Regulation of Transcriptional Activation of \textit{mdm2} Gene by p53 in Response to UV Radiation\textsuperscript{1}

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Abstract
The \textit{mdm2} oncogene is expressed at elevated levels in a variety of human tumors, and its product inactivates the p53 tumor suppressor protein. MDM2 forms an autoregulatory loop with p53, because the \textit{mdm2} gene contains a promoter that is responsive to p53. Synthesis of MDM2 protein increases in a p53-dependent manner in response to DNA-damaging agents such as UV light. Although this increase likely results from enhanced transcription, the amount of MDM2 protein does not correspond to the amount of \textit{mdm2} protein in cells exposed to UV light. Here we show that the p53-specific internal promoter in the \textit{mdm2} gene is induced after exposure to UV light, whereas the upstream constitutive promoter is not induced. The amount of the \textit{mdm2} transcript does not parallel the ability of p53 to bind DNA, indicating that transcription is regulated at a step distinct from activation of the DNA-binding function of p53.

Introduction
The function of the p53 tumor suppressor protein is important for prevention of cancer in people and in mice (1, 2). In more than 50% of human tumors, both alleles of p53 are mutated or deleted, indicating that loss of function of p53 contributes to tumorigenesis (2). Studies in mice substantiate a role for p53 in preventing cancer; the incidence of tumor formation approaches 100% in mice genetically engineered to lack p53 (1). The tumor-suppressive effects of p53 seem to be mediated through the ability of the protein to bind DNA, because most mutations in human tumors result in loss of this function (3). The sequence-specific DNA-binding function of p53 is essential for transcriptional activation (4) of a number of genes that contain sequences to which wild-type, but not tumor-derived, mutant p53 binds (5–8). Several of these genes encode proteins that seem to participate in the arrest of cell division or the stimulation of apoptosis induced by p53 (reviewed in Ref. 9). Expression of these genes may be regulated through interactions between p53 and components of the basal transcription factor TFIIID, because p53 mutants lacking the ability to interact with the TATA box-binding protein-associated factors TAFII32 and TAFII45 are unable to activate transcription, although they are able to bind DNA (10, 11). Alternatively, transcription may be influenced by interactions between p53 and coactivators such as p300 and CBP, because p53 mutants that fail to interact with these coactivators fail to transactivate (12). The mechanisms that normally regulate p53 function are not well understood.

Recent evidence indicates that tumors expressing wild-type p53 have evolved multiple mechanisms to inhibit the ability of p53 to transactivate gene expression (9). In 10–60% of sarcomas, leukemias, and breast cancers (13–15), an inhibitor of the transcriptional activation function of p53 is expressed at elevated levels. The inhibitory protein, the largest product of the human homologue of the murine double minute 2 gene (\textit{mdm2}), p90, binds to the transactivation domain of p53 and inhibits expression of constructs containing p53-responsive promoters (16–18). In other tumors, such as neuroblastomas and some breast cancers, the wild-type \textit{mdm2} protein is retained in the cytoplasm, where it cannot function as a transcriptional activator (19, 20). For other tumor types, the mode of inhibition of p53 is less clear. For example, in cell lines derived from teratocarcinomas, high levels of wild-type \textit{mdm2} protein are present in the nucleus, but there are low levels of expression of reporter and endogenous genes containing p53 REs\textsuperscript{6} (21). The specific activity of the p53 protein, defined as the transcriptional response per mole of p53 protein, seems to be regulated in teratocarcinoma cell lines, because expression of p53-responsive genes can be induced on differentiation of these cells, while the amount of p53 protein decreases (21). Differentiation of normal keratinocytes also results in decreased levels of p53 protein, concurrent with increased expression of p53-responsive genes (22). Together, these studies indicate that there are uncharacterized mechanisms that regulate the specific activity of p53 as an inducer of gene expression.

Treatment of cultured cells with UV light results in a discordance between the amount of p53 protein and the amount of mRNA expressed from p53-responsive genes (23).

\textsuperscript{6} The abbreviations used are: RE, response element; EMSA, electromobility shift assay; PBSBT, PBS containing 0.5% BSA and 0.05% Tween 20; Ab, antibody.
24). The response of cells to UV light provides an opportunity to study the regulation of the transcriptional activation function of p53, because the levels of p53 protein rise in a dose-dependent manner (24, 25). In addition, exposure to UV light results in enhanced expression of reporter and endogenous p53-responsive genes (24, 25). Although some p53-responsive genes such as GADD45 (26) and p21WAF1 (Ref. 27; this paper) do not require p53 for induction in response to UV light, others, such as mdm2, do require p53 function (23). Paradoxically, the magnitude of the induction in mdm2 expression, measured by Northern analysis and by pulse-labeling of MDM2 protein, does not correspond with the amount of p53 protein in cells exposed to different doses of UV light. These findings indicated that there may be regulation of the ability of p53 to mediate gene expression after exposure of cells to UV light. Indeed, Hupp et al. (28) reported that 16 h after exposure of mammalian cells to UV light, there was elevated expression of a p53-responsive reporter gene without an increase in the amount of p53 protein. A model was proposed in which the specific activity of the p53 protein as a sequence-specific DNA-binding protein is increased by exposure of cells to UV light (28). Consistent with this model is the observation that the DNA-binding activity of purified p53 protein can be stimulated by single-stranded DNA molecules similar in length to those oligonucleotides excised during repair of UV-induced DNA damage (29). In this study, we determined that exposure of cells to UV light results in regulation of the ability of p53 to transactivate gene expression at a level distinct from the ability of p53 to bind DNA in a sequence-specific manner. We examined the regulation of the mdm2 gene in response to UV light because this gene contains two promoters, one of which (P2) is dependent on p53 for activity (30). Thus, expression of transcripts initiated from the P2 promoter reflects the ability of p53 and the effectors of its function to stimulate transcription. We found that the P2 promoter in the mdm2 gene responds to UV light, whereas the basal upstream promoter does not respond. Although induction of the P2 promoter by UV light requires p53, there is a discrepancy between the amount of p53 and the amount of transcript initiated at the P2 promoter. Furthermore, we measured the ability of p53 to bind DNA in a sequence-specific manner and found that the amount of transcript initiated at the P2 promoter does not correlate with the capacity of p53 to bind DNA in a sequence-specific manner. Thus, the ability of p53 to stimulate the activity of the P2 promoter of mdm2 is mediated in part by changes distinct from those that regulate the DNA-binding function of p53.

Results

The Increase in the Rate of MDM2 Protein Synthesis after Exposure of Cells to UV Light Does Not Correlate with the Amount of p53 Protein in the Cell. The induction of expression of the MDM2 protein in response to UV light requires p53 function; however, the magnitude of the increase in the level of MDM2 protein does not parallel the magnitude of the increase in the level of p53 protein as determined by Western analysis (23). To determine whether the rate of synthesis of MDM2 protein correlates with the amount of p53 protein in cells exposed to UV light, we measured the increase in the amount of newly synthesized MDM2 and compared it to the increase in the total amount of p53 protein after exposure of cells to UV light. The p53 protein normally has a short half-life of about 20 min that becomes lengthened to several hours on exposure of cells to UV light (25). Thus, the amount of p53 can be tracked by radiolabeling cells for several half-lives before exposure to UV light, by which time p53 is labeled to steady state, and continuing to label for the length of time of interest. The continuous labeling procedure was used to track the amount of p53 protein, because it is more sensitive than Western analysis and allowed us to analyze several parameters in each cell lysate (see below). To ensure that the assay was capable of measuring the steady-state level of p53 over a 9-h labeling period, we radiolabeled cells in the presence of unlabeled methionine (2 × 10⁻⁴ M in DMEM) for 3 or 9 h, in the absence of UV exposure, and found comparable amounts of radiolabeled p53 in the resulting lysates. Thus, this method is capable of radiolabeling p53 to steady state in the absence of exposure to UV light. Furthermore, we compared the magnitude of the increases in the level of p53 measured by the continuous labeling method to those by Western analysis and found them to be similar.

We treated C127 cells with a dose of UV light that results in greater than 97% survival (4 J/m²) or a dose that results in only 10% survival (20 J/m²). For simplicity, we will refer to these doses as the low and high doses of UV light. The amount of p53 protein present after continuous labeling was compared to the amount of MDM2 protein synthesized during a 30-min pulse labeling (Fig. 1). Two h after treatment with the low dose of UV light, the level of p53 protein had risen 2-fold (Fig. 1A), and the rate of MDM2 protein synthesis had risen 6-fold (Fig. 1B). By 8 h, the amount of p53 protein had decreased, as had the rate of synthesis of MDM2 protein. In contrast, after exposure of cells to the high dose of UV light, p53 levels rose about 3-fold by 2 h, but MDM2 levels had not changed. Eight h after exposure, the amount of p53 protein had increased to 8 times the normal level (Fig. 1A), and the rate of synthesis of MDM2 was increased 13-fold (Fig. 1B). Therefore, although both p53 and MDM2 increase in response to low and high doses of UV light, there is not a correlation between the level of p53 protein and the rate of synthesis of MDM2 protein 2 h after exposure to UV light. Instead, there is a delay in the induction of MDM2 expression relative to the rise in p53 after exposure to the high dose of UV light (20 J/m²).

This discrepancy is not due to the decrease in total cellular protein synthesis after exposure to UV light, because equivalent amounts of radioactive protein were incubated with Abs to MDM2, yet less radiolabeled MDM2 protein was recovered 2 h after the high dose than 2 h after the low dose. Thus, relative to the rate of synthesis of other proteins, the synthesis of MDM2 protein was slower 2 h after exposure to a high dose of UV light than 2 h after exposure to a low dose of UV light. The lack of an induction of MDM2 2 h after exposure to the high dose could not be ascribed to death of

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6 M. E. Perry, unpublished observations.
Fig. 1. Comparison of the level of p53 (A) to the rate of synthesis of MDM2 (B) in UV-treated C127 cells. To determine the levels of p53 (A), cells were labeled with [35S]methionine for 1 h before exposure to a dose of UV light of 0, 4, or 20 J/m² and subsequently labeled for 2 or 8 h (h labeled). Equivalent amounts of total cell protein were incubated with the polyclonal Ab CM5 to immunoprecipitate p53. 14C-labeled molecular size markers are indicated in kilodatons (M). An Ab that recognizes SV40 large T antigen was used as a negative control (−). To measure the rate of synthesis of MDM2 protein (B), cells were incubated with [35S]methionine for 30 min either 2 or 8 h after exposure to a dose of UV light of 0, 4, or 20 J/m². Equivalent amounts of acid-precipitable counts were incubated with the polyclonal Ab 628 to immunoprecipitate MDM2. The markers (M) and negative control (−) are as described in A.

The cells, because MDM2 was induced 6 h later (Fig. 1B). These observations indicate that p53 may be less effective at stimulating MDM2 expression 2 h after exposure to a high dose of UV light than 2 h after exposure to a low dose of UV light.

A Specific p53-responsive Promoter Is Induced by UV Light. Expression of mdm2 RNA is induced by DNA damage (31, 32). If the delay in the increase in the rate of MDM2 protein synthesis after exposure to a high dose of UV light was due to a lag between the time p53 was stabilized by UV light and the time mdm2 RNA was induced, then the delay might reflect an inhibition of the ability of p53 to stimulate transcription. The first intron of the mdm2 gene contains two binding sites for p53 that confer responsiveness to p53 on reporter genes (7). When cells expressing a temperature-sensitive mutant of p53 (ala135val) are shifted from the nonpermissive to the permissive temperature, transcription of mdm2 from an internal promoter (P2) is stimulated (33), but transcription from an upstream constitutive promoter (P1) is not increased. Thus, the P2 promoter responds to p53 function, whereas the P1 promoter does not. To test whether the P2 promoter, but not the P1 promoter, was induced by UV light, we attempted to measure the amount of mdm2 mRNA initiating at each promoter using primer extension. The two transcripts differ by the presence of exon 1 (30), such that the amount of mdm2 mRNA initiated at each promoter should be revealed by primer extension analysis using an oligonucleotide that hybridizes to exon 3 (Fig. 2A). After exposure of C127 cells to either a low or a high dose of UV light, a mdm2-specific transcript initiating at bp 3–5 of exon 2 was induced (Fig. 2B), indicating that the P2 promoter was stimulated by exposure of cells to UV light. Transcripts initiating at P2 were not detected in untreated cells, confirming the correlation of P2 activity with p53 function, which is increased in cells exposed to UV light. No product corresponding to the size predicted for the transcript from the P1 promoter (283 bp) was detected by this assay before or after exposure of cells to UV light. Two products larger than that expected from a RNA initiated at the P1 promoter were...
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Fig. 3. Dependence of the induction of the P2 promoter by UV light on p53. The induction of the p53-dependent P2 promoter and the p53-independent P1 promoter were compared in cells expressing C127 or lacking [10]3 p53. Cells were treated as described in the Fig. 2 legend. Total RNA was protected from digestion with S1 nuclease after hybridization to a denatured 254 bp, radiolabeled DNA complementary to sequences from exons 1–3 of mdm2. Transcripts from the constitutive (P1) and p53-responsive (P2) promoters are indicated. The negative control (−) was a sample in which the probe was incubated with no RNA before digestion. The positive control (+) was a reaction in which the probe was hybridized to two RNAs transcribed in vitro from mdm2 cDNAs of different length. RNA from pGEM1F contains exon 1 and reflects a transcript initiated at the constitutive promoter (F), whereas RNA from pGEM1X2 reflects transcripts initiating from the P2 promoter (X2).

Table 1 Increase in levels of mdm2 expression and amount of p53 2 h after exposure of C127 cells to the low or high dose of UV light

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<th>2-h low dose (mean ± SE)*</th>
<th>2-h high dose (mean ± SE)</th>
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<tr>
<td>Synthesis of MDM2p</td>
<td>6.4 ± 0.06</td>
<td>1.0 ± 0.10</td>
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<tr>
<td>Amount of P1 RNAa</td>
<td>1.1 ± 0.04</td>
<td>0.78 ± 0.01</td>
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<tr>
<td>Amount of P2 RNAa</td>
<td>8.9 ± 0.5</td>
<td>3.5 ± 1.3</td>
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<tr>
<td>Amount of p53a</td>
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<td>3.3 ± 1.1</td>
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* Mean fold increase over no UV treatment (n = 3).

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The larger of these products was induced by UV light, whereas the smaller was not. We had estimated the size of the expected product from the P1 promoter to be 283 bases, based on the sequence of the longest cDNA for mdm2 (30, 34). However, there may be multiple start sites upstream of the 5′ end of the cDNA for transcription from the P1 promoter (35). To determine whether the long primer extension products contained exon 1, cDNAs from this region of the gel were isolated, amplified by 5′ rapid amplification of cDNA ends, and cloned.7 Sequence analysis revealed that none of the cDNAs contained exon 1 of mdm2; they were products of other genes or products of unspliced RNA from the P2 promoter of mdm2 (containing exon 2, intron 2, and exon 3 of mdm2). Use of oligodeoxynucleotidic acid-selected RNA resulted in loss of the largest primer extension product, consistent with unprocessed mdm2 RNA being the source of this species. The inability of primer extension to detect RNAs from the P1 promoter may be due to inhibition of reverse transcription through exon 1 by the high (73%) G-C content (34) or the use of multiple start sites, as indicated by the work of Jones et al. (35), in which case the signal from the P1 promoter would be diluted. Both of these possibilities are consistent with detection of products from P2, but not P1, using primers in exon 3 (Fig. 2B) or in exon 4.7

To determine whether activation of the P2 promoter by p53 was responsible for all of the increase in MDM2 expression in UV-treated cells, it was necessary to measure the amount of mdm2 mRNA initiated at both the p53-independent (P1) and p53-dependent (P2) promoters in p53-positive and p53-negative cells before and after exposure to UV light. S1 nuclease digestion of RNA hybridized to a cDNA including sequences from exons 1–3 of mdm2 might be expected to allow simultaneous detection of transcripts from the two promoters, because the probe contains sequences from exons 1–3 of mdm2. This method has two advantages over primer extension for detection of transcripts from the P1 promoter: (a) one digestion product would be obtained from transcripts initiated at multiple sites in the P1 promoter, because the 5′ end of the mdm2 cDNA sequences in the probe is 3′ of all mapped start sites; and (b) there is no requirement for reverse transcription through G-C-rich sequences in exon 1. This method was successful, as revealed by the pattern obtained from P1- and P2-specific transcripts synthesized in vitro (F and X2, respectively; Fig. 3). Transcripts initiated from the P2 promoter were increased by both doses of UV light, but transcripts from the P1 promoter were not increased, indicating that all of the increase in mdm2 RNA was due to enhanced transcription from the P2 promoter. Transcripts from the P2 promoter were not detected in cells lacking p53, even after exposure to UV light. These data indicate that the induction of mdm2 by UV light is regulated through the p53-specific P2 promoter. The amount of P2-specific transcript thus reflects the ability of p53 and its effectors to enhance transcription from this promoter.

Delayed Activation of the P2 Promoter by p53 in Response to a High Dose of UV Light. Both the primer extension (Fig. 2B) and S1 nuclease digestion (Fig. 3) analyses indicate that the amount of mdm2 transcript initiated at the p53-specific internal promoter (P2) was about 2.5 times higher 2 h after exposure to the low dose of UV light than it was 2 h after exposure to the high dose (Table 1). This pattern is paralleled by the greater increase in the rate of MDM2 protein synthesis 2 h after exposure to the low dose than 2 h after the high dose (Fig. 1B; Table 1). Thus, the changes in the rate of synthesis of MDM2 protein in response to UV light correspond to the amount of P2-specific transcript in the cells.

The magnitude of the increase in the P2-specific transcript was greater, relative to the level of p53 protein, 2 h after exposure to the low dose of UV light than it was 2 h after exposure to the high dose, indicating that p53 may be more effective as an activator of transcription after exposure of cells to the low dose of UV light than after exposure to the

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7 L. J. Saucedo, unpublished observations.
high dose (Table 1). The inefficiency of p53 in stimulating the P2 promoter 2 h after exposure to the high dose of UV light could be due to the general inhibition of RNA synthesis that occurs in cells exposed to UV light (36). To investigate whether the timing of mdm2 expression after exposure to the high dose of UV light corresponded to the timing of the recovery of general RNA synthesis, we measured the incorporation of [3H]uridine into C127 cells for 60 min at several times after exposure to UV light (36). We found that inhibition of general RNA synthesis occurred in cells exposed to both low and high doses of UV light, and that the inhibition was greater at the higher dose. Two h after exposure to the low dose of UV light, the rate of RNA synthesis was inhibited 14%, whereas after the high dose, it was inhibited 55%. The overall rate of RNA synthesis recovered by 5 h after exposure to the low dose of UV light, but it continued to decrease for up to 10 h after exposure to the high dose of UV light.8 Eight h after exposure to the high dose of UV light, the rate of RNA synthesis was further decreased to 30% of normal levels. At this time, the amount of mdm2 RNA transcribed from the P2 promoter was increased 8.9-fold. Thus, the induction of the P2 promoter after exposure to the high dose does not correspond to a recovery of the general rate of RNA synthesis. Instead, the P2 promoter is induced in spite of the general inhibition of RNA synthesis and seems to be regulated independently of the recovery of total RNA synthesis. Inhibition of general RNA synthesis might be expected to affect transcripts from both the P1 and P2 promoters equally; however, the ratio of the level of the P2 transcript to the P1 transcript is lower 2 h after exposure to the high dose of UV light than 2 h after exposure to the low dose (P = 0.0063). Thus, there is specific regulation of the amount of mdm2 transcription that initiates from the P2 promoter. These data indicate that in the presence of a general inhibition of RNA synthesis, the amount of transcript from the P2 promoter rises compared to the amount from the P1 promoter. In fact, the percentage of total RNA that is represented by the mdm2 transcript initiated at the P1 promoter is not significantly altered 2 or 8 h after exposure to the high dose.

The factors that contribute to the activity of the P2 promoter may be involved in the timing of induction of other promoters after exposure to high doses of UV light (37). For example, some cells are capable of inducing expression of immediate early genes such as c-fos and c-jun within 45 min of exposure to doses of UV light as high as 40 J/m² (38). In fact, the c-jun gene is induced to a greater extent after exposure to 40 J/m² than to 10 J/m² in HeLa cells (38). To test whether the factors required for induction of c-jun expression were rate-limiting after exposure of C127 cells to high doses of UV light, the levels of c-jun RNA were measured before and after exposure to 4 and 20 J/m². Expression of c-jun was induced to a greater extent 2 h after exposure to a high dose than 2 h after exposure to a low dose of UV light,7 indicating that C127 cells can induce expression of specific genes to a greater degree 2 h after treatment with the high dose than 2 h after treatment with the low dose. These results are consistent with those of others, indicating that specific factors regulate the activity of different UV-responsive promoters (37).

**Stabilized p53 is Localized to the Nucleus of UV-treated Cells.** The delay in expression of the P2-specific RNA after exposure to the high dose of UV light indicates that the ability of p53 to stimulate promoter activity may be inhibited during this period. The inhibition could result from a delay in transport of the p53 protein to the nucleus after stabilization in response to UV radiation. To determine whether there was a delay in transport of p53 from the cytoplasm to the nucleus after exposure of cells to a high dose of UV light, indirect immunofluorescence was used to locate p53. By 2 h after treatment with either the low or high dose of UV light, p53 had accumulated in the nucleus (Fig. 4). The amount of p53 in the nucleus appears higher 2 h after exposure to the high dose than 2 h after exposure to the low dose, as predicted from the continuous labeling experiment (Fig. 1). Therefore, the delayed induction of mdm2 transcription after exposure of C127 cells to the high dose of UV light is not explained by retention of p53 in the cytoplasm.

**Induction of Other p53-responsive Genes Seems Delayed after Exposure to the High Dose of UV Light.** The discrepancy between the level of p53 protein in the nucleus and the activity of the p53-dependent P2 promoter of mdm2 indicates that the ability of p53 to stimulate transcription is regulated. To determine whether other p53-responsive genes were induced to a greater extent 2 h after exposure to the low dose of UV light than 2 h after exposure to the high dose, we measured the levels of cyclin G1 (39) and p21WAF1 (5) RNAs before and after UV exposure. Expression of cyclin G1 was slightly greater 2 h after exposure to the low dose than 2 h after the high dose; however, the magnitude of the induction of cyclin G1 was only about 2-fold.7 Expression of p21WAF1 RNA was induced approximately 9-fold 2 h after exposure to the low dose of UV light and 4-fold 2 h after the high dose of UV light (Fig. 5), indicating that this p53-responsive gene is induced in a manner similar to that of mdm2. These data indicate that the activity of p53 may be modified by UV light in such a way as to affect all p53-responsive genes similarly.

To determine whether p53 function was required for the induction of p21WAF1 by UV light, we measured the levels of p21WAF1 mRNA after UV treatment of (10)3 cells that lack p53. Expression of p21WAF1 was induced in cells lacking p53 (Fig. 5), although to a lesser extent than in cells expressing p53. The length of the RNA induced by UV light is identical in cells expressing and lacking p53 (Fig. 5), indicating that there is not a p53-specific promoter in the p21WAF1 gene. Thus, the level of p21WAF1 RNA after exposure of cells to UV light cannot be used as a measure of p53 function. Indeed, our results are consistent with a recent report describing p53-independent induction of p21WAF1 expression after exposure of human cells to UV light (27).
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Fig. 4. Nuclear localization of p53 after exposure of C127 cells to UV light. Cells grown on coverslips were treated with a dose of UV light of 0, 4, or 20 J/m²; incubated at 37°C for 2 or 8 h; and then fixed in methanol. Indirect immunofluorescence was used to detect p53. The polyclonal Ab CM5 was incubated with the cells and followed by a FITC-conjugated goat antirabbit IgG. The negative control was a coverslip on which no CM5 Ab was placed before the secondary Ab (no primary Ab).

Fig. 5. Induction of p21WAF1 is delayed after exposure to a high dose of UV light. C127 cells were treated with a dose of UV light of 0, 4, or 20 J/m², and total RNA was harvested 2 or 8 h later. The (10)3 cells, which lack p53, were treated with 0 or 20 J/m² and harvested 8 h later. A radiolabeled primer complementary to exon 2 of the p21WAF1 gene was hybridized to RNA and extended by reverse transcriptase.

No specific binding of p53 to DNA could be detected in extracts from C127 cells before or after treatment with UV light unless the Ab 421, which can activate the DNA-binding function of p53 in an EMSA (41), was added to the reaction. The addition of Ab 421 resulted in greater DNA-binding activity by p53, such that DNA binding could be detected in extracts from both treated and untreated cells. A comparison of the amount of DNA bound by p53 from extracts of cells harvested 2 h after exposure to UV light reveals that there is more DNA-binding activity in cells after the high dose than after the low dose. Thus, the DNA-binding activity of p53
can be regulated allosterically (40). To determine whether the specific activity of p53 as a DNA-binding protein determines the activity of the P2 promoter of mdm2 after exposure of cells to UV light, the amount of p53-specific DNA-binding activity in nuclear extracts of C127 cells was measured by EMSA. Extracts were incubated with the consensus binding site for p53 (Fig. 6) or with sequences from the mdm2 gene.8

8 B. P. Carstens, unpublished observations.
Fig. 6. Activation of p53 DNA binding by Ab 421. Cells were treated as described in the Fig. 2 legend. Nuclear extracts were prepared 2 or 8 h after exposure to UV light. Equivalent amounts of nuclear protein were incubated with a radiolabeled double-stranded oligonucleotide with the sequence of the consensus binding site for p53. Extracts were incubated with the probe for 30 min on ice in the presence (+) or absence (−) of the 421 Ab, and the reactions were separated on a 5% denaturing gel. Bands corresponding to p53, as determined by both immunodepletion and competition with wild-type but not mutant oligonucleotides, are marked (+).

does not seem to be rate-limiting for transcription 2 h after exposure to the high dose of UV light.

The results of the EMSA could be complicated by the use of Ab 421, because addition of the 421 Ab to an EMSA is known to enhance the binding of certain forms of p53 that are thought to be inactive for DNA binding in the absence of Ab 421 (41, 42). Also, if exposure of C127 cells to UV light results in production of a form of p53 active for DNA binding but not recognized by Ab 421 (41, 43), the EMSA would not reveal the DNA-binding activity of such a protein. Therefore, we used a more sensitive DNA-binding assay to determine whether changes in the specific activity of p53 in response to UV light account for the delayed induction of the P2 promoter of mdm2.

An assay based on the immunoprecipitation method of McKay (44) allowed us to measure the ability of cellular p53 to bind DNA in the absence of the activating Ab 421 (Fig. 7). Whole-cell lysates were incubated with two radiolabeled restriction fragments from the 5’ end of the mdm2 gene. The smaller fragment included the two REs for p53 from the mdm2 gene; the larger fragment lacked sequences recognized by p53. Specific binding of p53 was reflected in an increased ratio of the fragment containing the REs (160 bp) to the fragment lacking the REs (450 bp) after immunoprecipitation of p53 with the polyclonal Ab CM5 (compare Lanes 3 and 4 of Fig. 7). This polyclonal Ab (CM5) recognizes several epitopes within the p53 protein, none of which is that recognized by Ab 421 (45). It follows that the CM5 Ab does not enhance the DNA-binding activity of p53 in an EMSA.

The sequence-specific DNA-binding activity of p53 was undetectable in extracts from cells that had not been exposed to UV light (Fig. 7); however, the activity of p53 was increased in cells exposed to UV light (Fig. 7). The amount of DNA-binding activity was higher 2 h after the high dose of UV light than 2 h after the low dose (1.9-fold, + 0.22).

To determine whether the specific activity of p53 as a DNA-binding protein correlated with the activity of the P2 promoter, we compared the DNA-binding activity with the amount of p53 protein present in each lysate. The same lysates were used to immunoprecipitate p53 in the presence (Fig. 7) and absence (Fig. 1) of DNA. For the McKay assay, the radiolabeled protein was extracted from the radiolabeled DNA before separation on the gel. The amount of DNA precipitated in the McKay assay increased in proportion to the amount of p53 in the extract. That is, the specific activity of p53 as a DNA-binding protein was not different in extracts from cells irradiated with either the low or high dose of UV light and harvested 2 h later (P = 0.27). Thus, the differential induction of mdm2 expression after exposure to low and high doses of UV light must be regulated, in part, at a step independent of the ability of p53 to bind DNA.

The Activity of the P2 Promoter Does Not Correlate with Changes in the Immunoreactivity of p53. The activity of the P2 promoter of mdm2 may be determined by a modification of the p53 protein or by the activity of a general transcription factor, a repressor, or a coactivator. To attempt to differentiate between these possibilities, we asked whether the reactivity of p53 to several conformation-specific Abs was different 2 h after exposure to the low and high doses of UV light.

Monoclonal Ab 421 recognizes an epitope in the carboxy-terminal region of p53 and has been reported to bind a form of p53 that lacks DNA-binding activity (46), but that can be activated for DNA binding by addition of Ab 421 to an EMSA (41). The 246 Ab recognizes an epitope between amino acids 88–109 of wild-type murine p53 (42), and reactivity of p53 with Ab 246 has been proposed to indicate that p53 is
functional as a DNA-binding protein (42). To determine whether the activity of the P2 promoter correlated with a change in the conformation of p53 from one reactive with Ab 421 to one reactive with Ab 246, p53 protein was radiolabeled and immunoprecipitated with either Ab (Fig. 8). The amount of p53 recognized by Abs 421 and 246 was compared to the amount of p53 precipitated by polyclonal Ab CM5 (see Fig. 1). The proportion of CM5-positive p53 that was positive for either Ab 421 or 246 did not change on treatment of cells with UV light. For example, the percentage of CM5-positive p53 recognized by Ab 421 was 82 (±4.4) and 88% (±6.3), on average, 2 h after exposure to the low and high doses of UV light, respectively. For Ab 246, the averages of the same samples were 85 (±13) and 89% (±8.3), respectively. Furthermore, sequential immunoprecipitation of p53 with the different Abs failed to reveal species of p53 that reacted with only Ab 246 or 421. Finally, a mixture of the three Abs, CM5, 421, and 246, failed to precipitate more p53 than did CM5 alone. Together, these experiments indicate that the majority of the p53 protein in C127 cells is reactive with both Abs 421 and 246 and that the activity of the P2 promoter does not correlate with changes in the conformational state of p53 as revealed by immunoreactivity with these Abs.

Discussion
The p53-dependent increase in expression of the MDM2 protein after exposure of cells to UV light is regulated through an increase in the activity of a p53-specific promoter in the mdm2 gene. The activity of this promoter is not induced in cells that lack p53 and is therefore a reflection of the ability of p53 and its effectors to regulate gene expression. A delay in stimulation of P2 promoter activity relative to a rise in the amount of p53 protein is evident after exposure to a high dose of UV light. Two DNA binding assays showed that the specific activity of p53 as a DNA-binding protein is not rate-limiting for stimulation of the P2 promoter of mdm2 after exposure of cells to the high dose of UV light. In most studies of endogenous p53, the DNA binding activity of p53 has been measured in the presence of Ab 421, which is known to increase the ability of p53 to bind DNA (6, 32, 47). Here, we have measured the ability of cellular p53 to bind DNA in cells that have been exposed to UV light without the use of an activating reagent. Our assay demonstrates that the activity of the P2 promoter of the mdm2 gene is not regulated solely by the amount of sequence-specific DNA-binding activity of p53 in the cell, indicating that other factors must contribute to regulate the timing of induction of mdm2. Such factors may be proteins that directly modify p53 or interact with the DNA to stimulate or retard transcription from the P2 promoter. Such proteins may regulate the ability of p53 to stimulate several different promoters, because induction of the p53-responsive p21WAF1 and cyclin G1, genes, like that of mdm2, is delayed relative to the increase in the level of p53. However, cyclin G expression is induced only about 2-fold, and p21WAF1 expression is induced in cells lacking p53. Thus, the level of the transcripts from these genes is not as reliable an indicator of the ability of p53 to stimulate transcription as the ratio of transcripts from the P2 and P1 promoters of mdm2.

The timing of induction of the P2 promoter of mdm2 seems to be specifically regulated in response to different doses of UV light, because it does not correspond to the recovery of the rate of total cellular RNA synthesis. Furthermore, our assays indicate that transcription from the P2 promoter is increased, whereas transcription from the P1 promoter is not increased after exposure to UV light. The high dose of UV light causes a delay in induction of the P2 promoter with no change in the level of transcription from the P1 promoter. This delay does not seem to be the result of direct DNA damage, because the template for transcription from the P1 promoter encompasses the template for transcription from P2 and includes more than 600 additional bp (34). The P1 template would be expected to suffer more DNA damage than the P2 template, resulting in an increased chance of inhibition of transcriptional elongation (48). Thus, exposure of cells to the high dose of UV light seems to affect the activity of the P2 promoter through a transacting factor that may be p53 itself or an unidentified protein that influences the effect of p53. The factor(s) influencing the ability of p53 to induce expression of mdm2 does not affect all UV-responsive genes similarly, because the c-jun gene, unlike mdm2, is induced to a greater extent 2 h after exposure to the high dose than 2 h after the low dose.

The factor(s) influencing the ability of p53 to induce expression of mdm2 is either inhibiting or rate-limiting at early times after exposure to the high dose of UV light. Several proteins, such as cyclin E and Ref-1, stimulate the ability of p53 to bind DNA and to activate transcription (49, 50). The activities of these proteins are unlikely to account for the delay in expression of mdm2 after exposure of cells to a high dose of UV light, because our data indicate that mdm2 expression is likely to be regulated at a step distinct from regulation of the ability of p53 to bind DNA. Some proteins stimulate the ability of p53 to induce transcription through mechanisms that are apparently independent of stimulating the DNA-binding activity of p53. For example, exogenous expression of either the p300 or CBP coactivator proteins increases the ability of p53 to induce the expression of reporter genes (12, 51, 52), including one regulated by the mdm2 promoter (12). It is unknown whether any of these proteins are required for p53 function in the response to UV
light. Recently, a repressor of mdm2 transcription has been proposed to be induced by high doses of UV light (53). This model is based on a transient decrease in total mdm2 RNA levels after exposure of primary cells to high doses of UV light (53). Evidence suggests that this effect is independent of p53 and therefore may be due to a decrease in the level of the P1 transcript, not an inhibition of induction of the P2 transcript. Because we did not detect a significant decrease in RNAs from either the P1 or P2 RNAs, we cannot distinguish between an induced repressor and an inactive (co)activator. Our data indicate that the delay in mdm2 expression at the high dose of UV light is due to a delayed induction, because the amount of transcript from the P1 and P2 promoters is not decreased.

The factors coordinating with p53 to regulate the activity of the P2 promoter may be intimately connected to the transcriptional machinery. In fact, several components of the multisubunit transcription factor TFIIH bind to p53, including two helicases mutated in the repair disorder xeroderma pigmentosum (54). Different forms of TFIIH are active in transcription and in repair (55). The subunit composition of TFIIH changes in response to UV light, and the activity of the kinase associated with TFIIH is reduced (55, 56). This kinase is required for transcription of some genes by RNA polymerase II (57). Thus, it has been proposed that the repair-competent form of TFIIH may down-regulate transcription in cells that have suffered large amounts of damage (58). The transcriptionally active form of TFIIH may be required for p53 to efficiently stimulate transcription from the P2 promoter of mdm2. Perhaps the higher levels of p53 protein seen 2 h after exposure to the high dose of UV light can compensate for a decrease in transcription-competent TFIIH. The signals regulating the conversion of repair-competent TFIIH to transcriptional active TFIIH after repair of DNA damage are unknown. These may be the same signals that regulate the recovery of the rate of total RNA synthesis; however, it is clear that the recovery of the rate of total RNA synthesis is not required for induction of P2 activity, because mdm2 is induced before the recovery of the rate of RNA synthesis. These observations indicate that factors independent of the recovery of total RNA synthesis may be rate-limiting for P2 function after exposure to the high dose of UV light. Although little is understood about the mechanisms governing the recovery of the rates of RNA synthesis in response to UV light, the amount of repair of UV-induced DNA damage is not the only factor, because cells derived from patients with Cockayne’s syndrome, which have normal global repair, fail to recover normal rates of general RNA synthesis after exposure to UV light (36).

It is possible that the induction of the P2 promoter is specifically delayed, because transcripts from this promoter may be more efficient than transcripts from the P1 promoter at directing translation of MDM2 (30). Because MDM2 inhibits p53 function (16) and stimulates p53 degradation (59, 60), cells severely damaged by UV light may forestall increasing the amount of MDM2 until p53 has carried out its required functions. Sunburned epidermal cells undergo p53-stimulated apoptosis (61), and it may be in the best interest of the organism to delay mdm2 expression until such time as cells have committed to apoptosis, because MDM2 can inhibit p53-dependent apoptosis (62). Studies of the requirements for induction of the p53-responsive P2 promoter of mdm2 may reveal unknown mechanisms of regulation between these two proteins after exposure to agents that damage DNA.

Materials and Methods

Cell Culture. C127 is a nontransformed murine cell line expressing wild-type p53 (63). Cells lacking p53 (1103 and 1101) were immortalized from 14-day-old embryos by BALB/c mice (64). V55 is a derivative of (101) and expresses a temperature-sensitive allele of p53 (ala135Val) that acts as a transcriptional activator at 32°C but not at 37°C (7). A1–5 cells are rat embryo fibroblasts transformed with Ha-ras and the temperature-sensitive allele of p53 (65). Cells were cultured in DMEM with 10% FCS (HyClone) in a humidified environment of 5% CO2.

Exposure to UV Light. C127 or 1103 cells were plated at a density of 2.1 × 10^5 cells/15-cm dish and exposed 24–36 h later to a Blak-Ray UV lamp with a wavelength of 254 nm (UVC). The distance between the plates and the lamp was adjusted so that the fluence of the light was 2 J/m^2/s, as measured by a Blak-Ray J-225 UV meter (UV Photoproducots, San Gabriel, CA).

Metabolic Labeling and Immunoprecipitation. For determination of the levels of p53 protein after stabilization by UV light, C127 cells were radiolabeled for 1 h (three p53 half-lives) before treatment with UV light and for 2 or 8 h after exposure to a dose of 0, 4, or 20 J/m^2. Cells were incubated for the length of time indicated with 100 μCi/ml 35S-Express (a mixture of 35S)methionine and 35S)lyssoleucine (DuPont New England Nuclear) in normal growth medium (DMEM plus 10% FCS). After lysis (66), the concentration of protein in each sample was determined using the Bio-Rad protein assay (67), and equivalent amounts of total protein (200 μg) were incubated with the CM-5 Ab (described below). For determination of the rate of synthesis of MDM2 protein, the cells were labeled for 30 min with 100 μCi/ml 35S-Express in DMEM lacking methionine supplemented with 2% dialyzed FCS. The amount of radiolabeled protein in each sample was quantified by trichloroacetic acid precipitation and used to normalize the volume of lysate added to the immunoprecipitation reactions such that each had 5 × 10^6 cpm. Quantitation was performed on a Molecular Dynamics PhosphorImager.

Abs. Polyclonal CM5 is a rabbit antisera specific for p53 (Novocastra); monoclonal Ab 421 recognizes the carboxyl terminus of wild-type and mutant p53 (45), and monoclonal Ab 246 recognizes wild-type murine p53 (45). The Ab used as a negative control was monoclonal Ab 419, which recognizes SV40 large T antigen (68), a protein not expressed in any of the cells used in this study. Hybridomas for Abs 421, 246, and 419 were from Arnold J. Levine (Princeton University). The polyclonal Ab 628 was used to immunoprecipitate MDM2. This Ab was raised in our laboratory against a histidine-tagged fusion protein of murine MDM2 purified from Escherichia coli (as first described in Ref. 69).

Plasmids. The plasmid encoding histidine-tagged MDM2 was provided by Donna George (University of Pennsylvania). Plasmids containing cDNAs from mdm2 (pGEM1F and pGEM1X2) were gifts from Moshe Oren (Weizmann Institute). pGEM1F contains a cDNA from a transcript including exons 1–12 of mdm2, whereas pGEM1X2 contains a cDNA from a transcript including exons 2–12 (30). To generate a source of a probe for the nucleic acid protection assay, an EcoRI fragment from pGEM1F containing the entire cDNA for mdm2 was excised and cloned into the EcoRI site of Bluescript (Stratagene) to generate pBSF.

Primer Extension. An oligonucleotide primer (100 ng) of the sequence 5′-TCGAAGCTTGTCTCTGAGACCA-3′, complementary to exon 3 of mdm2 (34), or an oligonucleotide (100 ng) of the sequence 5′-TCCAGCGATTGGACATG-3′ (5), complementary to exon 2 of p21WAF1, was end-labeled with [γ-32P]ATP using T4 kinase (New England Biolabs) and incubated with ethanol in the presence of RNA homopolymer (Pharmacia). Total RNA was harvested according to Sambrook et al. (70), and the primer extension reaction was modified from that described by Treiez et al. (71). Radiolabeled primer (3 × 10^5 cpm) was annealed to 20 μg of total RNA in a buffer containing 300 mM NaCl, 10 mM Tris (pH 7.5), 12.5 μg/ml actinomycin D (Calbiochem), and 1 μM EDTA by denaturation at 80°C for 1 min, followed by incubation at 62°C for 15 min. After annealing,
the buffer was adjusted to contain 10 mM Tris (pH 8.0), 8 mM MgCl₂, 10 mM DTT, 1 mM deoxyribonucleotide triphosphates, and 25 units of avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim). The reactions were incubated at 42°C for 90 min and then at 52°C for 30 min. The samples were subsequently treated with RNase A (Boehringer Mannheim), precipitated with ethanol, and separated on a 5% denaturing polyacrylamide gel. To generate a marker for mdm2, pGem1/F was sequenced with the primer described above using Sequenase version 2.0 (United States Biochemical) according to the manufacturer’s instructions. Products migrating more slowly than that from pGem1/F RNA were excised from the gel after electrophoresis and purified using the Wizard PCR Purification Kit (Promega). The eluted cDNA was then amplified in a 5'-rapid amplification of cDNA ends (RACE Technologies, Inc.) reaction using a nested primer (5'-ATTCGAGAGTGGTCATCA-3') for the PCR. The resultant PCR products of expected size were then gel purified, reamplified, and directly cloned into a TA vector [pT7Blue(R), Novagen]. Fourteen clones were sequenced as described above.

**S1 Nucleosome Protection Assays.** The probe was a 254-bp Accl restriction fragment from pBSF that includes sequences from exons 1, 2, and 3 of mdm2 as well as 27 bp from the plasmid vector at the 5’ end. Twenty µg of total RNA were analyzed in each reaction. End-labeling, hybridization, and nuclelease treatment were performed as described by Sambrook et al. (70) with a hybridization temperature of 48°C. The protected products were separated on a 5% denaturing polyacrylamide gel. For controls, the probe was digested after annealing to RNAs transcribed in vitro from pGem1/F and pGem1/X (Stratagene).

**Total RNA Synthesis.** RNA synthesis was measured 0, 2, 5, 8, and 10 h after exposure to a UV dose of 4 or 20 J/m². Cells were incubated with 10 µCi/ml [³²P]uridine (29 Ci/mM; Amersham) for 1 h after treatment, washed twice in PBS, and lysed in SDS. After reduction of viscosity of the samples by passage 10 times through an 18-gauge needle, duplicate samples were spotted onto Whatman 3 MM paper and precipitated with trichloroacetic acid as described previously (86). As a control for cell loss, the cells were prelabelled with 0.02 µCi/ml [³²P]thymidine (56.9 Ci/mM; Amersham) for 24 h before exposure to UV light; the results indicated no loss of cells during the time course of this assay.

**Indirect Immunofluorescence.** C127 cells were grown on coverslips, irradiated, and then incubated for the times indicated. The coverslips were then rinsed in PBS and fixed for 10 min in 100% methanol at room temperature. Coverslips were rinsed with PBSB, incubated for 1 h in the presence or absence of a polyclonal Ab specific for p53 (CMS), and then washed three times with 5 min each with PBSB. The coverslips were then incubated for 45 min with a FITC-conjugated goat antirabbit IgG (Vector Laboratories). After extensive washing with PBSB, the coverslips were mounted on slides and viewed and photographed with a Zeiss Axioskop fluorescence microscope.

**EMSA.** Nuclear extracts were prepared, and binding assays were conducted as described by Price and Calwooder (47) with the following modifications: the binding assays were performed with 0.2 µg of Ab and 10 µg of nuclear extract and incubated at room temperature for 30 min. An oligonucleotide identical to the consensus binding site for p53 defined by El-Deiry et al. (Ref. 72; 5'-GGTGGAAGACGGGCATGCGCCGGG-3') was end-labeled with [³²P]ATP, and 1 ng of the radiolabeled oligonucleotide (3 x 10⁴ cpm) was incubated with each sample. Protein-DNA complexes were separated on a 5% nondenaturing polyacrylamide gel. Loading dye was added only to the sample containing probe alone.

**McKay Assay.** C127 cells were lysed by the same method used for immunoprecipitation, aliquoted, and stored at -80°C. Binding reactions were based on the method of McKay (44) and contained two fragments from the mdm2 gene. The larger fragment contained sequences from upstream of the two REs for p53 (nonspecific probe) and the other contained sequences that include the two REs for p53 (specific probe). To generate these fragments, a 610-bp BglII-Aval fragment from plasmid CoaxC17 (7) was digested with HindIII. The resulting fragments, a 450-bp BglII-Aval fragment and a 160-bp HindIII-Aval fragment, were treated with calf intestinal phosphatase (Boehringer Mannheim) and end-labeled with T4 polynucleotide kinase (New England Biolabs). The 160-bp fragment contains both 20-bp REs for p53, whereas the 450-bp fragment contains no sequences recognized specifically by p53 (7). The immunoprecipitation reaction was modified from Kern et al. (73) to contain: 2 x 10⁵ cpm of ³²P labeled probe, 20 µg of lysate, 1.6 µg of CMS Ab, 60 µl of binding buffer, and sufficient lysate buffer to bring the total volume to 75 µl. Binding reactions were then incubated with protein A-Sepharose (Sigma) and poly(deoxyinosinie-deoxyctydilic acid) (Pharmacia) as described by Kern et al. (73). DNA was purified from proteins by digestion with 50 µg/ml proteinase K (Boehringer Mannheim) in 10 mM Tris (pH 7.8), 5 mM EDTA, and 0.5% SDS, followed by extraction with phenol and chloroform and precipitation with ethanol. The labeled DNA fragments were separated on a 5% nondenaturing polyacrylamide gel and quantified on a PhosphorImager.

**Statistical Analysis.** Data are expressed as means ± SE. The unequal Student's t test was used to determine the significance of differences between data.

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**References**


