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p53-Mediated DNA Repair Responses to UV Radiation: Studies of Mouse Cells Lacking *p53*, *p21*, and/or *gadd45* Genes

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Human cells lacking functional p53 exhibit a partial deficiency in nucleotide excision repair (NER), the pathway for repair of UV-induced DNA damage. The global genomic repair (GGR) subpathway of NER, but not transcription-coupled repair (TCR), is mainly affected by p53 loss or inactivation. We have utilized mouse embryo fibroblasts (MEFs) lacking *p53* genes or downstream effector genes of the p53 pathway, *gadd45* (*Gadd45a*) or *p21* (*Cdkn1a*), as well as MEFs lacking both *gadd45* and *p21* genes to address the potential contribution of these downstream effectors to p53-associated DNA repair. Loss of *p53* or *gadd45* had a pronounced effect on GGR, while *p21* loss had only a marginal effect, determined by measurements of repair synthesis (unscheduled DNA synthesis), by immunoassays to detect removal of UV photoproducts from genomic DNA, and by assays determining strand-specific removal of CPDs from the mouse *dhfr* gene. Taken together, the evidence suggests a role for Gadd45, but relatively little role for p21, in DNA repair responses to UV radiation. Recent evidence suggests that Gadd45 binds to UV-damaged chromatin and may affect lesion accessibility. MEFs lacking *p53* or *gadd45* genes exhibited decreased colony-forming ability after UV radiation and cisplatin compared to wild-type MEFs, indicating their sensitivity to DNA damage. We provide evidence that Gadd45 affects chromatin remodelling of templates concurrent with DNA repair, thus indicating that Gadd45 may participate in the coupling between chromatin assembly and DNA repair.

The tumor suppressor p53 is an important mediator of cellular responses to DNA damage in mammalian cells. In some cell types, p53 activation triggers apoptosis (30), while in other cell types, p53 serves a protective function, attributable not only to the activation of cell cycle checkpoints, but also enhancement of DNA repair (12; reviewed recently in reference 41). P53 plays a role in nucleotide excision repair (NER), the pathway for repair of UV-induced DNA damage, bulky carcinogen adducts, and DNA damage caused by cancer chemotherapy agents such as *cis*-dichloro-diammine-platinum (cisplatin) (see references 11–13 and 38–41 and references therein). Loss of p53 function, as occurs frequently in human cancer cells, leads to decreased DNA repair of these types of lesions, and in some cell types, this is reflected by increased cellular sensitivity to these agents (7, 9, 13, 16, 20, 39–41).

An important question concerning the connection between p53 and NER is to what extent the p53 protein participates directly in DNA repair (28, 48), versus p53 transcriptionally regulated gene products that contribute to the NER response. For example, p53 has been shown to directly associate with TFIIH, an NER component (28, 48), while genes implicated in repair, such as *DDB2* (23, 24) and *gadd45*, are p53 regulated (24, 25). In addition, *gadd45* is UV responsive, even in p53-deficient cells (51). In the case of *gadd45*, its protein product is known to associate with proliferating cell nuclear antigen (PCNA), core histones, p21, and MTK1 (6, 27, 38, 45, 47), and reduced repair, as measured by host cell reactivation of UV-damaged plasmid reporter, was observed in RKO cells expressing antisense Gadd45 (40). However, the conclusion that Gadd45 contributes to NER is complicated by several issues. First, the antisense approach only suppressed Gadd45 expression, but did not ablate it. Second, the study was carried out with a human tumor line that contained additional genetic changes. Third, one such mutation resulted in the mismatch repair-deficient phenotype, and mismatch repair has been implicated in damage recognition (32). Moreover, RKO cells express higher levels of Gadd45 than are observed for most human cell lines (40). Here we have used mouse embryo fibroblasts (MEFs) carrying homozygous deletions of the *p53* gene or deletions of known component genes (downstream effectors) of the p53 pathway *p21* (*Cdkn1a*, also known as *cip1*, *waf1*, or *sdi1*) and *gadd45* (*Gadd45a*, also known as *Gadd45*α) or MEFs lacking both *gadd45* and *p21* (*gadd45*/*p21*-null cells). The present study is the first to explore the components of the p53 pathway that contribute to DNA repair in an isogenic, primary cell system carrying only the defined genetic alterations. Rodent cells do, however, exhibit intrinsically slower global genomic repair (GGR) of cyclobutane pyrimidine dimers (CPDs) than human cells (14).

Recent studies using *gadd45*-null cells showed that Gadd45 contributes to maintenance of genomic stability, inasmuch as cells lacking *gadd45* genes exhibited multiple chromosome abnormalities, and *gadd45*-deficient mice showed increased radiation carcinogenesis (22). While perturbation in the control of G_2 cell cycle progression was also observed, control of G_1 checkpoints after either ionizing radiation (IR) or UV radia-

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tion as well as radiation-induced apoptosis was equivalent in *gadd45*$^{-/-}$ and wild-type cells (22). Although the components of the p53 pathway involved in apoptosis and cell cycle checkpoint control are well known, much less is known about p53 and the roles of its downstream effectors in the maintenance of genomic stability, including DNA repair. For example, p53 mutants have been isolated from human cancers that retain the apoptotic and cell cycle arrest properties of wild-type p53, but are nonetheless associated with genomic instability and carcinogenesis (43).

Two approaches were used to assess acute responses to UV irradiation in the MEF lines, measurements of DNA repair synthesis as unscheduled DNA synthesis (UDS) after UV irradiation and quantification of major photoproducts remaining at various times after UV irradiation. Long-term cytotoxicity responses in these cell lines after treatment with UV radiation or cisplatin suggest that p53-downstream effectors—Gadd45 in particular—contribute to NER responses and may influence cellular sensitivity to certain DNA-damaging agents.

A potential mechanism by which Gadd45 might interact with cellular NER was suggested by a recent study showing that Gadd45 interacts with chromatin (6). In this study, Gadd45 was found to have some similarity to other acidic chromatin-interacting proteins and was found to bind to UV-irradiated nucleosomes. Gadd45 also disrupted histone DNA associations in vitro (6). We now present evidence that Gadd45, in the presence of other nuclear factors, affects chromatin remodelling of damaged plasmid templates concurrent with DNA repair in vitro.

MATERIALS AND METHODS

MEFs. Mouse embryos at 13.5 days of gestation were isolated in utero and cells were dispersed by using a razor blade. Resultant cells were cultured overnight, and were frozen as soon as was practical. MEFs from matched littermates were screened for the presence or absence of the appropriate gene sequences by Southern blotting and/or by PCR for the presence of diagnostic restriction fragments. MEFs lacking *p53* genes were from previously published studies (25). The generation of mice lacking *p21* genes has been described previously (8). Mice hemizygous for the presence or absence of *p21* genes were bred to generate matched sets of *p21*$^{+/+}$ and *p21*$^{-/-}$ MEFs. The construction of *gadd45*$^{-/-}$ and *gadd45*/*p21*-null mice has been reported elsewhere (22). The *gadd45* gene disruption ablates all but 37 carboxy-terminal residues of the expected protein product and all of the essential promoter region. As controls in the UDS studies, we used the *rb*$^{-/-}$ MEFs (kindly provided by Allan Bradley, Baylor College of Medicine or *p16ink4a*$^{-/-}$ MEFs (kindly provided by Ronald DePinho, Dana-Farber Cancer Center). Cells were stored in liquid nitrogen, and upon thawing, were used within one to three passages. Results were obtained from two *p53*$^{-/-}$ MEF lines, three *gadd45*$^{-/-}$ lines, and two *p21*$^{-/-}$ lines. Only one MEF line was available carrying the *gadd45*/*p21*-null genotype. MEFs from matched littermates were used whenever possible.

UDS technique. UDS assays were carried out essentially as described previously (40). Primary MEFs grown on glass slides were transferred to 150-mm^2 dishes, and each respective nullizygous MEF line was irradiated side-by-side with corresponding wild-type (+/+) controls. Cells were irradiated with 20 J of UV radiation m^{-2} (254 nm) and were incubated for 3 h in serum-free medium containing [^{3}H]thymidine (10 μCi per ml). Experiments with lower doses yielded similar results, but fewer tritium grains. Alternatively, cells were exposed to 100 μM cisplatin for 5 h concurrent with [^{3}H]thymidine uptake. Cells were processed for autoradiography, and nuclei were photographed by using a ×100 objective (Olympus model AX70) under oil-immersion optics. The number of tritium grains per nucleus was determined from the photomicrographs by using a manual colony counter. Negative controls consisted of unirradiated MEFs, and the human xeroderma pigmentosum XP-A cell line XP12BE (40).

In experiments using serum-starved MEFs arrested in G_0, cells were incubated in serum-free RPMI 1640 lacking isoleucine for 48 h, and UDS assays were performed as described above. Alternatively, cells were arrested in G_1 and G_2 by staurosporine treatment (50 nM, 24 h [35]). UDS assays were used as described above.

PCNA immunostaining. Cells were grown on glass slides and irradiated with 20 J of UV radiation m^{-2} as for UDS studies. After 1.5, 3, or 6 h, slides were incubated in the presence of 1% Triton X-100 and methanol fixed. PCNA was detected with antibody PC10 (Oncogene Science, Inc.) followed by fluorescein-conjugated goat anti-mouse immunoglobulin G (Sigma).

Global genomic NER assay. The relative number of UV-induced photoproducts, and 6-4 pyrimidine-pyrimidone photoproducts (6-4 pps) in total unreplicated genomic DNA from cells collected at various times following UV irradiation was determined using an immunoblot assay, as previously described (12). Briefly, exponentially growing cells were labeled with [^{3}H]thymidine, washed with phosphate-buffered saline, and irradiated with 10 J of UV m^{-2} using a 15-W germicidal UV lamp delivering predominantly 254-nm light. Cells were either lysed immediately for an initial sample or were incubated in growth medium containing 5-bromodeoxyuridine (BrdU) to density label newly replicated DNA and then lysed at various times. Density labelling was performed during repair periods to allow unreplicated DNA to be isolated by cesium chloride isopycnic density gradient sedimentation. Equal amounts from each DNA sample were fixed to a Hybond N+ nylon membrane (Amersham) in triplicate by using a slot-blot apparatus. The membrane was incubated with mouse monoclonal antibodies specific for either CPDs or 6-4 pps (34) and horseradish peroxidase-conjugated secondary antibody. Enhanced chemiluminescence (Amersham) and phosphorimager analysis (Bio-Rad model GS-363) were employed for detecting the primary antibodies. Following antibody detection, equal DNA loading to each slot of the membrane was confirmed by scintillation spectrophotometry of the ^{3}H-DNA on individual pieces cut from the membrane.

Strand-specific repair assays. The rates of photoproduct removal were determined within the transcribed (TS) and nontranscribed (NTS) strand of a 14-kbp *Bam*HI restriction fragment spanning the central region of the mouse *dhfr* gene, as described previously (36). Cells were irradiated with 10 Jm^{-2} of UV radiation, lysed immediately for an initial sample, or incubated for the times indicated to allow photoproduct repair. The frequency of induction and rate of removal of CPDs from TS and NTS of the *dhfr* gene was measured by treating purified *Bam*HI-digested DNA with T4 endonuclease V (TEV), and then quantifying the reappearance of the full-length restriction fragments in DNA from cells allowed various times to remove the lesions. *Bam*HI-treated samples from each time point were treated or mock treated with TEV, electrophoresed in parallel under denaturing conditions, Southern-transferred to a membrane, and then hybridized to strand-specific RNA probes generated by in vitro transcription. The ratio of full-length restriction fragments in the TEV-treated and untreated samples was determined by phosphorimager analysis and was used to calculate the average number of TEV-sensitive sites (unrepaired lesions) per fragment by using the Poisson distribution (12).

Survival responses to UV irradiation or cisplatin. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assays were carried out as described previously (10), but were terminated at 7 days. For cisplatin, cells were treated in microtiter wells for 72 h (40). For UV radiation experiments, cells were irradiated in 60-cm^2 dishes and were dispensed in microtiter plates for quantitation on day 7. Assays were quantified by the use of a microtiter plate reader (E-max; Molecular Devices, Inc.). All survival data are expressed relative to untreated cells in the same experiment, as previously described (10).

Clonogenic survival experiments were conducted as described previously (38–40), except that irradiated feeder layers were used to alleviate the poor plating efficiency of MEFs. *p53*-null MEFs were used as feeder cells and were irradiated with 100 Gy of ionizing radiation. Approximately 5×10^5 feeder cells were applied per 10-cm^2 dish. Each respective MEF line was then plated on the feeder layer and treated with DNA-damaging agents (UV radiation or cisplatin). Some experiments were also performed without feeder layers. While irradiated feeder cells did not grow, colonies (>100 cells/colony) of each respective MEF line were visible after 12 days and were counted by a manual colony counter as described previously (38–40).

Cell cycle analysis. Wild-type or *gadd45*$^{-/-}$ MEFs were prepared for fluorescence-activated cell sorter (FACS) analysis as described previously (9, 10). Cells were arrested in G_0 by serum deprivation and irradiated with 10 J of UV radiation m^{-2} (254 nm) and were incubated in complete medium containing 10 μM BrdU for 24 h. BrdU incorporation was detected by an anti-BrdU fluorescein-conjugated antibody (Becton-Dickinson) (10). Propidium iodide (PI) staining for DNA content was as previously described (9, 10). Cell cycle analysis was performed using a Becton-Dickinson FACScan flow cytometer. The Cell-Quest software package (Becton-Dickinson) was used to analyze the data in which 15,000 BrdU-positive cells were analyzed in each individual sample. All available evidence from other studies showed a lack of effect of *gadd45* deficiency on the first G_1 (nor the second G_1) arrest after DNA damage (29, 49). Attention was therefore focused on S and G_2/M phases by the gating of BrdU-labeled cells. Only actively cycling cells are counted by this technique.

The S-phase fraction was also measured by [^{3}H]thymidine pulse labelling 15 h after UV irradiation. Asynchronous wild-type or *gadd45*$^{-/-}$ MEFs were irradiated with 10 J of UV radiation m^{-2}, incubated for 15 h in complete medium, and then labeled for 3 h with [^{3}H]thymidine. Autoradiography was conducted as for UDS experiments. Although UDS was not detected after 15 h (i.e., most of the UDS occurs within the first few hours), the percentage of S-phase cells 15 h after UV irradiation was determined from photographic fields similar to those shown in Fig. 1A.

In vitro NER-chromatin assembly assay. Whole-cell extracts (WCE) and nuclear (NUC) extracts were prepared from stimulated, growing wild-type or *gadd45*$^{-/-}$ mouse lymphoblasts (22). Assays were conducted in the presence of 100 μg of WCE and 50 μg of NUC extracts, utilizing a UV-damaged plasmid template (>20 lesions per plasmid molecule [38, 39]). WCE are fully competent

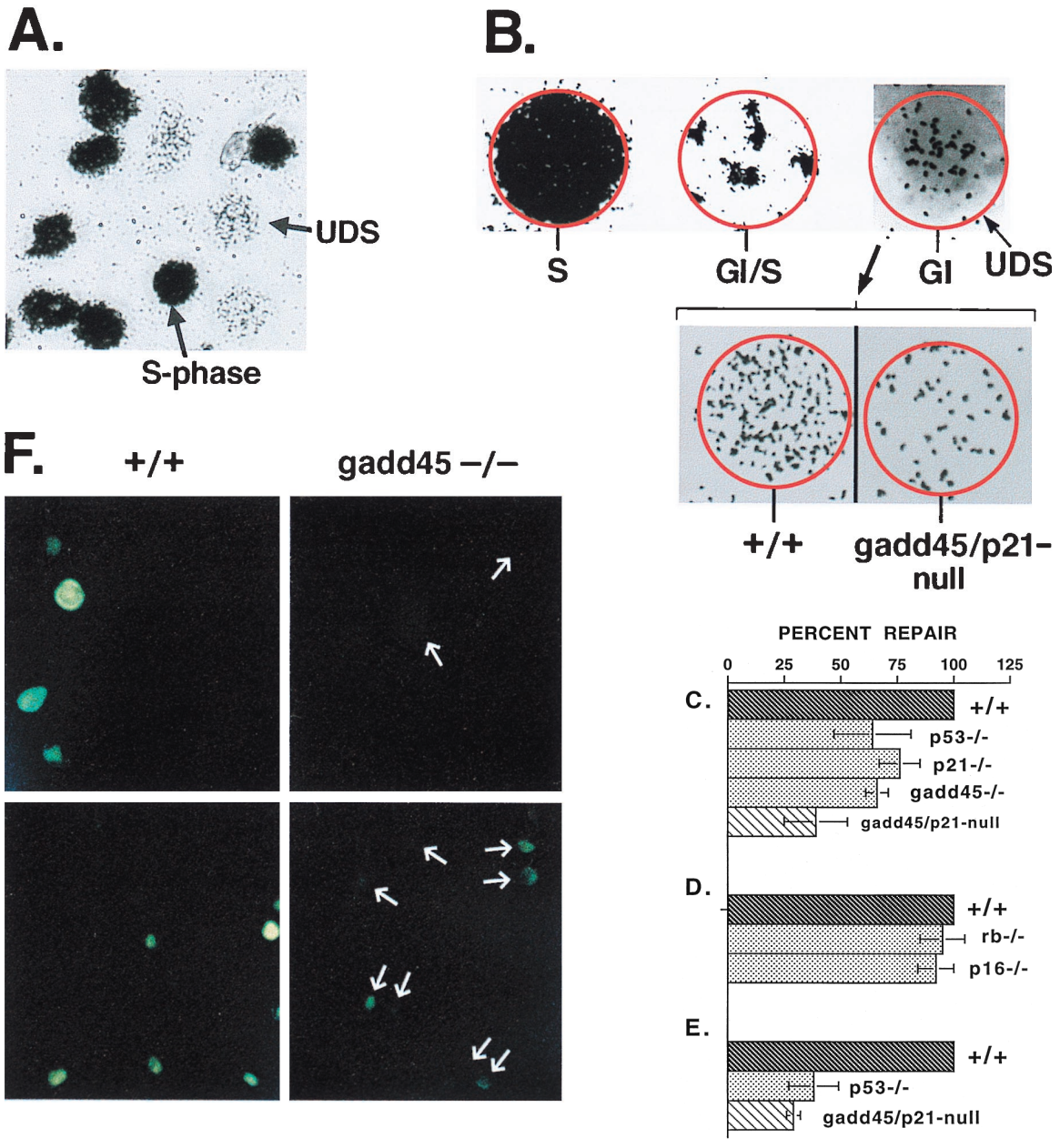

FIG. 1. DNA repair deficiency in cells lacking p53 or p53 effector genes, *gadd45* and *p21*, measured by UDS. (A) Illustration of UDS technique. A low-magnification (×20) field of cells irradiated with 20 J of UV radiation m^{-2} is shown. Nuclei were made visible by the incorporation of [^{3}H]thymidine during either replicative or repair DNA synthesis. S-phase nuclei appear black upon the photographic emulsion, while non-S-phase cells exhibit UDS. The $p21^{-/-}$ and *gadd45*/*p21*-null lines did exhibit a higher S-phase fraction (50%) compared to wild-type or $gadd45^{-/-}$ lines (15 to 20%). (B) Distinctions between replicative DNA synthesis and repair synthesis (UDS). S-phase or G_1/S transitional nuclei exhibited a different pattern of tritium incorporation from that observed in G_1 or G_2 nuclei (×100 oil immersion). Because nuclear membranes were not always visible on photomicrographs, the nucleus is outlined in red. Note the visualization of replicon clusters in G_1/S transitional nuclei, while a G_1 nucleus exhibits only UDS. The number of UDS grains per nucleus was quantitated, providing a measure of the number of sites of repair synthesis per nucleus. In comparing wild-type and (+/+) mutant MEFs, differences were observed in numbers of UDS grains per nucleus. UDS experiments were terminated 3 h after UV radiation (compare UDS results to the 4-h time point shown in Fig. 2). (C to E) Compiled data from several experiments such as those shown in panel B. Values obtained for wild-type cells were defined as 100%. (C) Compiled data for UDS after UV irradiation. The following numbers of nuclei were counted: wild-type, 652; $p53^{-/-}$, 88; $p21^{-/-}$, 129; $gadd45^{-/-}$, 177; *gadd45*/*p21*-null, 157. The values shown are means ± standard deviations. (D) Compiled data, control MEF lines, UDS after UV irradiation. The following numbers of nuclei were counted: wild-type, 51; $rb^{-/-}$, 22; $p16^{-/-}$, 20. The values shown are means ± standard deviations. (E) Compiled data for UDS after cisplatin treatment. The following numbers of nuclei were counted: wild-type, 116; $p53^{-/-}$, 93; *gadd45*/*p21*, 81. The values shown are means ± standard deviations. (F) PCNA immunostaining (Triton resistant) in wild-type and $gadd45^{-/-}$ MEFs 1.5 h after UV radiation (top) or 3 h after UV radiation (bottom). While all wild-type nuclei were clearly visible, $gadd45^{-/-}$ nuclei were much less visible; their positions are marked by arrows. The PCNA staining defect was most evident at 1.5 h and recovered to near normal after 6 h (not shown).

for NER, while NUC extracts provide chromatin assembly factors and DNA topoisomerases as described previously (15). Assays were carried out in 50-μl reaction mixtures as described previously (38, 39). Approximately 150 fmol of radioactive nucleotide was incorporated during repair synthesis and subsequent steps, as determined by phosphorimager quantitation. Recombinant Gadd45 (human; rGadd45) was added to *gadd45*-null extracts to result in increased recovery of repaired (labeled by ^{32}P) and subsequently remodeled (supercoiled and assembled into nucleosomes) plasmid template. rGadd45 was highly purified

by high-pressure liquid chromatography (6) (a gift from F. Carrier, University of Maryland). For micrococcal nuclease (MNase) studies, in vitro reactions were conducted in parallel to those above, except that ^{32}P-dCTP was omitted. MNase (0.001 U) (Sigma) was added directly to the reaction mixtures, incubated for an additional 5 min, and terminated by phenol-chloroform extraction, and ethanol precipitation.

The "uncoupling" of the repair reaction from the chromatin remodelling step was attempted. Damaged plasmid templates were first incubated with WCE in the presence of [^{32}P]dCTP (3 h). Reactions were terminated by phenol-chloroform extraction and ethanol precipitation. Templates were then incubated with NUC extracts in the absence of radiolabel (3 h). In accord with the results of Gaillard et al. (15), the two processes could not be dissociated.

RESULTS

Measurements of DNA repair synthesis in situ by UDS technique. DNA repair synthesis in UV-irradiated MEFs was quantified by unscheduled DNA synthesis (UDS; see Materials and Methods). UDS relies on the ability to distinquish replicative DNA synthesis from repair synthesis. Cells in S phase at the time of irradiation exhibit dense nuclear labelling (Fig. 1A). Cells that traverse the G_1/S boundary during the 3-h labelling period exhibit a characteristic pattern of "replicon initiation" synthesis (Fig. 1B). The G_1 phase of the cell cycle is 15 to 16 h in MEFs (21), while the duration of the UDS experiments is 3 h. Therefore, although MEFs spend a majority of the time in G_1, few cells would traverse the G_1/S boundary during this type of experiment. Repair synthesis (UDS) was detected in all non-S phase, and non-G_1/S transitional cells, irrespective of whether cells were in G_1 or in G_2 (Fig. 1B). NER-defective XP12BE cells showed little or no UDS (results not shown and reference 40). In NER-competent cells, the number of grains per nucleus is a direct measure of the number of sites of DNA repair synthesis per nucleus and is linear with UV dose (2, 5, 14, 40). When the wild-type and mutant MEFs were compared, there was a clear difference in the number of grains per nucleus (Fig. 1B). We assayed only G_1 nuclei in the current study for purposes of consistency; i.e., G_2 nuclei were assayed in separate experiments but yielded results similar to those shown for G_1 nuclei (G_2 results not shown). A number of independent experiments were conducted for each respective pair of wild-type and mutant MEF lines (in total, eight experiments were conducted for *p53*$^{-/-}$; four experiments for *p21*$^{-/-}$, five experiments for *gadd45*$^{-/-}$, and eight experiments for *gadd45*/*p21*-null MEFs). As negative controls, two experiments each were conducted in *rb*$^{-/-}$ and *p16*$^{-/-}$ MEFs. The data from all of these experiments (average relative number of grains per nucleus) are summarized in Fig. 1C to E.

Compared to the wild-type, *p53*$^{-/-}$ MEFs exhibited 35 to 70% of the normal UDS in response to UV irradiation, in agreement with other experimental approaches in which NER was found to be decreased in cells lacking functional p53 (11–13, 39, 40). Importantly, *gadd45*$^{-/-}$ MEFs exhibited UDS that was 61 to 71% of the wild-type level ($P < 0.0001$ by Student *t* test), while the UV-induced UDS response of *gadd45*/*p21*-null MEFs was 23 to 55% of the wild-type level ($P < 0.0001$ by Student *t* test) (Fig. 1C). An additional series of experiments utilized cisplatin as a DNA-damaging agent (Fig. 1E). In these experiments, the UDS response to cisplatin treatment in *p53*$^{-/-}$ MEFs was 25 to 50% of wild-type, while *gadd45*/*p21*-null MEFs showed 25 to 35% UDS compared to the wild type ($P < 0.0001$ by Student *t* test) (Fig. 1E).

Included in some studies, as controls, were MEFs lacking *rb* or *p16ink4a* tumor suppressor genes (see Materials and Methods for details). Neither *rb*$^{-/-}$ nor *p16ink4a*$^{-/-}$ MEFs exhibited an NER defect as measured by UDS (Fig. 1D), although they did exhibit deregulated cell cycle regulation. Therefore, reduced (UDS) labelling in G_1 (observed in *p53*$^{-/-}$ and *gadd45*$^{-/-}$ MEF lines) is not due to accelerated S-phase entry. Indeed, *p16*$^{-/-}$ MEFs exhibited a very high S-phase fraction (approximately 50%; results not shown), yet showed normal repair measured as UDS (Fig. 1D). UDS studies with serum-starved (G_0) wild-type or *p53*$^{-/-}$ MEFs yielded similar results (not shown), which indicated that the NER defect associated with p53 occurs even in noncycling cells. Because replicative DNA synthesis and repair synthesis are two distinct processes (Fig. 1B), the S-phase fraction of a given cell line does not appear to be relevant to UDS. Analysis of UDS data was confined to 2N (G_1) nuclei merely for purposes of consistency. There is evidence that NER does not differ between G_1 and G_2 cells (33 [discussed in reference 41]). Moreover, *p53*$^{-/-}$ MEFs that were synchronized or arrested in G_2 also exhibited reduced UDS (results not shown).

PCNA is recruited to sites of DNA damage, and PCNA immunostaining may be used to reflect DNA repair responses (2). PCNA immunostaining was reduced in *gadd45*-null MEFs, which is consistent with the UDS results (Fig. 1F). In the absence of DNA damage, there appeared to be no differences in PCNA expression between the two cell lines, as measured by Western blotting (results not shown).

Repair of CPDs and 6-4 pps in global genomic DNA. Experiments were performed to measure specifically the removal of the major UV-induced photoproducts CPDs or 6-4 pyrimidine-pyrimidone photoproducts (6-4 pps) from global genomic DNA by using an immunoblot assay with monoclonal antibodies to each of these photoproducts. The repair of 6-4 pps from global genomic DNA showed substantial differences between the different MEF lines, as shown in Fig. 2A. Wild-type MEFs demonstrated efficient removal of 6-4 pps, with 63% of the lesions repaired by 4 h and 75% of the lesions repaired by 8 h after UV irradiation. (The level of photoproducts in unirradiated cells is designated as 100% removal or repair.) p53-deficient MEFs exhibited a defect in repair of 6-4 pps, as well as CPDs (36), in line with previous studies conducted with human cells (11–13). Note that *gadd45*$^{-/-}$ and *gadd45*/*p21*-null MEFs exhibited the greatest defect, with only 18 to 27% of the lesions being repaired even after 24 h while *p21*$^{-/-}$ MEFs showed essentially normal repair of 6-4 pps after 24 h (Fig. 2A).

Strand-specific repair assays. Quantitative Southern blotting of TEV-treated DNA from UV-irradiated cells was used to examine CPD removal from TS and NTS strands of the *dhfr* gene. Previous studies of p53-deficient human cells showed that p53 deficiency did not significantly affect repair of the *dhfr* TS (repaired by TCR), but did markedly affect repair of the NTS (11–13) (repaired by GGR). Similar to p53-deficient human cells, MEFs lacking *p53* or *gadd45* genes exhibited nearly normal TCR of the transcribed strand of the *dhfr* gene, but defective GGR of the NTS of the *dhfr* gene (Fig. 2B). Importantly, *p21*$^{-/-}$ cells exhibited nearly normal levels of repair of either strand (Fig. 2B) (J. M. Ford, unpublished data).

Cellular sensitivity to UV irradiation or cisplatin. In some cell types, loss of p53 function and the corresponding decrease in NER capacity sensitize cells to agents that produce DNA damage that is repaired by NER (7, 9, 10, 13, 16, 20, 39, 40). These agents include, in addition to UV irradiation, many chemical cross-linking agents, such as cisplatin or nitrogen mustards. We tested *p53*$^{-/-}$, *p21*$^{-/-}$, *gadd45*$^{-/-}$, and *gadd45*/*p21*-null MEFs for sensitivity to some of these agents by using 7-day MTT (thiazolyl blue) cell survival assays. Each of the mutant MEF lines displayed enhanced sensitivity to UV radiation or cisplatin compared to wild-type MEFs (Table 1). Similar results were obtained using melphalan (a nitrogen mustard; results not shown). Table 1 shows 50% inhibitory concentration (IC_{50}) data, which is the dose or concen-

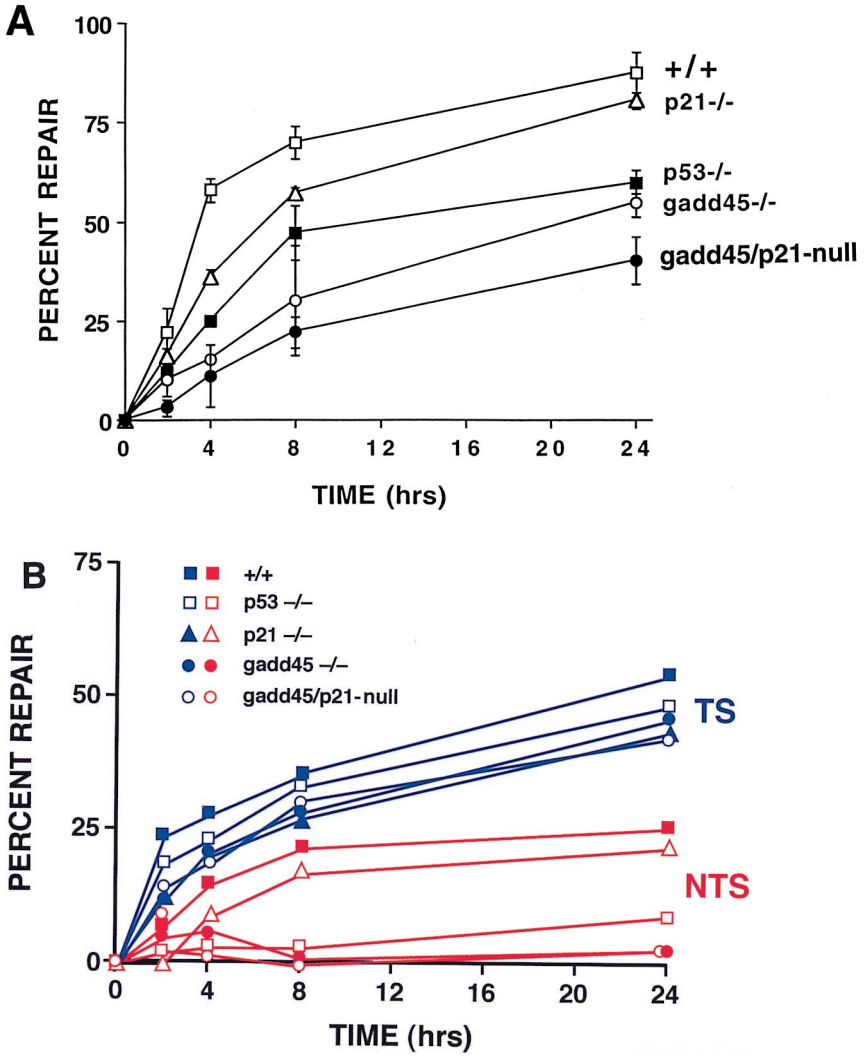

FIG. 2. Deficient UV photoproduct repair in genomic DNA isolated from *p53*- or *gadd45*-null MEF lines. (A) Kinetics of 6-4 pp removal in MEFs determined by immunoassays following 10 J of UV irradiation m^{-2}. Data were from two independent experiments conducted in triplicate. The values shown are means ± standard deviations. Note that 100% repair was defined relative to unirradiated cells. The bulk of the damage is repaired in wild-type cells within the first 3 to 4 h (compare the 4-h time point with the UDS data in Fig. 1). (B) Strand-specific repair of CPDs within the *dhfr* gene measured by TEV assays following 10 J of UV irradiation m^{-2}. Repair of the NTS (red) was markedly reduced in *gadd45*$^{-/-}$ or *gadd45*/*p21*-null MEFs. Each of the MEF lines exhibited near normal TCR repair of the TS (blue).

tration of a given agent required to give 50% survival, for each MEF line.

We also conducted clonogenic survival experiments (results not shown), as in previous studies (39, 40). MEFs exhibited poor plating efficiencies at low density, a problem alleviated through the use of feeder layers. Clonogenic survival after 10 of J UV radiation m^{-2} demonstrated a mean surviving fraction of 65% for wild-type cells and only 25% for *gadd45*/*p21*-null cells. After 20 J of UV radiation m^{-2}, wild-type cells exhibited 51% mean survival, compared to only 9% for *gadd45*/*p21*-null cells. Cisplatin yielded similar results (not shown). Thus, the mutant MEFs were more sensitive to UV radiation and cisplatin than the wild type, as determined by both types of assays, consistent with other reports (16, 20). There were, however, differences in the actual doses of DNA-damaging agents required, between the two types of survival assays, owing perhaps to differences in cell density, e.g., the presence or absence of feeder cells. (Higher doses of DNA damage were required to achieve equivalent cell killing in the presence of feeder layers, probably because feeder cells secrete cytokines that promote cell survival.)

Reduced S-phase progression in *gadd45*-null cells after UV radiation. The results shown in Fig. 1 and 2 provided strong evidence that Gadd45 is one component of the p53 pathway

TABLE 1. Sensitivity of MEFs to UV irradiation or cisplatin[a]

MEF line	IC_{50}	
	UV radiation (J m^{-2})	Cisplatin (μM)
+/+	12.0 ± 2.0	9.0 ± 0.5
gadd45$^{-/-}$	7.0 ± 2.0	4.0 ± 1.0
p21$^{-/-}$	8.0 ± 2.5	4.0 ± 1.0
gadd45/*p21*-null	2.0 ± 1.0	1.5 ± 0.5
p53$^{-/-}$	1.0 ± 0.5	1.0 ± 0.5

[a] Shown are IC_{50}s of the indicated agents (mean of five or more independent experiments ± standard deviations). Determinations were by 7-day MTT assays.

that contributes to DNA repair. In the present study, absence of Gadd45 resulted in the persistence of photolesions (Fig. 2), lesions that are known to interfere with replicative DNA synthesis. While *gadd45*$^{-/-}$ cells have already been shown to have normal p53-mediated G_1 checkpoints after IR or UV radiation (22), we focused on S-phase progression by employing FACS analyses of MEFs in the presence or absence of UV damage. The data are summarized in Fig. 3A and B. Cell cycle profiles of wild-type and *gadd45*$^{-/-}$ cells were similar in the absence of DNA damage and indicated that the cells were actively growing. However, after UV irradiation, a pronounced S-phase accumulation of *gadd45*$^{-/-}$ cells was observed. This result is similar to those reported by other groups regarding UV-irradiated *p53*-deficient cells (7, 10, 20) and is consistent with defective NER and subsequent replicative arrest. We also determined the S-phase fractions 15 h after UV irradiation by [^{3}H]thymidine labeling. Consistent with the FACS analysis, *gadd45*$^{-/-}$ MEFs exhibited a pronounced S-phase fraction 15 h after UV (Fig. 3C). Thus, *gadd45*$^{-/-}$ MEFs exhibit an S-phase delay after UV irradiation, due perhaps to persistence of UV photoproducts (Fig. 2), consistent with a slow DNA repair phenotype resulting in inhibition of DNA replication at damaged sites (Fig. 3).

Biochemical NER assays to explore Gadd45 function(s). The core NER reaction can be carried out in vitro in the presence of WCE (1, 37). However, WCE extracts may be relatively depleted of a number of nuclear proteins including DNA topoisomerases and chromatin remodeling factors, that while not required, may contribute to NER (14, 15, 50). The contributions of nuclear proteins to in vitro NER reactions, can be assessed by the addition of nuclear fractions (NUC) to WCE-NER reaction mixtures (15). By this approach, chromatin assembly and disassembly have been identified as processes closely linked to NER (15, 18, 23, 26, 50). WCE and NUC extracts were prepared from mouse lymphoblasts derived from either wild-type or *gadd45*$^{-/-}$ animals. The presence of Gadd45, either as an endogenous component of NUC extracts from wild-type mouse cells or added exogenously to *gadd45*$^{-/-}$ extracts, promoted chromatin assembly on plasmids undergoing or having undergone NER in vitro, although the amount of recombinant Gadd45 (400 ng) exceeded the amount endogenous to wild-type extracts (20 to 50 ng). Ethidium bromide (EtBr) staining was used to show approximate equal loading of the lanes, while repair synthesis was measured by ^{32}P incorporation (panels marked ^{32}P) into the damaged plasmids. Note that while very little form I DNA was recovered from the reactions (total plasmid DNA shown by EtBr staining), an appreciable amount of radiolabeled (repaired) plasmid DNA was recovered as form I DNA; i.e., repaired plasmids were preferentially assembled into nucleosome ladders, which showed a characteristic MNase digestion pattern (Fig. 4B). Recovery of (repaired) form I DNA was enhanced in the presence of Gadd45 (Fig. 4A). The chromatin remodeling reaction could not be uncoupled from the NER reaction (reference 15 and results not shown).

DISCUSSION

Effect of p53 pathway on the GGR subpathway of NER. NER can be classified into two broad subpathways: TCR and GGR. Recent studies show that p53 affects primarily GGR (11–13), although one study suggested a contribution of p53 to TCR in addition to GGR (46). In the present study, we examined both GGR and TCR, and as shown previously in human cells lacking p53 function, GGR was markedly affected. Defective GGR repair of 6-4 pps was more pronounced in the *gadd45*-deficient MEFs than in the *p53*-deficient MEFs (Fig. 2A), suggesting that Gadd45, like p48-XPE (DDB2, the p48 DNA-damage-binding protein that is defective in a subset of xeroderma pigmentosum group E patients [24]) contributes considerably to the p53-mediated NER response. The contributions of p53-regulated genes Gadd45 and p48-XPE to DNA repair responses define a new paradigm of p53 function, separable from other known p53 functions in cell cycle arrest and apoptosis. While p21 is a major mediator of p53-mediated G_1 cell cycle arrest, p21 contributes relatively little to DNA repair at early times (3- to 4-h time points in Fig. 1 and 2), and not at all at later time points (24 h; Fig. 2A). On the other hand, approximately 50% of the 6-4 lesions persist in the *gadd45*-deficient cells even after 24 h (Fig. 2A). We also assayed strand-specific repair of TS and NTS strands of the *dhfr* gene by using TEV assays. MEFs lacking *p53* or *gadd45* genes exhibited defective CPD repair of the NTS, but near normal TCR of the TS (Fig. 2B). Thus, a pronounced GGR defect associated with *p53* or *gadd45* loss is clearly shown by both types of assays.

Relationship of p53-mediated NER to G_1 checkpoint and p21. The p53-mediated NER response appears to be distinct from the G_1 cell cycle checkpoint, as indicated by several lines of evidence: Li-Fraumeni fibroblasts heterozygous for mutant or wild-type p53 genes retain the G_1 checkpoint, but nonetheless exhibit an NER defect (11); HPV16-E7 oncoprotein expression, which blocks Rb, and hence blocks G_1 cell cycle arrest, had no effect on NER (40). In the present study, *rb*- or *p16*-null MEFs exhibited altered G_1 cell cycle control, but not altered NER, while *gadd45*-null MEFs showed no G_1 checkpoint abnormality (22), but deficient NER (Fig. 1). Although the UDS experiments measured NER primarily in G_1 nuclei, these could be readily distinguished from G_1/S transitional nuclei (Fig. 1B), and, moreover, *p53*-null cells synchronized or arrested in G_1 or G_2 also showed an NER defect (results not shown). Although p21 is a major mediator of p53-induced G_1 arrest, *p21*-null MEFs exhibited only a very slight NER defect (discussed below), contrary to what one would predict were the NER defect dependent on or coincident with the G_1 checkpoint.

The potential contribution of p21 to DNA repair has been unclear. Some studies have shown reduced capacity for NER and UV sensitivity in *p21*-null HCT116 cells (31). In the present study, a modest but statistically significant decrease in UDS (75% of the wild-type level) was observed in *p21*$^{-/-}$ MEFs 3 h after UV irradiation, and *gadd45*/*p21*-null MEFs exhibited a greater defect than either of the single nullizygous mutants, which was again statistically significant. Indeed, *p21* and *gadd45* appear to be "additive" in their effects on NER measurements taken 3 h after irradiation (Fig. 1C), an interesting and novel observation that could possibly be due to subtle cell cycle differences, with due consideration of the caveats mentioned in the preceding paragraph. The 6-4 pp removal experiments are consistent with the UDS results (3 to 4 h after UV irradiation), but these experiments also showed that *p21*$^{-/-}$ cells recovered normal levels of NER after 24 h (Fig. 2A). TEV assays likewise showed essentially no effect of p21 deficiency on NER 24 h after UV irradiation (Fig. 2B). Thus, the effect of p21 on NER appears to be minimal, and Gadd45 contributes considerably to the p53-mediated NER response. Note that at the 3-h time point, where greater than 50% of the 6-4 pps lesions are removed from genomic DNA of wild-type cells, only 15% of the lesions were removed in *gadd45*$^{-/-}$ cells. After 24 h, wild-type and *p21*$^{-/-}$ cells were essentially identical in 6-4 lesion repair, while *gadd45*$^{-/-}$ cells still retained about 50% of the 6-4 lesions (Fig. 2A).

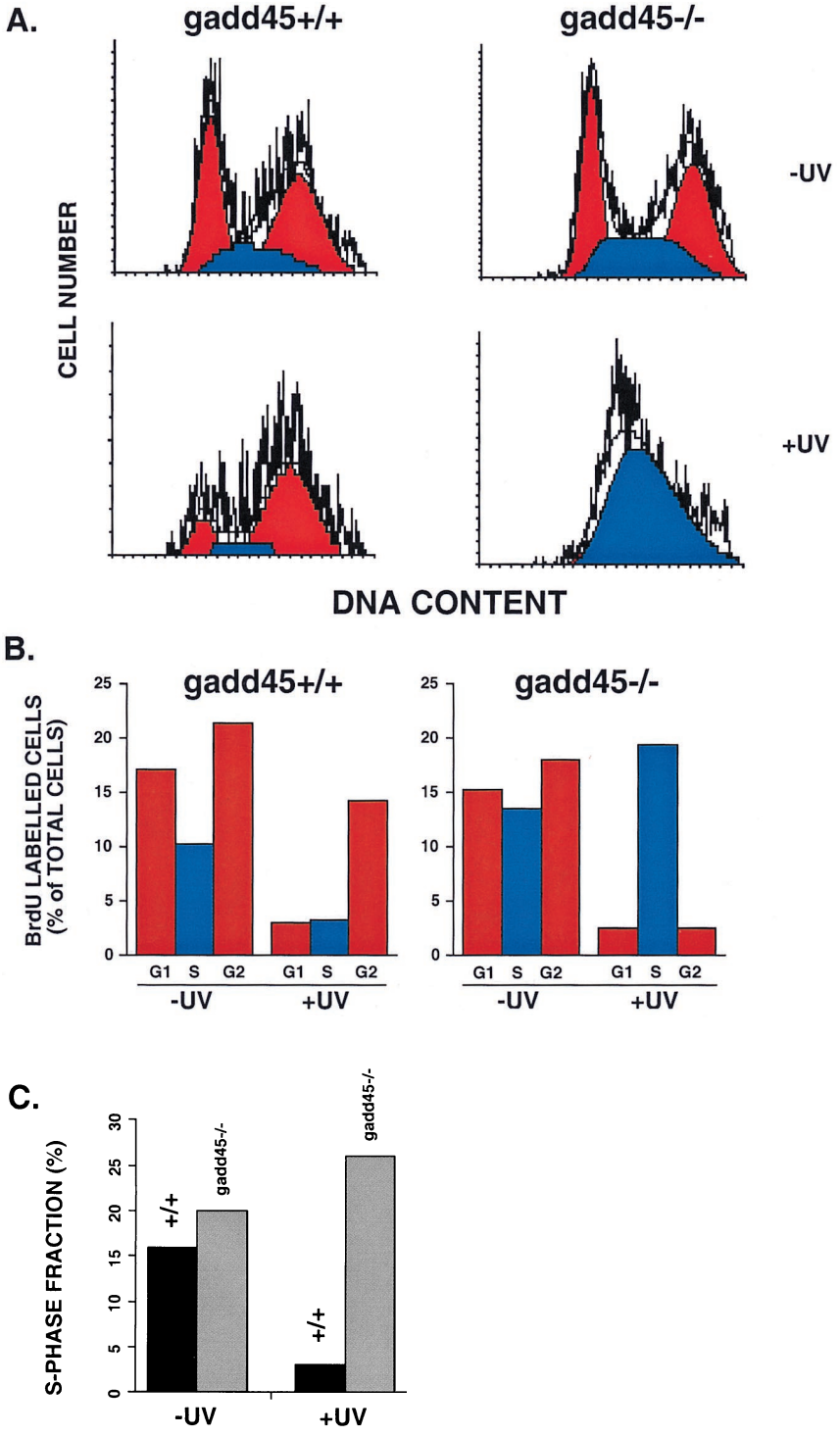

FIG. 3. Pronounced S-phase delay following UV-damage in *gadd45*$^{-/-}$ MEFs. (A) Flow cytometric profiles shown by PI staining. In the absence of UV irradiation, cell cycle profiles were similar (50 to 60% G_1, 10 to 25% S, 20 to 35% G_2/M). G_1 and G_2 peaks are in shown in red; the S-phase fraction is shown in blue. After UV irradiation, *gadd45*$^{-/-}$ MEFs exhibited a marked S-phase delay. The results shown correspond to 24 h after 10 J of UV irradiation m^{-2}. Only BrdU-positive cells that were actively cycling are shown (10). The gating of BrdU-positive cells was designed to exclude cells arrested in the first G_1, which as cited in other studies, was not affected by the presence or absence of Gadd45. The technique further distinquishes the p53/gadd45-mediated response to UV radiation from p53/p21-mediated G_1 arrest, as discussed in the text. (B) Summary of cell cycle profiles in MEFs in the absence or presence of UV radiation. The results shown in panel A are summarized by bar graphs. Again, *gadd45*$^{-/-}$ cells are delayed in S-phase progression. Results shown correspond to 24 h after 10 J of UV irradiation m^{-2}. Only BrdU-positive cells that were actively cycling are shown (10). (C) Determination of S-phase fractions 15 h after UV irradiation by [^{3}H]thymidine pulse-labelling. UV-irradiated *gadd45*$^{-/-}$ MEFs exhibit a pronounced S-phase fraction 15 h after UV damage. The results were obtained from two or more independent experiments.

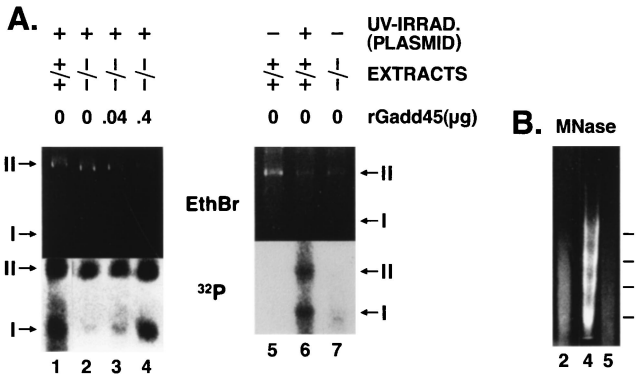

FIG. 4. (A) Gadd45 affects in vitro NER-chromatin assembly in experiments with mouse cell extracts. WCE and NUC extracts prepared from wild-type (+/+) or *gadd45*$^{-/-}$ (−/−) mouse lymphoblasts were incubated with UV-damaged plasmid template (left panel). While little form I DNA was recovered from the reactions, evidenced by EtBr staining (total DNA), a greater fraction of repaired (radiolabeled) DNA was recovered as form I. Gadd45 affects the recovery of form I (repaired, radiolabeled) DNA, which is ordered into nucleosome ladders, evidenced by MNase digestion (shown in panel B). Gadd45 was either endogenous to wild-type extracts, or recombinant Gadd45 (rGadd45) was added to *gadd45*$^{-/-}$ extracts in the amounts indicated (micrograms). (Right panel) Plasmid templates lacking UV damage exhibited low levels of ^{32}P labelling. Lanes show approximate ^{32}P incorporation as follows: 1 to 4, 150 fmol; 6, 190 fmol; 5 and 7, 11 fmol. Form II DNA was predominantly nicked, while form I DNA was predominantly supercoiled. (B) MNase digestion of plasmids recovered from in vitro NER-chromatin assembly assays, corresponding to lanes 2, 4, and 5 in panel A. In the absence of Gadd45 (lane 2), the predominantly form II plasmid DNA did not exhibit nucleosome ladders after MNase digestion. In the presence of Gadd45 (lane 4), laddering was observed, indicative of chromatin assembly. Undamaged plasmids were not assembled into chromatin (lane 5).

If p21 does contribute, albeit minimally, to NER, as suggested by Fig. 1, it is likely that this function is unrelated to p21 activity as a cyclin-dependent kinase inhibitor, because, again, neither HPV16-E7 expression, nor *rb* or *p16* deficiency recapitulates the effect of *p53* or *gadd45* deficiency on NER. Moreover, it is conceivable that any effect of p21 on NER may require the presence of additional genetic alterations, such as *mutS* deficiency in HCT116 cells or *gadd45* deficiency in the *gadd45*/*p21*-null MEFs. The complex relationship of p53-mediated DNA repair to the activation of cell cycle checkpoint(s) will be the subject of future studies.

Cell cycle checkpoint responses to UV radiation may differ from the well-known G_1 checkpoint response elicited by IR (and also G_1 arrest by UV radiation to some extent [25, 51]) and mediated by cyclin-dependent kinase inhibitor p21. In contrast, p53-mediated responses to UV radiation can involve the S phase and G_2/M delays, which may reflect the greater lesion frequency of UV photoproducts compared to IR-induced damages. The cell cycle analyses in Fig. 3 further dissociate the p53/gadd45-mediated response to UV radiation from the p53/p21-mediated response to IR, in that the presence or absence of Gadd45 showed no effect on G_1 arrest in the present and previous experiments (reference 22 and results not shown), and in fact, the experiments in Fig. 3A and B, employing BrdU incorporation, were specifically designed to exclude cells arrested in the first G_1 phase from the analysis.

Gadd45-deficient cells exhibit slow repair. It should be noted that p53 protein and a number of downstream effector proteins are present at appreciable basal levels in many cell types, including normal human and mouse fibroblasts (7). This has also been observed for the p48-XPE gene product. p48-XPE is expressed at higher basal levels in p53 wild-type cells than p53 mutant cells, but is induced after DNA damage only in p53 wild-type cells (24). This may explain why DNA repair functions are significantly enhanced in p53-wild-type cells at early times (3 to 4 h after UV irradiation; Fig. 1 and 2). On the other hand, MEFs nullizygous for *gadd45* genes did not achieve normal levels of 6-4 pp repair even after 24 h (Fig. 2A). TEV assays likewise showed a pronounced NER deficiency in *gadd45*-null cells (Fig. 2B). The persistence of lesions in *gadd45*-deficient cells led to S-phase delay 24 h after UV irradiation (Fig. 3). S-phase delay may represent an active checkpoint response or may reflect a blockage of replication fork progression at UV-induced lesions, as observed in other NER-defective cells (17). The cell cycle results shown in Fig. 3 are consistent with an NER defect causing replicon stalling at damaged nucleotide bases (17). Persistence of lesions (e.g., after 24 h as shown in Fig. 2) may continue to trigger cell cycle checkpoint responses, including p53, although *gadd45* deficiency produced no overt changes in p53 or p21 mRNA or protein expression at early times (3 to 12 h) following DNA damage (results not shown).

Cytotoxic responses to DNA-damaging agents. In this study and others, cells lacking p53 or components of the p53 pathway were sensitized to UV- or cisplatin-induced DNA damage (previously cited). These findings provide a counterpoint to the prevalent view that cells lacking functional p53 are often desensitized to DNA damage (attributed to escape from apoptosis). There are a number of implications that follow. (i) Many epithelial cell types may die by mechanisms other than apoptosis, e.g., cytotoxicity of DNA damage (3). (ii) In these cell types, loss of functional p53 either has no effect on cell death responses, or in the case of UV or cisplatin damage, can result in enhanced cell death. (iii) Downstream effectors of the p53 pathway can contribute to cell survival responses, although DNA repair is only one of multiple parameters that influence cytotoxic responses to DNA damage. For example, *p21*$^{-/-}$ cells also were more sensitive to UV radiation, due probably to G_1 checkpoint loss. It will be of interest to explore further the role of p53 in cell death or survival responses in epithelial and other cell types in which p53 activation is associated with DNA repair (survival) responses rather than apoptosis.

Coupling of later stages of NER to chromatin assembly. The mechanism of NER consists of several steps including incision of the DNA strand carrying the damage, displacement of an approximately 30-nucleotide oligomer containing the damaged base(s), resynthesis of the correct sequence using the complementary strand as a template, and ligation. Because mammalian cells package their genomes into chromatin, one may also consider that destabilization of the preexisting chromatin structure may affect early phases of NER, inasmuch as nucleosomes may impede NER (14). One may also consider that restoration of chromatin structure, i.e., repositioning of nucleosomes along newly repaired DNA, would be required to maintain proper regulation of genomic functions. In fact, other studies have shown that the NER process is tightly linked to chromatin assembly (15). Specifically, the passage of the DNA polymerase associated with repair synthesis was found to promote chromatin formation. This was demonstrated by the finding that plasmid templates undergoing NER in vitro were preferentially assembled into nucleosomes (over those that were not repaired, and therefore retain DNA damage). Stated another way, this means that the repair process promotes nucleosome assembly when all factors are available. The chromatin assembly factor CAF1 was implicated as a mediator of the chromatin remodelling process, because nuclear extracts containing CAF1 could carry out the nucleosome assembly step, while CAF1-deficient extracts could not (15). Moreover, the chromatin assembly step could not be uncoupled from NER, because plasmids that were repaired in WCE extracts,

recovered by extraction and ethanol precipitation, and then subsequently incubated with nuclear extracts did not exhibit nucleosome assembly (15). These findings implicated CAF1 in a late step of NER, in which repaired DNA is then repackaged into a native configuration. Supercoiled plasmids were consequently recovered from in vitro repair reactions (15). We show evidence that Gadd45 participates in late-stage NER steps involving chromatin assembly. As mentioned above, nucleosome assembly is inhibited by DNA damage, particularly in the form of bulky lesions or UV damage which may cause helical distortions. It is clear from Fig. 4 that Gadd45 promotes one or more activities associated with this process.

p53-regulated gene products involved in chromatin accessibility. A recent study showed that Gadd45 binds to UV-damaged chromatin and may affect accessibility to sites of DNA damage (6). A number of reports in the literature suggest that chromatin accessibility proteins such as yeast and human CAF-1 (mentioned above and in references 15 and 26), and *Saccharomyces RAD7* and *RAD16* (50), while not components of the core NER complex, can specifically contribute to the GGR subpathway of NER. XPC, which is also required for normal GGR (44), has also been implicated in damage recognition which could involve either DNA damage or chromatin damage (3, 44). Such a role for Gadd45 would be consistent with the present results, wherein MEFs lacking *gadd45* exhibit defective GGR. An additional link between p53 and the GGR subpathway of NER is suggested by the finding that the xeroderma pigmentosum group E (XP-E) p48 gene, like *gadd45*, is transcriptionally regulated by p53 (24). The NER defect in XP-E likewise affects the GGR subpathway (23, 24). Interestingly, the p48-XPE protein shares sequence homology with CAF-1 (23), while Gadd45 shares some homology to other chromatin accessibility proteins (6).

One means by which chromatin accessibility factors may contribute to NER is by facilitating the binding of damage recognition proteins and/or other proteins involved in DNA damage processing, to sites of DNA damage. One such protein, PCNA, is known to bind strongly to damaged chromatin after UV irradiation. The binding of PCNA to damaged chromatin is defective in XP cells, strongly suggesting that the "recruitment" of PCNA to damaged chromatin reflects its involvement in NER (determined by immunostaining of Triton-resistant PCNA [2]). In the present study, PCNA immunostaining after UV radiation was defective in Gadd45-deficient cells (Fig. 1F). This finding suggests that PCNA may be one such protein whose interaction with damage sites is affected by the presence or absence of Gadd45 (2), which interestingly has been shown to associate with PCNA (19, 38, 47).

Since naked plasmid DNA was introduced to the in vitro DNA repair reactions (Fig. 4), the assay may not address early events involving recognition of damaged DNA in chromatin. However, the present results clearly illustrate a role for Gadd45 in chromatin assembly. A possible role for Gadd45 in chromatin accessibility early in the reaction cannot be excluded. Indeed, the RAD7 and RAD16 proteins, previously shown to be chromatin accessibility factors, that enhanced NER, but were not required for NER (50), have more recently been shown to act as a part of a DNA damage sensor mechanism, by binding to UV-damaged chromatin as a component of the damage recognition step (18). One implication is that Gadd45 signalling may participate in DNA damage responses, at least in some cases, upstream of p53 in the damage-response pathway (49), as would be predicted were Gadd45 to play a role in damage recognition. Such a postulated role for Gadd45 early in the repair reaction would be consistent with the UDS experiments, in which *gadd45*-deficient cells exhibited a defect in the repair synthesis step of NER (Fig. 1), but could also be due to the temporal coupling of repair synthesis with latter-stage chromatin remodeling (Fig. 4) (15).

In summary, we have used a genetic approach to dissect components of the p53 pathway that contribute to DNA repair responses. In particular, Gadd45 contributes appreciably to DNA repair, while p21 contributes relatively little. One interpretation is that p53-associated NER may be independent of the G_1 cell cycle checkpoint mediated by p21 (25). Recent studies showed that Gadd45 binds to UV-damaged chromatin, perhaps facilitating access to regions of DNA damage (6). The p53-associated NER response may therefore be mediated at the level of chromatin accessibility to sites of DNA damage.

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