

Modulation of Transcriptional Activity of p53 by Ultraviolet Radiation: Linkage Between p53 Pathway and DNA Repair Through Damage Recognition

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The increase in the p53 activity in response to DNA damage is thought to be one of the important mechanisms by which p53 contributes to transcriptional activation of *p21^{waf1}*, *mdm2*, and other downstream regulatory genes. To investigate the p53 response to ultraviolet (UV) type of DNA damage, p53 protein level, its transcriptional activity and in vivo ubiquitination were compared in repair-proficient normal human fibroblasts (NHFs) and repair-deficient xeroderma pigmentosum (XP) group A and group C (XP-C) fibroblasts subsequent to irradiation with UV light. Accumulation of p53 protein level was observed with increasing UV doses in all the cell lines; however, discordance between p53 and *p21^{waf1}* and *mdm2* levels was observed in NHF and XP-A cells. Induction of *p21^{waf1}* and *mdm2* was inhibited by UV irradiation, requiring higher doses in NHF and lower doses in XP-A cells. However, inhibition of *p21^{waf1}* and *mdm2* induction was not observed in XP-C cells. Ubiquitin-p53 conjugates could be detected in irradiated or unirradiated NHF and XP-A cells but not in XP-C cells irradiated with 30 and 50 J/m² UV light. Using a p53 reporter assay, p53 transcriptional activities were found to be induced by 10 J/m² UV exposure and dramatically inhibited with increasing UV doses in NHF cells. Compared with repair-proficient NHF cells, UV inhibition of p53 transcriptional activity was relatively more sensitive in XP-A cells but resistant in XP-C cells. These results indicate that DNA damage by UV, in addition to inducing p53, acts as a trigger for inhibition of p53 transcriptional activity. Overall, recognition of DNA damage links both p53 induction and p53 degradation to DNA repair mechanisms. *Mol. Carcinog.* 28:215–224, 2000. © 2000 Wiley-Liss, Inc.

Key words: p53; p21; ultraviolet irradiation; transcriptional activation; ubiquitination; DNA repair

INTRODUCTION

Mutations in the *p53* tumor-suppressor gene are the most common genetic alterations in many types of cancer [1,2]. Roles for p53 in normal cellular proliferation have not been completely identified. Abundant evidence, however, indicates that p53 is critical for maintaining genomic stability and homeostasis [3–5]. It is known that p53 acts as a transcription activating factor [6] for the expression of its target genes, e.g., *p21^{waf1}*, *mdm2*, *Gadd45*, cyclin G and *bax* [6–11]. In response to various types of DNA damage, increase of p53 protein level and transcriptional activity of p53 has been reported [12–14], and it is believed that p53 activation signals the G1 cell-cycle checkpoint [7,15]. However, discordance between the accumulated p53 protein level and its transcriptional activity and inhibition of *p21^{waf1}* and *bax* expression by high ultraviolet (UV) fluences in normal human fibroblasts (NHFs) and in xeroderma pigmentosum (XP) group A cells has been observed by several investigators [16,17].

It is well known that wild-type p53 protein is relatively unstable because of a short protein half-

life. However, it is stabilized in response to DNA-damaging agents or irradiation [18]. The turnover of p53 in vivo is mediated by the ubiquitin-proteolysis system, which has been reported to be transiently suppressed after DNA damage [19]. Although the mechanisms of ubiquitin-proteolysis remain to be understood, *mdm2*, which is known to directly suppress transcriptional activity of p53 by protein-protein interaction [20,21], has been implicated as playing a role in p53 degradation [22–24]. *dm2* may act as a ubiquitin ligase E3 for p53 [25]. p300, a p53 coactivator, has also been shown to form complexes with *mdm2* by participating in *mdm2*-mediated p53 degradation [26].

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Abbreviations: UV, ultraviolet; NHF, normal human fibroblast; XP, xeroderma pigmentosum; NER, nucleotide excision repair; TFIID, transcription factor IID; TCR, transcription-coupled repair; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; GGR, global genomic repair; CBP, CREB-binding protein.

Nucleotide excision repair (NER) enzymes can recognize and remove a broad range of DNA lesions, including UV-induced dimers and bulky chemical adducts. The biochemical mechanisms of NER involves damage recognition and open complex formation by factors such as XPA, XPC, replication factor A, and transcription factor IIIH (TFIIH), dual incision of the damaged DNA strand by endonucleases XPF-ERCC1 and XPG, repair synthesis mediated by a proliferating cell nuclear antigen-dependent DNA polymerase, and ligation of new synthesized DNA strand [27]. The precise reaction mechanism of NER, including damage-recognition steps, has recently been established to a significant extent [28–30]. Nevertheless, how other cellular factors regulate the NER system and how NER is connected to the cell cycle and apoptosis remain unclear. p53, which contacts three subunits of TFIIH (p62, XPD, and XPB) [31,32], may play a role in linking NER to the cell cycle. Involvement of the p53 in NER of DNA damage has recently been examined by several investigators [33–37]. Interestingly, Li-Fraumeni fibroblasts, like XP-C fibroblasts, have been shown to be deficient in global DNA repair but not in transcription-coupled repair (TCR) [33]. Recently, RAD23, a yeast homolog of human hHR23A and hHR23B, has been demonstrated to link DNA repair to the ubiquitin-proteolysis pathway in yeast [38]. Given the fact that the evolutionary conserved protein RAD23 functions similarly in yeast and in mammalian cells, human hHR23A or hHR23B could also act to link DNA repair to the ubiquitin-proteolysis pathway. Results presented in this article, for the first time, provide evidence that recognition of damage might link p53 activation and p53 degradation to DNA-repair mechanisms.

MATERIALS AND METHODS

Cell Culture and Treatment

The NHFs were established as described previously [39,40]. Human XP-A fibroblasts, GM05509A (XP12BE), and human XP-C fibroblasts, GM02096 (XP1MI), were purchased from the NIGMS Human Genetic Mutant Cell Repository (Coriell Institute, Camden, NJ). These fibroblasts were cultured in minimal essential medium with 10% fetal calf serum and antibiotics. For western blot analysis, the exponentially growing cells (~60% confluent) in 150-mm dishes were washed and replaced with a thin layer of phosphate-buffered saline (PBS) and irradiated with different UV doses. Irradiation was performed from a germicidal lamp at a dose rate of 1.0 J/m²/s as measured by a Kettering model 65 radiometer (Col-palmer Instrument Co., Vernon Hills, IL). After irradiation, fresh medium was added to cell cultures, and incubation continued for desired periods.

Protein Analysis by Western Blotting

The cells, irradiated or unirradiated, were incubated for 8 h and recovered by trypsinization. Cells were lysed by boiling for 10 min in a sample buffer (2% sodium dodecyl sulfate (SDS), 10% glycerol, 62 mM Tris-HCl, pH 6.8, 10 µg/mL pepstatin, and 10 µg/mL leupeptin). Protein concentration was determined with the Bio-Rad Protein Assay (Richmond, CA) according to the manufacturer's manual. Dithiothreitol (10 mM final concentration) was added to samples just before loading. Proteins were separated by electrophoresis in 8% or 14% SDS-polyacrylamide gels. Protein transfer to PDVF membrane and immunodetection with suitable antibody were performed as previously described [41]. p53-specific antibody Ab-6, p21-specific antibody Ab-3, and mdm2-specific antibody Ab-1 (clone SMP14) were used for immunodetection of p53, p21^{waf1}, and mdm2, respectively.

Immunoprecipitation and Western Blot Analysis of Ubiquitinated p53 Protein

In all experiments, exponentially growing cells were used either as the control or for treatment with UV radiation. At 4 h postirradiation, cells were washed three times with PBS buffer. Cells were lysed in RIPA buffer (2 mM Tris-HCl pH 7.5; 5 mM EDTA; 150 mM NaCl; 1.0% NP40; 1.0% deoxycholate; 0.025% SDS; 1 mM phenylmethylsulfonyl fluoride; 10 µg/mL pepstatin; and 10 µg/mL leupeptin). p53 was immunoprecipitated from approximately 2 mg of extracts by incubating with 20 µL of p53-specific antibody Ab-6 (200 µg/mL) and 20 µL of protein A agarose beads (Oncogene Research, Cambridge, MA) overnight at 4°C. Immunoprecipitates were resolved in 8% SDS-polyacrylamide gel and transferred to a PVDF membrane, and the membrane was autoclaved in water for 15 min. For p53 detection, the membrane was cut at approximately 60 kDa, and the upper portion of the membrane was immunodetected by anti-p53 antibody Ab-1801. The blots were stripped of all proteins with stripper buffer (62.5 mM Tris-HCl; pH 6.7; 100 mM β-mercaptoethanol; and 2% SDS) and reused for immunodetection by monoclonal anti-ubiquitin antibody against ubiquitin purified from bovine erythrocytes (Calbiochem, San Diego, CA).

Plasmids, Transfection, and Reporter Assay

Plasmid PG13-*luc*, a luciferase reporter containing 13 copies of a synthetic p53 consensus site derived from the promoter of p21^{waf1}, and plasmid MG15-*luc*, which contains 15 copies of a mutated sequence that does not bind p53, were provided by Dr. B. Vogelstein (Johns Hopkins University, Baltimore, MD). These constructs are the same as those previously described [42], except that a firefly luciferase gene replaces the chloramphenicol acetyl-

transferase gene. The pCMV-Tag 2 control plasmid (Stratagene, La Jolla, CA) contains the firefly luciferase reporter gene fused in reading frame with a FLAG tag and is directed by the cytomegalovirus immediate-early enhancer/promotor. Exponentially growing cells (1 or 2×10^5) were plated in 35-mm dishes 18–20 h before plasmid transfection. The nonliposomal formulation FuGENE Transfection Reagent (Roche Molecular Biochemicals, Indianapolis, IN) was chosen because of its minimal cytotoxicity for many eukaryotic cells. Cells were transfected with p53 reporter or pCMV-Tag 2 control plasmid DNA by using FuGENE 6 transfection reagent according to the manufacturer's manual. Three or four hours after transfection, cells were washed with PBS buffer and irradiated with UV, as described earlier. Eight hours after UV irradiation, cells were washed four times with PBS buffer and lysed in 150 μ L of luciferase cell culture lysis reagent (Promega, Madison, WI). Luciferase activity from 20 μ L of cell lysates was assayed with the luciferase assay system (Promega) by using the Luminat LB 9510 luminometer (Wallac, Gaithersburg, MD).

RESULTS

UV Dose-Dependent p53 Response of NHFs and XP-A and XP-C Fibroblasts

To study whether the p53-induction response is related to the DNA-repair proficiency of cells, the effects of UV light-induced DNA damage on the p53 level and p53-regulated genes were examined in repair-proficient NHFs and repair-deficient XP-A and XP-C fibroblasts. The protein levels of p53, p21^{waf1}, and mdm2 were assessed by western blot analysis 8 h after irradiation of the fibroblasts with increasing doses of UV light ranging from 10 to 50 J/m² (Figure 1). The previous time-course experiments indicated that the rapid p53-induction response, detectable within a few hours and peaking at about 6–8 h, is sustained up to a period of at least 24 h [40]. In addition, no significant cell loss was seen during the 8 h after irradiation of the three cell lines with 50 J/m². Thus, in these experiments, an 8-h post-irradiation time point was chosen for comparison of dose-dependent induction of various proteins in different cell lines. NHF cells exhibited a higher basal level of p21^{waf1}, whereas the basal mdm2 levels, like those of the p53 protein, were very low. In contrast, both XP-A and XP-C fibroblasts showed a higher basal level of mdm2 protein. More importantly, the basal p21^{waf1} level remained lower in both the repair-deficient XP-A and XP-C cells. After low UV exposures, e.g., 10 or 20 J/m², all cell types demonstrated augmented p53 protein. Nevertheless, in response to damage-induced elevated p53 protein, significant increases in the levels of p21^{waf1} and mdm2 were only seen in TCR-proficient NHF

and XP-C cells but not in global genomic repair (GGR)-deficient XP-A cells. Consistent with results of previous reports [16], these results indicate that persistent UV lesions could be responsible for triggering the p53 response [43,44] and that lower damage levels, e.g., less than 10 J/m², of UV exposure were sufficient for such induction in TCR- and GGR-deficient XP-A fibroblasts [16].

At high doses of UV, e.g., 30 and 50 J/m², p53 continued to accumulate in all the cell types. Surprisingly, expression of p21^{waf1} and mdm2 did not show a proportionate increase in response to p53 accumulation but was seen to significantly decrease at high-dose exposures of NHF and XP-A fibroblasts. Nevertheless, the level of p21^{waf1} and mdm2 proteins showed a distinct increase in XP-C fibroblasts exposed to high UV doses (Figure 1). An examination of UV dose dependency showed that lower UV exposures were sufficient for blocking the expression of p21^{waf1} in XP-A cells. Complete lack of expression of p21^{waf1} was observed in NHFs and XP-A fibroblasts at UV exposures of 50 and 20 J/m², respectively. These results indicate that UV lesions, which promptly trigger induction of p53 protein level in the cells, must also cause the inactivation of p53 protein function. A similar type of differential response has been seen in NIH/3T3 cells, T22 cells, human colon cancer cell line RKO, and E6-transformed RKO cells [17,45].

Effects of High UV Exposures on the Ubiquitination of p53 in NHFs and XP-A and XP-C Fibroblasts

It is well known that mdm2 regulates transcriptional activity of p53 through a negative feedback-loop mechanism. Overexpression of p53 protein induces the expression of *mdm2*, and in turn the mdm2 protein binds p53 to inhibit its ability to stimulate target gene transcription [20,21]. The observations noted in the preceding section raise a question about the lack of effectiveness of this feedback loop in regulating the transcriptional activity in XP-C cells irradiated with UV light. Because recent studies have shown that mdm2 is involved in degradation of p53 via the ubiquitin proteasome pathway [22,23], our next set of experiments examined the in vivo ubiquitination of p53 protein in NHF, XP-A, and XP-C cells. Only a limited set of studies has reported the detection of in vivo ubiquitinated p53 protein in cells [19,46]. It has been difficult to detect ubiquitin-p53 conjugates because they are intermediates of degradation and are only present in minute quantities in vivo. We were able to detect ubiquitin-p53 conjugates by western blot analysis in untreated and UV-treated cells after establishing the extensive washing and longer exposure conditions of detection through several trial and error experiments. As shown in Figure 2, ubiquitin-p53 could be detected in NHF and XP-A cells under both UV-treated and untreated

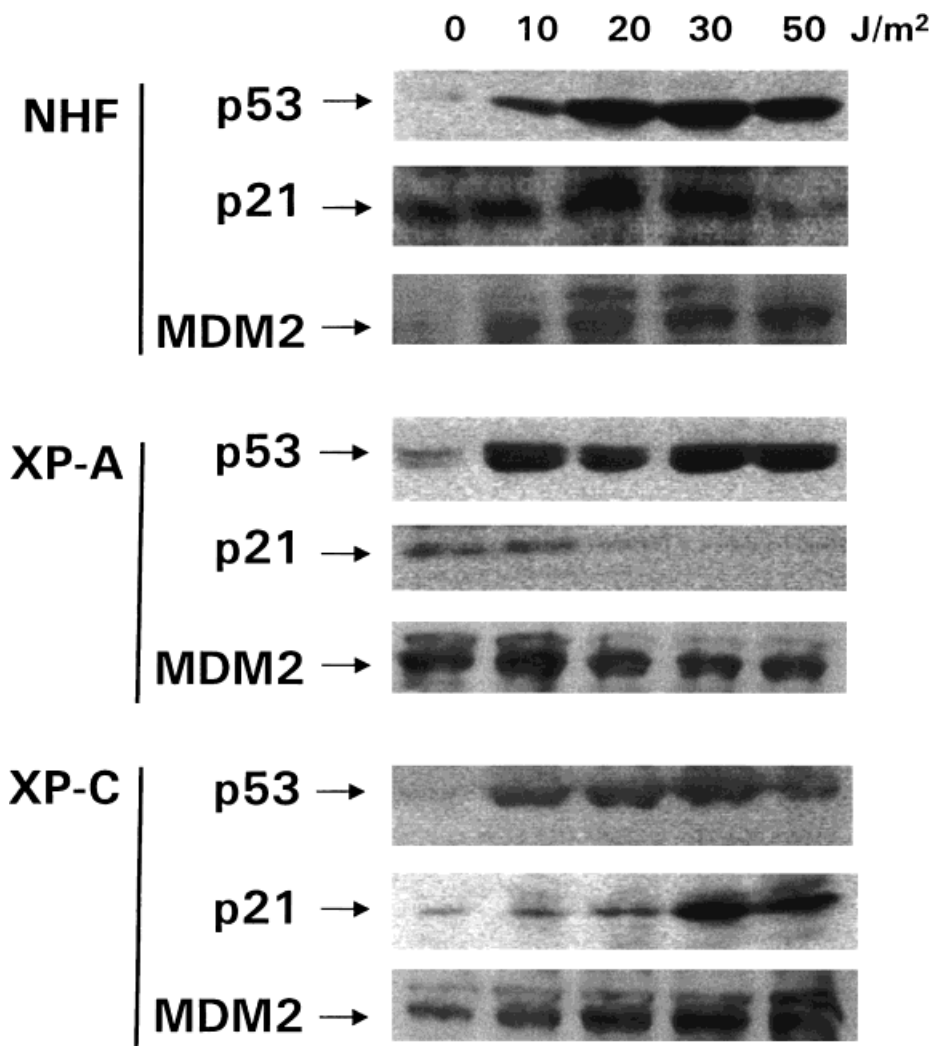


Figure 1. p53, p21^{waf1}, and mdm2 expression after exposure to UV irradiation. Exponentially growing NHFs or XP-A or XP-C fibroblasts were either unirradiated or exposed to UV. Eight hours after exposure, cells were collected and protein extracts were prepared. Thirty micrograms or 50 μ g (for mdm2) of each extract was

resolved on 8% (p53 and mdm2) or 14% (p21^{waf1}) polyacrylamide gels. p53, p21^{waf1}, and mdm2 levels were determined by western blotting by using specific anti-p53, anti-p21^{waf1}, and anti-mdm2 antibodies, as described in Materials and Methods.

conditions. The sizes of bands of ubiquitin-p53, ranging from M_r \sim 69 000 to \sim 90 000, were recognized by the p53-specific antibody Ab-1801. According to the molecular size of ubiquitin, two to five ubiquitin moieties seemed to be added to one p53 molecule. An ubiquitinated p53 molecule of M_r \sim 90 000 was the most obvious band detected (Figure 2). These bands of ubiquitin-p53 conjugates could also be recognized by monoclonal anti-ubiquitin antibody developed against ubiquitin purified from bovine erythrocytes. An ubiquitinated p53 molecule of M_r \sim 90 000 could be more clearly viewed by western blot analysis when using anti-ubiquitin antibody (results not shown). However, smaller ubiquitinated p53 molecules were poorly recognized and barely detectable by the anti-ubiquitin antibody used in these experiments.

Ubiquitin-p53 conjugates in XP-C cells could be detected only when cells received low UV irradiation at 10 or 20 J/m² but could not be detected when cells were treated with either 30 or 50 J/m² of UV (Figure 2). With longer exposure and higher concentration of p53 antibody, ubiquitin-p53 conjugates were faintly detectable in unirradiated XP-C cells. Such exposure conditions, however, failed to depict any bands in XP-C cells exposed to high UV dosages. Obviously, this could not be due to a low amount of p53 protein because p53 was seen to accumulate significantly in these cells with UV irradiation. In addition, mdm2 induction is not inhibited in these cells. Taken together, these results indicate that ubiquitination of p53 is inhibited or limited in XP-C cells after higher UV fluences, reflecting the fact that mdm2-mediated degradation

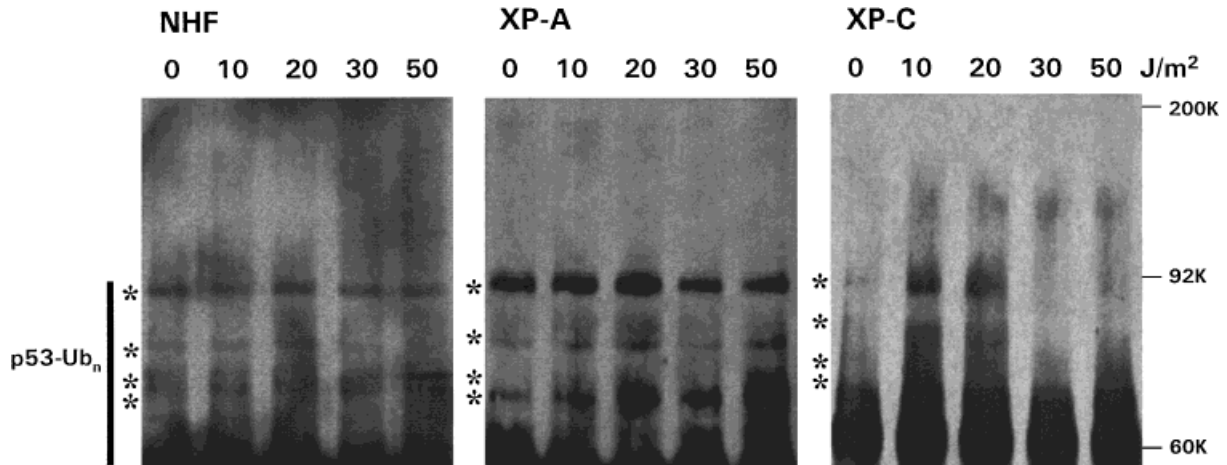


Figure 2. Effect of UV irradiation on the in vivo ubiquitination of p53 in NHFs and XP-A and XP-C fibroblasts. NHF, XP-A, and XP-C cells were either unirradiated or exposed to UV at a dosage between 10 and 50 J/m². Four hours after UV irradiation, cell lysates were prepared in RIPA buffer, and p53 was immunoprecipitated from extracts by using the p53-specific monoclonal antibody Ab-6. Immunoprecipitates were resolved on 8% polyacrylamide gel and transferred to a PDVF membrane. The membrane was cut at ~60

kDa to avoid detection of the antibody heavy chain (50–55 kDa). The upper portion of the membrane was examined by western blot analysis with p53-specific monoclonal antibody 1801. The blot was stripped and reprobbed with an anti-ubiquitin (Ub) monoclonal antibody. The asterisks indicate a ladder of bands (p53-Ub_n). Ubiquitinated p53 molecules with migration consistent with a size (M_r~90 000) corresponding to the addition of five ubiquitin molecules to p53 were prominently recognized by both antibodies.

of the p53 pathway is negatively affected in these cells.

Inhibition of p53 Transcriptional Activity in NHFs and XP-A and XP-C Fibroblasts by UV Exposures

Previous investigations on discordance between the transcriptional activity of p53 and its protein level suggested that the transcriptional activity might be inhibited by high UV fluences [17]. A cell line harboring p53 reporter plasmid RGCΔ*fosLucZ* was used in those experiments. To investigate the effects of UV exposure on the transcriptional activity of p53, we conducted transient-transfection and reporter-activity assays in NHF, XP-A, and XP-C cells. As expected, controls using plasmid MG15-*luc*, which contains 15 copies of mutated sequence unable to bind p53 protein, showed a very low level of luciferase activity. Furthermore, UV treatment of cells containing this construct did not significantly alter the levels of luciferase activity (data not shown). In contrast, with the PG13-*luc* p53 reporter, which contains 13 copies of a synthetic p53 consensus site derived from the promoter of *p21^{waf1}*, all the three cell lines exhibited significant basal transcriptional activity of p53, with the highest activity present in NHF cells (Figure 3A). This observation is consistent with higher basal levels of p21^{waf1} in NHF cells as determined by western blot analysis.

A clear induction of transcriptional activity of p53 was seen in NHF cells after exposure to 10 J/m². Nevertheless, a dramatic decrease of transcriptional activity of p53 occurred in NHF cells that received

20 J/m² UV, and this activity of p53 decreased further with higher doses of UV exposure. Only 42%, 19%, and 10% of basal activity could be visualized in NHF cell irradiated with 20, 30, and 50 J/m² UV exposure, respectively (Figure 3A and B).

Inhibition of p53 activity by UV exposure was more dramatic in XP-A cells. Unlike NHF cells, no induction of transcriptional activity of p53 was seen in XP-A cells irradiated with a low UV dose of 10 J/m². Instead, 10 J/m² UV exposure reduced p53 activity to 15% of basal activity in these cells (Figure 3B). These results do not necessarily reflect that UV exposure is totally unable to induce p53 activity in XP-A cells that may be demonstrable at a somewhat lower level of UV exposure. In fact, other studies have reported an induction of p53 and enhanced expression of p53 target genes, e.g., *p21^{waf1}* and *bax*, in XP-A cells with 5 J/m² UV exposure [16].

As in XP-A cells, lower basal p53 transcriptional activity was found in XP-C cells (Figure 3A). However, transcriptional activity of p53 in XP-C cells was resistant to inhibition by high UV exposure. In XP-C cells irradiated with 10 J/m² UV, induction of transcriptional activity of p53 was similar to that observed in NHF. Furthermore, exposure to 20 J/m² UV radiation did not show a measurable inhibitory effect on transcriptional activity of p53. XP-C cells irradiated with 30 J/m² UV still had 64% of basal transcriptional activity, whereas 50 J/m² UV exposure reduced this activity to about 27%. Thus, after receiving high doses of UV exposure, XP-C cells clearly had much higher p53 activity than NHF cells (Figure 3A and B).

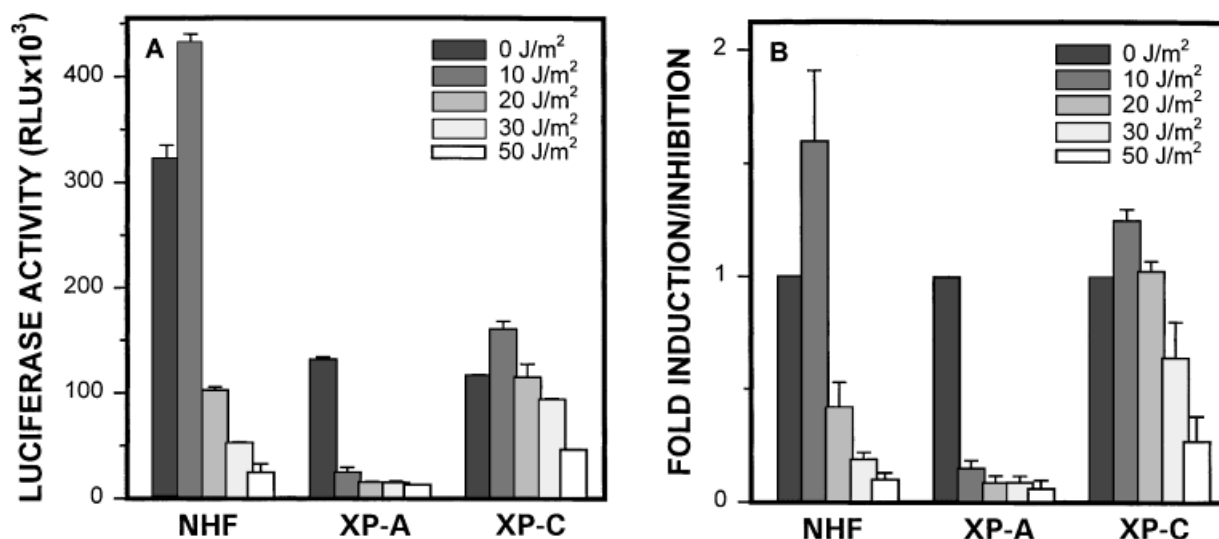


Figure 3. Induction and inhibition of transcriptional activity by UV irradiation. NHFs and XP-A and XP-C fibroblasts (2.0×10^5) were plated in 35-mm dishes 18–20 h before transfection. Cells were transfected with 2.0 μ g of plasmid pG13-*luc* using FuGENE 6 transfection reagents (Boehringer Mannheim). Three hours after transfection, the FuGENE 6–DNA complex was removed, and cells were either unirradiated or irradiated with UV at a dosage between 10 and 50 J/m². Eight hours after exposure, cell extracts were prepared and luciferase activity was assayed as described in Materials

and Methods. Equal amounts of cell lysates were assayed for luciferase activity at the indicated UV dose for each cell line. (A) Results are expressed as fold induction or inhibition of UV-irradiated versus unirradiated control cells. Results shown are the average of two to three independent experiments, each performed in duplicate. (B) Results are expressed as relative luciferase activity for easy comparison of activity levels. Data represent at least three independent transfections (error bars = SE).

Inhibition of General Gene Transcription in NHFs and XP-A and XP-C Fibroblasts by UV Exposures

It has been reported that UV light-induced pyrimidine dimers and 6–4 photoproducts in DNA efficiently block the progression of RNA polymerase to effect the inhibition of the general gene transcription [47,48]. To examine whether different UV exposures differentially inhibit the general gene transcription in repair-proficient and repair-deficient cells, we conducted a transient transfection and reporter assay by using a different plasmid construct that contains a firefly luciferase reporter gene directed by the cytomegalovirus immediate-early enhancer/promotor sequence. Figure 4 shows that luciferase reporter activity in NHF cells decreased to about 50% and 34% after 8 h of exposure to 10 and 20 J/m² of UV irradiation, respectively. Such a decrease is consistent with results of previous reports in which mRNA synthesis was assayed directly to reflect the inhibition of general gene transcription [44]. Although decreases in the luciferase reporter activity were dose dependent in all the three cell lines, UV inhibition of general gene transcription was found to be more drastic in NHF than in XP-A or XP-C fibroblasts (Figure 4). Because XPA protein is an early participant of the NER process, its involvement is also indicated by the significant transcription inhibition at a level seen between NHF and XP-C cells. These results clearly cannot be simply explained by the blockage of RNA polymerase progression due to unrepaired lesions in DNA, because XP-A cells are

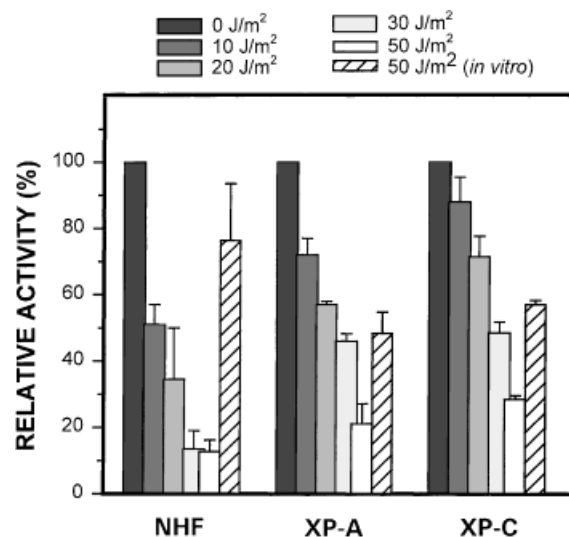


Figure 4. Inhibition of general transcription by UV irradiation. NHFs and XP-A and XP-C fibroblasts (1.0×10^5) were plated in 35-mm dishes 18–20 h before transfection. Cells were transfected with 1.0 μ g of pCMV-Tag2 control or UV-irradiated plasmid DNA using FuGENE 6 transfection reagents (Boehringer Mannheim). Four hours after transfection, the FuGENE 6–DNA complex was removed, and cells were either unirradiated or irradiated with UV at a dosage between 10 and 50 J/m². Luciferase activity from lysates of each cell line was assayed 8 h after exposure, as described in Materials and Methods. Results shown are the average of two or three independent experiments, each performed in duplicate (error bars = SE).

known to be deficient in both GGR and TCR, whereas XP-C cells are deficient in GGR only. Moreover, in parallel experiments, native plasmid DNA was UV irradiated at a dose of 50 J/m² before transfection and assaying for the reporter activity.

Unlike the *in vivo* treatment, transfection of this *in vitro*-irradiated plasmid DNA demonstrated a much higher luciferase activity, e.g., 76%, 48.5%, and 57% of control unirradiated plasmid DNA in NHF, XP-A, and XP-C cells, respectively. From these data, it can be clearly inferred that the overall extent of excision repair of UV-induced lesions plays a prominent role in the recovery of transcription from *in vitro* or *in vivo* damaged transcribed strands in transfected genes. Compared with *in vitro* treatment, *in vivo* exposure to the identical UV dose of the same plasmid target within the cells caused a more severe inhibition of reporter gene transcription. For example, the exposure of transfected cells to 50 J/m² decreased the luciferase reporter activity to about 12%, 21%, and 28.5% in NHF, XP-A, and XP-C cells, respectively. Because more damage will be expected to occur in *in vitro*-modified than in *in vivo*-irradiated plasmid DNA, these differences, showing higher inhibition under *in vivo*-irradiated conditions, cannot be simply accounted for by the blockage of transcription by UV photoproducts in the transfected gene. Thus, inhibition of general gene transcription seems to be intimately associated with other processes involved in the damage recognition and repair at global genomic or individual strand level rather than a simple lesion blockage of RNA polymerase.

DISCUSSION

Induction of p53 Protein and Inhibition of p53 Transcriptional Activity by UV-Induced DNA Damage

These data and data reported by others [17] have shown that p53 transcriptional activity is not necessarily in accordance with p53 protein accumulation observed with DNA damage. Thus, the concept of "p53 response" should be advanced with caution because it relates to the multiple p53 activated downstream effects and to p53 accumulation. Triggering of the p53 response with low-dose UV exposures of TCR-deficient fibroblasts has been reported by many investigators [16,43,44]. Blockage of transcription by helix-distorting UV dimers has been promulgated as the model to explain this and the conclusion that DNA damage within the transcribed strand triggers p53 response. Accordingly, the recovery of RNA synthesis resulting from the removal of UV-induced dimers by TCR has been suggested to eliminate the trigger for p53 accumulation [43,44,49]. If the general shutdown of transcription by UV-induced lesions in DNA was the actual cause of decreased expression of *p21^{waf1}* and *mdm2*, then the expression of *mdm2* would have been more sensitive to UV inhibition than the expression of *p21^{waf1}* because the *mdm2* gene is much larger than the *p21^{waf1}* gene. However, such a decrease in *mdm2* expression was not observed in the present study. UV exposures above 20 J/m² only

showed a gradual inhibition of *mdm2* expression. Further comparisons showed that 50 J/m² UV exposure blocked the expression of *p21^{waf1}* but not of *mdm2* in NHFs. Similarly, a lower dose of 20 J/m² UV exposure blocked the expression of *p21^{waf1}* but not of *mdm2* in XP-A cells. On the contrary, elevated *p21^{waf1}* and *mdm2* expressions were seen in XP-C cells irradiated with increased doses of UV, up to 50 J/m². It is unlikely that increased expression of *p21^{waf1}* and *mdm2* in repair-deficient XP-C cells is due to the recovery of DNA damage-blocked RNA synthesis. In fact, Lu et al. [17] showed that the expression of even a small gene such as *lacZ*, in cells harboring the p53 reporter plasmid, is inhibited by high UV exposures. Furthermore, *in vivo* exposure of cells to UV decreased the transcription from transfected reporter gene more dramatically than did *in vitro* exposure of plasmid DNA before transfection. Moreover, UV exposure inhibited the general gene transcription more effectively in repair-proficient NHF than in repair-deficient XP-A and XP-C fibroblasts (Figure 4). Thus, inhibition of *p21^{waf1}* and *mdm2* expression by high UV doses reflects a decrease of transcriptional activity of p53 rather than a direct blockage of transcription by UV-induced lesions in DNA. The present data clearly demonstrate that p53 transcriptional activity can be inhibited by UV damage and that DNA repair-deficient XP-A cells are more sensitive to such an inhibition. Therefore, DNA damage, which triggers induction of p53 and its transcriptional activity, may also trigger inhibition of p53 transcriptional activity depending on how much UV dosage was received by the cells and, more importantly, the DNA-repair proficiency of the cells being exposed.

Linkage of DNA Repair to Induction, Activation, and Degradation of p53 Through DNA-Damage Recognition

This study confirms previous observations on discordance between accumulated p53 protein level and its transcriptional activity [17]. In addition, the data indicate that the inhibition of p53 transcriptional activity is related to the DNA-repair status of the cells. An examination of *in vivo* ubiquitination of p53 showed that high UV exposures inhibit ubiquitination of p53 in XP-C but not in NHFs and XP-A fibroblasts. Integrating these observations in a plausible scheme requires a precise understanding of the potential interconnections between the pathways operating for DNA-damage recognition, p53 induction, p53 degradation, and DNA repair. Despite the extreme complexity of these pathways, several recent studies have shed a bright light on the nature of such multiprotein and multipathway interactions. It has been shown that *mdm2*, which negatively regulates p53 activity, is also involved in p53 degradation via the ubiquitin-proteolysis pathway [reviewed in 50]. This process involves complexing through the C/H1 domain of the p300/

CREB-binding protein (CBP) [26], a well-known transcription coactivator for p53 and other transcription factors. Furthermore, stabilization of p53 by adenovirus E1A has been demonstrated to occur through the modification of the ubiquitin-proteolysis pathway, suggesting the key involvement of p300/CBP in p53 ubiquitination [51]. These studies raise a possibility that transcription activation and degradation of p53 may use the components of common machinery. This suggestion is supported by the recent demonstration of an overlap between the activation and degron domains at amino acids 1–42 within the N terminus of p53 [22]. In a search for a possible link between DNA repair and the ubiquitin-proteolysis pathway, RAD23 and RAD4, a yeast homolog of XPC, were recently identified to complex Cim5, an ATPase subunit of yeast 26 S proteasome [38]. Interaction of hHR23B with the S5a subunit of human 26 S proteasome has also been demonstrated [52]. It should be noted that XPC protein, when complexing with hHR23B, plays an important role in early stages of DNA-damage recognition [53–55]. A recent study has shown that TFIIH can be recruited to DNA-damage sites through interaction with XPC-hHR23B [56]. In a separate study, we demonstrated the functional and physical interaction between hHR23A and p300/CBP [57]. These observations provide a strong basis for connecting DNA-damage recognition, p53 induction, p53 degradation, and DNA repair as an integrated system designed to work together for the maintenance of genomic stability.

The present data could be attributed to the biological consequences from the network of multiple interactions discussed in the previous paragraph. The use of common machinery for transcription activation and degradation of p53 affords cells a special advantage for keeping p53 activity under tight regulatory control. NER-repairable DNA damage would instigate the recruitment of DNA-damage recognition factors, e.g., XPA and XPC/hHR23, and a p53 transcription/degradation complex to the lesion sites. Therefore, such recruitment, on the one hand, would lead to the reassembly of the complex of transcription/degradation of p53 and consequent stabilization of p53 protein and, on the other, would lead to the formation of a p53 transcription/DNA recognition complex comprising interactions between p300, hHR23A/B, XPC, TFIIH, and p53 protein. This entire interaction causes the fluctuations of p53 activity observed in irradiated cells. At low UV fluences, the stabilization of p53 overcomes the tendency for decreased p53 activation. However, at high UV fluences, concomitant inhibition of p53 activity results in discordance between p53 level and its transcriptional activity. In XP-C cells, limited function of XPC protein results in the resistance of p53 activity to inhibition by high UV exposure. This

model of the regulation of p53 activity does not exclude other possible pathways of p53 regulation, e.g., via mdm2 feedback or wild-type p53 adopting a “mutant” conformation [58]. In fact, consistent high levels of mdm2 could also contribute to the inhibition of p53 activity observed in XP-A cells. Thus, in essence, recruiting TFIIH to DNA damage would cause a transitory shutdown of general transcription. From the overall transcription inhibition data of the three cell types, it seems logical to conclude that, although recognition of DNA damage by XPA contributes to the process, it is not necessarily required for recruiting TFIIH to DNA damage (Figure 4).

Requirement of XPC Function for Ubiquitination of p53

The present data indicated that the small amount of ubiquitinated p53 present in XP-C cells under normal unirradiated conditions was promptly elevated with low-dose UV exposures. However, there was a complete lack of p53 ubiquitination in XPC cells at higher doses of UV. As a part of the RAD23/Cim5 complex, the function of RAD4 seems to be required to some extent for the process of ubiquitination. Reduced XPC mRNA levels and point mutation in XPC protein were both characterized in the XP-C fibroblasts, XP1MI, used in this study [59]. Mutation was not, however, located in its binding domain for hHR23B and hHR23A. This cell line has the highest degree of UV sensitivity of any reported group C cell line [60]. Whether dysfunction of XPC is caused by such a point mutation or lower expression of XPC remains to be investigated. Nonetheless, in both cases, it is conceivable that the capability of damage recognition by the XPC-hHR23B complex would be limited in this cell line. Mechanistically, therefore, with high UV exposure, such limited capability would be used up for damage recognition, such that it is no longer available for the ubiquitin-proteolysis pathway and the ubiquitination of p53 would be inhibited, as observed in this study. Inhibition of ubiquitination of p53, however, was not seen in NHF and XP-A cells, as suggested by Maki and Howley [19]. Such differences could be attributed to the use of different cell lines used in the two studies. The RKO cell line, used in the previous study, is a carcinoma cell and may have unidentified characteristics, making *in vivo* ubiquitination of p53 in this cell line more sensitive to UV inhibition.

In summary, our results demonstrate that, although DNA damage triggers induction of p53 protein, it also acts as a trigger for the inhibition of p53 transcriptional activity and that recognition of DNA damage could link p53 induction and p53 degradation to DNA-repair mechanisms. Regulation of the level and transcriptional activity of p53 is an integrated cellular process in mammalian cells.

Accumulating evidence indicates that mdm2, p300/CBP, p19^{ARF} and DNA-dependent protein kinase C are all involved in such a cellular process [26,51,61]. Nevertheless, to rule out the possibility that the observed effects were not merely due to cell line-specific effects and interindividual variation, additional cell lines representing each genotype must be evaluated for p53-specific responses. Further in-depth knowledge of regulation of p53 activity and its interacting molecules will clarify the nature of p53 function in transcriptional events and the critical regulatory parameters associated with the processing of DNA damage.

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