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A DNA Damage Signal Is Required for p53 to Activate *gadd45*[1]

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ABSTRACT

We provide direct evidence that overexpression of p53 is not sufficient for robust p53-dependent activation of the endogenous *gadd45* gene. When p53 was induced in TR9-7 cells in the absence of DNA damage, waf1/p21 and mdm2 mRNA levels were increased, but a change in gadd45 mRNA was barely detectable. Activation of the *gadd45* gene was observed when camptothecin was added to cells containing p53 in the absence of a further increase in the p53 level. Phosphorylation of p53 at serine 15 and acetylation at lysine 382 were detected after drug treatment. It has been suggested that p53 posttranslational modification is critical during activation. However, inhibition of these modifications by wortmannin was not sufficient to block the transactivation of *gadd45*. Interestingly, after camptothecin treatment, increased DNase I sensitivity was detected at the *gadd45* promoter, suggesting that an undetermined DNA damage signal is involved in inducing chromatin remodeling at the *gadd45* promoter while cooperating with p53 to activate *gadd45* transcription.

INTRODUCTION

The p53 tumor suppressor is a nuclear protein that responds to DNA damage to preserve genomic integrity. Upon DNA damage, p53 is stabilized and posttranslationally modified (1). p53 is a transcription factor that can activate transcription of many target genes in the absence of DNA damage. Three p53 target genes that have been well described include *waf1/p21*, *gadd45*, and *mdm2*. waf1/p21 is an inhibitor of cyclin-dependent kinases. It inhibits the phosphorylation of Rb and blocks cell cycle progression from G_1 to S phase (2). *gadd45* (growth arrest and DNA damage 45) was first identified as a gene that was rapidly induced after IR[3] of lymphoblasts and fibroblasts (3, 4). The gadd45 protein can interact directly with the essential replication factor proliferating cell nuclear antigen, may block DNA replication, and may coordinately enhance nucleotide excision repair of damaged DNA (5). An auto-regulatory feedback loop is formed between p53 and mdm2, whereby Mdm2 protein keeps p53 under negative control (6–9). The protein products of the p53 target genes have an intriguingly diverse number of functions. Does p53 turn on all its target genes at the same time? Do posttranslational modifications play a role in controlling the regulation of different targets? These are questions that are being addressed by a number of laboratories working in the p53 field. Recent evidence suggests that p53 target genes can be differentially regulated. The topoisomerase poison etoposide has been shown to inhibit mdm2 synthesis while increasing *waf1/p21* and *gadd45* gene expression (10).

Two model systems have been used to study transcriptional regulation by p53: (*a*) one is the stabilization of p53 through DNA damage; and (*b*) the other is the overexpression of p53 by either inducible promoters or temperature-sensitive alleles. Increased p53 levels are found in both systems, but DNA damage also triggers posttranslational modifications of p53. DNA damage has also been shown to trigger p53-mediated transcription of the *gadd45* gene, but it has not been determined whether this is the result of an increase in the p53 level or a change in the p53 posttranslational modification. In the p53 inducible cell line TR9-7, the removal of tetracycline causes an increase in p53 that elicits the transactivation of the *p21* gene. This evokes both a G_1-S and a G_2-M cell cycle arrest (11). Because high levels of p53 in this cell line are able to rapidly transactivate *waf1/p21*, it has been assumed that p53 would also rapidly activate other p53 inducible genes, including *gadd45*. It is known that DNA damage activates signal transduction pathways that, in turn, activate p53 by posttranslational modification (12). However, *waf1/p21* can be activated in the absence of these signals. Members of the PI3k-related kinase superfamily can phosphorylate p53 at serine 15 and serine 37 (12). *In vitro* studies have demonstrated that ATM phosphorylates p53 at a single residue, serine 15. They also suggest a direct role for ATM and related kinases in the DNA damage-induced phosphorylation of serine 15 (13–15). Therefore, it was important for a study to be done that would examine the activation of p53 target genes by constant p53 levels in both the presence and absence of DNA damage to determine whether combinatorial signal transduction pathways could modify the regulation of any p53 target genes. Such a study is presented here and shows that differences in the ability of p53 to activate *gadd45* in the presence or absence of DNA damage were not dependent on a change in the nuclear protein level of the tetracycline-regulated overexpressed wt p53. gadd45 mRNA was only significantly accumulated when the cell line containing wt p53 was treated with CPT. Phosphorylation of p53 at serine 15 and acetylation at lysine 382 were detected after CPT treatment, but inhibition of phosphorylation at serine 15 and acetylation at lysine 382 were not sufficient to block the transactivation of the *gadd45* gene. These data suggest that after DNA damage, a signal triggered functional p53 to rapidly activate the *gadd45* gene for increased transcription, but this signal did not require the phosphorylation-acetylation cascade previously thought to be critical.

MATERIALS AND METHODS

Cell Lines. The MDAH041 cell line is a human fibroblast cell line lacking functional p53 protein due to a frameshift mutation of one p53 allele at codon 184 and loss of the normal p53 allele (11). The TR9-7 cell line is an isogenic cell line derived from MDAH041 that contains tetracycline-regulated wt p53 (both were generously provided by Munna Agarwal, The Cleveland Clinic Foundation, Cleveland) (11). The 184A1 cell line is an immortalized human mammary epithelial cell line that contains wt p53 (16). This cell line was obtained from American Type Culture Collection.

DNase I Treatment of Nuclei. 184A1 cells were grown on 150-mm plates at 37°C in a 1:1 mixture of Ham's F-12 medium and DMEM containing 0.1 μg/ml cholera enterotoxin, 10 μg/ml insulin, 0.5 μg/ml hydrocortisol, 20 ng/ml epidermal growth factor, and 5% horse serum. TR9-7 cells were grown on 150-mm plates at 37°C in DMEM containing 10% fetal bovine serum and 2 μg/ml tetracycline until 70% confluence. Subconfluent plates were grown either in media containing tetracycline (control) for 24 h, withdrawal of

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[3] The abbreviations used are: IR, irradiation; PI3k, phosphatidylinositol 3′-kinase; CPT, camptothecin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT, reverse transcription; ECL, enhanced chemiluminescence; EMSA, electrophoretic mobility shift assay; SCS, super consensus sequence; wt, wild-type; ATM, ataxia telangiectasia mutated; DNA-PK, DNA-dependent protein kinase; AMV, avian myeloblastosis virus.

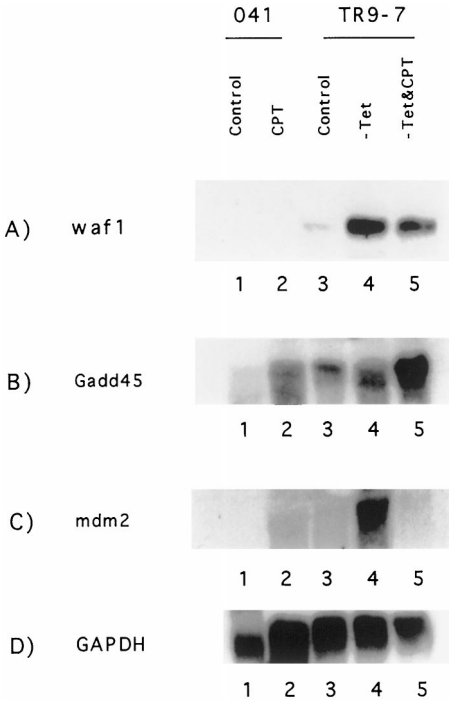

Fig. 1. Differential activation of wt p53 target genes occurs after CPT-induced DNA damage. The RNA extraction was made from cells grown in media containing tetracycline (*Control*), after a 24-h withdrawal of tetracycline (−*Tet*), or after a 24-h withdrawal of tetracycline with overlapping 0.1 mM CPT treatment for the last 4 h (−*Tet & CPT*). Northern blot analysis of the wt p53-responsive genes was carried out by separating 25 μg of cytoplasmic RNA in a 1% formaldehyde-agarose gel and transferring the RNA to a nylon membrane. The blot was hybridized with full-length cDNA probes for waf1, gadd45, mdm2, and GAPDH as indicated. The results were reproducibly obtained in multiple blots using both mRNA and total cytoplasmic RNA. The signals were analyzed using a Molecular Dynamics PhosphorImager with Image Quant software.

tetracycline (−Tet) for 24 h, or after 24 h withdrawal of tetracycline with overlapping 0.1 mM CPT treatment for the last 4 h (−Tet & CPT). The isolated nuclei were treated with increasing amounts of DNase I (Worthington; 2932 units/mg) for 10 min at 37°C as described previously (17).

DNA and RNA Analysis. DNase I-treated genomic DNA (20 μg) was digested with *Xba*I to completion. The digested DNA was electrophoresed on a 0.8% agarose gel and transferred to nylon membrane. The blots were hybridized with a *Xba*I-*Msc*I gadd45 probe (see Fig. 6). Cytoplasmic RNA was isolated from MDAH041 and TR9-7 cells according to Maniatis as described previously (17). Polyadenylated RNA was prepared using mini-oligo dT spin columns. Either 1.5 μg of polyadenylated RNA or 25 μg of cytoplasmic RNA from each sample were electrophoresed on a 1% formaldehyde-agarose gel and transferred onto nylon membrane. The blot was hybridized with full-length cDNA probes for human gadd45, mdm2, and waf1/p21. The GAPDH probe was obtained as a 1.25-kb cDNA *Pst*I fragment (18). All probes were labeled with ^{32}P by random prime labeling.

Real-Time PCR with Molecular Beacon. For RT of each sample, 5 μg of cytoplasmic RNA were incubated at 65°C for 10 min with 250 μmol of oligo(dT)$_{15}$ primer (Boehringer Mannheim) in a total volume of 10 μl. After cooling on ice, 10 μl of RT mix [2× AMV, 15 units of AMV reverse transcriptase (Amersham), 2.5 mM deoxynucleotide triphosphate, and 20 units of RNase inhibitor RNasin (Promega)] were added. Samples were incubated for 1 h at 37°C. The reaction was stopped by heating at 94°C for 2 min. Samples were stored at −80°C.

For PCR with molecular beacons, PCR primer pairs were designed to anneal to their target at the same temperature (55°C) and to amplify DNA fragments of approximately 100 bp. Molecular beacons were designed with a DNA folding program[4] to have a hairpin stem that dissociates at a temperature 10°C higher than the detection temperature. The molecular beacons were synthesized as described previously (19).

[4] http:www.ibc.wustl.edu/~zuker/dna/form1.cgi.

Two μl of RT products were used in the PCR reaction carried out under the following conditions: 1× TaqMan buffer (Perkin-Elmer), 2.5 mM $MgCl_2$, 250 μM deoxynucleotide triphosphate, 15 pmol of each primer, 2.5 units of AmpliTaq Gold polymerase (Perkin-Elmer), and 125 ng of the appropriate molecular beacon. Forty cycles of amplification (94°C denaturation for 30 s, 55°C annealing for 1 min, and 72°C elongation for 30 s) were carried out in sealed tubes in an Applied Biosystems 7700 Prism spectrofluorometric thermal cycler (Perkin-Elmer). Fluorescence was measured during the annealing step and plotted automatically for each sample.

The primer pairs used for PCR reaction were synthesized by Operon and were as follows: (*a*) gadd45, 5′-CCATGCAGGAAGGAAAACTATG-3′ (forward primer) and 5′-CCCAAACTATGGCTGCACACT-3′ (antisense primer); and (*b*) GAPDH, 5′-AGAGCACAAGAGGAAGAGAGAGACC-3′ (forward primer) and 5′-AACTGTGAGGAGGGGAGATTCAG-3′ (antisense primer).

The sequences of the molecular beacons were as follows: (*a*) gadd45, 5′-CGCTGCAGAATGGTTGAGTTACATTAAAATAAACCGCAGCG-3′; and (b) GAPDH, 5′-GGACGCGGTGGGGGACTGAGTGTGGCGTCC-3′.

Preparation of Nuclear Protein Extracts. Nuclear lysates were prepared as described previously (17). To prevent rapid deacetylation, trichostatin A, an inhibitor of histone deacetylases, was added in the nuclear extraction buffer at a final concentration of 5 μM (20). Wortmannin is a fungal metabolite that has been shown to act as a selective inhibitor of PI3k family members (21). TR9-7 cells were incubated in 5 μM wortmannin for 4 h before cells were lysed.

Western Blot Analysis. Nuclear lysates were prepared from cells maintained as described above. Samples were electrophoresed on a 10% SDS-PAGE and electrotransferred to nitrocellulose membrane. The blots were probed with specific antibodies as described and visualized by incubation with either goat antimouse or goat antirabbit secondary antibody followed by ECL solutions. The p53-specific anti-phosphoserine-15 antibody and anti-acetylated lysine 382 antibody were produced as described previously (22).

Ligation-mediated PCR for *in Vivo* Footprinting. The ligation-mediated PCR *in vivo* footprinting was carried out as described previously (17). The following primers were used: (*a*) oligonucleotide 1, 5′-CCCTGAAAACATAACTTCCC-3′; (*b*) oligonucleotide 2, 5′-GAAGCTGACTCCTTAATGAGGG-3′; and (*c*) oligonucleotide 3, 5′-TGACTCCTTAATGAGGGGTGAGCCAG-3′.

EMSA. The SCS synthetic oligonucleotide used in this study contained consensus p53 binding sites. The sequence of this oligonucleotide was 5′-TCGAGCCGGGCATGTCCGGGCATGTCCGGGCATGTC- 3′.

Labeling of the oligonucleotides was performed with the large fragment of DNA polymerase and [^{32}P]dCTP. Reaction mixtures for EMSA experiments

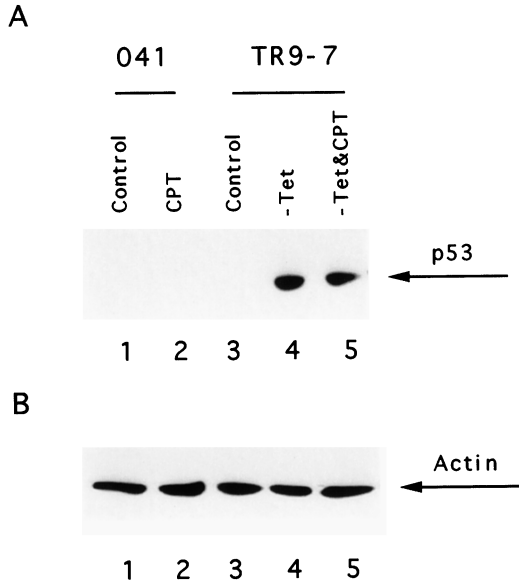

Fig. 2. p53 protein levels in MDAH041 and TR9-7 nuclear extract. *A*, 100 μg of nuclear protein were resolved by electrophoresis on a 10% SDS-PAGE. The p53 in samples was visualized by Western blotting with the p53-specific monoclonal antibody PAb240 and detected with ECL reagent. *B*, the blot was reprobed with anti-actin antibody as a control of loading.

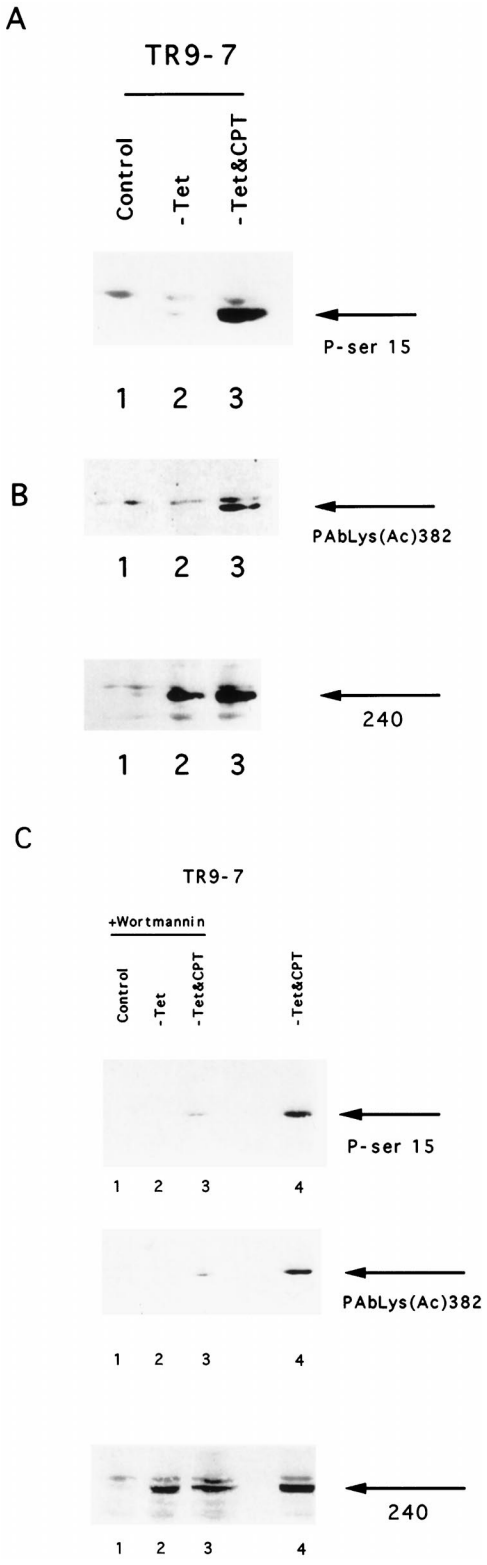

Fig. 3. Wortmannin inhibits CPT-activated phosphorylation of p53 at serine 15 and acetylation at lysine 382 *in vivo*. *A*, 100 μg of nuclear protein (same samples as Fig. 2) were resolved by electrophoresis on a 10% SDS-PAGE. The phosphorylated serine 15 in samples was visualized by Western blotting with a specific antibody (anti-phosphorylated-serine 15) and detected with ECL reagent. *B*, the acetylated lysine 382 in samples was detected by Western blotting with a specific antibody (anti-acetylated lysine 382). The nuclear protein extraction buffer contained 5 μM trichostatin A. The blot was also probed with PAb240 to detect total p53. *C*, nuclear extract was made from the TR9-7 cells grown in media containing 5 μM wortmannin for the last 4 h. Nuclear extract (100 μg) was resolved by electrophoresis on a 10% SDS-PAGE and transferred to nitrocellous paper. The blot was probed with anti-phosphorylated serine15, anti-acetylated lysine 382, and PAb240 as indicated.

(30 μl) were composed of 0.1 pmol of oligonucleotide, 20 mM HEPES (pH 7.8), 100 mM KCl, 1 mM EDTA, 1 mM DTT, 1 μg of sonicated salmon sperm DNA, and 10% glycerol. In addition to 2 μg of MDAH041 or TR9-7 nuclear protein extract, 0.5 μg of PAb1801 was added to each reaction. All samples were incubated at room temperature for 20 min. The protein-DNA complexes were resolved on a 4% acrylamide gel.

RESULTS

Differential Activation of p53-responsive Genes *waf1*, *mdm2*, and *gadd45*. The tetracycline-regulated wt p53-expressing cell line TR9-7 and its isogenic p53-negative partner MDAH041 (11) were used to analyze p53-mediated transactivation of *waf/p21*, *mdm2*, and gadd45 in the presence and absence of DNA damage (Fig. 1). It has been shown that increased p53 DNA binding activity was detected when fibroblasts were treated with 100 μM CPT for 4 h by a time course assay (23). Barely detectable expression of the p53 target genes was observed in RNA samples derived from MDAH041 cells before drug treatment (Fig. 1, *Lane 1*). No change was observed for waf1/p21 after CPT treatment in the absence of p53, whereas a slight increase was observed for both mdm2 and gadd45 (Fig. 1, *Lane 2*). The activation resulting from the induced p53 or the induced p53 in the presence of CPT was examined using the inducible cell line TR9-7. Induction of p53 by the withdrawal of tetracycline caused a 5-fold increase for both waf1/p21 and mdm2 RNAs above those seen in the controls (Fig. 1, *A* and *C*, compare *Lanes 3* and *4*). The *waf1/p21* gene was activated to the same extent with or without the addition of CPT (Fig. 1*A*, *Lanes 4* and *5*). Interestingly, in the TR9-7 cells, although no activation of gadd45 RNA was detected after tetracycline withdrawal, an 8.8-fold activation was observed when the induced cells were incubated with CPT (Fig. 1*B*, compare *Lanes 3–5*). In addition, the *mdm2* gene was activated in a p53-dependent manner when tetracycline was removed (Fig. 1*C*, *Lane 4*), but the mdm2 RNA level decreased when the cells were treated with CPT (Fig. 1*D*, *Lane 5*). A similar result showing inhibition of *mdm2* in the presence of etoposide was recently reported (10).

p53 Nuclear Protein Level Does Not Correlate with Transcription Activity. Increased levels of nuclear p53 protein often result from treating cells with DNA-damaging agents (1). We examined the p53 protein level in the presence and absence of drug to see whether the differential activation of the p53-responsive gene *gadd45* corresponded to an increase in nuclear p53 (Fig. 2). The protein level was detected by Western blotting with the p53-specific monoclonal antibody PAb240. Nuclear p53 protein was induced after the withdrawal of tetracycline for 24 h, and the protein level did not change after CPT treatment (Fig. 2*A*, *Lanes 4* and *5*). Therefore, it appeared that an increased level of p53 protein in the nucleus was not required for the p53-dependent CPT-mediated induction of *gadd45*.

p53 Is Phosphorylated at Serine 15 and Acetylated at Lysine 382 in Response to CPT Treatment, and These Modifications Can Be Inhibited by Wortmannin *in Vivo*. Posttranslational modification of p53 has been suggested as one mechanism that regulates p53 activity. *gadd45* can be activated by an ATM and p53-dependent mechanism mediated by the p53-RE in intron 3 (4). *In vitro* studies have demonstrated that ATM phosphorylates p53 at a single residue, serine 15 (13, 14). We analyzed the DNA damage-induced phosphorylation status of p53 using the p53 specific anti-phosphoserine-15 antibody. Similarly, we analyzed the DNA damage-induced acetylation of p53 using a p53-specific anti-acetylated lysine 382 antibody. Western blot results demonstrated that p53 was phosphorylated at serine 15 only when CPT was added (Fig. 3*A*, *Lane 3*). Likewise, acetylation at lysine 382 was induced after treatment with CPT (Fig. 3*B*). These results correlate differential posttranslational modification

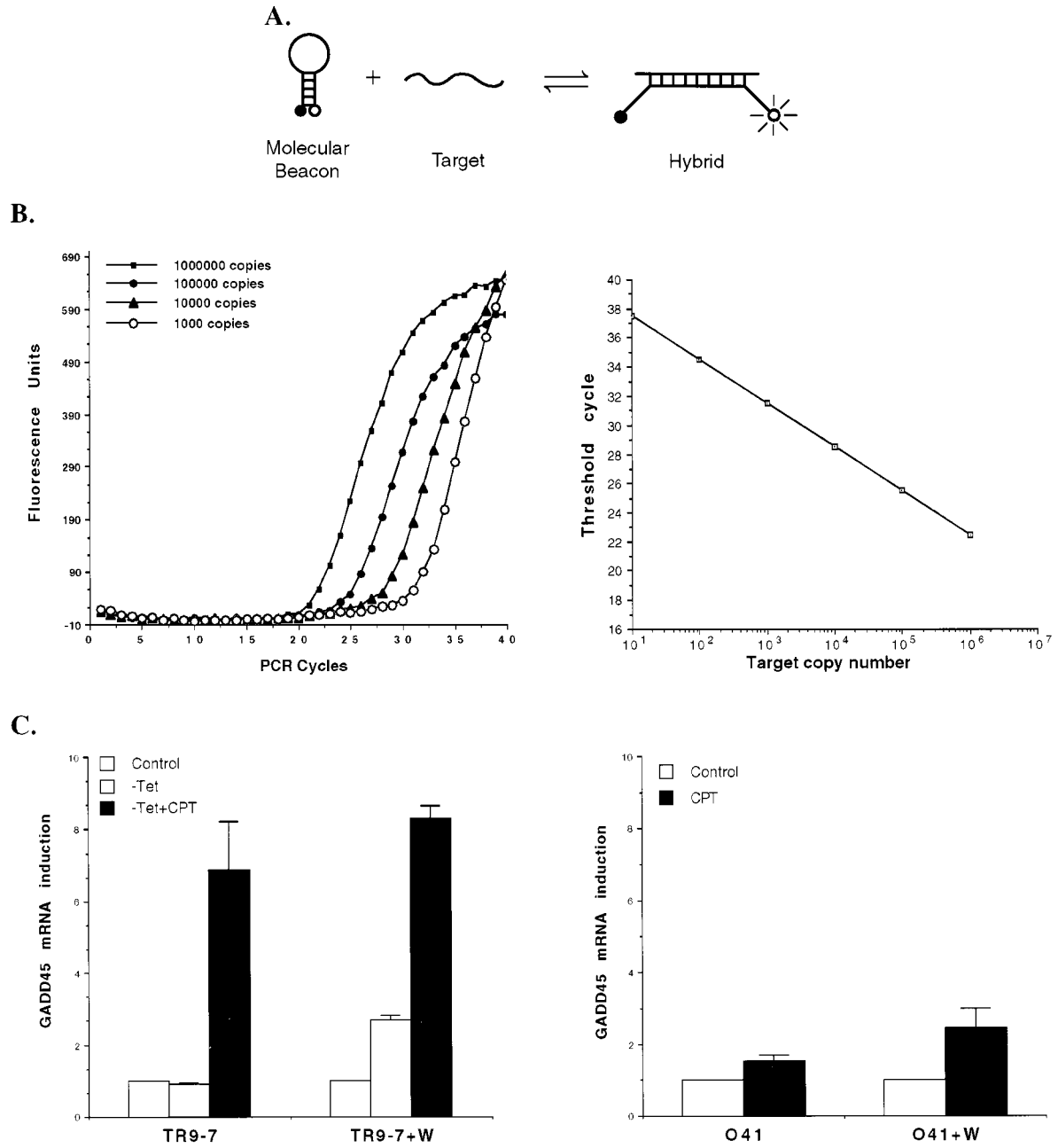

Fig. 4. Wortmannin does not block DNA damage induction of the *gadd45* gene. Analysis of gadd45 mRNA expression was analyzed by real-time RT-PCR using specific sequence molecular beacons. *A*, molecular beacons are hairpin-shaped oligonucleotide probes that consist of a central part complementary to the target mRNA, flanked by two 6-bp inverted repeats that can form a stable stem. The 5′-end of the beacon is coupled to a fluorophore, whereas the 3′-end is coupled to a quencher. In the absence of the target, the stem is closed, and the fluorophore is quenched, whereas in the presence of the target, an opened conformation allows the fluorophore to fluoresce. *B*, the PCR was carried out in a spectrofluorometric thermal cycler that monitored the fluorescence in each reaction tube at the annealing stage of each thermal cycle. The four reactions shown in the *left panel* correspond to PCR done with increasing amounts of GAPDH-plasmid DNA (10^3-10^6 copies). The cycle at which the fluorescence signal becomes detectable above the background gives the threshold cycle. An inverse relationship between the threshold cycle and the logarithm of the initial number of template was observed (*right panel*). *C*, The RT reactions were carried out with 5 μg of cytoplasmic RNA, and 1 of 10 of the RT products was used in the PCR reactions specific for Gadd45 and GAPDH. The initial number of targets in each sample was calculated according to the threshold cycle. The results were normalized using the control samples and the GAPDH values to give relative units of mRNA induction.

at these amino acid residues with the differential activation of the *gadd45* gene.

An inhibitor that could block phosphorylation of p53 was added to examine whether phosphorylation of p53 at serine 15 was required for activation of *gadd45*. *In vitro* studies have shown previously that wortmannin can inhibit phosphorylation of p53 at serine 15 by blocking both DNA-PK and ATM kinase activity (22, 13). Additionally, it was reported that wortmannin inhibits actinomycin D-induced activation of a p53 chloramphenicol acetyltransferase reporter construct (24). Wortmannin is a specific inhibitor of PI3k but does not affect protein kinase C, cAMP- or cGMP-dependent kinase, c-src, phospholipase C, or calmodulin-dependent protein kinase (25). The posttranslational modification of p53 derived from cells grown in media containing wortmannin for the last 4 h of growth before protein extraction was analyzed (Fig. 3*C*, *Lanes 1–3*). Wortmannin reduced both *in vivo* phosphorylation of serine 15 and acetylation of lysine 382 (Fig. 3*C*, compare *Lane 3* with *Lane 4*). Wortmannin did not alter the induction or level of p53 in the TR9-7 cells (Fig. 3*C*, with antibody PAb240). It has been shown *in vitro* that acetylation of p53 at lysine 382 is dependent on NH_2-terminal phosphorylation (20). Our results

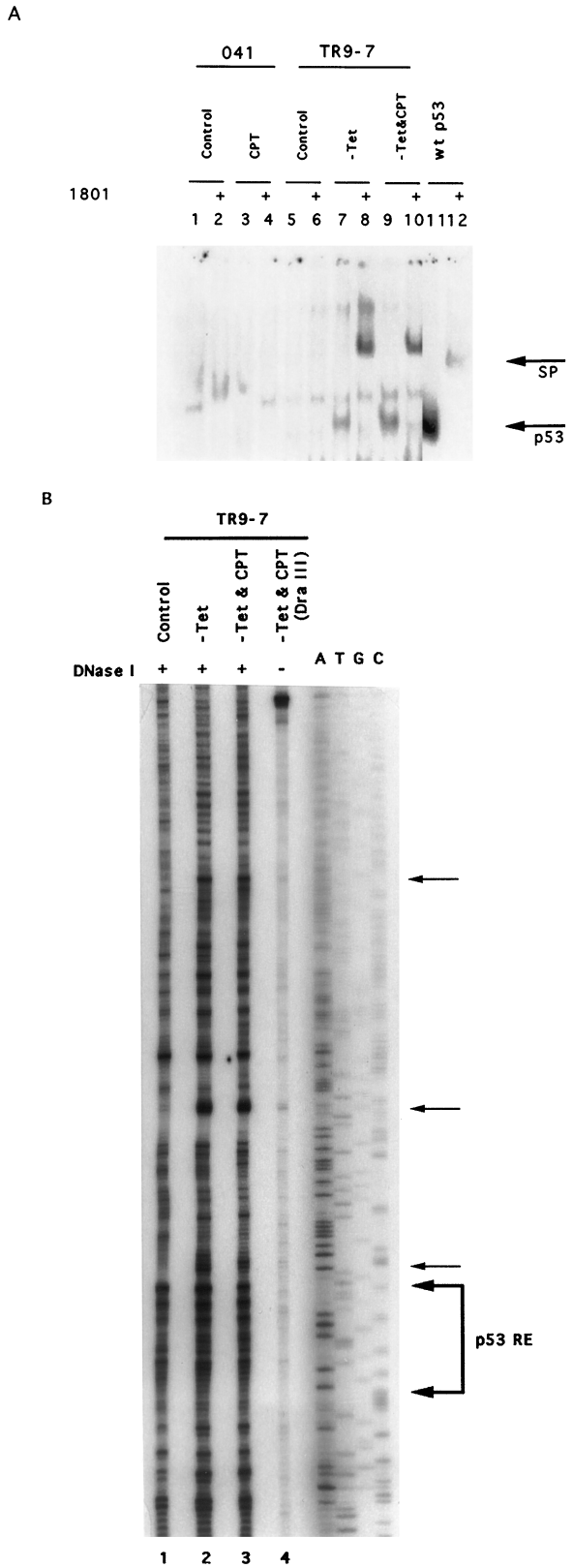

Fig. 5. p53 DNA binding activity was not enhanced after DNA damage. *A*, the electrophoretic mobility shift assay was carried out in the absence (*Lanes 1, 3, 5,7,* and *9*) or presence of p53 antibody PAb1801 (*Lanes 2, 4, 6, 8,* and *10*). *B*, Genomic DNase I footprinting was carried out on TR9-7 cells treated with 0.1 μg of DNase I. The published putative p53-RE was identified by sequencing genomic *gadd45* in a plasmid clone (*Lanes A, T, G,* and *C*). Ligation-mediated PCR was performed, followed by primer extension with ^{32}P-labeled oligonucleotide 3, which hybridized approximately 150 bp downstream from the *gadd45* putative p53 binding site. Purified DNA digested with *Dra*III from DNase I-untreated nuclei of TR9-7 (*−Tet & CPT*) cells was shown in *Lane 6*. Samples were electrophoresed on a 6% urea sequencing gel.

suggest that this is also the case *in vivo* and that CPT can induce this cascade.

Phosphorylation at Serine 15 and Acetylation at Lysine 382 Are Not Necessary to Control p53-mediated Activation of *gadd45* Expression. It has recently become clear that real-time RT-PCR analysis with novel fluorescent molecular beacon probes is a more rapid method for quantitative analysis of mRNA accumulation (19, 26). Therefore, a molecular beacon designed for the *gadd45* gene was used to investigate the levels of gadd45 mRNAs in the presence or absence of wortmannin (Fig. 4). We investigated whether there was any reduction in p53-mediated *gadd45* gene expression when wortmannin was present because wortmannin reduced CPT-induced phosphorylation of p53 at serine 15 and acetylation at lysine 382 (Fig. 3). Cytoplasmic RNA was extracted from TR9-7 cells incubated with or without wortmannin for the last 4 h of growth. The mRNA level of gadd45 in the TR9-7 cells was observed not to increase when p53 was induced by the removal of tetracycline (Fig. 4*C*). However, a 7-fold increase was detected when cells with induced p53 were treated with CPT (Fig. 4*C*). The addition of wortmannin did not inhibit the p53-mediated transcriptional activation of *gadd45* (Fig. 4*C*). This result suggests that CPT-induced DNA damage does not require the phosphorylation of p53 at serine 15 to effect the activation of gadd45. CPT-induced damage activates a yet to be determined component of the p53-dependent signal transduction cascade that does not appear to be a PI3k family member. In fact, the addition of wortmanin facilitated the ability of p53 to activate *gadd45* in the absence of DNA damage, suggesting that a PI3k member can act to inhibit *gadd45* activation. Only a slight increase in gadd45 accumulation was detected in the MDAH041 cell line without p53 in the presence of either CPT or wortmannin (Fig. 4*C*).

DNA Binding by p53 Was Not Stimulated by CPT Treatment. It is believed that p53 activates its target genes in part by the interaction of p53 with its recognition sequences. In fact, *in vitro* studies have demonstrated that the acetylation of p53 at its COOH terminus can stimulate the sequence-specific DNA binding activity of p53 (27). To see whether activation of p53 DNA binding resulted after CPT induced DNA damage, EMSAs were carried out with an oligonucleotide containing the p53 SCS (28). The same p53-dependent gel shift species was observed in both the −Tet and −Tet + CPT samples (Fig. 5*A, p53 arrow*). In addition the p53-specific antibody PAb1801 was able to supershift this protein-DNA complex, further indicating that this complex was p53 dependent (Fig. 5*A, SP arrow*). The EMSA results also showed that p53, similar to the SCS oligonucleotide, bound in both the presence and absence of CPT (Fig. 5*A, Lanes 7–10*). Interestingly, the p53-specific antibody PAb421 could not supershift the p53-DNA complex. This result suggests that there was another modification at the COOH terminus, such as phosphorylation by protein kinase C (29). We also observed the loss of PAb421 reactivity by Western blot analysis. Because this loss of PAb421 reactivity was detected using denatured and immobilized p53, it can be attributed to the direct steric effect of an added phosphate (30). Similarly, we did not observe increased p53 DNA binding activity to the gadd45 oligonucleotide after drug treatment. Although the p53 gel shift results were identical with both the SCS and gadd45 oligonucleotides, the level of background obtained when the gadd45 oligonucleotide was used was high; therefore, the SCS results are presented as the representative sample.

Ligation-mediated PCR genomic footprinting was performed to determine whether p53 bound to the *gadd45* p53-RE *in vivo* and whether binding changed on CPT treatment. We have previously detected protection of the *mdm2* p53-RE using this technique (17). The putative p53-RE was identified by sequencing genomic *gadd45* (Fig. 5*B, Lanes A, T, G,* and *C*). There was no DNase I protection of

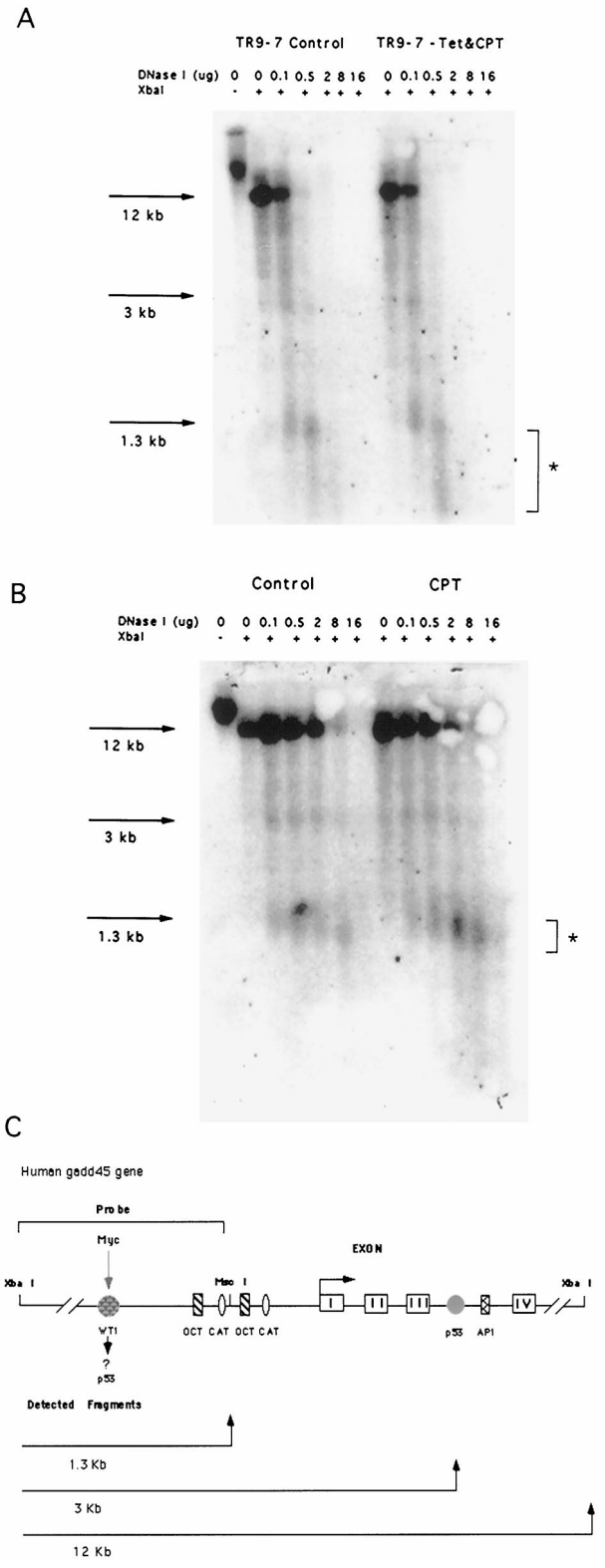

Fig. 6. p53-mediated activation of the *gadd45* gene occurs from a nucleosome-free region. DNA in 2×10^6 isolated nuclei was digested with increasing amounts of DNase I (0, 0.1, 0.5, 2, 8, or 16 μg as indicated *above* each lane). *A*, nuclei were isolated from TR9-7 cells containing tetracycline (control) or after a 24-h withdrawal of tetracycline with overlapping 0.1 mM CPT treatment for the last 4 h (−Tet & CPT). *B*, nuclei were isolated from 184A1 cells with (*CPT*) or without (*Control*) CPT for 4 h. Purified DNA was restricted with *Xba*I, electrophoresed on a 0.8% agarose gel, and probed with a ^{32}P-labeled *Xba*I-*Msc*I genomic *gadd45* probe fragment. *C*, schematic diagram of the genomic structure of the human *gadd45* gene showing the known and putative transcription factor binding sites. The corresponding length detected by *Xba*I-*Msc*I probing to the *gadd45* promoter region or putative p53-RE is also shown.

the putative p53-RE observed when p53 was either induced or induced and activated by CPT (Fig. 5*B*, compare *Lanes 2* and *3* with *Lane 1*). Several DNase I hypercutting sites did emerge in a p53-dependent manner (Fig. 5*B*, *Lanes 2* and *3*, *arrow*), which suggests that p53 might bind transiently to the site and might be involved in a modification of the nucleosomal structure in this area. Interestingly, no change in the hypercutting sites was observed when CPT was added. CPT forms a ternary complex with topoisomerase I and the DNA, covalently trapping the topoisomerase I on the DNA (31). gadd45 appears to facilitate topoisomerase activity *in vivo* (32). Therefore, technically, we cannot rule out the possibility that there might have been CPT-mediated DNA cleavage interfering with our DNase I footprints when CPT was present. Clearly no increased protection of the putative p53-RE in the *gadd45* gene was detected on activation of *gadd45* transcription, although protection of the *mdm2* p53-RE was detected under these conditions.

Increased DNase I Sensitivity Was Detected at the *gadd45* Promoter Region when CPT Was Present. To determine whether remodeling of the *gadd45* gene chromatin was associated with gene activation, Southern blot analysis using a probe to the far end of the expected fragment was carried out under various conditions (Fig. 6*C*). Selective, gene-specific changes in chromatin structure have emerged from DNase I mapping studies that focused on the arrangement of nucleosomes around specific genes as a function of their state of transcriptional activity (33). We have previously shown that the p53-RE of the *mdm2* gene is constitutively DNase I sensitive (17). We screened for the appearance of DNase I-hypersensitive sites at the promoter and the putative p53-RE of the *gadd45* gene. The DNase I-treated genomes isolated from the nuclei of TR9-7 cells grown with or without CPT treatment were analyzed (Fig. 6*A*). Two constitutive DNase I-hypersensitive sites were observed in the *gadd45* gene. One hypersensitive site was observed 3 kb from the *Xba*I cutting site and corresponded to the location of the putative p53-RE (Fig. 6). The other hypersensitive site was observed 1.3 kb from the *Xba*I cutting site and corresponded to the *gadd45* promoter region (Fig. 6). The overall DNase I sensitivity at the putative p53 binding sites did not change in the presence of CPT. However, DNase I sensitivity at the *gadd45* promoter region was increased when CPT was added to the TR9-7 cells (Fig. 6*A*). The 184A1 cell line is an immortalized human mammary epithelial cell line that contains wt p53 (16). The 184A1 cell line was used to rule out the possibility that the increased DNase I sensitivity that resulted in the TR9-7 cells was not due to the artificial nature of the overexpressed p53. In the 184A1 cell line, the p53 was only activated by the DNA damage induced by CPT, and once again the same two constitutive DNase I-hypersensitive sites were observed in the *gadd45* gene, with increased sensitivity at the *gadd45* promoter resulting on CPT treatment (Fig. 6*B*). It has been suggested that increased DNase I sensitivity can result as a consequence of the absence of a canonical nucleosome or, alternatively, it can result from binding of transcription factors that locally distort the DNA within or adjacent to a site (34). Several transcription factors have been found that are involved in the regulation of *gadd45* gene expression. Sequence analysis of the *gadd45* promoter demonstrates a GC-rich region that contains a consensus sequence for one WT1 and three overlapping Egr-1 sites (35). It has been reported that this GC-rich region is necessary for p53/WT1-dependent activation of the *gadd45* promoter (36). In addition, myc-mediated repression of *gadd45* also requires this GC-rich region (35). Our results demonstrate that both the promoter and the putative p53-RE of the *gadd45* gene are constitutively hypersensitive to DNase I, indicating that these two regions have accessible chromatin structures. In addition, when *gadd45* was turned on, the promoter region appeared to be more dynamic than the putative p53-RE. This suggested that chromatin

remodeling and differential association of transcription factors might have been involved in *gadd45* gene activation from the promoter when the DNA damage signal was present. The *mdm2* P2 promoter is constitutively sensitive to DNase I, but when p53 activates *mdm2* transcription, no increase in this sensitivity occurs (17). Therefore, increased DNase I sensitivity at the *gadd45* promoter suggests that p53 might function differently at various target genes. Chromatin remodeling may act as one modulator to regulate gene expression of p53-inducible genes.

DISCUSSION

p53 Requires a Damage Signal to Efficiently Activate *gadd45*. DNA damage induces activation of p53 as a transcription factor and also causes the activation of a number of p53-responsive genes. The specific mechanism of how p53 is regulated to activate its many downstream target genes remains unclear. The general paradigm is that on activation, p53 levels increase concomitant with changes in p53 posttranslational modification. DNA damage induces p53 to activate *gadd45* transcription, but it has been difficult to dissect the type of p53 activation required because DNA damage generally causes both an increase in the level of p53 and a number of p53 posttranslational modifications (37). Interestingly, we have found that induced p53 in the absence of DNA damage does not significantly activate the *gadd45* gene. On the other hand, both the *waf1/p21* and *mdm2* genes were activated in the absence of DNA damage. Transcriptional activation of the *gadd45* gene required both signals induced by DNA damage and increased p53 levels. Using the p53-inducible cell line TR9-7, we have demonstrated that the addition of the topoisomerase I-targeted DNA-damaging agent CPT allowed for the rapid activation of *gadd45* without an increase in the intracellular p53 level. It has previously been shown that an increase in p53 that occurs without DNA damage is unable to activate *gadd45* (38). Therefore, this phenomenon is not restricted to one cell type. We also demonstrated that CPT treatment of TR9-7 cells induced phosphorylation of p53 at serine 15 and acetylation at lysine 382. These changes correlated with the accumulation of gadd45 mRNA. However, inhibition of these posttranslational modifications by the addition of wortmannin did not inhibit *gadd45* activation. This is the first time that efficient gadd45 mRNA induction has been shown to require a signal initiated by drug-induced cellular DNA damage to augment wt p53 activity.

Are the p53 Posttranslational Modifications Induced by DNA Damage Necessary for *gadd45* Activation? ATM kinase activity is increased after DNA damage, and this causes phosphorylation of p53 at serine 15 *in vitro* (13, 14). Phosphorylation of serine 15 in AT minus cells is delayed after IR (39). ATR and DNA-PK can phosphorylate p53 at both serine 15 and serine 37 *in vitro* (14, 15, 40). Furthermore, it has been shown that phosphorylation of serine 15 *in vivo* is induced by γIR, CPT, and UV IR (22, 39). DNA damage-induced phosphorylation at the NH_2 terminus is known to act in two separate pathways. One pathway works at the level of reducing the binding of MDM2 to the p53 protein (22), which inhibits the ability of MDM2 to promote the degradation of p53 (8, 9). The second pathway is involved in increasing the ability of p53 to recruit CBP/p300, followed by increasing the overall level of acetylation of the COOH terminus of p53 (20, 41). It has been suggested that DNA damage can activate p53 as a transcription factor through signaling for an NH_2-terminal phosphorylation and COOH-terminal acetylation cascade (20). *gadd45* gene induction by IR is blocked by the protein kinase inhibitor H7, suggesting that *gadd45* gene activation is mediated by a kinase (3). In our study, inhibition of phosphorylation at serine 15 by wortmannin was observed not to inhibit the DNA damage-induced accumulation of gadd45 mRNA. This observation suggests that blocking the phosphorylation of p53 serine 15 by either DNA-PK or ATM is not sufficient to inhibit p53-mediated *gadd45* gene activation. Previous studies have demonstrated that single mutations of individual NH_2-terminal serines do not have significant effects on the p53 transactivation capacity (42). The possibility cannot be ruled out that wortmannin does not inhibit phosphorylation of the critical amino acids of p53 required for activation of the *gadd45* gene. It will be interesting to investigate what effect inhibiting modification at other phosphorylation sites has on the regulation of *gadd45* gene activation. The block of acetylation of p53 at lysine 382 also did not have an inhibiting effect on *gadd45* gene activation. This suggests that the acetyltransferase activity of p300 associated with p53 may not be necessary to initiate *gadd45* induction.

No Increase in p53 DNA Binding Activity Occurs Coincident with the Activation of *gadd45*. Posttranslational modification of p53 by acetylation is thought to disrupt the interaction between the COOH-terminal domain and the central domain of p53 (27). This may allow p53 to adopt an active conformation, which enhances the sequence-specific DNA binding activity of the protein. p53 was acetylated at lysine 382 after CPT treatment. Increased DNA binding by p53 after CPT treatment was not observed. The increased DNA binding data, documented previously, were obtained by comparing latent bacterially or baculovirus produced p53 to *in vitro* acetylated p53 (27, 20). The observation that p53 in the TR9-7 cells was not reactive with PAb421 suggests that the protein was phosphorylated at the COOH terminus (30). *In vitro* phosphorylation of the p53 COOH-terminal region by protein kinase C can stimulate sequence-specific DNA binding ability while inhibiting PAb421 reactivity (29). In this way, the p53 protein in TR9-7 cells is conformationally different as compared with latent p53. The COOH-terminal modification of the p53 in the TR9-7 cell line may be involved in the inability of the p53 to activate gadd45 significantly in the absence of DNA damage. However, PAb421 reactivity did not emerge after CPT treatment and is therefore not required for the activation of the *gadd45* gene.

Direct DNA binding of p53 to the *gadd45* putative p53-RE was not observed. This suggests that either direct p53 binding to the putative binding element is not necessary for p53-mediated activation or that the interaction of p53 with the *gadd45* p53-RE is transient. This was in agreement with a previous *in vivo* footprinting study (43) that was unable to detect clear protection at the *gadd45* p53-RE. The hypercutting seen in the footprinting analysis that resulted when p53 was induced strengthens the argument for a transient interaction of p53 with the *gadd45* gene. The *gadd45* putative p53 binding site was confirmed by comparing homology to a published p53 consensus sequence (38) as well as by testing in mobility shift assay, immunoprecipitation, and transient transfection assays (4). It is possible that p53 may bind to a different region of the *gadd45* gene or that p53 binds to the putative binding site before the 4 h drug treatment time point that we examined. Another possibility is that the *gadd45* p53-RE is only bound by p53 in a specific cell cycle stage. The TR9-7 cells with induced p53 undergo growth arrest at both G_1-S phase and G_2-M phase, resulting in a mixed population of cells (11). If the p53 DNA binding assay were carried out at each cell cycle stage instead of in an exponentially growing population, the answer of whether p53 is bound to its putative binding site would be more definitive. p53 can also participate in transcriptional induction of the *gadd45* promoter in the absence of direct DNA binding (36). Moreover, whereas some genotoxic stress does not require p53 to activate *gadd45*, p53 has been shown to always have a cooperative activation effect (44). Interestingly, p53 cooperates with WT1 as well as BRCA1 to activate the transcription of the *gadd45* gene (36, 45). Here barely detectable *gadd45* induction by genotoxic stress was observed in the absence of

p53, and barely detectable induction was observed in the presence of p53 without genotoxic stress. Therefore, p53 must normally be cooperating with some other signal to initiate the rapid and robust activation of *gadd45*.

A Change in DNase I Sensitivity at the *gadd45* Promoter Occurs during DNA Damage Induction. Constitutive DNase I hypersensitivity at the *gadd45* promoter and the p53-RE was observed. This suggests that this gene is "preprimed" for activation and that both these regions require a relaxed chromatin structure for the gene to function properly. The observation of increased accessibility for DNase I at the *gadd45* promoter region on DNA damage suggests that chromatin changes occur at the promoter region, but the nature of these changes is unclear. It is possible that changes occur because repressing factors are released or because new factors are bound.

Activation of *gadd45* expression occurs in a c-myc knockout cell line (46). The promoter of the *gadd45* gene can be activated by p53 in the absence of the putative p53-RE when WT1 is present (36). The GC-rich region of the promoter required for WT1/p53-mediated activation is also required for myc-mediated *gadd45* repression (35, 47). The *gadd45* promoter lacks a TATA box (4, 47). The TATA-less promoter organization differs from that of many other p53 target genes, such as *waf1*, *mdm2*, and *bax*, which may influence how *gadd45* is regulated. It is possible that the activation of *gadd45* by stress is effected through the release of c-myc repression in addition to p53 recruitment of basal transcription machinery in the absence of a TATA box. This would be reminiscent of the inducible lactose operon system in *Escherichia coli* that is both positively and negatively regulated.

Differential Expression of p53-inducible Genes. *mdm2* transcription is reduced by the topoisomerase poison etoposide (10). We have demonstrated that reduction of *mdm2* expression occurs after CPT treatment as well. Arriola *et al.* (10) proposed that *mdm2* expression may be inhibited by bulky adduct damage to the template because both the P1 and P2 promoters of *mdm2* are DNase I hypersensitive. However, this suggestion is not consistent with our observation that the *gadd45* promoter (which is also constitutively DNase I hypersensitive) is turned on in the presence of CPT. The model proposed by Wu and Levine (48) is preferred, in which DNA damage induces a repressor specific for *mdm2*, which will inhibit *mdm2* transcription. It should be noted that to fully analyze p53 transcriptional activity, the analysis of one target gene (or one means of p53 activation) is not enough. Coordinate repression and activation may work to differentially regulate the complex pattern of expression of p53 target genes. In an inducible BRCA1 system, *gadd45* induction is coincident with BRCA1 expression (45). This suggests that BRCA1 may be a more powerful activator of *gadd45* than p53. Additionally, DNA damage may activate endogenous BRCA1 so that it readily cooperates with p53 to activate *gadd45* transcription. Many hypotheses for the mechanisms of p53-mediated *gadd45* gene activation remain to be tested. The phosphorylation-acetylation cascade involving p53 posttranslational modifications at serine 15 and lysine 382 does not appear to be critical for the regulation of p53-mediated activation of *gadd45*, although it may be critical for other functions of p53. gadd45 has recently been reported to bind and activate an upstream regulator of c-Jun-NH_2-terminal kinase/stress-activated protein kinase, thus triggering c-Jun-NH_2-terminal kinase/stress-activated protein kinase-dependent apoptosis (49, 45). When p53 is induced by the withdrawal of tetracycline in TR9-7 cells, it directs the cells to undergo G_1-S-phase and G_2-M-phase arrest (11). Therefore, it is not surprising that no detectable activation of *gadd45* was observed in cells with induced p53. It is possible that in the presence of CPT, a program directing the cells toward apoptosis is turned on. This might necessitate the activation of *gadd45*.

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