case of UV-irradiated MEFKO p53−/− cells (40 to 50% cells were attached to plates by 24 hours after 20 J/M^2 UV irradiation), Mdm2 protein levels were initially reduced within 12 hours and then recovered to the original level by 24 hours after treatment, presumably after DNA repair of the remaining living cells. On the other hand, the majority of H1299 and Saos-2 cells died by 24 hours following 20 J/M^2 UV irradiation. In these two cell types, Mdm2 protein lev-

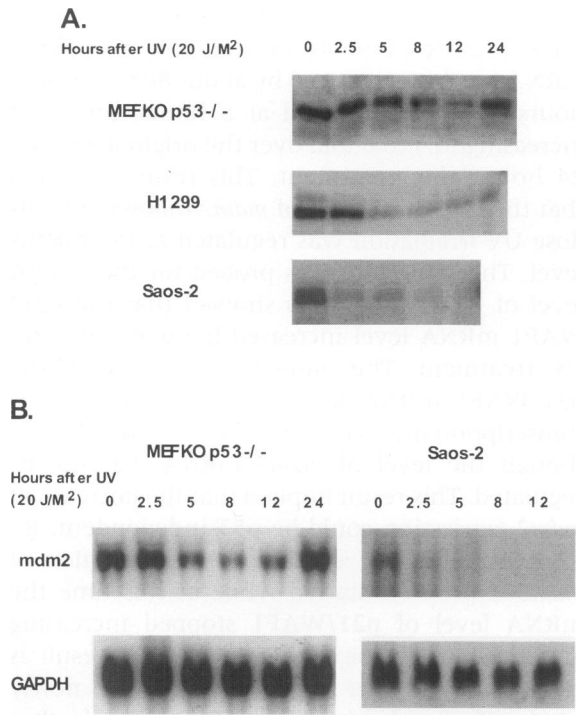

FIG. 4. The initial decrease of Mdm2 expression after high-dose UV irradiation is p53-independent

The MEF from p53 knockout mice (MEFKO p53−/−), H1299 cells and Saos-2 cells, all of which lack the endogenous expression of p53, were treated with a dose of 20 J/M^2 UV and harvested at the indicated times. The protein levels of Mdm2 were analyzed by IP-Western (A) and the mRNA levels of mdm2 were examined by Northern analysis (B) as described in Figures 1 and 3.

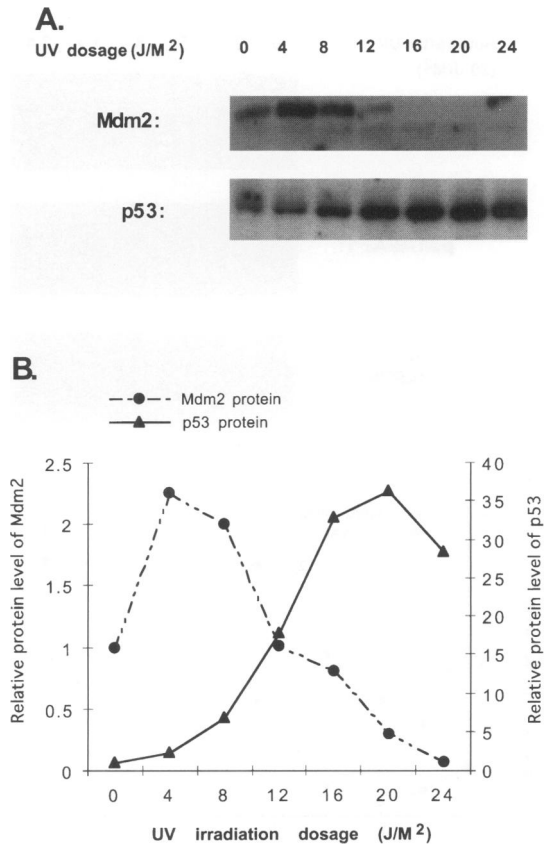

FIG. 5. Expression of *mdm2* changes as a function of UV dose

Primary REF cells were irradiated with UV light of the indicated doses, harvested at 6 hours after treatment and analyzed as in Figure 1. (A) IP-Western analysis of Mdm2 and p53. (B) Quantitation of Mdm2 and p53 protein levels.

els, which fell early, never recovered to the original level. Northern analysis (Fig. 4B) revealed that the *mdm2* mRNA levels also declined in MEFKO p53−/− cells and Saos-2 cells shortly after high-dose UV irradiation. These results confirmed that the increase of the *mdm2* expression at late time after exposure to high-dose UV irradiation relied on the presence of p53. Furthermore, these results indicate that the early decline in *mdm2* gene expression following high-dose UV treatment is p53-independent.

Initial Decline of *mdm2* Expression Is UV Dosage-Dependent

Because the initial repression of *mdm2* mRNA in response to irradiation was only tested when the cells were treated with a single high dose (20 J/M^2) or low dose (4 J/M^2) of UV irradiation, a dose-response curve between 0 and 24 J/M^2 was carried out. REF cells were irradiated with a series of UV doses and harvested after 6 hours. The levels of Mdm2 and p53 proteins were examined by an immunoprecipitation—Western blot analysis. At 6 hours after UV irradiation, the level of p53 increased in a UV-dosage dependent manner, to more than 30-fold when the cells were treated with high-dose UV irradiation (>16 J/M^2) (Fig. 5). Mdm2 protein levels responded in quite a different manner. When the UV dose was below 8 J/M^2, the expression of *mdm2* protein was induced; when the UV dose was increased to 12 J/M^2, the expression of *mdm2* protein began to decline at 6 hours after treatment. Mdm2 protein levels dropped by 95% of its original level when the UV dose was 20 J/M^2 or higher. The induction of *mdm2* following low-dose UV

irradiation was mediated by p53 because the cells lacking the endogenous wild-type p53 did not show the elevated Mdm2 level (data not shown). Following high-dose UV treatment, the *mdm2* expression decreased even in the presence of much higher levels of p53. These results suggest that the *mdm2* expression following high-dose UV irradiation may be initially controlled by a factor(s) which selectively prevents expression of the *mdm2* gene by p53 or other basal transcription factors. The net result is a repression of *mdm2* gene expression.

Mdm2 Down-Regulation following Irradiation with High Doses of UV Occurs at the Level of Transcription

The lower levels of *mdm2* mRNA and protein could be due to increased *mdmd2* mRNA instability or a decreased rate of *mdm2* gene transcription. In an effort to explore the mechanism by which the *mdm2* expression was down-regulated by high-dose UV treatment, murine SP2/0 cells were employed. These cells have very high levels of *mdm2* gene expression due to a translocation event that brings the mdm2 promoter in contact with the immunoglobulin enhancer region. The mdm2 promoter element remains intact in these cells. IP-western analysis (Fig. 6A) and Northern blot analysis (Fig. 6B) indicated that these cells, like all of the cell lines tested in this study, showed an 80 to 90% decline of *mdm2* mRNA and protein at 8 hours following treatment with 20 J/M^2 of UV.

In order to test the effect of UV on the stability of the *mdm2* mRNA, the SP2/0 cells were first treated with actinomycin D to inhibit transcription by RNA polymerase II. Fifteen minutes later, the cells were irradiated with 20 J/M^2 of UV light or were left untreated, and then these cells were harvested at various times after UV exposure. Total cellular RNA was isolated from these cells for Northern analysis (Fig. 6C), and the amount of *mdm2* mRNA after UV irradiation was quantified as shown in Figure 6D. The half-life of *mdm2* mRNA in SP2/0 cells without UV radiation is about 1.5 hours. After UV irradiation of 20 J/M^2, the half-life of *mdm2* mRNA is even slightly extended to 2 to 2.5 hours. The same blots were reprobed with a β-actin cDNA, showing no changes in mRNA stability of β-actin. These results indicated that UV irradiation does not destabilize the *mdm2* mRNA.

To test the transcriptional regulation of the *mdm2* gene after UV irradiation, run-on experiments were performed with the nuclei isolated from the SP2/0 cells at 6 hours after treatment of 20 J/M^2 UV. Equal amounts of newly synthesized total RNA by untreated and treated nuclei were probed for transcription of *mdm2*, p21/WAF1, and the control genes GAPDH and β-actin. These results are shown in Figure 6E. At 6 hours after 20 J/M^2 UV treatment, the transcription rates of the control genes GAPDH and β-actin were only slightly reduced as compared to the untreated nuclei. However, the transcription rate of the *mdm2* gene was significantly reduced while the transcription rate of the p21/WAF1 gene was substantially increased. After normalization to the GAPDH gene, the impact of UV irradiation on transcription of the other genes was compared and the results presented in Figure 6F. At 6 hours after UV irradiation, the transcription rate of the *mdm2* gene was reduced by 50% of the original level. On the other hand, the transcription rate of the p21/WAF1 gene was shown to be 2-fold higher than in untreated cells. Since the run-on experiments only test the initiation of transcription, the differences may not quantitatively reflect all the changes observed in the steady-state levels shown by Northern analysis (Figure 6B). The steady-state level of mRNA is the balance of many determinants such as the rate of initiation of mRNA synthesis, the rate of propagation of mRNA synthesis, the rate of mRNA degradation, and the rate of transporting mRNA from the nuclei to the cytoplasm, all of which could vary at different times after UV irradiation. It is clear, however, that these results suggest that the initial decline of the *mdm2* mRNA after high-dose UV irradiation is controlled at least in part at the level of transcriptional initiation, possibly by a UV-responsive transcription factor or repressor of the *mdm2* gene that has the ability to override the positive regulation of transcriptional initiation by the p53 protein.

DISCUSSION

The results presented here demonstrated that the expression profile of the *mdm2* gene in response to UV irradiation varies as a function of UV dosage. In response to low-dose UV irradiation, the transcription of the *mdm2* gene is induced as early as 2.5 hours after treatment (Fig. 2). However, after high-dose UV irradiation, the expression of the *mdm2* gene is initially repressed to a significant extent before it is finally induced in a

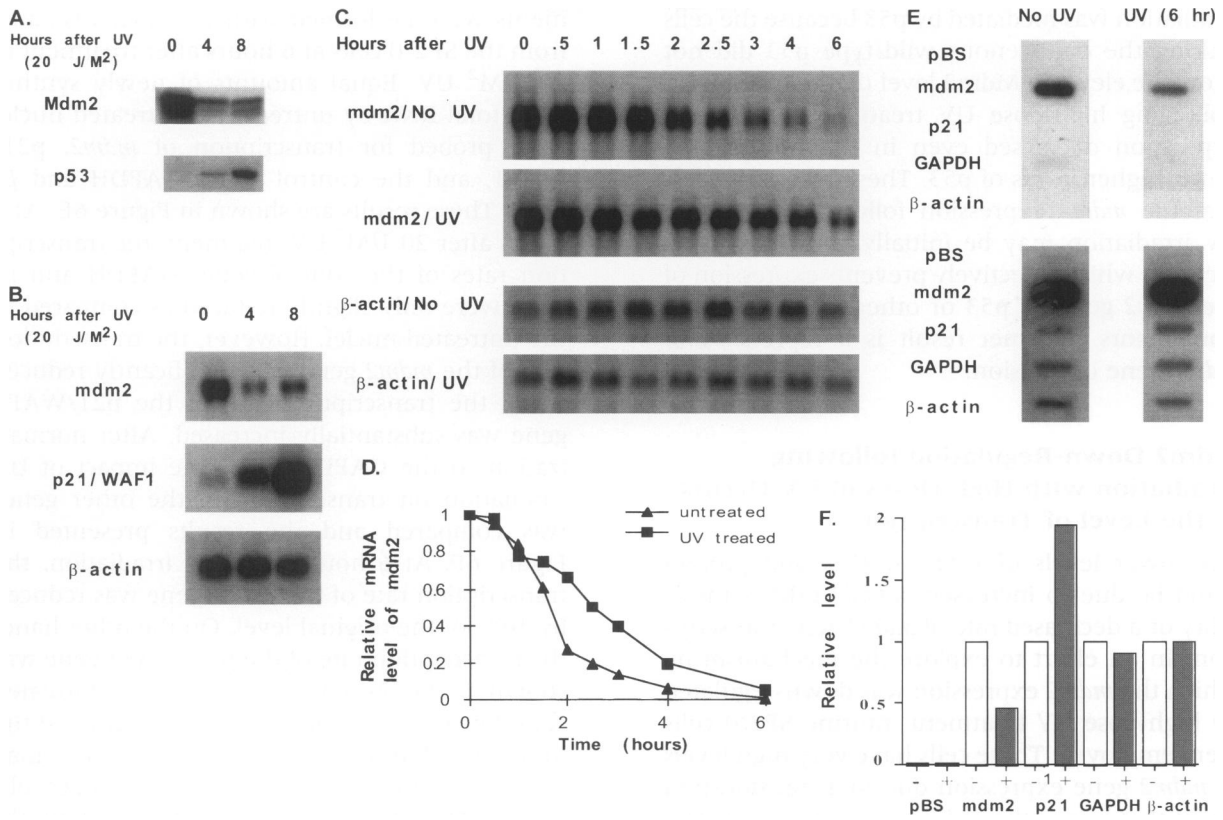

FIG. 6. The initial decrease of mdm2 expression following a high dose of UV irradiation is transcriptionally regulated

SP2/0 cells were treated with a UV dose of 20 J/M^2 and harvested at the indicated times. The protein levels of Mdm2 and p53 were analyzed by IP-Western (A) and the mRNA levels of mdm2, p21/WAF1, and β-actin were examined by Northern analysis (B). (C) Northern analysis of stability of the mdm2 mRNA in SP2/0 cells treated with or without UV (20 J/M^2). SP2/0 cells were first treated with actinomycin D at a concentration of 0.5 μg/ml for 15 min and then were either irradiated with 20 J/M^2 UV or were left untreated. Total RNA was isolated from the cells at the indicated times. 10 μg of each total RNA was used for Northern blots probed with cDNAs of mdm2 and β-actin. (D) The relative amounts of mdm2 mRNA levels to the un-irradiated level were plotted as a function of time. (E) Nuclear run-on assays. The experimental procedure was described in Materials and Methods. The membranes were blotted with cDNAs of mdm2, p21/WAF1, GAPDH, β-actin, and vector pBluescript. Both light (top) and dark exposures (bottom) were taken for showing different intensities of signals on the same blots. The mdm2 signal as indicated was shown on the light exposure while the signals of p21/WAF1, GAPDH, and β-actin only appeared on the dark exposure. (F) Effects of UV on transcription of various genes.

p53-dependent fashion (Figs. 1, 3). In contrast to the induction of *mdm2*, which is mediated by p53, the decline in the expression of the *mdm2* gene after high-dose UV irradiation is p53-independent (Fig. 4). These results demonstrate that this decline in *mdm2* expression is UV dosage-dependent (Fig. 5) and appears to be controlled at the level of transcriptional initiation (Fig. 6).

The response to DNA damage in mammalian cells is very complex, involving changes in the expression of many different cellular genes. Among these, the p53 protein level is increased and it functions as a cell-cycle checkpoint by causing cell-cycle arrest or inducing apoptosis (25). Loss of p53 function contributes to tumorigenesis, likely because cells that fail to arrest in the G1 phase or pursue apoptosis after DNA damage are more likely to accumulate mutations and display genomic instability. The mechanism by which p53 functions is believed to be at least in part through its transcriptional activity, inducing p21/WAF1 which contributes to cell-cycle arrest (14), or inducing Bax which facilitates apoptosis (16). Mdm2 is a negative regulator of p53 (11); its level is therefore important in controlling p53 checkpoint function in response to DNA

damage. This paper demonstrates that high-dose UV irradiation induces a *mdm2* gene repressor in a p53 independent fashion and it blocks the synthesis of *mdm2* mRNA. This presumably allows time for p53 protein to selectively enhance transcription of genes involved in cell-cycle arrest (p21/WAF1) permitting DNA repair prior to reversal of p53-mediated effects of *mdm2*. Conceivably, DNA damage by a low dose of UV irradiation is limited and takes less time to repair. Therefore, it is beneficial for the cell if p53 activity is controlled by Mdm2 shortly after low-dose UV irradiation. Both the early decline and delayed up-regulation of *mdm2* expression following a high dose of UV irradiation could well be important for modulating the transcriptional activity of p53, and thereby effectively controlling p53 function as a cell-cycle checkpoint.

Human Mdm2 has been reported to bind to the retinoblastoma (pRb) tumor suppressor protein and inhibit pRb growth suppression function by perturbing its regulation of E2F (28). It also has been shown that human Mdm2 directly interacts with E2F and DP1 transcription factors and promotes proliferation by stimulating the activity of E2F/DP1 (29). These studies suggest that *mdm2* may play other roles in addition to regulation of p53 activity in controlling cell-cycle progression. It will be interesting to see whether the down-regulation of the *mdm2* gene shortly after UV irradiation may also have important effects on the cell by reducing the stimulation of E2F/DP1 activities and diminishing growth to allow DNA repair.

Understanding how expression of the *mdm2* gene is down-regulated in response to DNA damage will advance our knowledge of the cell-cycle regulation following DNA damaging treatment. Under normal circumstances, the expression of *mdm2* is controlled by two promoters, P_1 and P_2 (30). P_2 promotor is located near the 3′ end of the first intron of the *mdm2* gene and contains two p53-responsive elements. Transcripts from either promotor both contain the translation initiation site which is located in the exon 2. It has been shown that transcription from the P_2 promotor can be activated by either the introduction of overexpressed wild-type p53 into cells or the induction of endogenous wild-type p53 by ionizing radiation while the upstream P_1 promotor is only mildly affected by p53 (30). The results presented here demonstrate that the initial down-regulation of *mdm2* expression in response to a high dose of UV is controlled transcriptionally, possibly by a UV-responsive repressor or other factor(s). This putative UV-responsive factor becomes active immediately following high dosages of UV, represses the expression of the *mdm2* gene, and prevents the activation by p53 even in the presence of high levels of p53 protein and the transcriptional activation of the p21/WAF1 gene. Later, presumably after DNA repair, this protein no longer represses *mdm2* transcription allowing the induction of *mdm2* by p53. Therefore, it is likely that a putative UV-responsive factor might well bind to a DNA sequence near or overlapping with the p53-responsive elements. Experiments which will allow the identification of such DNA sequence(s) are presently in progress. Through that DNA sequence(s), it would then be possible to identify the putative factor that binds to it. Because this factor can sense the signals from high dosages of UV which cause DNA damage, it could be an important player in the signaling pathways between DNA damage and downstream gene expression. Such a repressor is also an example of a protein that can modify p53-mediated response in selected genes. If Mdm2 was overproduced too early after DNA damage, it could inactivate p53-mediated functions. Thus, a mutation in this putative *mdm2* repressor protein could act like a suppressor of p53 function and possibly contribute to the cancerous phenotype. For these reasons it will be useful to identify this putative activity.

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