[CANCER RESEARCH 63, 6583–6594, October 15, 2003]

The Human Ribonucleotide Reductase Subunit hRRM2 Complements p53R2 in Response to UV-Induced DNA Repair in Cells with Mutant p53[1]

Bingsen Zhou, Xiyong Liu, Xueli Mo, Lijun Xue, Dana Darwish, Weihua Qiu, Jennifer Shih, Edward B. Hwu, Frank Luh, and Yun Yen[2]

Department of Medical Oncology and Therapeutics Research, City of Hope National Medical Center, Duarte, California 91010

ABSTRACT

Ribonucleotide reductase (RR) is responsible for the *de novo* conversion of the ribonucleoside diphosphates to deoxyribonucleoside diphosphates, which are essential for DNA synthesis and repair. RR consists of two subunits, hRRM1 and hRRM2. p53R2 is a new RR family member. Because the majority of human tumors possess mutant *p53*, it is important to know the molecular mechanism by which mutant *p53* regulates RR and to what extent. In this study, we investigated the expression and function of p53R2 and hRRM2 after UV treatment in human prostate cancer PC3 cells, which possess mutant *p53* with a truncated COOH-terminal, and in human oropharyngeal cancer KB cells, which possess wild-type *p53*. p53R2 (analyzed by Western blot and standardized relative to Coomassie Blue-stained band) was down-regulated in PC3 cells and up-regulated in KB cells after UV exposure. In contrast, hRRM2 was up-regulated by UV in both PC3 cells and KB cells. hRRM2 and p53R2 mRNA levels were assessed by Northern blot, and the results paralleled that of the Western blot. Coimmunoprecipitation assays using agarose-conjugated goat anti-human RRM1 antibody confirmed that the p53R2 binding to hRRM1 decreased in PC3 cells but increased in KB cells after UV treatment. hRRM2 binding to hRRM1 increased in both cell lines under the same conditions. These results suggest that PC3 cells are deficient in both transcription of p53R2 and binding to hRRM1 in response to UV irradiation. Confocal microscopy further confirmed that these findings were not due to translocation of hRRM2 and p53R2 from the cytoplasm to the nucleus. RR activity was measured following UV treatment and shown to increase in PC3 cells. It was unchanged in proportional of KB cells. The RR activity is consistent with the expression of hRRM2 seen in the Western blots. Thus, we hypothesize that hRRM2 complements p53R2 to form RR holoenzyme and maintain RR activity in PC3 cells after UV treatment. To further confirm this hypothesis, we examined the effect of RRM2 inhibitors on cells exposed to UV. In PC3 cells, hydroxyurea inhibited hRRM2 and resulted in increased sensitivity to UV irradiation. We also examined the effect of UV treatment on the colony-forming ability of cells transfected with hRRM2 as well as p53R2 sense or antisense expression vectors. Expression of antisense hRRM2 in PC3 cells led to decreased hRRM2 expression and resulted in greater sensitivity to UV than observed in wild-type PC3 cells. Taken together, we conclude that UV-induced activation of p53R2 transcription and binding of p53R2 to hRRM1 to form RR holoenzyme are impaired in the p53-mutant cell line PC3.

INTRODUCTION

RR[3] is a highly regulated enzyme in the deoxyribonucleotide synthesis pathway in human, bacterial, yeast, and others (1). RR is responsible for the *de novo* conversion of ribonucleoside diphosphates to deoxyribonucleoside diphosphates that are essential for DNA synthesis and repair (1, 2). RR consists of two subunits, M1 and M2. M1 (hRRM1) is a M_r 170,000 dimer with a binding site for enzyme regulators (3). M2 (hRRM2) is a M_r 88,000 dimer containing a tyrosine free radical and a non-heme iron for enzyme activity (3). The enzyme is expressed specifically in S phase and is rate-limiting for DNA synthesis. Therefore, RR plays an important role in the regulation of cell proliferation (1–5). Recently, a new RR family member, p53R2, was cloned by Tanaka *et al.* (6). p53R2 contains a *p53*-binding site in intron 1 and encodes a 351-amino acid peptide with striking similarity to hRRM2 (6). Expression of p53R2, but not hRRM2, is induced by UV light, γ-irradiation, or Adriamycin treatment in a *p53*-dependent manner (6–8). The discovery of p53R2 suggests a relationship between RR activity and repair of damaged DNA (6). Tanaka *et al.* (6) reported that p53R2 supports DNA repair by increasing the dNTP pool needed for repair. Inhibition of endogenous p53R2 expression in cells that have a *p53*-dependent DNA damage checkpoint led to reduced RR activity, DNA repair, and cell survival after exposure to various genotoxins. Guittet *et al.* (7) further reported that p53R2 and hRRM1 generate a RR holoenzyme that is directly involved in *p53*-directed repair of damaged DNA. Although the role of p53R2 in the DNA repair pathway has been defined, it is still unclear whether this function of p53R2 is altered in cell with a mutant *p53* phenotype.

p53 has been shown to participate in several cellular responses that could contribute to the suppression of tumor development, including cell cycle arrest and apoptosis (9–13). Activation of *p53* in response to stress signals such as DNA damage is thought to prevent the replication of abnormal cells by either allowing their repair or targeting them for elimination. However, it was not clear whether *p53* contributed directly to DNA repair until p53R2 was cloned. In most cells, wt *p53* exists in a latent form and in very low concentrations. In response to stress, upstream signals activate *p53* to respond to the need for DNA repair. The amount of wt *p53* is rapidly increased in cells; it activates transcription of target genes. The increased *p53* level may be due to an increased half-life or to an increase in the rate of transcription. It has been reported that wt *p53* may activate transcription of p53R2 in response to UV light (6). However, it has never been clear whether mutant *p53* directs transcription of p53R2 or other RR subunits or alters their interaction (14).

Exposure of mammalian cells to UV irradiation leads to the introduction of a number of photoproducts in cellular DNA. These are removed by the NER pathway. Damaged nucleotides are excised together with a number of adjacent nucleotides (15), and the resulting gaps are filled in by DNA polymerase and sealed by ligase. To fill these gaps, cells need precursors for DNA synthesis. RR is a unique enzyme that can supply these precursors (16, 17). It has been shown that NER normally occurs within 3 h of DNA damage (18). Therefore, cells need to have rapid mechanisms to supply precursors for prompt DNA repair. Regulation of p53R2 by *p53* may fill this need. Rapid induction of RR activity may be achieved by releasing p53R2 from an inactive complex with *p53*, freeing it to bind to hRRM1 and form an active holoenzyme. Activation of p53R2 transcription by *p53* may then provide additional p53R2 in a slower fashion to maintain RR

Received 11/8/02; revised 7/14/03; accepted 7/28/03.

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[1] Supported by National Cancer Institute Grant CA 72767.

[2] To whom requests for reprints should be addressed, at Department of Medical Oncology and Therapeutics Research, City of Hope National Medical Center, 1500 East Duarte Road, Duarte, CA 91010-3000. Phone: (626) 359-8111, ext. 62307; Fax: (626) 301-8233; E-mail: yyen@coh.org.

[3] The abbreviations used are: RR, ribonucleotide reductase; dNTP, deoxynucleoside triphosphate; NER, nucleotide excision repair; wt, wild-type; HU, hydroxyurea; IPTG, isopropyl-1-thio-β-D-galactopyranoside; RPA, RNA protection assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GGR, global genomic repair; CBB, Coomassie Blue-stained band.

activity at later times. This hypothesis was suggested by our previous report that p53R2 binds to *p53* but is released within 3 h of UV irradiation, whereupon it binds to hRRM1 and forms an active holoenzyme (14). This mechanism would be more rapid than a transcriptional mechanism. Therefore, it is important to examine the regulation of p53R2 after UV exposure in a time-dependent manner.

The role of hRRM2 in DNA repair has lately become controversial. In a mouse study, the level of RRM2 decreased in G_1- and G_2-phase cells but increased during S phase, suggesting that it may not be involved in DNA repair (19, 20). Others have suggested that hRRM2 only supplies dNTPs for DNA replication in S phase, whereas p53R2 replaces it to supply dNTPs needed for DNA repair during G_1 and G_2 phases (6). *p53* contributes to the activation of G_2 cell cycle arrest in response to DNA damage. However, cells transfected with p53R2 show only a slightly elevated number of cells in S-G_2-phase after UV treatment (21), suggesting that cell cycle arrest in response to DNA damage requires signals in addition to p53R2 (6, 19). The hRRM2 gene is expressed during the cell cycle in S-G_2 phase (22–25). It has been reported that hRRM2 plays a role in repairing UV-induced DNA damage outside of S phase in yeast (26). The response of hRRM2 to ionizing radiation in human cervical carcinoma cells has also been examined (27). Therefore, it remains unclear whether the hRRM2 gene plays a role in DNA repair in *p53* wt or mutant lines. It has been known that the process of carcinogenesis results from a long-term exposure to a low dosage of the DNA damage agent. Our rationale, then, was to apply sublethal-dose UV irradiation rather than high-dose γ-irradiation to demonstrate how hRRM2 and p53R2 are involved in DNA repair.

Because the majority of human tumors possess mutant *p53*, it is important to know the molecular mechanism by which mutant *p53* regulates RR in response to UV-induced DNA damage. In this study, we investigated the expression and function of p53R2 and hRRM2 after UV treatment in human prostate cancer cells (PC3), which possess mutant *p53* with a truncated COOH-terminal.

MATERIALS AND METHODS

Cells. Human oropharyngeal carcinoma KB cells (*p53* wt), human prostate cancer PC3 cells (*p53* mutant), human prostate cancer DU145 cells (p53 mutant), human hepatoma HepG2 cells (p53 wt), and human hepatoma Hep3B cells (*p53* null) were purchased from American Type Culture Collection. Cells were cultured on plastic tissue culture plates in RPMI 1640 (DMEM for HepG2 and Hep3B cells) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin in a 5% CO_2 atmosphere at 37°C. KB cells were transfected with hRRM2 sense (KB-M2S) or antisense (KB-M2AS) cDNA by an inducible vector system as described previously (28), and PC3 cells were transfected with hRRM2 sense (PC3-M2S) or antisense (PC3-M2AS) cDNA in the same vector system and cultured on plastic tissue culture plates in RPMI 1640 supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 300 μg/ml G418, and 200 μg/ml hygromycin. To induce DNA damage, exponentially growing cells were treated with UV (20, 10, and 5 J/m^2 for KB; 5, 2.5, and 1.25 J/m^2 for PC3) and then returned to culture for varying times.

Plasmid Construction and Stable Clone Selection. To construct the p53R2 expression vector, a full-length p53R2 cDNA (translation region only) was synthesized by reverse transcription-PCR. Then, the cDNA was cloned into *Bam*HI/*Not*I-digested plasmids pcDNA3.1(+) and pcDNA3.1(−) (Invitrogen, San Diego, CA). Both plasmids were transfected into wt KB cells by electroporation. Forty-eight h after transfection, the selection medium (300 μg/ml G418 in RPMI 1640) was added to select stabilized clones. After a month's selection, both sense and antisense stable clones were selected and designed as KB-p53R2S and KB-p53R2AS.

Colony Forming Assay. The colony-forming ability of KB, KB-M2S, KB-M2AS, KB-p53R2S, KB-p53R2AS, PC3, PC3-M2S, and PC3-M2AS cells treated with varying doses of UV was determined. Logarithmically growing cells (1000 cells/well) were plated in 60-mm tissue culture dishes. Cells were treated with UV or UV plus HU and then incubated at 37°C for 8 generation times (6 days), and the number of colonies (>50 cells) was counted. For KB-M2S, KB-M2AS, PC3-M2S, and PC3-M2AS cells, 5 μM IPTG was added to induce M2 cDNA expression before UV treatment.

Cell Cycle Analysis. Exponentially growing PC3 and KB cells (1×10^6) were treated with UV (10 J/m^2 for PC3 cells; 20 J/m^2 for KB cells), and cells were harvested after 1, 2, 3, 6, 12, 16, 24, and 48 h. Cells were then washed with PBS, fixed with 70% ethanol, and pretreated with 10 μg/ml RNase (Sigma). Cells were further stained with propidium iodide (10 μg/ml; Sigma) and analyzed by flow cytometry. The cell cycle profile was determined by using program M software on an Epics flow cytometer (City of Hope Core Facility).

Northern Blot Analysis and RPA. Exponentially growing PC3 and KB cells were treated with UV (10 J/m^2 for PC3 cells; 20 J/m^2 for KB cells), and total RNA was extracted after the designated number of hours. RNA was separated by formaldehyde-agarose gel electrophoresis and blotted onto Hybond-N membrane. Hybridization was performed under stringent conditions using radioactive probes that were prepared as described previously (5). Blots were probed with PCR products consisting of full-length hRRM2 or p53R2 cDNA, and RNA loading was normalized by probing for GAPDH expression. Multihuman tissue mRNA blots were purchased from BD Clontech (Palo Alto, CA), and hybridization conditions followed the manufacturer's recommended protocol. For the RPA, the Multi-Probe Template Set containing Human Cell Cycle-related gene DNA templates (hCC templates) was purchased from PharMingen International (San Diego, CA). The manufacturer's protocol was followed in this study.

Antibodies. Polyclonal rabbit antihuman R2 antibody was a gift from Dr. T. J. Kinsella's laboratory. Monoclonal mouse antihuman *p53* (sc-126), polyclonal goat antihuman R2 (sc-10844), polyclonal goat antihuman p53R2 (sc-10840), polyclonal goat antihuman R1 (sc-11733), agarose-conjugated normal goat IgG (sc-2346), agarose-conjugated polyclonal goat antihuman R1 (sc-11733 AC), FITC-conjugated goat antirabbit IgG (sc-2012), rhodamine-conjugated bovine antigoat IgG (sc-2349), alkaline phosphatase-conjugated goat antimouse IgG (sc-2008), and alkaline phosphatase-conjugated bovine antigoat IgG (sc-2351) were all purchased from Santa Cruz Biotechnology, Inc.

Immunoprecipitation and Western Blot. Approximately 1×10^7 cells were washed twice with PBS and lysed in 0.65 ml of ice-cold radioimmunoprecipitation assay buffer (1× PBS, 1% NP40, 0.5% sodium deoxycholate, and 0.1% SDS) with freshly added protease inhibitors (100 μg/ml phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 30 μl/ml aprotinin). The lysate was passed through a 27-gauge needle, debris was removed by centrifugation (10,000 rpm, 10 min, 4°C), and the protein concentration was measured (Bio-Rad protein assay). Ten mg of each lysate were precleared with agarose-conjugated normal goat IgG (30 min at 4°C) and subsequently incubated overnight at 4°C with agarose-conjugated polyclonal goat antihuman R1 antibody (15 μl). Beads were collected by centrifugation (6000 rpm, 10 min, 4°C), and the immunoprecipitates were washed four times with lysis buffer (4°C) and then solubilized in 60 μl of SDS-PAGE sample buffer. Seventy μg of each cell lysate and 12 μl of each immunoprecipitate were separated by SDS-PAGE (14%) and transferred to polyvinylidene difluoride membrane (Amersham Pharmacia Biotech). After transfer, membranes were kept in 1% I-block blocking buffer (Applied Biosystems) until detection. Polyclonal goat antihuman RRM2 or p53R2 antibody was diluted 1:200 in blocking buffer containing 1% I-block reagent and 0.1% Tween 20 (Applied Biosystems). Polyvinylidene difluoride membranes were incubated with the antibodies for 1 h at room temperature and then washed six times with 0.5% I-block blocking buffer. Washed membranes were incubated with 1% I-block blocking buffer containing alkaline phosphatase-conjugated secondary antibody (bovine antigoat IgG, diluted 1:2000) for 30–60 min and then washed with 0.5% I-block blocking buffer and assay buffer (200 mM Tris and 10 mM $MgCl_2$). A thin layer of CSPD Ready-to-Use substrate solution (Applied Biosystem) was pipetted onto the membrane, incubated for 5 min, and then exposed to X-ray film for 3 min. Multihuman tissue protein samples were purchased from BD Clontech.

Confocal Microscopy. Cells were grown on sterile glass coverslips at 37°C for 24 h, exposed to UV irradiation, and then harvested at the indicated times. Cells were washed briefly with PBS and fixed with 100% methanol for 5 min at −20°C. After air drying, fixed cells were washed three times with PBS, blocked for 2 h in blocking buffer (10% BSA in PBS), and then incubated for another hour in blocking buffer containing goat antihuman p53R2 IgG,

rabbit antihuman hRRM2 IgG, or both antibodies together. After washing three times in PBS, cells were incubated with secondary antibodies (FITC-conjugated goat antirabbit for hRRM2, rhodamine-conjugated bovine antigoat for p53R2, or both antibodies) in blocking buffer for 45 min in the dark at 37°C. Glass slides were washed three times in PBS and mounted with a coverslip with 90% glycerol in PBS. Images were acquired using a confocal microscope (Zeiss).

RR Activity Assay. The RR activity assay was performed as described previously. In brief, 1×10^7 cells were plated in a 150-mm dish and incubated for 24 h. Then the cells were treated with UV irradiation (10 J/m^2 for PC3 cells; 20 J/m^2 for KB cells) and harvested at the indicated time points. Cells were washed twice with cold PBS and detached by trypsin and a cell scraper. Cells were transferred to a 15-ml tube and pelleted by centrifugation at $300 \times g$ for 10 min at 4°C. The pellets were washed again with PBS. One volume of low-salt homogenization buffer [10 mM HEPES (pH7.2) and 2 mM DTT] was added to the cell pellets, and the cell suspension was passed through a needle 20 times on ice. After homogenization, one volume of high-salt buffer [1 M HEPES (pH 7.2) and 2 mM DTT] was added, and the cell suspension was again passed through a needle 20 times on ice. Cell debris was removed by centrifugation at $16{,}000 \times g$ at 4°C for 20 min. The supernatant was passed through a Sephadex G25 spin column pre-equilibrated with buffer [50 mM HEPES (pH 7.2) and 2 mM of DTT] to remove endogenous nucleotides. Protein concentration was measured by the Bio-Rad protein assay. The reaction mixture contained 0.15 mM [^{3}H]CDP (0.02 μCi), 2 mM ATP, 0.05 mM CDP, 50 mM HEPES (pH 7.2), 6 mM DTT, 4 mM magnesium acetate, and differing amounts of cell extract in a final volume of 0.15 ml. After a 20-min incubation, the dCDP formed was dephosphorylated by phosphodiesterase, and C and dC were separated by high-performance liquid chromatography with a C_{18} ion-exchange column. The cytidine and deoxycytidine peaks were collected in scintillation vials, and the amount of radioactivity was measured with a Beckman LS 5000CE liquid scintillation counter. Specific activity was calculated as nmol CDP/h/mg protein.

RESULTS

Expression of RR Subunits in Different Normal Human Tissues. We have examined the expression of hRRM2, p53R2, and hRRM1 mRNA in different normal human tissues by Northern blot. β-Actin was used as a control. As shown in Fig. 1*A*, hRRM1 mRNA was found to be highly expressed in most tissues, whereas hRRM2 mRNA was readily detected primarily in proliferating tissues including placenta, lung, thymus, testis, small intestine, and colon. Like hRRM1, p53R2 mRNA was detected in most tissues but was in-

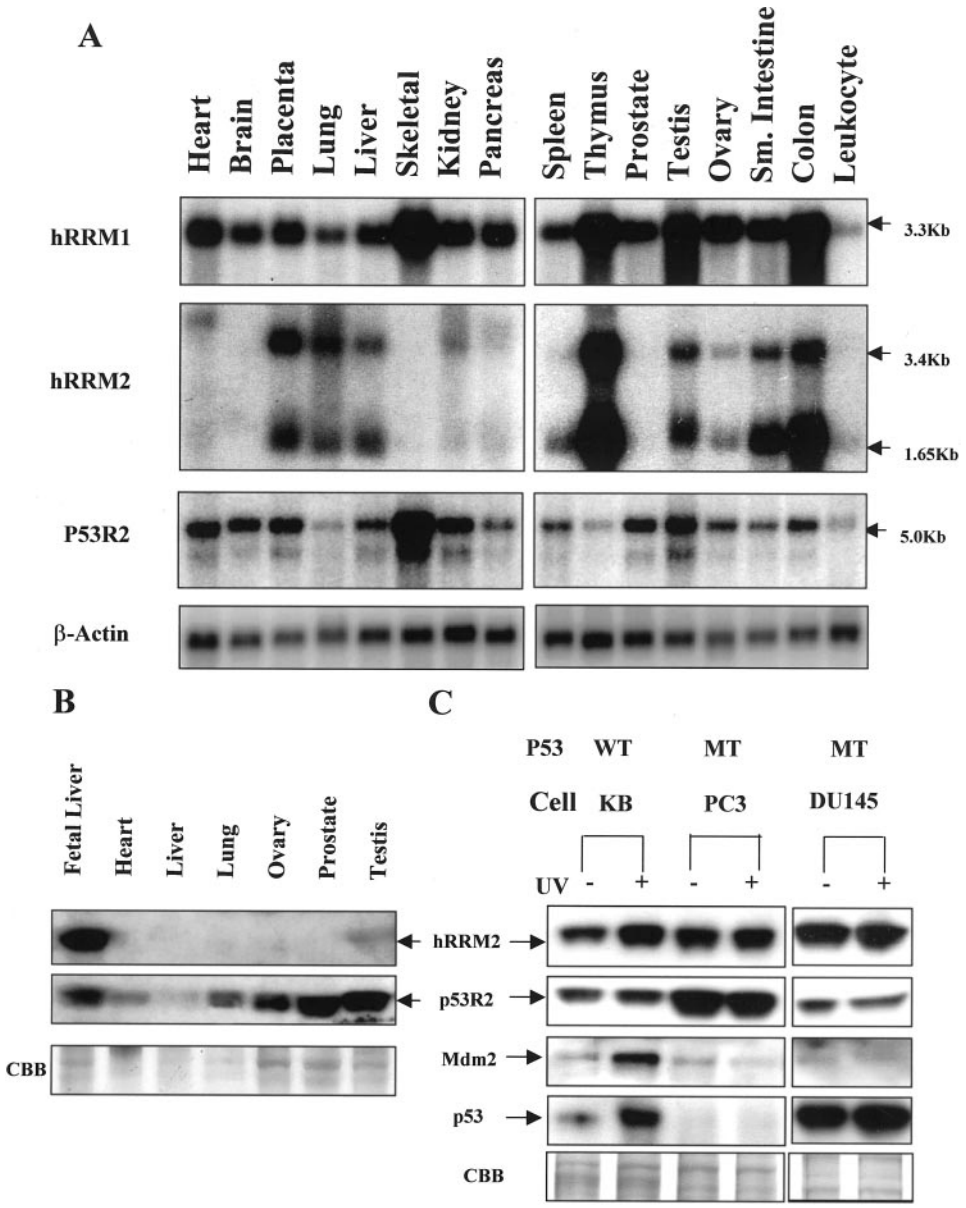

Fig. 1. Expression of RR subunits in different normal human tissues and tumor cell lines. *A* shows a Northern blot of multiple human tissues. The blot was probed to detect expression of hRRM1, hRRM2, and p53R2. β-Actin was used to control for the total amount of mRNA present in each lane. *B* shows a Western blot of multiple human tissues that was hybridized with antibodies to detect hRRM2 and p53R2. The CBB was used to control for the total amount of protein present in each lane. *C* shows a Western blot detecting p53R2, hRRM2, and Mdm2 protein expression before and after UV irradiation (24 h) in five human tumor cell lines. p53 status was demonstrated as wt (*WT*), mutant (*MT*), or null.

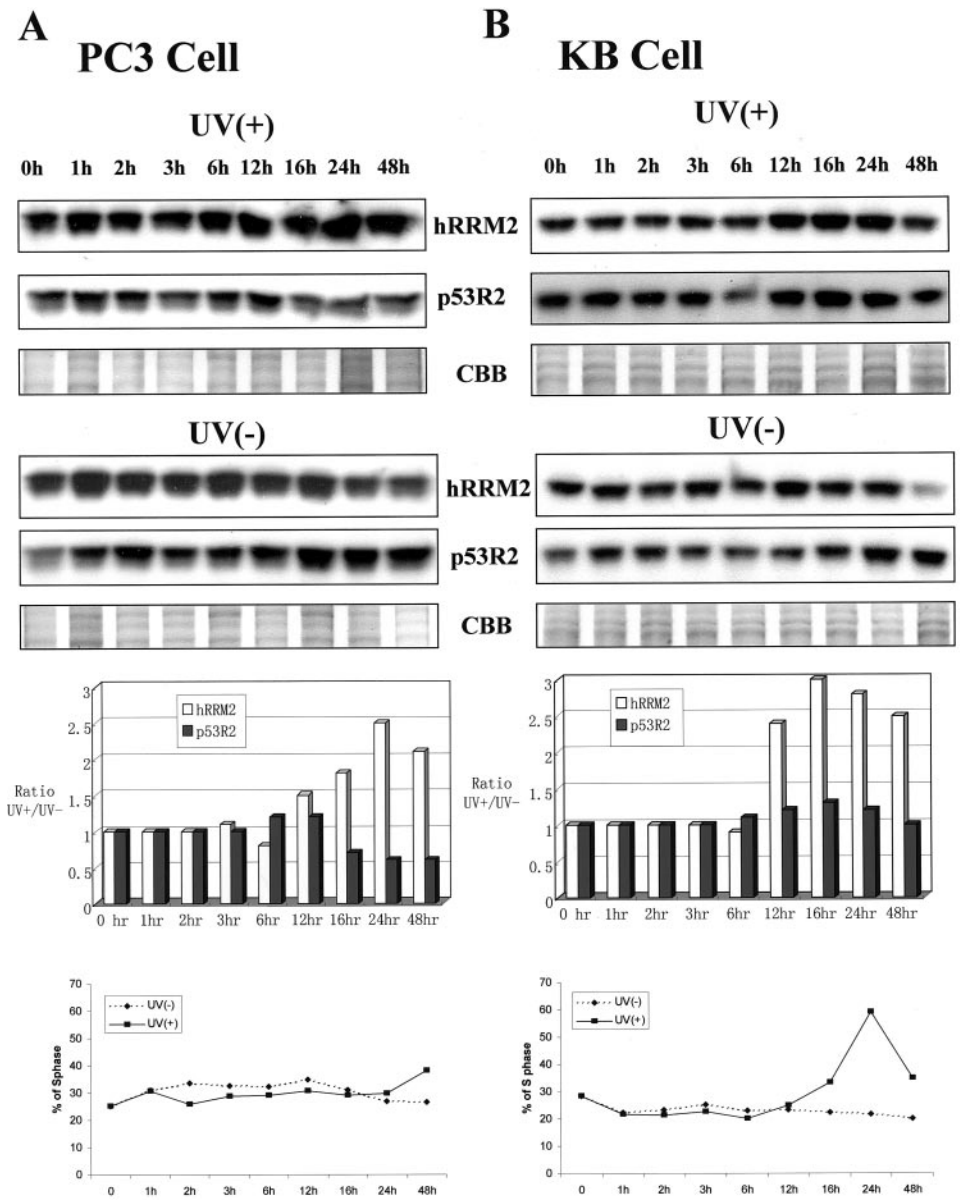

Fig. 2. Western blot and cell cycle profile analysis of hRRM2 and p53R2 protein expression in PC3 and KB cells after UV irradiation. Total cellular protein (100 μg/lane) was extracted from PC3 and KB cells after UV irradiation and from parallel control cells not exposed to UV and analyzed by Western blot using anti-hRRM2 and -p53R2 antibodies. The CBB was used to control for the total amount of protein present in each lane. *A* and *B* show the amount of hRRM2 and p53R2 detected in PC3 and KB cells at the indicated times. The intensity of each band was measured by densitometry and standardized by the CBB control. The effect of UV treatment is shown as the ratio of UV(+)/UV(−) hRRM2 or p53R2 protein. *C* and *D* show the cell cycle profile of PC3 and KB cells at the indicated times.

creased in skeletal muscle tissue and decreased in thymus tissue. In Fig. 1*B*, protein expression detected by Western blot showed a high level of hRRM2 expression in fetal liver and moderate expression in testis, but low expression in other tissues. p53R2 was expressed relatively highly in all tissues except liver (Fig. 1*B*). These results indicate that expression of p53R2 is much more widespread than that of hRRM2. Moreover, posttranscriptional regulation of hRRM2 and p53R2 might explain the inconsistent expression between mRNA and protein levels. Because most human tumor cells possess mutant *p53*, we assessed the expression of p53R2 in a range of tumor cell lines by Western blot (Fig. 1*C*). KB and HepG2 cells, which all possess wt *p53*, expressed an equally high level of p53R2 and hRRM2. Interestingly, both PC3 and DU145 cells possess mutant *p53*, but they express hRRM2 and p53R2 proteins in different ratios. However, expression of p53R2 and hRRM2 was nearly undetectable in Hep3B cells, which are *p53* null. This result suggests that the status of *p53* does not directly influence the expression of p53R2 or hRRM2 unless it remains null. Furthermore, UV irradiation (20 J/m^2) was used to induce hRRM2 and p53R2 expression in five cell lines. After 24 h of UV irradiation, hRRM2 increased in all five cell lines. The increased expression of hRRM2 is less significant in *p53*-mutant PC3 and DU145 cells but more prominent in *p53* wt KB and HepG2 cells. Of interest, p53R2 was only up-regulated in KB and HepG2 cells, and not in *p53*-mutant PC3 and DU145 cells. Our results suggested that hRRM2 and p53R2 can be induced by a sublethal dose of UV irradiation when the cells possess *p53* wt status, whereas hRRM2 and p53R2 are barely induced by UV irradiation in *p53*-mutant cells. *Mdm2*, the known *p53* direct target gene, was also used as control. *Mdm2* was induced by UV irradiation in KB and HepG2 cells but decreased in PC3 and DU145 cells. Thus, KB and PC3 cells were selected for the following studies based on the different *p53* status and equally high expression of both hRRM2 and p53R2 protein.

Expression of p53R2 Is Not Induced by a Sublethal Dose of UV Irradiation in PC3 Cells. Because expression of p53R2 has been reported to be associated with *p53* status and up-regulated by UV irradiation, we hypothesized that exposure to UV irradiation might affect expression of hRRM2 and p53R2 in PC3 cells differently than expression in KB cells. Western blot analyses were used to assay the levels of hRRM2 and p53R2 at various times after exposure to UV. PC3 and KB cells, growing in logarithmic phase, were exposed to UV

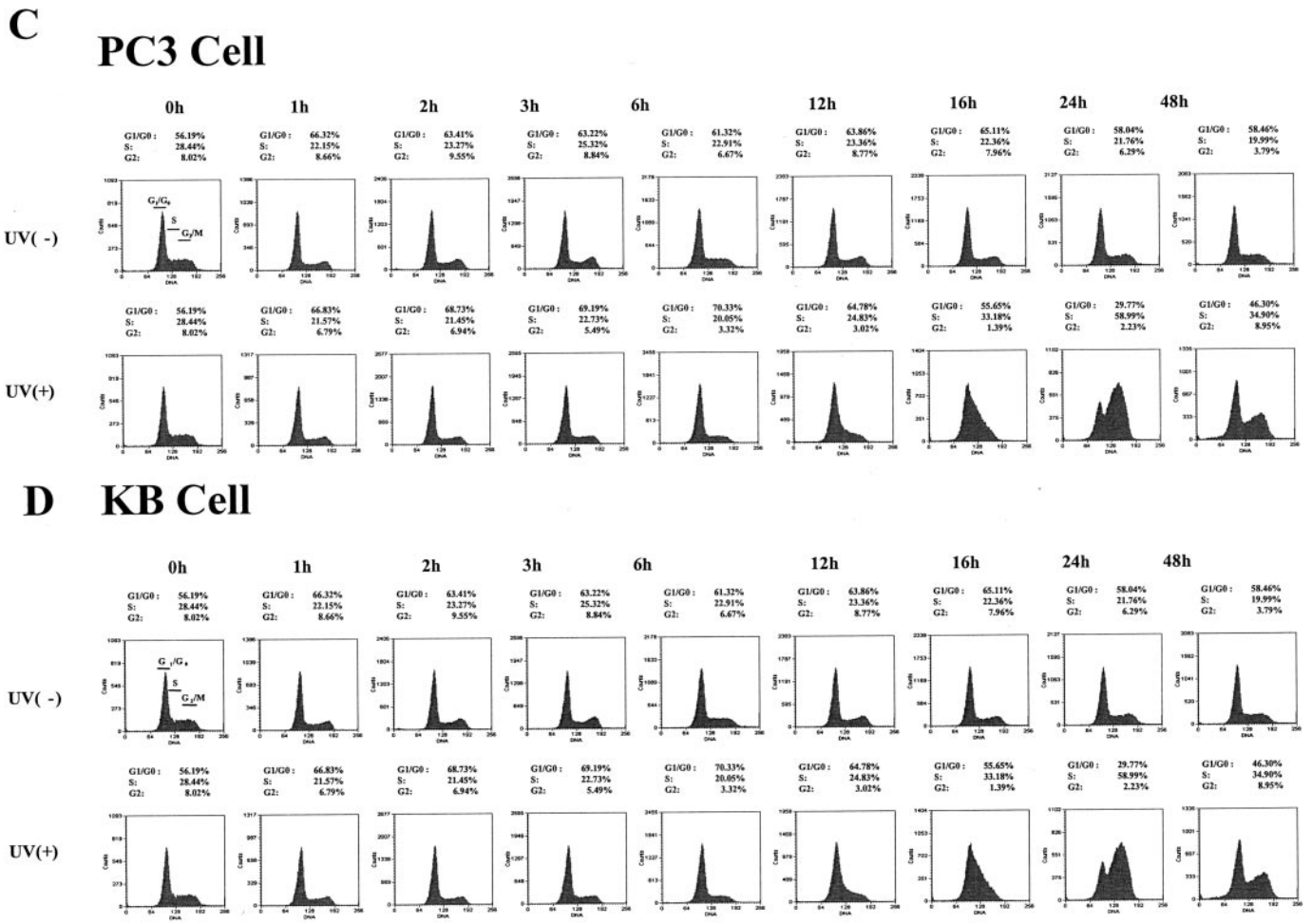

Fig. 2. *Continued*

irradiation (10 J/m^2 for PC3; 20 J/m^2 for KB) and harvested after 1, 2, 3, 6, 12, 16, 24, and 48 h (Fig. 2). Total cellular proteins were extracted and analyzed by Western blot. Fig. 2*A* shows the effect of UV irradiation on hRRM2 and p53R2, respectively, in PC3 cells, whereas Fig. 2*B* shows the same results for KB cells. Overall, the amount of hRRM2 and p53R2 was unchanged in both cell lines for up to 3 h after exposure to UV. By 6 h after UV exposure, the amount of hRRM2 protein in PC3 cells decreased slightly compared with that in the untreated control and then gradually increased from 12 to 24 h. At the 24 h time point, the hRRM2 protein increased 2.5-fold relative to the control and remained elevated (2.1-fold) after 48 h (Fig. 2*A*). In contrast to hRRM2, the amount of p53R2 protein in PC3 cells increased slightly (1.2-fold) relative to the untreated sample after 6 and 12 h and then decreased from 16 to 48 h. It was 60% of the control after 24 h and remained suppressed after 48 h (Fig. 2*A*). In KB cells, the amount of hRRM2 increased from 12 to 48 h after UV exposure, as expected, whereas p53R2 increased slightly from 6 to 24 h (Fig. 2*B*). Quantitative analysis was performed by fluorescence imaging and summarized in a *bar graph* (Fig. 2, *A* and *B*, *bottom panel*).

To confirm that hRRM2 and p53R2 induction by UV irradiation was not simply dependent on cell cycle redistribution, flow cytometry analysis was used. The results are summarized in Fig. 2, *C* and *D*. The cell cycle distribution of PC3 and KB cells did not change significantly at various time points in the UV(−) panel. After a sublethal dose of UV irradiation, no cell cycle redistribution could be seen in PC3 cells from the 0 to 24 h time points. S-phase augmentation was detected at 48 h. After UV irradiation, an S-phase increase could be seen, starting from 16 h in KB cells and continuing to increase significantly at 24 and 48 h. These results indicate that p53R2 and hRRM2 induction by UV irradiation was not concordant with cell cycle redistribution in PC3 cells. The different expression pattern of hRRM2 and p53R2 induced by a sublethal dose of UV might be related to p53 status.

To further confirm this phenomenon, Northern blot analysis of hRRM2 and p53R2 expression was performed (Fig. 3). Because no change in mRNA level was detected until 6 h after UV treatment, we only show the amount of mRNA detected after 6 h. The 12 and 16 h time points were similar to the 24 h time point. The result demonstrated here only included the 0, 6, 24 and 48 h time points. In PC3 cells, the pattern of hRRM2 mRNA expression was consistent with the Western blot, showing a slight decrease after 6 h and then increasing to more than 2.8-fold greater than the untreated control after 24 h and remaining increased 1.2-fold after 48 h (Fig. 3*A*). In contrast to hRRM2, the amount of p53R2 mRNA decreased to half of the control levels after 24 h and kept decreasing to 24% of the control after 48 h (Fig. 3*A*). In KB cells, hRRM2 mRNA gradually increased to 2.3-fold greater than the control 24 h after UV treatment and then decreased at the 48 h time point, whereas p53R2 mRNA expression decreased at 6 h but increased 3-fold as compared with control at 24 h and then decreased at 48 h (Fig. 3*B*). To further confirm that *p53* directed hRRM2 and p53R2 in response to UV irradiation, we analyzed the *p53* and *p21* mRNA expression level using RPA. In Fig. 3*C*, *p53* mutated PC3 cells; the *p53* mRNA was barely detectable even after exposure to UV, whereas *p21* increased significantly at 6 h after

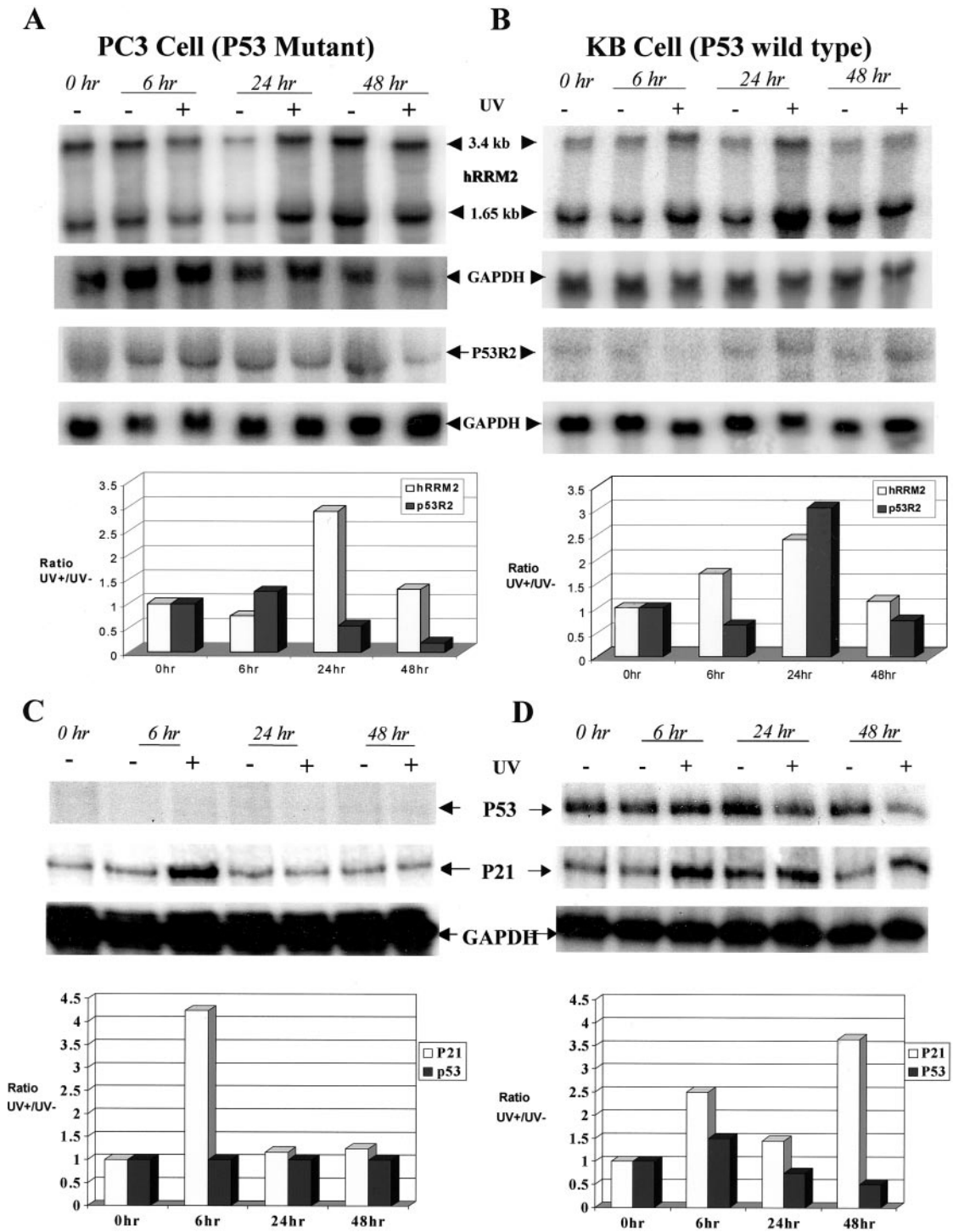

Fig. 3. Northern blot and RPA analysis of hRRM2, p53R2, p53, and p21 mRNA expression in PC3 and KB cells after UV irradiation. Total cellular RNA (20 μg/lane) was extracted from PC3 and KB cells after UV irradiation and from parallel control cells not exposed to UV. In addition to hRRM2 and p53R2 mRNA, GAPDH cDNA probes were used to control for the total amount of mRNA present in each lane. *A* and *B* show the amount of hRRM2 and p53R2 mRNA detected in PC3 and KB cells at the indicated times. The intensity of each band was measured by phosphorimager, and the amount of hRRM2 or p53R2 mRNA is presented as a ratio of UV(+)/UV(−) after normalization to the GAPDH control. *C* and *D* show the amount of p53 and p21 mRNA detected in PC3 and KB cells at the indicated times by RPA. The intensity of each band was measured by phosphorimager, and the amount of p53 or p21 mRNA is presented as a ratio of UV(+)/UV(−) after normalization to the GAPDH control.

UV irradiation. In Fig. 3*D*, *p53* and *p21* increased after 6 h of UV irradiation in KB cells. *p21* decreased slightly at 24 h but increased significantly at 48 h. *p53*, however, remained without much change between 24 and 48 h. Thus, the hRRM2 response to UV seems *p53* independent, whereas p53R2 could only be induced when the cell possessed wt *p53*. The increase of *p21* during treatment with a sublethal dose of UV irradiation demonstrated integrity of *p53* function. Overall, the mRNA expression was consistent with protein expression for all conditions.

UV Irradiation Induces Binding of hRRM2 but not p53R2 to hRRM1 in PC3 Cells. RR holoenzyme is formed by the binding of hRRM2 or p53R2 to hRRM1. To further explore the function of p53R2 in PC3 cells, immunoprecipitation using hRRM1 antibody-conjugated agarose beads was used to investigate the interaction

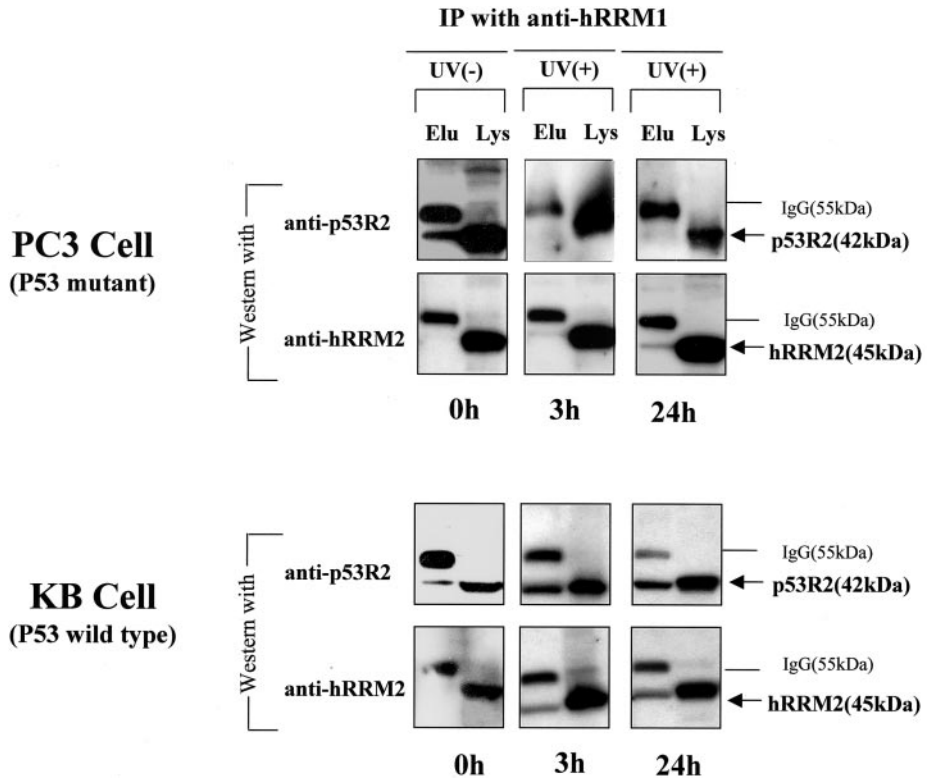

Fig. 4. Immunoprecipitation of proteins bound to hRRM1. PC3 and KB cells were irradiated with 5 J/m^2 UV. Cells were harvested 24 h later, and protein lysates were prepared. Antibodies to hRRM1 were used to immunoprecipitate lysates from both control and UV-irradiated cells, and then protein-bound p53R2 (*top panels*) and hRRM2 (*bottom panels*) was detected by Western blot. Proteins eluted from the immunoprecipitated anti-hRRM1 antibody beads (*Elu*) and from the total lysates (*Lys*) were detected for each sample.

between RR subunits after UV exposure. As shown in Fig. 4, coimmunoprecipitation of p53R2 (*top panels*) and hRRM2 (*bottom panels*) was detected by Western blot (described in "Materials and Methods"). In both PC3 and KB cells, p53R2 is strongly associated with hRRM1 in the absence of UV treatment, whereas binding of hRRM2 to hRRM1 is barely detectable, as expected. Although binding to hRRM1 was significantly different in untreated cells, nearly equal amounts of p53R2 and hRRM2 were detected in the total protein lysates. In PC3 cells, the binding of p53R2 to hRRM1 decreased, whereas the binding of hRRM2 increased slightly 3 h after UV exposure. By 24 h after UV treatment, binding of p53R2 remained suppressed, whereas binding of hRRM2 increased further. The amount of total p53R2 in the PC3 cell lysates was slightly decreased relative to the control but was still present, whereas the amount of hRRM2 increased over time, consistent with the previous Western blots (Fig. 2). In KB cells, the binding of both p53R2 and hRRM2 to hRRM1 increased after UV treatment. These finding suggest that the defect in p53R2 binding to hRRM1 to form RR holoenzyme after UV treatment is *p53* dependent. Furthermore, the observation that p53R2 and hRRM2 each bind to hRRM1 after UV treatment in the presence of wt *p53* suggests that both of these subunits may contribute to RR activity in response to the need for DNA repair.

Deficiency of p53R2 Binding with hRRM1 Is Not due to Cytoplasmic-Nuclear Shuttling of p53R2 after UV Irradiation in PC3 Cells. To further explore the underlying mechanism of *p53*-mediated binding of p53R2 to hRRM1 observed by immunoprecipitation, we examined the effect of UV irradiation on the localization of p53R2 and hRRM2 in PC3 cells by confocal scanning microscopy (Fig. 5). Before UV treatment, both hRRM2 and p53R2 are localized throughout the cytoplasm. Both subunits translocated from the cytoplasm to the nucleus as early as 1 h after UV irradiation and continued to accumulate in the nucleus for at least 3 h. By 24 h after UV exposure, both hRRM2 and p53R2 were still in the nucleus of some cells, but they had reverted to cytoplasmic localization on most cells. Similar results were obtained for KB cells (data not shown). These results indicate that both hRRM2 and p53R2 move rapidly from the cytoplasm to the nucleus in response to UV exposure but mostly revert to cytoplasmic localization after 24 h. The appearance of both p53R2 and hRRM2 in the nucleus of PC3 cells suggests that the deficiency of p53R2 binding to hRRM1 observed by immunoprecipitation is unrelated to their ability to translocate.

RR Activity Is Not Altered in PC3 Cells Compared with KB Cells in Response to UV Treatment, Suggesting That hRRM2 Complements p53R2 to Form RR Holoenzyme. Specific RR activity was examined in untreated KB and PC3 cells and at various times after UV treatment and is summarized in Fig. 6. RR activity was significantly greater in KB cells than in PC3 cells (3-fold higher). RR activity in PC3 cells increased 2-fold 3 h after UV exposure and then decreased slightly after 6 h, consistent with the protein levels observed in the Western blot. It is interesting to note that RR activity in PC3 cells peaked after 24 h, which is inconsistent with the level of p53R2 but consistent with the amount of hRRM2 seen by Western blot. These results also correspond to the increased binding of hRRM2 to hRRM1 detected in the coimmunoprecipitation study. Taken together, this suggests that the increased RR activity in PC3 cells after 24 h is due to increased expression and binding of hRRM2 to hRRM1 independent of p53R2 and that in the presence of mutant *p53*, hRRM2 is able to complement p53R2 to maintain RR activity needed for DNA repair. RR activity in KB cells was proportionately higher than that observed in PC3 cells. After 2 h, RR activity was increased 1.4-fold relative to the control, and after 24 h, it was increased 2.3-fold. These results are consistent with the up-regulation of hRRM2 expression seen in KB cells as well as the coimmunoprecipitation results. Therefore, we conclude that hRRM2 does contribute to RR activity associated with DNA repair and that it is able to complement defects in p53R2 expression associated with *p53* mutation.

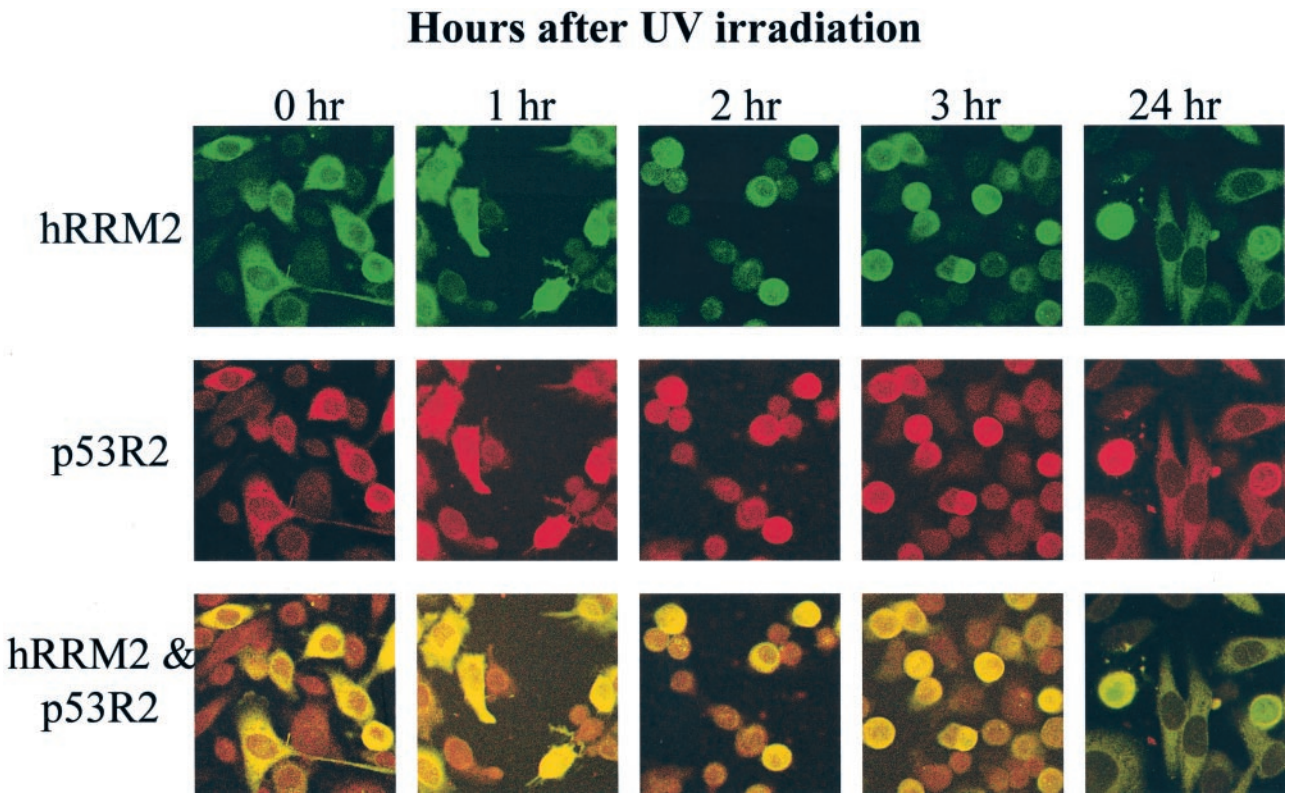

Fig. 5. Localization of hRRM2 and p53R2 in PC3 cells treated with UV. PC3 cells were irradiated with 5 J/m^2 UV and then returned to culture. At the indicated times, cells were fixed and stained for hRRM2 (FITC-conjugated goat antirabbit hRRM2; *green*) or p53R2 (rhodamine-conjugated bovine antigoat p53R2; *red*) and visualized by confocal microscopy. Colocalization of hRRM2 and p53R2 is indicated by the *yellow color* in the *merged images*.

Inhibition of Colony Formation by HU Results in Increased Cytotoxicity to PC3 Cells Treated with UV. To further confirm that hRRM2 complements p53R2 to provide nucleotides needed in cells with mutant *p53*, we treated PC3 cells with HU, a specific inhibitor of hRRM2, and then exposed them to UV. The results, shown in Fig. 7, indicate that inhibition of hRRM2 led to enhanced sensitivity to UV in PC3 cells. Colony formation was suppressed by 10% for cells exposed to 1.25 J/m^2 UV in the presence of HU relative to the same cells without HU. Suppression increased to 80% when the UV dose was increased to 2.5 J/m^2. These results provide further evidence that hRRM2 contributes to RR activity in response to DNA damage, complementing defects in p53R2 in cells with mutant *p53*. Our preliminary finding suggested that HU also inhibited p53R2.[4] However, dysfunctional p53R2 in PC3 cells showed no influence in the current experiment setting.

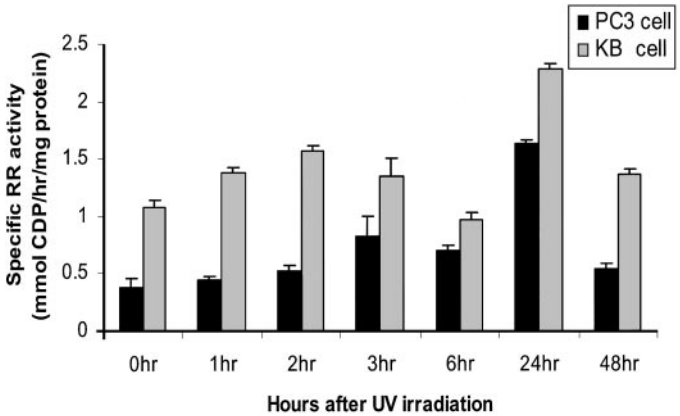

Fig. 6. RR activity in PC3 cells and KB cells after UV treatment. RR activity was measured for PC3 and KB cells at various times after UV treatment. Total protein lysates were prepared, and RR activity was assessed as described in "Materials and Methods."

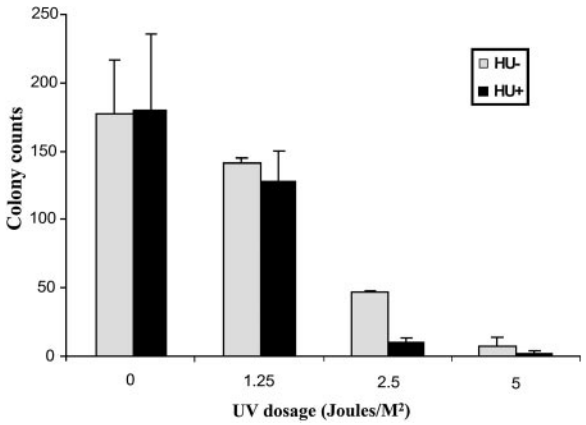

Fig. 7. Effect of hRRM2 inhibition by HU on colony formation of PC3 cells after UV treatment. PC3 cells were exposed to varying doses of UV (1.25, 2.5, and 5 J/m^2) in the presence or absence of 0.1 mM HU, a specific inhibitor of hRRM2. After UV treatment, cells were returned to culture for eight generations, and the number of resulting colonies (>50 cells) was assessed. The data shown here represent the mean ± SE of three independent experiments, each carried out in duplicate.

Inhibition of hRRM2 by Expression of Antisense hRRM2 Results in Increased Cytotoxicity to PC3 Cells Treated with UV. To further confirm the effect of hRRM2 inhibition on response to DNA damage, we examined the effect of UV treatment on colony formation

[4] B. Zhou, X. Liu, X. Mo, L. Xue, D. Darwish, W. Qiu, J. Shih, E. Hwu, F. Luh, and Y. Yen, manuscript in preparation.

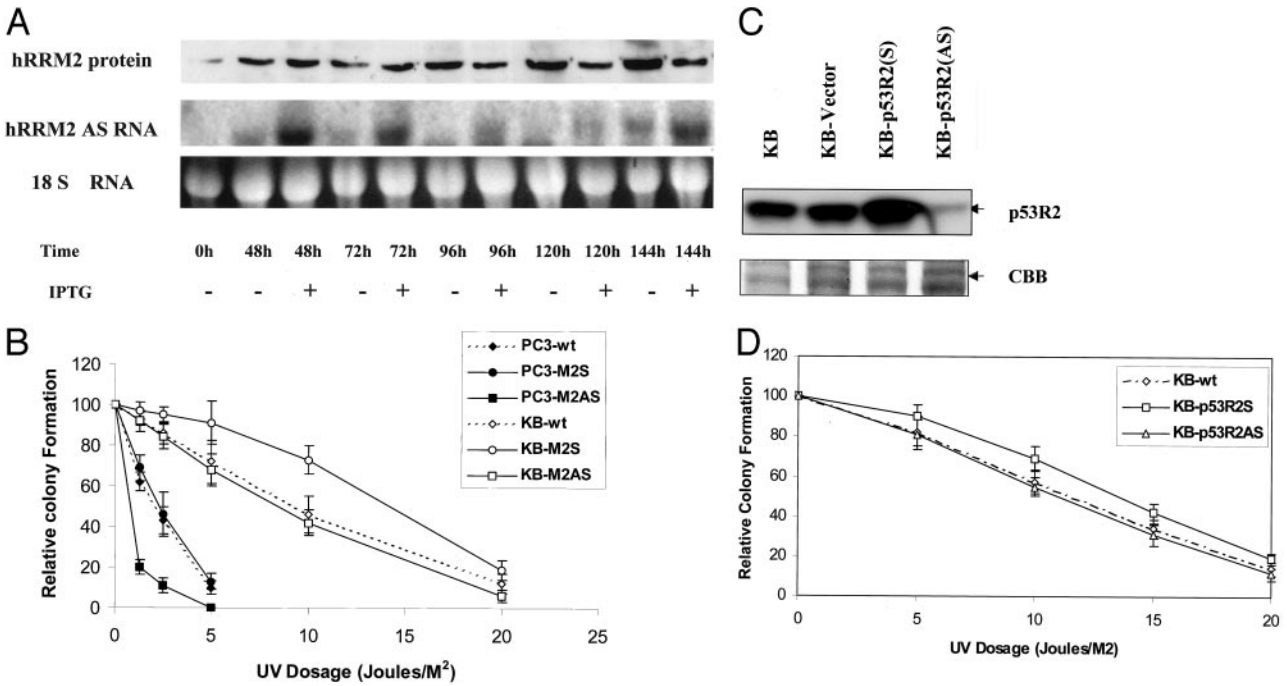

Fig. 8. Colony formation of PC3 and KB cells transfected with hRRM2 or p53R2 sense/antisense cDNA under UV irradiation. *A*, expression of antisense hRRM2 was induced by 5 mM IPTG at various time points. The hRRM2 protein and hRRM2 AS RNA level were determined here with 18S RNA control. *B*, PC3 and KB cells and clones transduced to express sense or antisense hRRM2 were assayed for colony formation after exposure to different doses of UV. Expression of sense or antisense hRRM2 was induced by 5 mM IPTG before UV exposure. *C*, expression of p53R2 protein in wt KB cell and KB-pcDNA3.1 (vector only), KB-p53R2S, and KB-p53R2AS clones. *D*, KB cells and clones transduced to express sense or antisense p53R2 were assayed for colony formation after exposure to different doses of UV. The number of resulting colonies was determined after eight generations, and values presented are the mean ± SE based on three independent experiments, each carried out in duplicate.

of PC3 and KB cells transfected with inducible hRRM2 antisense (PC3-M2AS and KB-M2AS) or sense (PC3-M2S and KB-M2S) expression vectors. As shown in Fig. 8*A*, hRRM2 protein decreased under IPTG induction of hRRM2 antisense at the 96, 120, and 144 h time points in KB-M2AS clone. hRRM2 AS RNA expression can be identified at all time points under IPTG induction. The PC3 cells transfected with hRRM2 antisense revealed the same expression status as KB cells (data not shown). Both KB M2 sense and PC3 M2 sense clones demonstrated overexpression of M2 RNA and protein (data not shown). As shown in Fig. 8*B*, the *p53*-mutated PC3 cells were more sensitive to UV irradiation than the *p53* wt KB cells. At a dosage of 5 J/m^2, the colony-forming ability of the PC3 cell was inhibited >90%, whereas the KB cells remained at >80% viability. The colony-forming ability of PC3 cells exposed to UV is suppressed for cells expressing antisense hRRM2. When exposed to 1.25 J/m^2 UV, the colony-forming ability of PC3-M2AS cells decreased to 20% of the untreated control. At a dose of 2.5 J/m^2 UV, the colony-forming ability of wt PC3 cells decreased to 42.5% of the untreated control, whereas the colony-forming ability of PC3-M2AS decreased to 10.5% of the untreated control. These results suggest that inhibition of hRRM2 by antisense RNA will significantly affect the ability of hRRM2 to complement for p53R2 in DNA repair in PC3 cells. Colony formation for PC3 cells transfected with the sense construct, with increased expression of hRRM2, was essentially the same as that for wt PC3 cells or slightly elevated. The colony-forming ability of KB cells expressing antisense hRRM2 was nearly the same as that of wt KB cells or slightly decreased, whereas enhanced expression of hRRM2 enhanced survival of transfected cells. These results further confirm that hRRM2 coordinates with p53R2 to contribute to RR activity in response to DNA damage in cells with functional *p53*, but that in the absence of functional *p53*-mediated induction of p53R2 activity, hRRM2 complements this lack to provide the necessary activity.

To further investigate the role of p53R2 in UV-induced DNA repair, we constructed pcDNA-p53R2S and pcDNA-p53R2AS with p53R2 sense or antisense fragments and delivered it into KB cells. Fig. 8*C* demonstrates p53R2 expression from wt KB, KB-pcDNA3.1 (vector only), KB-p53R2S (sense-transfected clone), and KB-p53R2AS (antisense-transfected clone). The KB-p53R2S demonstrated a 3-fold expression of p53R2 compared with the control, whereas KB-p53R2AS revealed a significant decrease compared with the control. Colony-forming ability was evaluated in wt KB, KB-p53R2S, and KB-p53R2AS under different dosages of UV irradiation. From the results shown in Fig. 8*D*, the colony-forming ability of wt KB, KB-p53R2S, and KB-p53R2AS was steadily inhibited with increasing UV doses from 0 to 20 J/m^2. KB-p53R2S seemed to have more resistance to UV stress. However, KB-p53R2AS was not significantly different from control. This finding further strengthened our hypothesis that hRRM2 complements p53R2 when p53R2 is inhibited by antisense. These results suggested that p53R2 expression may enhance repair and lead to resistance to UV damage in *p53* wt KB cells, whereas hRRM2 might be more dominant when p53R2 is inhibited by antisense in response to UV irradiation.

DISCUSSION

Here we report that the function of p53R2 in response to sublethal UV damage is *p53* dependent. Mutant *p53* is associated with decreased formation of RR holoenzyme by binding of p53R2 to hRRM1 and a failure to activate transcription of p53R2. In addition, we have shown that hRRM2, which is regulated independently of p53, can complement the p53R2 defect to enable DNA repair. An important implication of these studies is that the role of hRRM2 in mediating DNA repair is broader than that of p53R2 because it is independent of *p53* status. We have shown previously that both p53R2 and hRRM2 bind to *p53* (14). In addition, we have shown that p53R2 is able to

bind to mutant p53 but is not released in response to UV stress, preventing the formation of RR holoenzyme (14). Because hRRM1 and hRRM2 do not have *p53*-binding sites in their promoter regions, their expression is independent of *p53* status, allowing them to respond to UV-induced DNA damage in the absence of *p53*. The observation that p53R2 and hRRM2 were similarly induced by UV irradiation in KB cells suggests that both subunits play an important role in DNA repair in cells with functional *p53*. In this study, we further confirmed that hRRM2 can compensate for the reduced binding of p53R2 to hRRM1 in cells with mutant *p53*. This was shown in the immunoprecipitation experiment. In PC3 cells, hRRM1 did not bring down p53R2, whereas its interaction with hRRM2 was appreciably induced in response to UV irradiation. These experiments also showed that p53R2 binds to hRRM1 in cells with wt *p53* and that binding is further induced upon UV treatment, which would allow for a rapid increase in RR activity. The effect of increased hRRM2 expression in sense-transfected cells was most apparent in cells with intact *p53*. Decreased hRRM2 expression had a minimal effect on antisense-transfected cells with intact *p53* but resulted in increased cell toxicity under UV treatment in cells with mutant *p53*. Because inhibition of hRRM2 in cells with wt *p53* has little effect on sensitivity to UV, it is likely that RR activity required for DNA repair is primarily carried out by p53R2. However, the enhanced survival of cells expressing increased hRRM2 implies that hRRM2 also contributes to this activity. It has been reported previously that cells lacking functional *p53* exhibited defective repair of UV damage and were more sensitive to UV irradiation than their wt *p53* counterparts (6, 21). Our result supports these findings and suggests that this may be due to defects in the ability to release bound p53R2 and in induction of p53R2 transcription.

It has been reported that *p53* plays a role in NER (29–33). This pathway is required for the repair of UV-induced DNA damage, removal of bulky carcinogen adducts, and repair of DNA damage caused by chemotherapeutic agents such as cisplatin. Loss of *p53* function leads to decreased repair of these lesions and is reflected by increased sensitivity to these agents (29, 33). However, the extent of *p53* involvement in NER is not entirely clear. It has been shown that *p53* interacts directly with TFIIH, a NER component, and that other genes implicated in repair, such as GADD45, are regulated by *p53* (34–38). The details of p53R2 involvement in NER and its regulation need further elucidation. It has also been shown that UV-induced DNA damage involves the GGR subpathway of NER, but not transcription-coupled repair (30–32, 39). In GGR, acute response to UV irradiation can be measured as unscheduled DNA synthesis for photoproducts such as 6-4 pyrimidine-pyrimidine (31, 32, 39). Release of p53R2 from a complex with *p53* provides for rapid activation of RR, as we have shown here. Thus, our finding suggests that p53R2 participates in GGR repair at early times (3–4 h after UV irradiation) in cells with wt p53. On the other hand, in cells with mutant *p53*, p53R2 was not released to mediate repair. In addition, transcription of p53R2 was not induced, leading to a deficiency in repair and S-phase delay 24 h after UV treatment. S-phase delay may represent an active checkpoint response or may reflect a blockage of replication (40, 41). In cells with deficient *p53*, synthesis of hRRM2 is triggered at delayed times to complement the defect in p53R2. It will be of interest to further explore the role of p53R2 and hRRM2 in cell cycle checkpoint responses to UV radiation and the possible interaction with cyclin or cyclin-dependent kinase.

Our observations suggest that *p53* status does not influence hRRM2 response to UV irradiation, including its expression or binding to hRRM1. However, hRRM2 and p53R2 expression and binding to hRRM1 increased in response to UV irradiation (14). The role of *p53* in these responses is unclear. One possibility is that *p53* binds hRRM2 to block binding to hRRM1, inhibiting RR activity and keeping synthesis of dNTPs low. This could explain why hRRM1 did not bind to hRRM2 in the absence of UV irradiation. A less likely scenario is that *p53* might compete directly with hRRM1 to bind hRRM2. The argument could also be made that posttranslational modifications are needed for hRRM2, but not p53R2, to bind to hRRM1. Alternatively, p53R2 may interact directly with *p53*, whereas the interaction of hRRM2 with *p53* may be indirect through one or more intervening proteins. It is also possible that after UV irradiation, *p53* affects some other protein bound to hRRM2. It has been shown that *p53* affects transcriptional coactivator proteins such as CCAAT box-binding protein (CBP) and p300 (42–44). These proteins function by interacting with cellular activators, probably with multiple components of the transcriptional machinery, and modulate *p53* transcriptional activity. More work will be necessary to identify the different mechanisms involved in the role of *p53* on the regulation of hRRM2 and p53R2 in response to DNA damage. The differential responses in cells with mutant *p53* make it clear that the mechanisms are not the same for hRRM2 and p53R2.

Interestingly, confocal microscopy showed that mutation of *p53* did not affect the ability of hRRM2 and p53R2 to shift from the cytoplasm to the nucleus in response to UV irradiation. Therefore, the previous hypothesis that hRRM2 resides primarily in the cytoplasm and that p53R2 is primarily nuclear (6, 45, 46) becomes less likely. The dynamic shifting from the cytoplasm to the nucleus and back to the cytoplasm was the same for hRRM2 and p53R2. Interestingly, RR activity was found to be increased both at times when the localization of hRRM2 and p53R2 was primarily nuclear (3 h) and at times when it was cytoplasmic for most cells (24 h). It has been shown that RR subunits transfer to the nucleus individually and then assemble in the nucleus to form the holoenzyme and provide enzyme activity (47). Whereas our results are consistent with this at the 3 h time point, the discrepancy between localization and RR activity after 24 h requires further investigation. One possibility is that the activity of RR in the small percentage of cells where it remained nuclear was high enough to account for the total measured activity. Alternatively, RR holoenzyme may function in the cytoplasm in addition to the nucleus. We have preliminary results that suggest that RR may be active in the mitochondria. This is plausible because mitochondria are also a site of DNA synthesis and repair and therefore require a pool of dNTPs.

It has been reported that p53R2 is a *p53*-dependent RR small subunit (6, 48). However, the function and regulation of p53R2 are not very clear thus far. Because more than half of all cancer cells possess dysfunctional *p53*, it is important to know whether the regulation of p53R2 in *p53*-mutated cells might be different than that in *p53* wt cells in response to DNA damage stress. According to the results of Tanaka *et al.* (6), p53R2/hRRM2 could be induced/reduced after 24 h in response to DNA damage in *p53* wt cells. However, DNA damage caused by a sublethal dose of UV (20 J/m^2) irradiation could be detected before 8 h in HeLa cells (49, 50). In *p53*-mutated cells, the DNA repair process would be delayed, but DNA could still be repaired before 24 h (49–51). Hence, the dramatic increase in p53R2 and the decrease in hRRM2 after 24 h of γ-irradiation may be due to high-dose radiation-related apoptosis and cell death. p53R2 increased for repair, whereas hRRM2 decreased due to lack of DNA replication. However, our results demonstrated that mRNA and protein levels of both p53R2 and hRRM2 have only changed in response to UV irradiation when *p53* remains intact. This may be due to the sublethal dose of UV irradiation we used here rather than γ-irradiation (Figs. 2 and 3).

It has been known that *p21* and *Mdm2* could be induced though up-regulation of *p53* transactivation. Here we also demonstrated that the *p53* downstream gene, *p21* mRNA, could be induced after 6 h of

UV irradiation (20 J/m^2) in both KB and PC3 cells (Fig. 3, *C* and *D*). Of interest, our results seem to suggest that *p21* is antagonistic with hRRM2 and possibly p53R2 induced by UV. Our laboratory is currently investigating the detailed mechanism in *p21* and cell cycle regulation. Moreover, *Mdm2* can be induced by 24 h after UV irradiation in KB cells (Fig. 1*C*), which is compatible with decreased *p53* at this time point (Fig. 3*D*). This finding is consistent with previous reports that Mdm2 feedback inhibits *p53* under UV stress (52, 53). In addition, our preliminary study noticed that hRRM2 rather than p53R2 could be induced with H_2O_2 in KB cells after 24 h (data not shown). Furthermore, Western blot results showed that the baseline expression of p53R2 was not simply based on *p53* status (Fig. 1, *B* and *C*). Therefore, our result suggested that *p53* might not be the only factor involved in transcription regulation of p53R2 in response to DNA damage.

The defect in p53R2 binding to hRRM1 revealed by immunoprecipitation also requires further explanation. One possible explanation is that mutant *p53* is unable to release p53R2, preventing it from binding hRRM1, as we have shown in our previous study (14). It is also possible that additional factors affect the ability of p53R2 to bind to hRRM1 in response to the need for DNA repair. It has been reported that *p73*, an isoform of the *p53* family, plays a role in inducing p53R2 expression in p53-independent DNA repair (21). Furthermore, in the absence of *p53*, $p14^{AFR}$ may act in its place (21). Therefore, the interaction between $p14^{AFR}$ and p53R2 may be critical for response to DNA damage. The interaction of p53R2 with *p73* and $p14^{AFR}$ requires more study and may suggest that it has functions in addition to its role in DNA repair. Taken together, whereas p53 status clearly affects the ability of p53R2 to respond to the need for DNA repair, the complicated regulation of this activity may involve other factors associated with cell cycle progression or programmed cell death, and these need to be taken into consideration. To further confirm the relationship between *p53* status and p53R2 function, we will transfect *p53*-null cells with wt *p53* expression vectors to confirm that the observed defects were directly attributable to lack of p53 and not to some other unidentified defect.

In summary, our results demonstrate that both p53R2 and hRRMR contribute to DNA repair in response to UV is *p53* dependent. In the absence of functional *p53*, p53R2 is unable to respond, and hRRM2 takes on the entire activity. The inability to induce transcription of p53R2 in response to UV damage in cells with mutant *p53* results in growth retardation. The additional defect in p53R2 binding to hRRM1 in these cells further interferes with the process of DNA repair. However, expression of hRRM2 can complement this loss and allow for DNA repair in a *p53*-independent manner. The specific mechanism by which *p53* interferes with p53R2-mediated RR activity is not yet understood. These results suggest that the regulation by *p53* or other upstream regulators of RR is complicated and unique for each subunit.

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Bingsen Zhou, Xiyong Liu, Xueli Mo, et al.

Cancer Res 2003;63:6583-6594.